Factors Limiting Denitrification in Soils from Mature and Disturbed Southeastern Hardwood Forests

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ABSTRACT. The relative importance of $O_2$, $NO_3^-$, organic carbon, and acidity on denitrification was studied in a full 2⁶ factorial experiment. Soils were collected from two forested watersheds (WS) (disturbed and reference) and at two slope positions (stream-bank and hillside). Combinations of four incubation treatments (with and without $H_2O$ flooding, $NO_3^-$ amendment, glucose amendment, and at ~pH 5 or ~pH 6) were applied in a full factorial design. ANOVA of $N_2O$ production during 24-hr aerobic incubations under 10kPa acetylene revealed that all main effects were significant. Flooding with $H_2O$ increased $N_2O$ production by three orders of magnitude in disturbed WS soils exhibiting high ambient $NO_3^-$. Both flooding and $NO_3^-$ amendment were necessary to increase $N_2O$ production in reference WS soils exhibiting low ambient $NO_3^-$. Glucose amendment increased $N_2O$ production in the mineral soil sampled at 6-15 cm depth more than in the 0-6 cm sample. Carbon limitation is probably unimportant for denitrification in surface horizons at Coweeta, but C-limitation occurs with increasing soil depth. Although acid treatment had a minor inhibitory effect on denitrification rates, acid forest soils apparently support denitrifier populations capable of appreciable $NO_3^-$ reduction. For. Sci. 33(1):135-144.

ADDITIONAL KEY WORDS. Nitrogen cycle, nitrate reduction, organic carbon, soil acidity.

THE EFFECTS OF $O_2$, $NO_3^-$, organic carbon, acidity, and temperature on denitrifying activity in agricultural soils are well known (Firestone 1982). Unfavorable conditions for any one factor can completely inhibit denitrification, whereas high $NO_3^-$ reduction rates occur only when all factors are at optimal levels. However, a complex of microsites within a soil system creates a continuum of levels for each of these factors. Therefore, partial inhibition or stimulation by each factor may occur. Furthermore, the relative importance of these factors may vary among soil systems.

Two factors distinguishing forest soils from most agricultural soils are particularly germane to denitrification: (1) the Utter layer affects both quantity and quality of organic matter incorporated into mineral soil and therefore influences heterotrophic processes such as denitrification; and (2) acidic conditions...
TABLE 1. Selected properties of composite soil samples.

<table>
<thead>
<tr>
<th>WS slope position</th>
<th>Depth (cm)</th>
<th>Series</th>
<th>Classification</th>
<th>Acidity</th>
<th>( \text{NO}_3^- ) mg-N/ mg-C/ kg</th>
<th>pH</th>
<th>CWS- C/ kg</th>
<th>WFPSb %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hillside</td>
<td>0–6</td>
<td>Trimont loam</td>
<td>Typic</td>
<td>5.1</td>
<td>0.1</td>
<td>25</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Stream site</td>
<td>0–6</td>
<td>Haywood loam</td>
<td>Hapludults</td>
<td>5.4</td>
<td>0.1</td>
<td>42</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Disturbed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hillside</td>
<td>0–6</td>
<td>Tusquitee loam</td>
<td>Umbric</td>
<td>6.3</td>
<td>8.7</td>
<td>23</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6–15</td>
<td></td>
<td>Dystrochrepts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stream site A</td>
<td>0–6</td>
<td>Spivey loam</td>
<td>Typic</td>
<td>6.3</td>
<td>4.4</td>
<td>17</td>
<td>NDb</td>
<td></td>
</tr>
<tr>
<td>(unsaturated)</td>
<td></td>
<td></td>
<td>Haplumbrepts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(saturated) B</td>
<td>0–6</td>
<td>stoney loam</td>
<td></td>
<td>5.8</td>
<td>12</td>
<td>25</td>
<td>92</td>
<td></td>
</tr>
</tbody>
</table>

a Cold-water-soluble carbon.
b Water-filled pore space.
c No data.

denitrifying bacteria (Valera and Alexander 1961). Denitrification in forest systems has received limited attention, and the few studies that exist have been mostly observational (Davidson and Swank, 1986; Goodroad and Keeney 1984; Melillo et al. 1983; Robertson and Tiedje 1984). As intensive silviculture emulates agriculture by becoming increasingly reliant on N fertilizers (Allen 1987), an understanding of the processes regulating nitrification and denitrification in forest soils will become more important. The objective of the present study was to experimentally assess the relative importance of soil moisture, \( \text{NO}_3^- \), available carbon, and acidity on denitrification in soils from two southern Appalachian forested watersheds.

STUDY SITE

Two north-facing watersheds (WS) at the USDA Forest Service Coweeta Hydrologic Laboratory were studied: (1) WS18, a 12.6 ha reference WS supporting an uneven-aged, aggrading, mixed-hardwood forest that has not undergone major disturbance since the chestnut blight in the late 1920s; and (2) WS6, an 8.9 ha disturbed WS that was clearcut, limed, fertilized, and planted with fescue grass (Festuca arundinacea Schreb.) in the late 1950s (Hibbert 1969), was treated with herbicide in 1966 and 1967 (Douglass et al. 1969), and was then allowed to regenerate naturally. Black locust (Robinia pseudo-acacia L., Fabaceae) was the dominant woody species during early stages of succession, but was declining in this 18-yr-old forest due to natural successional processes, including a recent infestation by the locust stem borer (Megacyllene robiniae Forster). Within each of these two WSs, soil sampling areas at two slope positions were selected: (1) a well-drained hillside position and (2) an area within 5 m of a stream, near the base of the WS. Selected properties of these soils are given in Table 1.

METHODS

SOIL INCUBATIONS

A composite sample consisting of 16 mineral soil cores (2.5 cm diam. x 6 cm deep) was collected from each of the four sampling areas in May 1985. A sample
from 6–15 cm depth was also collected at the well-drained hillside position of the disturbed WS. Samples were returned to the laboratory on ice and stored at 4°C. Drying at 105°C for 24 hr was begun immediately on 20 g subsamples to determine gravimetric moisture content. On the following day, the field-moist equivalent of 10 g dry weight was calculated for each composite sample and this amount of moist unsieved soil was measured into forty-eight 125 mL serum bottles. A full 2^4 factorial design with three replications was used to apply the following amendments to the 48 incubation bottles for each composite sample:

1. 10 mL deionized H_2O
2. 100 µL KNO_3 solution providing 5 mg N kg^{-1} dry soil
3. 100 µL glucose solution providing 100 mg C kg^{-1} dry soil
4. 100–150 µL H_2SO_4 or NaOH solutions (1.0–1.5 N)

The appropriate volume and normality of H_2SO_4 and NaOH solutions necessary to change the bulk pH value of each sample by 1 pH unit was determined by trial and error on 10 g dry-weight-equivalent subsamples. After adding an aliquot of base or acid, 10 mL deionized H_2O was added; the slurry was stirred and permitted to stand for 10 min; and acidity was measured by glass electrode. Soils from the disturbed WS, which exhibited ambient acidity of ~pH 6, were left untreated for pH 6 incubations and treated with H_2SO_4 for pH 5 incubations. Soils from the reference WS, which exhibited ambient acidity of ~pH 5, were treated with NaOH for pH 6 incubations and left untreated for pH 5 incubations. Each incubation bottle either did or did not receive the H_2O, KNO_3, and glucose amendments, according to a full factorial design. Added H_2O completely saturated the soils. A 5 mg N kg^{-1} level was chosen for the NO_3^- amendment because denitrification rates have been limited by NO_3^- below this concentration (Ryden 1983). The glucose amendment of 100 mg C kg^{-1} dry soil more than doubled the cold-water-soluble carbon levels for all samples.

Incubation bottles were stoppered, and 10% of the head space air was replaced with C_2H_2 generated from CaC_2 and H_2O. Acetylene inhibits reduction of N_2O to N_2; thus N_2O becomes the major endproduct of denitrification (Tiedje 1983). The bottles were incubated aerobically in the dark at 20°C for 24 hr. A headspace gas sample was then removed by syringe and analyzed for N_2O, using a Varian 3700 gas chromatograph, equipped with an electron capture detector at 330°C, a Porapak-Q analytical column and a Porapak-R precolumn at 65°C, and operated with a carrier gas (95% argon; 5% methane) flow rate of 30–40 cm^3 min^{-1}. A 10-port valve permitted the heavier C_2H_2 and H_2O vapor to be backflushed from the precolumn while N_2O and lighter gases were separated on the analytical column.

STATISTICAL ANALYSES

Square root transformations of the data were necessary to equalize variances across treatment combination means. Combining WS and slope position with the four treatments (H_2O flooding, NO_3^-, glucose, and acidity) yields a full 2^6 factorial, which was analyzed using PROC ANOVA of SAS (SAS Institute 1982). The four-treatment factorial was repeated on soil samples collected from 0–6 cm and 6–15 cm depths at the hillside position of the disturbed WS. These data were analyzed as a full 2^5 factorial with depth as the fifth main effect. Similarly, the experiment was repeated on surface soils collected from two sites near the stream of the disturbed WS: one sample from within a braided channel area that was water-

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*The use of trade names does not imply endorsement by the North Carolina Agricultural Research Service of the product, nor criticism of similar products not mentioned.
FIGURE 1. Main effects of a full factorial experiment. Each bar represents mean \( \text{N}_2\text{O} \) production (dry wt basis) of 96 soil samples that received that treatment. Pluses (+) indicate amendment was provided; minuses (-) that it was withheld.

Saturated and another taken 2 m from the stream bank that was not saturated. Ambient moisture status became the fifth main effect for these data.

It should be noted that replication only applies strictly to the four amendments, since only one composite sample was collected from each site. The number of samples required by a full factorial design did not permit repeated soil sampling. Although a single sample precluded estimating within-site variation, compositing usually provides a reasonable estimate of the site mean (Keeney 1980). Comparisons of WS, slope position, depth, and ambient moisture status assume that composite samples adequately represent their respective sites.

SOIL ANALYSES

Ambient \( \text{NO}_3^- \) was determined by shaking triplicate subsamples for 1 hr in 2 M KCl (5:1 vol/wt ratio of solution: moist soil), filtering with Whatman \(^1\) No. 40 paper, and analyzing extracts colorimetrically with a Technicon Autoanalyzer. The method of Burford and Bremner (1975) for measuring cold-water-soluble carbon was modified: triplicate subsamples were shaken for 15 min in 25°C water (2:1 vol/wt ratio of water: moist soil) and centrifuged for 30 min at 19,000 g; the supernatant was filtered through a Gelman Metricel syringe filter (0.2 \( \mu \)m pore); and extracts were analyzed with a Beckman 915 B Total Organic Carbon Analyzer. Water-filled pore space was estimated by gravimetric moisture content, bulk density measurements of intact cores, and an assumed 2.65 particle density (Vomocil 1965).

RESULTS

Flooding with \( \text{H}_2\text{O} \) resulted in the most pronounced main effect (Figure 1). Non-flooded samples exhibited \( \text{N}_2\text{O} \) production 3 orders of magnitude lower than flooded samples (Figure 2). The WS and \( \text{NO}_3^- \) main effects were next in importance.
and were similar in magnitude. The glucose and acidity main effects were small, but all main effects were statistically significant at $\alpha = 0.0001$ (Figure 1).

Several 2, 3, and 4 order interactions were significant, only a few of which can be presented here. The WS $\times$ flooding $\times$ NO$_3^-$ interaction revealed that both flooding and NO$_3^-$ amendment were necessary for mg-range N$_2$O production in reference WS soils (Figure 2). In contrast, ambient NO$_3^-$ was sufficiently high in disturbed WS soils so that only flooding was necessary to stimulate high levels of N$_2$O production. For the disturbed WS, added NO$_3^-$ had little effect on N$_2$O production in soils from the well-drained hillside position, but did increase N$_2$O production from stream-site soils (Figure 3).

Glucose amendment did not affect N$_2$O production in reference WS soils (Figure 4). For the disturbed WS, glucose amendment increased N$_2$O production slightly in stream-site soils, caused a larger increase in surface hillside soils, and caused the largest increase in the 6-15 cm soil sample from the hillside site (Figure 4).

**TABLE 2. Watershed $\times$ acidity interaction**

<table>
<thead>
<tr>
<th>Watershed</th>
<th>Acidity treatment</th>
<th>$\sim$pH 6 $\sim$pH 5</th>
<th>mg N$_2$O-Nkg$^{-1}$ dry soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disturbed (ambient pH 6.3)</td>
<td></td>
<td>3.72$^b$</td>
<td>2.93</td>
</tr>
<tr>
<td>Reference (ambient pH 5.1-5.4)</td>
<td></td>
<td>1.87</td>
<td>1.83</td>
</tr>
</tbody>
</table>

* Significant at $\alpha = 0.0001$.

$^b$ Means of 12 samples that received both H$_2$O flooding and NO$_3^-$ amendment.
The magnitude of the glucose effect increased with decreasing ambient cold-water-soluble carbon in the disturbed WS soils.

When H\textsubscript{2}O flooding and NO\textsubscript{3}\textsuperscript{-} amendment were provided, reference WS soils exhibited mg-range N\textsubscript{2}O production, regardless of acidity level (Table 2). A modest inhibition was observed when disturbed WS soils were brought to ~pH 5 by acid amendment.

The effect of H\textsubscript{2}O flooding was less pronounced when a water-saturated sample from the disturbed WS was examined (Table 3). This soil also exhibited lower ambient NO\textsubscript{3}\textsuperscript{-} than other disturbed WS soils (Table 1), suggesting that NO\textsubscript{3}\textsuperscript{-} reduction was occurring rapidly in situ prior to sampling.

**TABLE 3. Ambient moisture x flooding interaction.**

<table>
<thead>
<tr>
<th>Soil sample</th>
<th>Ambient moisture</th>
<th>H\textsubscript{2}O treatment</th>
<th>% WFPS\textsuperscript{c}</th>
<th>mg N\textsubscript{2}O-N kg\textsuperscript{-1} dry soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flooded</td>
<td>Not flooded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td>92</td>
<td>2.05\textsuperscript{d}</td>
</tr>
<tr>
<td>Not saturated</td>
<td></td>
<td></td>
<td>64</td>
<td>3.25</td>
</tr>
</tbody>
</table>

\* Significant at \(a = 0.0001\).
\*\* Samples from stream site of disturbed WS.
\*\*\* Water-filled pore space.
\*\*\*\* Means of 24 samples.
FIGURE 4. Soil sample x glucose interaction (WS x slope position x glucose significant at $a = 0.0001$; glucose x depth of soil sample significant at $a = 0.01$). Each bar represents mean $\text{N}_2\text{O}$ production (dry wt basis) of 24 soil subsamples that received that treatment. Ambient levels of cold-water-soluble carbon (dry wt basis) are given in parentheses.

DISCUSSION

SOIL MOISTURE AND AERATION

Headspace gas in the incubation bottles remained aerobic, but flooding with $\text{H}_2\text{O}$ restricted $\text{O}_2$ diffusion into soil samples. Although $\text{NO}_3^-$, available carbon, and acidity levels were conducive to denitrification in many of the treatment combinations, mg-range $\text{N}_2\text{O}$ production did not occur unless $\text{O}_2$ diffusion was restricted either by the flooding treatment or, in the case of the water-saturated stream-site sample from the disturbed WS, by high ambient moisture content. In agricultural systems, the importance of soil moisture has been demonstrated by correlating denitrifying activity with rainfall patterns (Ryden 1983) and irrigation schedules (Rolston et al. 1984). The importance of slope position, and thus drainage, has been suggested in field studies of the same forested WSs sampled for the present study (Davidson and Swank 1986). Results of the present experiment support earlier observations of high $\text{NO}_3^-$ reduction potential in stream-site soils of the disturbed WS and on the importance of soil moisture in regulating denitrification.

NITRATE

The magnitude of WS and $\text{NO}_3^-$ main effects are similar (Figure 1), probably because ambient $\text{NO}_3^-$ levels reveal the most important difference between these two WSs. The necessity of both $\text{H}_2\text{O}$ flooding and $\text{NO}_3^-$ amendment in the reference WS soils (Figure 2) indicates the importance of substrate as well as $\text{O}_2$ restriction. This observation is consistent with positive correlations between denitrifying activity and nitrification potentials (Robertson and Tiedje 1984).
CARBON
Nitrate limitation in the reference WS probably overwhelmed any effect of glucose in these soils. However, where NO$_3^-$ was available in the disturbed WS soils, an inverse relationship between ambient cold-water-soluble carbon and response to glucose amendment was observed (Figure 4). Although drying and sieving were avoided, mixing of composite soil samples could cause a release of both available carbon and nitrogen. In the present study, NO$_3^-$ availability generally appeared to be more important than carbon limitation. The surface horizons of these forest soils probably possess sufficient available carbon for denitrifying activity, although some carbon limitation may develop along a topographic gradient and with increasing soil depth. Decreasing denitrifier MPN enumerations and denitrifying potentials with increasing depth (Bailey and Beauchamp 1973, Brar et al. 1978, Davidson et al. 1985, Wickramasinghe and Talibudeen 1981) may result from lack of energy sources to support heterotrophic organisms such as denitrifying bacteria.

ACIDITY
Studies on denitrifying bacteria in pure culture indicated that circumneutral and slightly alkaline conditions favor denitrification, whereas acidic conditions are inhibitory (Valera and Alexander 1961). However, results of the present study support recent observations of populations apparently adapted to acidic conditions, albeit at reduced levels of activity (Parkin et al. 1985, Waring and Gilliam 1983, Wickramasinghe and Talibudeen 1981).

The present method for changing acidity was less than ideal, since small aliquots of base or acid solutions were probably not evenly distributed throughout the soil samples that were not flooded. Buffer solutions, however, would have confounded H$_2$O flooding and perhaps glucose amendments. Our goal was to change H$_3$O$^+$ concentrations by approximately an order of magnitude between treated and untreated samples. Post-incubation pH values are difficult to interpret because the denitrification process consumes H$_3$O$^+$ ions, thus potentially causing an increase in pH values for some samples. Nevertheless, the difference between treated and untreated samples generally remained between 0.8 to 1.0 pH units following the incubation. The acidity treatment appears to have met our goal, but the effect of acidity on denitrification was minor (Figure 1 and Table 2). Indeed, reference WS soils at their ambient bulk ~pH 5 reduced added NO$_3^-$ in the mg-range. Acidity may have more profound effects on nitrification (Davidson and Swank 1986) and thus affect NO$_3^-$ availability for denitrification, but denitrifying bacteria appear capable of surviving and reducing NO$_3^-$ at acidity levels typical of mature forest soils at Coweeta.

IMPLICATIONS OF RESULTS TO WATERSHED-LEVEL ECOLOGY
The experimental approach of the present study was designed to examine factors limiting denitrification under controlled laboratory conditions. Although extrapolation of our laboratory studies of composite soil samples to field conditions is difficult, the results of the present study are consistent with field studies conducted at the same sites (Davidson and Swank 1986). The disturbance history of WS6, which includes liming and now turnover of N-rich black locust tissue, has created conditions ideal for nitrification. High levels of NO$_3^-$ and intermittent saturation of stream-site soils also create ideal conditions for denitrifying bacteria. Indeed, most probable number (MPN) enumerations have revealed large denitrifier populations relative to the reference WS soils (Davidson et al. 1985). The stream-site soils appear to possess populations capable of rapidly reducing both
ambient and added NO$_3^-$, provided that O$_2$ is restricted by flooding (Figure 3). Hillside soils from the disturbed WS and soils from the reference WS also possess populations capable of reducing either ambient or added NO$_3^-$, but apparently at lower rates than stream-site soils from the disturbed WS.

The presence of denitrifier populations capable of reducing added NO$_3^-$ in the reference WS soils was surprising in light of infrequent occurrence of conditions favorable for denitrification. Ambient NO$_3^-$ and nitrifying potentials were extremely low in the reference WS (Davidson and Swank, in press), which is expected for a mid-aged, aggrading forest (Vitousek and Reiners 1975). Ambient denitrifying enzyme concentrations determined by 2 hr incubations were below detection limits in this WS (Davidson et al. 1985). Although populations may be small and/ or not actively denitrifying, denitrifiers are apparently ubiquitous in these forest soils and are capable of sufficiently rapid multiplication and de novo enzyme synthesis to produce the N$_2$O observed in the present study within 24 hr. This rapid response to NO$_3^-$ amendment in the laboratory suggests that the denitrifiers in this soil are well adapted to temporary conditions conducive to denitrification. Nitrate production may be occasionally important at a microsite level in the reference WS following drying and wetting cycles. Bulk precipitation (4 kg N ha$^{-1}$ yr$^{-1}$ at Coweeta) may also provide both sufficient NO$_3^-$ input and moisture to temporarily favor denitrification.

SUMMARY

Denitrification is most likely to be important in surface horizons of wet soils within forested systems that exhibit high rates of nitrification or receive some other source of NO$_3^-$. Both O$_2$ restriction and presence of NO$_3^-$ are necessary for appreciable denitrification. Carbon may not be severely limiting in surface horizons of forest soils, but low levels of available carbon affect rates of denitrification with increasing soil depth and may impede denitrification at some slope positions. Acidity may affect relative rates of denitrifying activity, but denitrification in forest soils should not be dismissed because of acidic conditions.

LITERATURE CITED


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