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Identification of Heterotrophic Nitrification in a Sierran Forest Soil

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A potential for heterotrophic nitrification was identified in soil from a mature conifer forest and from a clear-cut site. Potential rates of NO_2^- production were determined separately from those of NO_3^- by using acetylene to block autotrophic NH_4^+ oxidation and chlorate to block NO_2^- oxidation to NO_3^- in soil slurries. Rates of NO_2^- production were similar in soil from the forest and the clear-cut site and were strongly inhibited by acetylene. The rate of NO_3^- production was much greater than that of NO_2^- production, and NO_3^- production was not significantly affected by acetylene or chlorate. Nitrate production was partially inhibited by cycloheximide, but was not significantly reduced by streptomycin. Neither the addition of ammonium nor the addition of peptone stimulated NO_3^- production. ^{15}N labeling of the NH_4^+ pool demonstrated that NO_3^- was not coming from NH_4^+. The potential for heterotrophic nitrification in these forest soils was greater than that for autotrophic nitrification.

Since nitrogen frequently limits productivity in forest ecosystems (17), the processes controlling nitrogen availability in forests and the effects of management practices on these processes have been widely studied (7, 14, 31). Nitrification has been one of the most intensively studied processes in forest N cycling, yet it is still poorly understood. Nitrate rarely accumulates in undisturbed forest soils, but commonly accumulates after clear cutting (18, 29, 30); the mechanisms responsible for this are still under discussion (7, 19, 28–31).

Few studies have addressed the relative importance of autotrophic versus heterotrophic nitrification in forest ecosystems. Nitrate production by a fungus was first reported in 1896 (24), and many heterotropic organisms have since been reported to oxidize various nitrogen compounds in culture (6). Although heterotrophic nitrification has been hypothesized to be important in some forest soils (13, 23), the actual extend to which it occurs in soils and its ecological significance are unknown.

Most studies on NO_3^- production in forests have used hydrological techniques such as watershed input-output budgets and lysimetry (9, 18, 30) or long-term soil incubations (19–21, 29). These techniques may provide the best available data for ecosystem budgets and models. However, hydrological analyses provide no information on the gross production of NO_3^- or on the mechanism of its production. Most incubation studies have used long time periods, disturbed the soil, and removed active roots, which may substantially change the processes from those occurring in situ. The usefulness of these techniques in examining the mechanisms and controls of nitrate production in soil is therefore limited.

Short-term assays may be preferable for quantifying the rate of a process before disturbance by the assay conditions has caused changes in the activity or size of the microbial population. Short-term assays have been done with soil slurries to measure maximum or potential rates of nitrification (2). This potential rate of nitrification may not be realized in the field; however, such assays are useful for identifying the processes occurring and for estimating ranges of possible activity. Potentials may also provide a measure of active population size (1, 3). Soil slurries allow rigorously controlled conditions and uniform substrate distribution.

In the present study, short-term assay with slurries of freshly sampled soil were used to quantify potential fluxes through the NO_2^- and NO_3^- pools in a forest soil and to examine the effect of clear cutting on these flows. Two inhibitors (chlorate and acetylene) were used to block the oxidation of NO_2^- and NH_4^+ (2, 10), chemically isolating the processes of interest (Fig. 1). By separately measuring the production rates of NO_2^- and NO_3^-, it was possible to identify the pathways responsible. Thus, both autotrophic nitrification of NH_4^+ and heterotrophic production of NO_3^- from organic nitrogen were measured.

MATERIALS AND METHODS

Sites and sample collection. Soil samples were collected in Blodgett Experimental Forest near Georgetown, Calif. in the foothills of the Sierra Nevada mountains. The elevation is 1,400 m, and the climate is Mediterranean with a xeric moisture regime. The soils are well-drained, sandy loams of the Holland series of Ultic haploxeralfs. Two sites were studied: a 60-year-old mature mixed confier stand and an adjacent stand clear cut in 1980. In the clear-cut site, litter and slash had been raked together and burned after harvest. Soil from both the forest A-horizon and the clear-cut surface had pH values of 5.8; the total organic matter levels were 5.2 and 3.9%, respectively.

Soils were collected in September and October 1982 and in May and June 1983. Mineral soil from the top 10 cm was composited and stored at 5°C until assayed (generally 3 days or less).

Assays. Soil (10 g) was slurried in 50 ml of 1 mM phosphate buffer (pH 7.2) amended with $(NH_4)_2SO_4$ (0.5 mM). The slurry was agitated in stoppered 250-ml Erlenmeyer flasks. A glass tube through the stopper was fitted with a tygon sleeve and capped with a rubber septum between samplings. Over the 6- to 7-h assay, uniform samples of the slurry (8 ml) were periodically taken through this tube. Samples were centrifuged and filtered, and the filtrate was frozen until analysis. Analyses for NO_2^- and NO_3^- were performed colorimetrically with a Lachat System IV flow injection autoanalyzer. Both NO_2^- and NO_3^- were quantified by a modified Griess-

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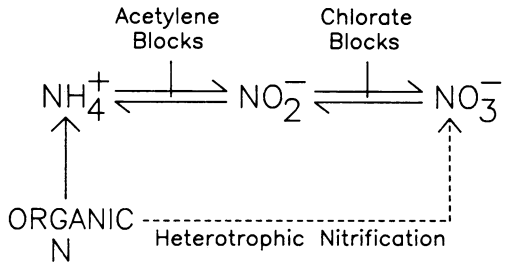

FIG. 1. Acetylene and chlorate inhibition of autotrophic and heterotrophic nitrification.

Ilosvay method (15). Nitrate was converted to NO_2^- by reduction with copperized cadmium before diazotization.

The effects of chlorate and acetylene on NO_2^- and NO_3^- production were examined. Sodium chlorate (10 mM) was added to the phosphate buffer to inhibit the chemoautotrophic oxidation of NO_2^- to NO_3^- (2, 11). Acetylene (15 ml) was injected into the flask headspace (6 KPa) to inhibit autotrophic oxidation of NH_4^+ to NO_2^- (4, 10).

To determine whether the various processes observed in this study were biological in nature, assays were performed on soils that had been sterilized by fumigation with propylene oxide for at least 12 h.

The roles of bacteria and fungi were examined by adding specific biocides (1,000 mg/liter) to the slurry at the beginning of the assay. The biocides used were streptomycin sulfate and cycloheximide (Sigma Chemical Co.) Nitrite and nitrate production were then assayed as described above.

Nitrate production from various nitrogen sources was assayed by treating the slurry with three nitrogen ammendments: no added nitrogen, $(NH_4)_2SO_4$ (0.5 mM), or peptone (100 mg/liter or approximately 1 mM N; Nutritional Biochemical Corp.). Peptone was used as a substrate because it provides a source of readily available organic N, and it has been commonly used as a substrate in studies on heterotrophic nitrification (26, 27).

$^{15}NH_4$ tracer study. To determine the source of NO_3^-, the NH_4^+ pool was labeled with $^{15}NH_4^+$. This allowed the measurement of NO_3^- and NO_2^- resulting from NH_4^+ oxidation, as compared with the total production of NO_3^- from all sources. In this study the slurry (15 g of soil in 75 ml of buffer) was amended with 70% atom abundance $(^{15}NH_4)_2SO_4$ (0.5 mM). Total NH_4^+ was quantified at 0 and 6 h to allow calculation of the atom percent ^{15}N in the NH_4^+ pool. Samples were taken periodically as above to determine the increase in total NO_3^- and NO_2^-. After the 6 h, the slurry was filtered and analyzed for $^{15}NO_3^-$ and $^{15}NO_2^-$, which were quantified as one pool. The filtrates were steam distilled with MgO to drive off NH_4^+, and the still was steamed with acetic acid and ethanol to remove residual $^{15}NH_4^+$. Devarda's alloy was then added, and the sample was redistilled to collect $^{15}NO_2^-$ and $^{15}NO_3^-$ as $^{15}NH_4^+$. As the processed samples contained 10 μg or less of ^{15}N, they were diluted with 1 mg of ^{14}N to provide sufficient N for analysis and to reduce the samples to less than 1% ^{15}N. Control samples contained 1 mM $^{15}NH_4^+$ in buffer; less than 1 μg of $^{15}NH_4$ was carried over in the second distillation. Samples were then analyzed for atom percent ^{15}N with a MAT GD 150 isotope ratio mass spectrometer. N_2 gas was used as a calibration standard for the 28:29 ratio (8).

TABLE 1. Effect of acetylene on nitrate production by *A. flavus*

Nitrogen source	Nitrate production[a] at the following acetylene concentrations (KPa):		
	0	1	5
Ammonium	5.0 (0.2)	4.5 (0.3)	5.6 (0.6)
Peptone	8.0 (0.4)	9.7 (0.7)	7.3 (0.02)

[a] Nitrate production is given as micrograms of NO_3^- N per gram (dry weight) of cells over 10-day growth period. Numbers within parentheses are the standard errors of three replicates.

TABLE 2. Rates of nitrite and nitrate production in soil samples from clear-cut and forested sites

Sampling date	Rate of production (ng of N per g per h)[a]			
	Clear-cut site		Forest	
	NO_2^-	NO_3^-	NO_2^-	NO_3^-
12 September 1982	10.7 (0.5)	490 (58)	6.7 (3.1)	89 (39)
3 October 1982	4.6 (1.2)	270 (48)	ND[b]	ND
28 October 1982	9.0 (1.6)	117 (24)	5.9 (0.4)	57 (10)
8 May 1983	2.0 (1.0)	37 (10)	12.4 (0.3)	46 (2)
10 June 1983	27.1 (3.4)	50 (15)	3.4 (2.0)	45 (17)

[a] Numbers within parentheses are the standard errors of three replicates.
[b] ND, Not determined.

Fungal nitrification. The effect of acetylene on nitrate production by *Aspergillus flavus* (ACTT 26214) growing on NH_4^+ and peptone nitrogen sources was examined. Hyphal disks were inoculated into medium (100 ml) in a 500-ml Erlenmeyer flask. The peptone media contained the following: peptone (4 g/liter), KH_2PO_4 (2 g/liter), $MgSO_4 \cdot 7H_2O$ (0.2 g/liter), $CaCl_2$ (0.1 g/liter), $FeSO_4 \cdot 7H_2O$ (1 mg/liter), $MnSO_4 \cdot 4H_2O$ (1 mg/liter), yeast extract (1 g/liter), and glucose (5 g/liter). The ammonia medium was identical to that of the peptone, except that it contained $(NH_4)_2HPO_4$ (2 g/liter) in place of peptone and contained no yeast extract. The flasks were stoppered, and sufficient headspace gas was replaced with acetylene to provide concentrations of 0, 1, and 5 KPa of acetylene. Flasks were incubated on an orbital shaker at 25°C for 10 days. After this time, media were analyzed for NO_3^- and NO_2^-.

Analyses of data. The rates of NO_3^- and NO_2^- production were determined by linear regression for each of three replicate samples. All treatment effects were analyzed by

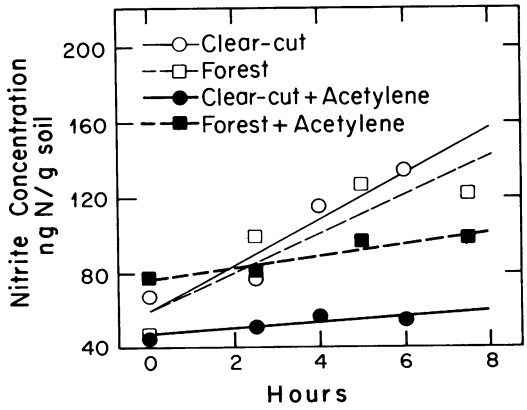

FIG. 2. Nitrite production in the presence and absence of 6 KPa of acetylene in soils from a forested and clear-cut site (12 September 1982 sampling). Each point is the mean of three replicates. Rates with acetylene were significantly lower ($P \leq 0.05$) than those without acetylene.

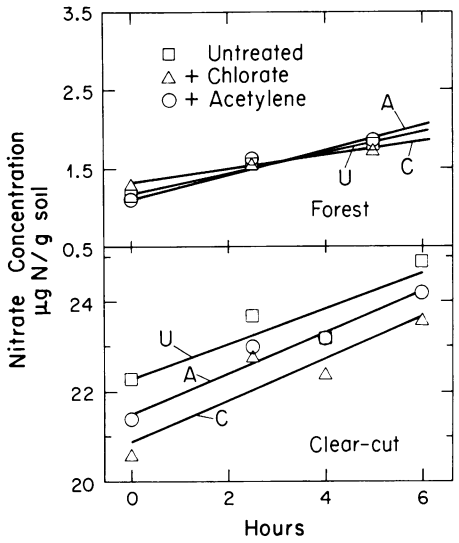

FIG. 3. Nitrate production in the presence and absence of 6 KPa of acetylene and 10 mM chlorate in soils from a forested and clear-cut site (12 September 1982 sampling). Each point is the mean of three replicates. There were no significant treatment effects.

one-way analysis of variance at the 5% significance level, except where specifically noted.

RESULTS

Acetylene effects on fungal nitrification. Acetylene concentrations up to 5 KPa did not significantly inhibit production of NO_3^- by *A. flavus* growing on either NH_4^+ or peptone nitrogen sources (Table 1). Nitrite production was less than 10% of NO_3^- production and was not affected by acetylene (data not shown).

NO_2^- production. The average rates of NO_2^- production assayed in the presence of chlorate varied considerably among samplings, although the rates were fairly consistent within each sampling (Table 2). The rates of NO_2^- production in samples from the clear-cut site were not consistently different than those from the forest. The addition of acetylene significantly ($P \leq 0.05$) reduced the rate of NO_2^- production (Fig. 2) from 16.2 and 11.5 ng of NO_2^- N per g per h in samples from the forest and clear-cut sites, respectively, to less than 3 ng of NO_2^- N per g per h. Acetylene effectively blocked NO_2^- production in soils sampled on all dates.

NO_3^- production. The rates of NO_3^- production were always much greater than the rates of NO_2^- production (Table 2). The rates of NO_3^- production were significantly ($P \leq 0.05$) higher in soil from the clear-cut site than in soil from the forest site in samples from September 1982. The rates of NO_3^- production were not significantly ($P \leq 0.05$) affected by either chlorate or acetylene on any of the six sampling dates. The data from the 12 September 1982 sampling are shown in Fig. 3. Nitrate was not produced in soil that had been sterilized before the assay, indicating that the process was biological.

TABLE 3. Effect on streptomycin and cycloheximide on nitrate production in soil from the clear-cut site

Biocide treatment	Nitrate produced[a] (µg of NO_3^- N per g of soil)
None	1.07 (0.11)
Streptomycin	1.00 (0.05)
Cycloheximide	0.82 (0.03)
Both	0.64 (0.05)

[a] Nitrate produced over a 4-h period. Numbers within parentheses are the standard errors of three replicates.

TABLE 4. Effect of ammonium and peptone additions on nitrate production by soil from the clear-cut site

Added nitrogen source	Nitrate produced[a] (µg of NO_3^- N per g of soil)
None	0.79 (0.04)
$(NH_4)_2SO_4$	1.07 (0.11)
Peptone	0.94 (0.08)

[a] Nitrate produced over a 4-h period. Numbers within parentheses are the standard errors of three replicates.

Table 3 shows the effect of streptomycin (an inhibitor of protein synthesis in bacteria) and cycloheximide (an inhibitor of protein synthesis in fungi) on NO_3^- production by soil from the clear-cut site. Streptomycin did not significantly affect NO_3^- production. The addition of cycloheximide significantly ($P \leq 0.05$) reduced the rate of NO_3^- production, as determined by the student range test used to contrast samples with cycloheximide to those without cycloheximide (32).

To determine possible substrates for NO_3^- production, two nitrogen sources were added to soil samples, and nitrate production was quantified. There were no significant ($P \leq 0.05$) effects on NO_3^- production due to the addition of either ammonium or peptone (Table 4). In both the presence and absence of an added nitrogen source, the rate of NO_3^- production declined over the course of the assay (data not shown).

NO_3^- production from ^{15}N-labeled NH_4^+ pool. The total NO_3^- production in both forest and clear-cut soils was significantly greater than the NO_3^- produced from the ^{15}N-labeled NH_4^+ pool (Fig. 4). The difference between the total NO_3^- and the NO_3^- produced from the labeled NH_4^+ pool was that which presumably resulted from heterotrophic nitrification of an organic nitrogen source.

DISCUSSION

In recent years, acetylene has become a useful tool in the study of nitrification since it is a powerful inhibitor of autotrophic ammonium oxidation (4, 10). However, acetylene has been found not to inhibit heterotrophic nitrification in a bacterial isolate, *Arthrobacter* sp., growing with NH_4^+

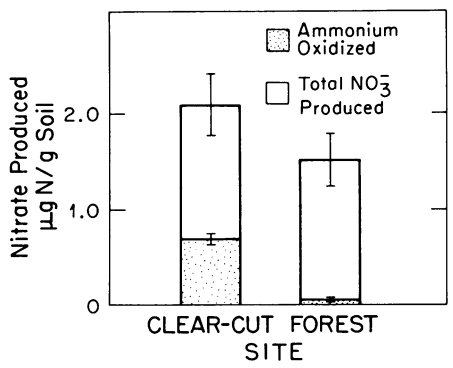

FIG. 4. Source of NO_3^- in soil slurrys in which the NH_4^+ pool was labeled with $^{15}NH_4^+$ (10 June 1983 sampling). Error bars are the standard errors of four replicates.

as a nitrogen source (10). In the present study, nitrification by *A. flavus* was not inhibited by acetylene with either NH_4^+ or peptone as the nitrogen source. The lack of inhibition of heterotrophic nitrification by acetylene may provide a useful means of distinguishing autotrophic and heterotrophic nitrification.

An effective means of quantifying heterotrophic nitrification in soil has been lacking. One approach to studying this process has been to use most probable number counting techniques to demonstrate that autotrophic nitrifiers were not present in sufficient numbers to account for the observed NO_3^- production (23, 25). This approach can have problems, which include low most probable number counting efficiencies and bacterial growth yields and efficiencies that differ between the laboratory and the field (1, 3). Other studies have used long-term incubations of soils ammended with different nitrogen sources to determine the substrate for NO_3^- formation. If the addition of organic N stimulated NO_3^- production and the addition of NH_4^+ did not, it was assumed that NO_3^- was produced heterotrophically (12, 26). Other soil incubation studies have also presented indirect evidence that implicates heterotrophic nitrification (5, 16).

In the present study, NO_3^- production was not inhibited by either acetylene or chlorate, which suggests that the origin of the NO_3^- was heterotrophic nitrification. Short-term assays on freshly sampled soil were used to minimize changes in the microbial population. The potential for heterotrophic nitrification identified should therefore exist in situ in these forest soils. The biocide experiment showed some inhibition of NO_3^- production by cycloheximide, indicating that at least a portion of the observed NO_3^- production was mediated by fungi. It is not surprising that the inhibition by cycloheximide was incomplete, as cycloheximide is an inhibitor of protein synthesis and should not have been fast acting.

Neither added NH_4^+ nor added peptone significantly stimulated nitrate production. Either these compounds were not useable substrates, or the rate of the process was not substrate limited. The decreasing rate of production during the assay period indicates that NH_4^+ and peptone were not suitable substrates for NO_3^- production. The observed NO_3^- was therefore produced from an unidentified substrate that was present in the soil, possibly in limited quantities. Slurrying the soil may have increased the availability of this substrate to heterotrophic nitrifiers. It is possible that the substrate already contained oxidized organic nitrogen, and this N was cleaved off as NO_3^- when the substrate became available. Hydroxamate siderophores might constitute one such substrate.

The $^{15}NH_4^+$ tracer study was used to demonstrate that the source of NO_3^- was not NH_4^+. In the ^{15}N assay, as in the others, the potential rate for heterotrophic NO_3^- production was greater than that for autotrophic NO_3^- production in soils from both the clear-cut and forested soils.

In summary, a potential for heterotrophic nitrification exists in the forest soils studied; NO_3^- appears to be released directly from soil organic nitrogen.

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LITERATURE CITED

1. **Belser, L. W.** 1979. Population ecology of nitrifying bacteria. Annu. Rev. Microbiol. **33**:309–333.
2. **Belser, L. W., and E. L. Mays.** 1980. Specific inhibition of nitrite oxidation by chlorate and its use in assessing nitrification in soils and sediments. Appl. Environ. Microbiol. **39**:505–510.
3. **Belser, L. W., and E. L. Mays.** 1982. Use of nitrifier activity measurements to estimate the efficiency of viable nitrifer counts in soils and sediments. Appl. Environ. Microbiol. **43**:945–948.
4. **Berg, P., L. Klemedtsson, and T. Rosswall.** 1982. Inhibitory effect of low partial pressures of acetylene on nitrification. Soil Biol. Biochem. **14**:301–303.
5. **Etinger-Tulczynska, R.** 1969. A comparative study of nitrification in soils from arid and semi-arid areas of Israel. J. Soil Sci. **20**:307–317.
6. **Focht, D. D., and W. Verstraete.** 1977. Biochemical ecology of nitrification and denitrification. Adv. Microbial Ecol. **1**:135–214.
7. **Gosz, J. R.** 1981. Nitrogen cycling in coniferous ecosystems. Ecol. Bull. (Stockholm) **33**:405–426.
8. **Hauk, R. D.** 1982. Nitrogen-isotope ratio analysis, p. 735–779. *In* A. L. Page, R. H. Mille, and D. R. Keeney (ed.), Methods of soil analysis, part 2, 2nd ed. Agronomy Monograph no. 9. ASA-SSSA, Madison, Wis.
9. **Henderson, G. S., W. T. Swank, J. B. Waide, and C. C. Grier.** 1978. Nutrient budgets of Appalachian and Cascade region watersheds: a comparison. Forest Sci. **24**:385–397.
10. **Hynes, R. K., and R. Knowles.** 1982. Effect of acetylene on autorophic and heterotrophic nitrification. Can. J. Microbiol. **28**:334–340.
11. **Hynes, R. K., and R. Knowles.** 1983. Inhibition of chemoautotrophic nitrification by sodium chlorate and sodium chlorite: a reexamination. Appl. Environ. Microbiol. **45**:1178–1182.
12. **Ishaque, M., and A. H. Cornfield.** 1976. Evidence for heterotrophic nitrification in an acid Bangladesh soil lacking autotrohic nitrifying organisms. Trop. Agric. **53**:157–160.
13. **Johnsrud, S. C.** 1978. Heterotrophic nitrification in acid forest soils. Holarctic Ecol. **1**:27–30.
14. **Keeney, D. R.** 1980. Prediction of soil nitrogen availability in forest ecosystems: a literature review. Forest Sci. **26**:159–171.
15. **Keeney, D. R., and D. W. Nelson.** 1982. Nitrogen—inorganic forms. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, part 2, 2nd ed. Agronomy Monograph no. 9 ASA-SSSA, Madison, Wis.
16. **Klein, T. M., J. P. Kreitinger, and M. Alexander.** 1983. Nitrate formation in acid forest soils from the Adirondacks. Soil Sci. Soc. Am. J. **47**:506–508.
17. **Kramer, P. J.** 1981. Carbon dioxide concentration, photosynthesis, and dry matter production. BioScience **31**:29–33.
18. **Likens, G. E., F. H. Bormann, and N. M. Johnson.** 1969. Nitrification: importance to nutrient losses from a cut-over forest ecosystem. Science **163**:1205–1206.
19. **Matson, P. A., and P. M. Vitousek.** 1981. Nitrogen mineralization and nitrification potentials following clearcutting in the Hoosier National Forest, Indiana. Forest Sci. **27**:781–791.
20. **Montes, R. A., and N. L. Christensen.** 1979. Nitrification and succession in the Piedmont of North Carolina. Forest Sci. **25**:287–297.
21. **Nadelhoffer, K. J., J. D. Aber, and J. M. Melillo.** 1983. Leaf litter production and soil organic matter dynamics along a nitrogen-availability gradient in southern Wisconsin (USA). Can. J. Forest Res. **13**:12–21.
22. **Powell, P. E., G. R. Cline, C. P. P. Reid, and P. J. Szaniszlo.** 1980. Occurrence of hydroxamate siderophore iron chelators in soils. Nature (London) **287**:833–834.
23. **Remacle, J.** 1977. The role of heterotrophic nitrification in acid forest soils—preliminary results. Ecol. Bull. (Stockholm) **25**:560–561.
24. **Stutzer, A., and R. Hartleb.** 1896. Ueber nitratbildung. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2 **2**:701.
25. **Tate, R. L.** 1977. Nitrification in histosols: a potential role for the heterotrophic nitrifier. Appl. Environ. Microbiol. **33**:911–914.

26. **Van de Dijk, S. J., and S. R. Troelstra.** 1980. Heterotrophic nitrification in a heath soil demonstrated by an *in situ* method. Plant Soil **57**:11–21.
27. **Van Gool, A. P., and E. L. Schmidt.** 1973. Nitrification in relation to growth in *Aspergillus flavus*. Soil Biol. Biochem. **5**:259–265.
28. **Verstraete, W.** 1981. Nitrification. Ecol. Bull. (Stockholm) **33**:303–314.
29. **Vitousek, P. M., J. R. Gosz, C. C. Grier, J. M. Melillo, and W. A. Reiners.** 1982. A comparative analysis of potential nitrification and nitrate mobility in forest ecosystems. Ecol. Monogr. **52**:155–177.
30. **Vitousek, P. M., J. R. Gosz, C. C. Grier, J. M. Melillo, W. A. Reiners, and R. L. Todd.** 1979. Nitrate losses from disturbed ecosystems. Science **204**:469–474.
31. **Vitousek, P. M., and J. M. Melillo.** 1979. Nitrate losses from disturbed forests: patterns and mechanisms. Forest Sci. **25**:605–619.
32. **Zar, J. H.** 1974. Biostatistical analysis. Prentice-Hall, Inc., Englewood Cliffs, N.J.