Fate of $^{15}$N labelled nitrate and ammonium in a fertilized forest soil

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Abstract

The possibility that long-term atmospheric nitrogen pollution and fertilization of forest soil may serve as a basis for adaptation for enhanced transformation rates of NO$_3^-$ and NH$_4^+$ in soil bacteria was elucidated in a laboratory bioassay. Bacteria extracted from soils that had been fertilized at various rates for the last 30 yr were characterised with respect to their capability to reduce or oxidise different nitrogen sources. The same soils were used under oxic or anoxic conditions to quantify denitrification, dissimilatory nitrate reduction to ammonium (DNRA) and nitrification. $^{15}$NO$_3^-$ and $^{15}$NH$_4^+$ were added as tracers to the soils, which were incubated in bottles for 3 to 5 d. Concentrations of $^{15}$N$_2$O in headspace and $^{15}$NO$_3^-$ and $^{15}$NH$_4^+$ in soil extracts were determined by gas chromatography–mass spectrometry. Total numbers of bacteria were similar in all soils and ranged from 3 to 4 x 10$^8$ cells g$^{-1}$ d.wt. of soil. Between 50 and 70% of the isolated strains were capable of reducing nitrate and the majority of them reduced nitrate to ammonium. About 0.01% of all isolates were classified as nitrifiers. Both nitrate reducers and nitrifiers were more common in fertilized soils than in the unfertilized control soil. The foremost fate of added $^{15}$NO$_3^-$ and $^{15}$NH$_4^+$ in all soils was immobilisation. More than 85% was immobilised in anoxic soils and between 64 and 97% in the oxic soils, with the lowest quantities in fertilized soils. As regards the remaining, non-immobilised N, DNRA dominated over denitrification, as could be expected from the higher frequency of ammonifying bacteria compared with denitrifiers. There was no obvious relationship between NH$_4^+$ produced and the amount of fertilizer applied, whereas denitrification was negatively correlated with the amount of fertilizer applied. Nitrifying activity was low in all soils with no obvious relationship between NO$_3^-$ produced and fertilizer applied. Hence, no correlation was found between the relative abundance of N transforming bacteria and the transformation activity. The N flux followed essentially the same pattern as that seen for product formation. The DNRA flux was higher than that of both denitrification and nitrification. DNRA and denitrification fluxes were highest in the control soil, whereas the nitrification flux was low in all soils. The absence of evidence for adaptation to enhanced rates of transformation of NO$_3^-$ and NH$_4^+$ in soil bacteria exposed to long-term N fertilization is reflected by the low concentration of extractable inorganic N in the fertilized soils. As a consequence of the quantitative importance of immobilization of added N, differences in physiological capacity evolved in soil bacteria to immobilize and mobilise N may determine the rates by which inorganic N is available for plant growth or lost to groundwater and air.

Keywords: Nitrogen; Mobilization; Immobilization; Denitrification; Nitrification; Soil bacteria; Gas chromatography; Mass spectrometry; Spruce

1. Introduction

Even if primary production in temperate forest soils is often N limited (Tamm, 1991), increased provision by atmospheric N pollution and by fertilization may have adverse effects on forest vegetation and promote soil acidification and leaching of nitrate and base cations to surface water and groundwater (Aber et al., 1989; Murdoch and Stoddard, 1992). The extent to which vegetation and water quality is affected depends on the retention and transformation of N in the soil. Larger amounts of deposited and added N tend to be immobilized in the soil, notably by microbial assimila-
tion, than taken up by plants (Nömmik, 1966; Nadelhofer et al., 1995; Buchmann et al., 1996) and remain unavailable to plants for years (Cheng and Kurtz, 1963; Hart et al., 1993). Mobilization and transformations may ensure that N is present in the soil as NO$_3^-$, NO$_2^-$ and NH$_4^+$ or removed by leaching and denitrification to N$_2$ and N$_2$O. Whereas the availability of inorganic N tends to control transformation rates in forest soils, other factors, such as the concentration of organic carbon ($C_{org}$) and O$_2$ and pH, may be responsible for the discrimination between specific transformations. Hence, it has been proposed that the ratio of available C to electron acceptor concentration determines whether NO$_3^-$ is reduced in a dissimilatory pathway to NH$_4^+$ or denitrified (Tiedje et al., 1982). In environments with high C and low NO$_3^-$ concentrations, such as most forest soils, NH$_4^+$ is predicted to be the main product of NO$_3^-$ reduction, supplying autotrophic nitrifiers with an energy source and plants and heterotrophs with a favoured nutrient. Observations useful for testing such predictions are scarce and quantification of the processes regulating the internal cycling of N in forests still have a good deal of uncertainty (Davidson et al., 1992; Groffman et al., 1993; Hart et al., 1993; Buchmann et al., 1996).

Although the fate of N imported to forest ecosystems by atmospheric N deposition and fertilization is of major concern in environmental management, the inability to foresee chemical and biological reactions in the soil precludes efforts to accurately estimate long-term effects on forest ecosystems. Data accumulated from long-term fertilizer experiments in forest ecosystems may be helpful in the assessment of the fate of long-term atmospheric N deposition. Priha and Smolander (1995) used plots in acid spruce forests that had been fertilized with 900 kg N ha$^{-1}$ for 30 yr to indicate long-term effects of N deposition on forest soils. NH$_4^+$ was the main inorganic N product formed during incubation of soil samples: 1.5–2 times as much NH$_4^+$ was produced in the fertilized plots than in the controls, both with the same and marginal NO$_3^-$ concentrations. However, the immobilisation of N by microorganisms was reduced in the fertilized soils.

One possible explanation for enhanced transformation of mineral N in soils that have been amended or polluted with N for many years is selection for enzymes with higher transformation activities. Such a phenomenon has been demonstrated in bacteria in estuarine sediments (King and Nedwell, 1987) and in shallow groundwater aquifers (Bengtsson and Bergwall, 1995) and is likely to occur in fertilized forest soils with high concentrations of organic carbon, in which increased capacity of the electron transport system should promote survival and fitness of the cells.

The objectives of this work were to determine the effect of fertilization on transformation rates and fate of inorganic N in a forest soil as a basis for an evaluation of potential adaptations to higher denitrifying, ammonifying (DNRA) and nitrifying activities among soil bacteria. This is of long-term consequences of atmospheric N deposition on N turnover in forest soils. Three hypotheses were tested:

1. The frequency of denitrifying, DNRA and nitrifying strains was expected to be higher in the fertilized forest soils compared with a control soil receiving no nitrogen fertilizer. This would be the consequence of the release of inorganic nitrogen as the major abiotic constraint for growth and, hence, density, of those groups in forest soils.

2. Denitrifying, DNRA and nitrifying activities were assumed to be higher in the fertilized forest soils compared to the control in response to the increased availability and concentration of inorganic nitrogen. The long-term nitrogen amendment of the fertilized soils may constitute a basis for selection of nitrogen transformers with altered enzyme activities. Since organic carbon is present in surplus, a selection of efficient nitrogen transforming enzymes may result in increased nitrogen transformation activity.

3. The C/N ratio determines the fate of the added nitrogen as per the discussion above. DNRA activity may dominate over denitrification as the major electron transport system, since the forest soils studied were characterized as electron donor rich and electron acceptor poor (e.g. high C/N ratio).

2. Materials and methods

2.1. Site description and soil sampling

The soil samples were taken from a Norway spruce (Picea abies Karst.) forest at Lake Stråsan, central Sweden. The soil is a glacial till dominated by medium and fine sand. The mean annual temperature is 3.1°C and the mean annual precipitation is 745 mm. The trees were planted in 1958 and the site was used for optimum nutrition experiments since 1967, when the average tree height was 1.3 m (Tamm et al., 1974). A total of 56 plots (30 x 30 m) were treated in different ways: four rates of N, three rates of P, two rates of K+Mg+micronutrients treatments and combinations of these. Each treatment was replicated in two plots, except for the control, which was replicated five times. NH$_4$NO$_3$ has been added yearly from 1967 at rates varying between 30 and 180 kg ha$^{-1}$ yr$^{-1}$. The total additions of fertilizer N ha$^{-1}$ until 1994 were 0, 930, 1620 and 2610 kg for the N0, N1, N2 and N3 treatments, respectively. The N2 treatments were suspended.
in 1990, the N3 treatments in 1992, whereas the N1 treatments are still in progress. Total N concentrations of needles had increased from ~1.3 to ~1.7% in the N2 and N3 treatments in 1982 and the annual growth rate in the N1–N3 plots was 12–13 m³ ha⁻¹ yr⁻¹ compared with 5 m³ ha⁻¹ yr⁻¹ in the N0 plots (Tamm, 1985).

Soil samples were taken in October 1995 from four of the plots: 13 (N0), 14 (N1), 52 (N2) and 33 (N3). A sterile planting trowel was used to collect soil from 30 × 30 cm squares of the O horizon, randomly located within the plot, into autoclaved 500 ml brown glass jars. Six jars were filled with soil from each plot and shipped to the laboratory overnight in a refrigerated box. The jars were opened in a sterile hood and visible roots removed aseptically. Approximately 50% of the volume collected consisted of roots. The soil was stored for approximately 2 weeks at 4°C prior to isolation of bacteria, physiological characterization and denitrification and nitrification bioassays.

2.2. Isolation and characterization of bacteria

Soil samples (2 g wet weight) were suspended in 20 ml of sterile 0.2% Calgon (sodiumhexametaphosphate) and 0.1% peptone solution and shaken for 30 min at 160 rpm by use of a wrist action shaker (Griffin and George). Aliquots of 0.1 ml of dilutions of the samples (10×, 100× and 1000× using sterile Calgon and peptone solution) were inoculated on the surface of three different agar media, one separate aliquot for each, by means of L-shaped glass rods. For heterotrophic bacteria (potential denitrifiers) peptone–yeast–glucose (PYG) agar was used. The PYG agar contained 1.25 g peptone, 1.25 g yeast extract, 1.25 g glucose, 0.15 g MgSO₄ × 7H₂O, 0.015 g CaCl₂ and 15 g agar 1⁻¹ sterile distilled water. The plates were incubated at 17°C. After 7 d of growth on PYG agar, colonies were picked from plates, streaked several times on duplicate PYG agar plates for purification and incubated at 17°C. The purity of strains was examined by colony check with a stereo microscope (10× and 30×). Pure cultures were stored in cryo tubes supplied with PYG and 20% sterile glycerol at −80°C. The isolated heterotrophic bacteria were tested for nitrate reducing, denitrifying and DNRA capability by means of microtiter plate described by Köhlbel-Boelke et al. (1988) and Daniels et al. (1994). Physiological tests included nitrate reduction (NO₃⁻→NO₂⁻), denitrification (NO₂⁻→N₂) and DNRA (NO₃⁻→NH₄⁺, ammonia by Nessler reaction). For chemolithotrophic bacteria (nitrifiers), media for ammonia oxidizers and nitrite oxidizers were used. Ammonia oxidizers were enumerated on 1.0 g K₂HPO₄, 0.2 g MgSO₄ × 7H₂O, 50 mg FeSO₄ × 7H₂O, 20 mg CaCl₂ × 2H₂O, 2 mg MnCl₂ × 4H₂O, 1 mg Na₂MoO₄ × 2H₂O, 5 g CaCO₃, 1.5 g NH₄Cl and 15 g agar (Sigma purified) 1⁻¹ of distilled water. The nitrite oxidizers were incubated on 1.0 g K₂HPO₄, 0.2 g MgSO₄ × 7H₂O, 5 mg FeSO₄ × 7H₂O, 2 mg CaCl₂ × 2H₂O, 2 mg MnCl₂ × 4H₂O, 1 mg Na₂MoO₄ × 2H₂O, 2.0 g NaNO₂ and 15 g agar (Sigma purified) 1⁻¹ of distilled water. The plates were incubated at 27°C for 3–4 weeks. The colonies were counted with the aid of a stereo microscope (10× and 30×) and then transferred to PYG plates to test for heterotrophic contaminants.

2.3. Total numbers of bacteria

Soil samples of 2.0 g (wet weight) from each site were suspended in 50 ml of sterile 0.2% Calgon (sodiumhexametaphosphate) and 0.1% peptone solution and homogenized for 2 min in an Omni mixer homogenizer (Omni Int.). Acidine orange solution (50 μl of a 0.1% solution) was added after dilution (10×). The mixtures were left for 2 min at room temperature and then slowly filtered through a black polycarbonate 0.22 μm membrane (Poretics Corp.). The number of fluorescent cells was counted in a Zeiss Axioskop 20 equipped for fluorescence observations (Carl Zeiss, Germany).

2.4. Bioassays

Soil samples (5 g for the denitrification assay and 3 g for the nitrification assay) from the four plots were transferred to 25 ml glass flasks. The soils were amended with 5 μg of Na¹⁵NO₃-N g⁻¹ soil (99 at%¹⁵N) and 1 μg of ¹⁵NH₄Cl-N g⁻¹ soil (98 at%¹⁵N), respectively, both obtained from Cambridge Isotope Laboratories, Andover, MA. Flasks for the denitrification assay were flushed with He for 5 min to remove O₂ and C₂H₂ added to the gas phase to a final concentration of 10%. The flasks were sealed with rubber stoppers and incubated for 3 d at 15°C. Three replicate flasks per soil sample were used. Headspace gas (0.5 ml) was withdrawn with a gastight syringe once every 24 h and analysed for N₂O. After the last injection on the GC/MS, the soil was extracted with 0.5 M K₂SO₄ for analysis of NO₃⁻ and NH₄⁺.

Flasks for the nitrification assay were sealed with cotton plugs and incubated for 5 d at 15°C. Three replicates were also used. After incubation the soil was directly extracted with 0.5 M K₂SO₄.

2.5. Analytical procedures

2.5.1. Sample preparations

NO₃⁻ and NH₄⁺ were extracted from the soil with 0.5 M K₂SO₄ at 5:1 extractant to soil ratios. The soil used in the bioassays was transferred to 50 ml test tubes. All test tubes had Teflon lined screw caps. The
soil residues in the 25 ml bioassay flasks were washed with 2 x 10 ml 0.5 M K₂SO₄ and transferred to the test tubes. The extraction was performed on a rotary shaker for 6 h at 17°C. Following extraction, the test tubes were centrifuged at 2000 rpm for 30 min and 7 ml aliquots of solution transferred to two 12 ml test tubes. The solution was evaporated in a Savant Speed Vac (Savant Instruments), redissolved in 1.5 ml of deionized water and used for the derivatization of NO₃⁻ and NH₄⁺. In addition, soil samples from each plot were directly extracted to obtain the initial concentrations of NO₃⁻ and NH₄⁺.

2.5.2. Derivatization of NO₃⁻ and NH₄⁺

We used convenient and sensitive methods to determine concentrations of ¹⁵NO₃⁻ and ¹⁵NH₄⁺ by GC–MS. Nitrate was determined by modification of a method developed by Tanaka et al. (1985). The sample was derivatized using 2-sec-butylphenol (purity > 98%, Aldrich) and N,O-Bis(trimethylsilyl)acetamide (BSA, purity > 97%, Fluka) to yield the trimethylsilyl ester of 4-NO₂ 2-sec-butylphenol. The aqueous sample (1 ml) was transferred to an 8-ml test tube and cooled in ice. Then 0.2 ml of Ag₂SO₄ solution (5% in deionized water) and 1.4 ml of concentrated H₂SO₄ were added followed by 100 μl 2-SBP (5% in 99.5% ethanol). The test tubes were now transferred to a heating block for 15 min at 30°C with shaking every five minute. The aqueous phase was extracted with 1 ml of toluene by the use of a wrist action shaker for 5 min. The water was removed after centrifugation (3500 rpm) and the organic phase washed twice with 2.0 ml of deionized water and dried with anhydrous Na₂SO₄. 30 μl of BSA was added to the test tubes which were allowed to stand in room temperature for 10 min. Finally, the toluene was evaporated under a stream of N₂ and the residue redissolved in 200 μl ethyl acetate.

Ammonium was derivatized using pentafluorobenzoyl chloride (PFBOCl, purity > 99%, Fluka) to yield pentafluorobenzamide. The procedure was based on modifications of a method originally used by Fujihara et al. (1986) to measure NH₄⁺ production in putrescine oxidation by human plasma. The aqueous sample (1 ml) was transferred to a test tube containing 1 ml NaHCO₃ (5% in deionized water), 15 μl PFBOCl and spiked with 2 μg of methyl amine as internal standard. The test tube was shaken for 3 min using a wrist action shaker. The aqueous phase was extracted with 1 ml ethyl acetate for 5 min. The water was removed after centrifugation (3500 rpm) and anhydrous Na₂SO₄ was added to the organic phase. The ethyl acetate was evaporated at 30°C under a stream of N₂ and the residue redissolved in 0.5 ml diethyl ether:hexane (15:85% v/v). The silicic acid columns (clean up step, 100 mg Bond Elute, Analytichem Int.) were conditioned with 3 ml of hexane. The sample was then added to the column which was subsequently washed with 3 ml diethyl ether:hexane (15:85% v/v). The PFBONH₂ derivative was eluted with 2 ml diethyl ether:hexane (50:50% v/v). The eluate was evaporated to dryness under N₂ and the residue redissolved in 1 ml ethyl acetate.

2.5.3. GC/MS

The mass spectrometer was a Hewlett-Packard 5890A MS-Engine coupled to Hewlett-Packard 5890 Series II gas chromatograph. For N₂O, a 25-m plot fused silica capillary column (Poraplot Q, film thickness 10 μm, i.d. 0.32 mm, Chrompack, The Netherlands) was used with He as the carrier gas at a flow rate of 2.9 ml min⁻¹. The injector and interface temperatures of the GC were 175 and 270°C, respectively. The oven temperature was 40°C. For derivatized NO₃⁻, a DB-17 15-m fused silica capillary column (film thickness 0.25 μm, i.d. 0.25 mm, J&W Scientific, Folsom, CA) was used with He as the carrier gas at a flow rate of 1.5 ml min⁻¹ and the split was opened 40 s after injection. The injector and interface temperatures of the GC were 130 and 270°C, respectively. The oven temperature was initially held at 100°C for 1 min and then increased by 20°C min⁻¹ to 230°C. For derivatized NH₄⁺, a DB-Wax 30-m fused silica capillary column (film thickness 0.25 μm, i.d. 0.25 mm, J&W Scientific, Folsom, CA) with He as the carrier gas at a flow rate of 1.2 ml min⁻¹ and the split was opened 40 s after injection. The injector and interface temperatures of the GC were 250 and 260°C, respectively. The oven temperature was initially held at 100°C for 1 min and then increased by 20°C min⁻¹ to 200°C. Electron-impact (EI) mass spectra were obtained using 70 eV at an ion source temperature of 200°C and at a quadrupole temperature of 100°C. The mass spectrometer was operated in the selected ion monitoring mode (SIM). Measured ions were of m/z 44 [¹⁴N¹⁵NO], m/z 45 [¹⁴N¹⁴NO], m/z 46 [¹⁵N¹⁵NO], m/z 238.1 [TMS ester of 4-¹⁴NO₂ 2-SBP], m/z 239.1 [TMS ester of 4-¹⁵NO₂ 2-SBP], m/z 211.0 [PFBO¹⁴NH₂], m/z 212.0 [PFBO¹⁵NH₂] and m/z 225.0 [PFBO¹⁴CH₃⁺]. The isotope-enrichment calculations for NO₃⁻ and NH₄⁺ were made by using the m/z ratio 239.1/238.1 and 212.0/211.0 of calibration solutions, with a natural isotopic distribution, subtracting them from the measured m/z 239.1/238.1 and 212.0/211.0 ratios of samples. The isotope-enrichment calculation for N₂O is described in Section 2.5.4.

2.5.4. Determination of the N₂O flux

The production of N₂O by denitrification was calculated from the abundance of the ions m/z 44, m/z 45 and m/z 46, which correspond to different combinations of the isotopes: ¹⁴N¹⁴N, ¹⁴N¹⁵N and ¹⁵N¹⁵N, respectively. We assumed that ¹⁴N and ¹⁵N were in isotopic equilibrium in the samples so that their frac-
tions could be described by a binomial distribution: 
\[ \frac{44[N_2O]}{\text{mol}} = (1 - 15a)^2, \]
\[ \frac{45[N_2O]}{\text{mol}} = 2\times 15a(1 - 15a), \]
\[ \frac{46[N_2O]}{\text{mol}} = (15a)^2, \]
where \( \frac{44[N_2O]}{\text{mol}} \), etc. are the fractions of \( N_2O \) molecules with molecular weights 44, etc. and \( 15a \) is the fraction of \( ^{15}N \) in the sample. The value for \( 15a \) was determined as \( \frac{44[N_2O] + \frac{1}{2}45[N_2O] + 46[N_2O]}{44[N_2O] + 45[N_2O] + 46[N_2O]} \). Since the molecular fractions as \( m/z \) 45 and 46 may be contaminated by the heavy oxygen isotopes \( ^{17}O \) and \( ^{18}O \) and traces of oxygen and nitrogen may react in the ion source to produce \( NO_2 \), it was necessary to correct the abundances of ions measured. We considered \( 44[N_2O] \) to be correctly measured since more than 99.2% of its abundance consists of only \( ^{14}N^{14}N^{16}O \) and used its abundance to calculate the abundance of \( N_2O \) molecules without heavy oxygen isotopes: 
\[ \frac{[N_2O]}{\text{mol}} = \frac{44[N_2O]}{44[N_2O]}/(1 - 15a)^2. \]
The correct abundances of the \( 45N_2O \) and \( 46N_2O \) molecules were then calculated by multiplying their binomial isotope distributions with the abundance of \( N_2O \) without heavy oxygen isotopes. A corrected value for \( 15a \) was then calculated from the correct abundances of the three \( N_2O \) molecules.

A standard curve for unlabeled \( N_2O \) was obtained by injecting between 200 and 1000 \( \mu l \) in triplicate in the mass spectrometer. The dose–response curve was nonlinear but accurately described by a power function. This equation was used to calculate the concentration of \( N_2O \) in the samples, which all had total and isotopic abundances in the range covered by the standards. The total concentration of \( N_2O \) in the closed flask \( y \) was calculated from an expression of Henry’s law: 
\[ y = z \times x \times \text{soil water volume/gas volume}, \]
where \( z \) is the solubility of \( N_2O \) in \( cm^2 \ cm^{-2} \) of water and \( x \) is \( mg \) \( N_2O \) in the gas phase (Moraghan and Buresh, 1977). The correct abundances at \( m/z \) 44, 45 and 46 of the gas standard were also used to calculate the natural abundance of \( 15a \). It was found to be 0.003633, regardless of the concentration of \( N_2O \). This value was subtracted from the calculated values for \( 15a \) in the samples to obtain the atom excess of \( ^{15}N \) in \( N_2O \).

Finally, the flux of \( N_2O \) derived from denitrification of added \( ^{15}NO_3^- \) was calculated: 
\[ ^{15}N-N_2O \times N_2O/M \times T \times ^{15}N-N_2O, \]
where \( ^{15}N-N_2O \) is atom excess of \( ^{15}N \), \( N_2O \) is the total corrected concentration of \( N_2O \) \( (44N_2O + 45N_2O + 46N_2O) \) in the flask, \( M \) is the weight of soil in grams, \( T \) is incubation time in hours and \( ^{15}N-N_2O \) is the atom excess of \( ^{15}N-N_2O \) in the soil. Denitrification coupled to nitrification that was immobilized was calculated in a similar way but with reference to atom excess of \( ^{15}N-NH_4^+ \) in the soil.

2.5.5. Other calculations and statistics

The relative concentration of the added \( ^{15}NO_3^- \) that was immobilized was calculated as the mean percentage of the tracer that was not recovered as \( ^{15}N_2O \) or extractable \( ^{15}NH_4^+ \) and \( ^{15}NO_3^- \). Likewise, the relative concentration of immobilized added \( ^{15}NH_4^+ \) was calculated as the mean percentage of an added tracer that was not recovered as extractable \( ^{15}NH_4^+ \) and \( ^{15}NO_3^- \).

The contribution of added \( ^{15}NO_3^- \) to denitrification was calculated as the ratio between the observed release of \( ^{15}N_2O-N \) and the product of observed \( N_2O-N \) and the initial added \( ^{15}NO_3^- \). The contribution of added \( ^{15}NO_3^- \) to the production of \( NH_4^+ \) was calculated as the production of \( ^{15}NH_4^+ -N \) divided by the production of \( NH_4^+ -N \). Nitrification from the added \( ^{15}NH_4^+ \) source was calculated as the ratio of produced \( ^{15}NO_3^- \) and produced \( NO_3^- \). We assumed that isotope discrimination, if present, was undetectable by the quadrupole mass spectrometer due to the high enrichment. Analysis of variance was used to determine the

![Fig. 1](image-url)
Table 1
Fate of $^{15}$N incubated in soil samples with different history of fertilization. Fertilizer was added yearly to field plots from 1967. Mean and standard deviation (S.D.) are given for measurements made at the end of laboratory incubations of soil sampled in 1995.

<table>
<thead>
<tr>
<th>Fertilizer applied (kg ha$^{-1}$)</th>
<th>Treatment suspended in year</th>
<th>$^{15}$N-NO$_3$, initial (mg kg$^{-1}$) mean</th>
<th>S.D.</th>
<th>$^{15}$N-NO$_3$ after incubation (mg kg$^{-1}$) mean</th>
<th>S.D.</th>
<th>$^{15}$N-NH$_4$, initial (mg kg$^{-1}$) mean</th>
<th>S.D.</th>
<th>$^{15}$N-NH$_4$ after incubation (mg kg$^{-1}$) mean</th>
<th>S.D.</th>
<th>$^{15}$N-N$_2$O after incubation (mg kg$^{-1}$) mean</th>
<th>S.D.</th>
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<tbody>
<tr>
<td>Nitrate reduction</td>
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<td>4.953</td>
<td>3.15E–06</td>
<td>0.187</td>
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<td>0.205</td>
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<td>1.94E–06</td>
<td>0.138</td>
<td>0.0124</td>
<td>0.169</td>
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<td>0.417</td>
<td>0.0097</td>
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effects of treatments on N transfer and to examine differences between means of different turnover products, e.g. between \( \text{NH}_4^+ \) and \( \text{N}_2\text{O} \) (post-hoc tests using Scheffé’s \( F \)). Analysis of variance and calculations of linear correlations were made in StatView 4.5 (Abacus Concepts).

3. Results

The dominant fate of \( ^{15}\text{NO}_3^- \) and \( ^{15}\text{NH}_4^+ \) in the soils was immobilization, regardless of the amounts of fertilizer added during the past 30 yr. Less than 13% of added \( ^{15}\text{NO}_3^- \) was extractable after 3 d of incubation (Table 1). More than 85% of it was immobilized, whereas less than 5% was reduced in a dissimilatory pathway (Fig. 1). The immobilization was related to the intensity of fertilization, in as much as \( ^{15}\text{NO}_3^- \) added to soil from the two plots receiving the largest doses of fertilizer was less immobilized than \( ^{15}\text{NO}_3^- \) added to the control plot soil. Between 64 and 97% of added \( ^{15}\text{NH}_4^+ \) was immobilized and less than 0.5% was nitrified (Fig. 2 and Table 1). Again, \( ^{15}\text{NH}_4^+ \) added to soil from the two most fertilized plots was less immobilized than \( ^{15}\text{NH}_4^+ \) added to the control soil.

Most of the dissimilatory reduced \( \text{NO}_3^- \) ended up as \( \text{NH}_4^+ \) (Fig. 1 and Table 1, \( r \ll 0.0001 \)), but there was no obvious pattern for the relationship between \( \text{NH}_4^+ \) produced and the amount of fertilizer applied or the concentration of extractable \( \text{NO}_3^- \) in the soil. However, the relationship between the reduction of \( ^{15}\text{NO}_3^- \) to \( ^{15}\text{NH}_4^+ \) and the concentration of extractable \( \text{NH}_4^+ \) was slightly negative (\( r = -0.105 \)). Of the \( ^{15}\text{NO}_3^- \) added to the soil, 0.5% or less was recovered as \( ^{15}\text{N}_2\text{O} \). Denitrification was negatively correlated with the amount of fertilizer added (\( r = -0.690, P < 0.0001 \)) and the concentration of extractable \( \text{NO}_3^- \) (\( r = -0.640, P < 0.0001 \)). Only traces of the nitrification detected, \(< 0.22\%\), were attributed to oxidation of extractable \( \text{NH}_4^+ \) (Table 2). Less than 1% of the denitrification could be traced to extractable \( \text{NO}_3^- \) and less than 4% of \( \text{NH}_4^+ \) produced during nitrate reduction resulted from extractable \( \text{NO}_3^- \). Most of the tracer-derived nitrate reduction and nitrification was found in the control soil (Table 2). The observations indicate that the main N source for nitrate reduction and nitrification was organic or non-extractable inorganic.

Years of fertilization had a marginal influence on the concentration of extractable \( \text{NO}_3^- \) in the soil (Table 3). The \( \text{NO}_3^- \) concentration had increased by 40% in the plot receiving the largest amount of fertilizer compared with the control, which is order of magnitude less than the corresponding difference in the dose of fertilizer applied. The concentration of extractable \( \text{NH}_4^+ \) in the soil had increased by 3- to 10-fold in the fertilized plots compared with the control (Table 3). The highest concentrations were found in the plots still fertilized (14) or most recently excluded from fertilization (33), but even the plot in which fertilization was discontinued 5 yr ago (52) had about 4-fold more extractable \( \text{NH}_4^+ \) concentrations than the control plot, suggesting that the effects of fertilization on extractable \( \text{NH}_4^+ \) concentrations have lasted for at least 5 yr.

![Fig. 2. The percentage loss of extractable \( \text{NH}_4^+ \) (reduction of at% excess \( ^{15}\text{NH}_4^+ \times \text{concentration of } ^{14+15}\text{NH}_4^+ \) (left axis) and the percentage thereof that was nitrified (right axis) in soils from plots treated with different doses of \( \text{NH}_4\text{NO}_3 \) fertilizer is given in the figure. Note the different scales on the axes.](image)

![Fig. 3. Fluxes of denitrification and nitrification (left axis) and of \( \text{NH}_4^+ \) (right axis) in soils from plots treated with different doses of fertilizers. The fluxes were calculated using isotope ratios as described in the methods.](image)
The N flux, calculated as the concentration of a product formed per atom % excess precursor added, followed essentially the same pattern of variation among the plots as described for the product formation (Fig. 3). The flux of NH$_4^+$ was three orders of magnitude faster than the flux of denitrification and nitrification and negatively correlated with the concentration of extractable NH$_4^-$ (r = −0.429, P = 0.0004). The denitrification flux was faster in the control soil than in the fertilized soils (P < 0.0001, Scheffé’s F), whereas the opposite was true for the nitrification flux (P = 0.0015, Scheffé’s F).

The total number of bacteria extracted from the soils were 3.0, 3.3, 4.0 and 3.7 × 10$^8$ g$^{-1}$ in the soils treated with 0, 930, 1620 and 2610 kg ha$^{-1}$ fertilizer, respectively. More than 50% of the colonies appearing after incubation of soil extracts on PYG agar were capable of reducing NO$_3^-$ in one or the other way (Fig. 4). A majority of them reduced NO$_3^-$ to NH$_4^+$ and they, as well as the denitrifiers, were more numerous in the fertilized soils than in the control soil. The density of nitrifiers, calculated as the number of colonies observed on the minimal medium agar after incubation of aliquots of soil extract for 3–4 weeks, was higher in the fertilized plots than in the control (Fig. 5) and NH$_4^+$ oxidizers were more common than NO$_2^-$ oxidisers. The frequency of nitrifiers observed within the total count of bacteria on plates was 0.01%, with at most a factor of two in difference between the control and fertilized plot (52).

In summary, we found no direct evidence for enhanced rates of NO$_3^-$ reduction in soils fertilized with up to 2 600 kg ha$^{-1}$ of NH$_4$NO$_3$ for tens of years. It is possible that the fertilized soils have selected for a greater number of strains with high rates of ammonification and denitrification, whose characteristics are hidden by the low concentrations of extractable NO$_3^-$. Even if that is true, their contribution to the turnover of nitrogen in the Stråsan soils seems to be subordinate to that of strains immobilising inorganic nitrogen and mobilising nitrogen bound by microorganisms, clays and organic material. Strains controlling immobilisation and mobilisation may be adapted to higher rates by fertilization, and the fate of nitrogen added to the Stråsan soil determined by their activity.

4. Discussion

The weak relationship revealed by the experiments between long-term forest soil fertilization and microbial transformation rates of inorganic nitrogen may be attributed to several causes, of which the low concentration of extractable NO$_3^-$ following fertilization is suggested to be the most important. The finding of < 1 mg NO$_3^-$-N kg$^{-1}$ in the experimental soils regardless of the amount of fertilizer applied (Table 3) reflects the common view about soils in coniferous forests as being low in NO$_3^-$ (Robertson, 1982; Vitousek et al., 1982), suggesting a combination of insignificant net nitrification and significant leaching and immobilization.

The dominance of immobilization of added $^{15}$N over inorganic transformations suggests that fertilization of the Stråsan soils has not supported any build up of sufficiently high concentrations of NH$_4^+$ and NO$_3^-$ to

<table>
<thead>
<tr>
<th>Fertilizer applied (kg ha$^{-1}$)</th>
<th>Added $^{15}$NO$_3^-$ as a source of</th>
<th>Added $^{15}$NO$_3^-$ as a source of</th>
<th>Added $^{15}$NH$_4^+$ as a source of</th>
</tr>
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<tr>
<td></td>
<td>denitrification (%)</td>
<td>produced NH$_4^+$ (%)</td>
<td>nitrification (%)</td>
</tr>
<tr>
<td>0</td>
<td>0.96</td>
<td>3.52</td>
<td>0.22</td>
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<td>0.81</td>
<td>0.95</td>
<td>0.10</td>
</tr>
<tr>
<td>1620</td>
<td>0.76</td>
<td>2.68</td>
<td>0.11</td>
</tr>
<tr>
<td>2610</td>
<td>0.70</td>
<td>0.89</td>
<td>0.15</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Fertilizer applied (kg ha$^{-1}$)</th>
<th>Plot</th>
<th>Extractable NO$_3^-$-N (mg kg$^{-1}$)</th>
<th>Extractable NH$_4^+$-N (mg kg$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>mean  S.D.</td>
<td>mean  S.D.</td>
</tr>
<tr>
<td>0</td>
<td>13</td>
<td>0.48  0.13</td>
<td>3.47  1.56</td>
</tr>
<tr>
<td>930</td>
<td>14</td>
<td>0.51  0.19</td>
<td>29.48  8.94</td>
</tr>
<tr>
<td>1620</td>
<td>52</td>
<td>0.70  0.78</td>
<td>13.30  8.21</td>
</tr>
<tr>
<td>2610</td>
<td>33</td>
<td>0.66  0.59</td>
<td>31.12  0.43</td>
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select for enhanced rates of their transformations. The N pool of the soil (litter layer, mor and mineral soil) at the N2 site is estimated to 3.5 t ha\(^{-1}\) (Sjögren and Persson, Swedish University of Agricultural Sciences, personal communication), of which extractable inorganic N represents about 0.01\%.

The N pool of the N2 site is 1.4 t ha\(^{-1}\) larger than that of the control site, which corresponds reasonably well with the total dose of N fertilizer added to N2, 1.6 t ha\(^{-1}\). The increase of organic N is confined to the litter and FH layer: the C-to-N ratio drops from 45 in the control site litter to 23 and in the N2 site and from 32.6 to 23.6, respectively, in the FH layer. The organic N concentration of the mineral soil does not seem to have been influenced by fertilization, the C-to-N ratio at 10–30 cm depth is higher at the N2 site (27.8) than at the control site (25.4). Immobilization in the soil organic matter pool has also been recognized as the dominant fate of NH\(_4^+\) and NO\(_3^-\) added to other forest soils (Davidson et al., 1992; Groffman et al., 1993; Hart et al., 1993; McKenney et al., 1995). Observations by Davidson et al. (1992), Hart et al. (1993) and Nadelhoffer et al. (1995) suggest that most of the added and retained inorganic N in a soil is assimilated by bacteria and fungi during the growth season, resulting in small amounts of added N being nitrified and denitrified, at least in well-drained soils (Groffman et al., 1993). Soils can fix NH\(_4^+\)-N by the interlayer of 2:1 clay minerals (Nömmik, 1957; Moore, 1965), especially when the fixation sites are not blocked by soil organic matter (Hinnman, 1966). The mean residence time of an N atom may be a few days in the inorganic pools, 1 or 2 months in the microbial biomass pool (Davidson et al., 1992) and probably much longer in the clay mineral and organic matter pools.

The observation that fertilized Stråsan soils immobilize less of the added inorganic N than the unfertilized control soil is consistent with results obtained for microbial N by Smolander et al. (1994) and Priha and Smolander (1995) with soils from fertilized and unfertilized Norway spruce forests. These forests were fertilized for 30 yr and the decreased immobilization in microbial biomass coincided with an increased net formation of mineral N. However, the relationship between fertilization and net formation of mineral N is not consistently positive but seems to be influenced by such variables as the source of fertilizer and soil. Priha and Smolander (1995) made their observations in soils treated with ammonium sulphate, urea and ammonium nitrate in succession. Martikainen et al. (1989) observed no significant effects on net formation of N in pine forest soils treated with urea or ammonium nitrate, whereas our own observations of the spruce forest soils at Stråsan indicated a net 8-fold increase of (NH\(_4^+\)-N + NO\(_3^-\)-N) after 25 yr of addition of ammonium nitrate to the N3 plots.

Although fertilization had increased the concentrations of NH\(_4^+\) relative to the control (Table 3), they were probably too low to have anything but a marginal influence on the nitrification rates (Fig. 3). The largest mean concentration of mineral N (NH\(_4^+\)-
N + NO$_3^-$-N) we found in the Stråsan soils was ~32 mg kg$^{-1}$ (Table 3), which is below the threshold for mineral N (60–90 mg kg$^{-1}$) in forest floor material at which nitrification ceases (Vitousek et al., 1982). Blew and Parkinson (1993) referred the low nitrification rate (10 ng N g$^{-1}$ h$^{-1}$) that they measured after 4 weeks of incubation of samples from the F–H horizon of a 120-yr-old spruce forest to the low concentration of mineral N (45 mg kg$^{-1}$). Yet, that rate was two orders of magnitude higher than the rates calculated from 72 h of incubation of Stråsan soils with $^{15}$N. The different rates are partly due to different methods of calculation. When calculated rates are based on extracted NO$_3^-$, regardless of the source ($^{15}$N) or concentration of extractable NH$_4^+$, the nitrification rates at Stråsan are only 2–5 times lower than at the Canadian site. This latter difference may be attributed to differences in incubation time, differences in pH and the contribution of mobilized NH$_4^+$ to nitrification.

The pH$_{H_2O}$ of the Stråsan soil was low (between 3.60 and 3.87 in the O-horizon of the plots) compared with the Canadian soil (5.9 on average in the FH-horizon). Both acid-sensitive and acid-tolerant strains occur among nitrifiers (de Boer et al., 1990), but Persson and Wirén (1995) were unable to detect net nitrification at pH values lower than 4.0 in Scandinavian spruce forest soils. Similarly, very low net nitrification was found by Johnsrud (1978) in spruce forests at pH below 4.3. Although the relative importance of autotrophic and heterotrophic nitrification in forest soils has been addressed in few studies, it seems as if mobilized NH$_4^+$ can be a significant source of NO$_3^-$ in some soils. Schimmel et al. (1984) estimated, for instance, that less than 5% of the nitrification rates, 45–90 ng N g$^{-1}$ h$^{-1}$, measured in slurries of mixed coniferous forest soils incubated for 6 h was due to autotrophic oxidation of NH$_4^+$. The measured nitrification in the Stråsan soils was net rates, which may underestimate the gross rates if some of the added $^{15}$NH$_4^+$ was oxidized to $^{15}$NO$_3^-$ and subsequently immobilized (Stark and Hart, 1997).

Nitrifiers may produce N$_2$O, either from various intermediates during NH$_4^+$ oxidation (Ritchie and Nicholas, 1972) or from the reduction of NO$_3^-$ coupled to the oxidation of NH$_4^+$ (Poth and Focht, 1985). The latter is confined to soils with limited aeration, e.g. due to slow O$_2$ diffusion in interstitial pore water, whereas the former may be substantial in pH neutral, aerobic agricultural soils and be responsible for anything less than 10% to more than 50% of the N$_2$O flux, depending on, for example, soil moisture conditions (Bremner and Blackmer, 1979; Freney et al., 1979; Skiba et al., 1993; Webster and Hopkins, 1996; Stevens et al., 1997). The nitrifier production of N$_2$O in forest soil seems to be more variable and a soil with pH 3.8 produced virtually no nitrifier derived N$_2$O (Robertson and Tiedje, 1987). A preliminary 6 h bioassay with Stråsan soil samples initially flushed with air and then sealed in the incubation flasks with rubber stopper showed no detectable N$_2$O peak and it was decided to assay nitrification with continuous aeration without stoppers, with the loss of data on traces of N$_2$O from the nitrification.

The concentrations of NO$_3^-$ in the Stråsan soils are also considerably lower than those found in aquifers (>20 mg l$^{-1}$), in which denitrifiers have evolved for enhanced rates of nitrate reduction (Bengtsson and Bergwall, 1995). However, even concentrations of that magnitude in the forest soil may have been insufficient to select for enhanced NO$_3^-$ reduction because of the lower accessibility of NO$_3^-$ ions to cells on soil particles compared with pore water. Murray et al. (1989) found, for example, no correlation between bulk soil NO$_3^-$ concentration and denitrification rates in agricultural soils that were fertilized and where NO$_3^-$-N concentrations occasionally were as high as 350 mg kg$^{-1}$.

The relative contribution of denitrifying and ammonifying bacteria to NO$_3^-$ reduction in a soil or a sediment is difficult to predict and the generally acknowledged qualitative characteristics that tend to distinguish the two processes are diluted by exceptions. Some observations suggest that dissimilatory reduction of NO$_3^-$ to NH$_4^+$ (ammonification) is quantitatively as important in soil and sediment as denitrification (Sørenson, 1978), whereas others find that NO$_3^-$ ammonification is insignificant (Binnerup et al., 1992). Based on the high capacity of electron acceptors per NO$_3^-$ reduced to NH$_4^+$ (8e$^-$ per N atom), Tiedje et al. (1982) suggested that DNRA bacteria should be competitively successful in electron donor rich and electron acceptor poor habitats, where selection should favour cells with a maximum electron acceptor capacity. Organic C concentrations are known for the N0 and N2 treatments (43.8 and 48.4% in the FH-layer, respectively (Sjögren, Persson, Swedish University of Agricultural Science, personal communication)) and those values as well as the corresponding C-to-N ratios (32.9 and 23.2, respectively) are sufficiently high to qualify the soils as electron donor rich and potentially favourable for ammonification.

Denitrification in soils is often limited by high oxygen concentrations and low nitrate and carbon concentrations (Tiedje, 1988). Some observations suggest that the denitrification rate depends on the NO$_3^-$ concentration (Murray et al., 1989; Weier et al., 1991) and others suggest the opposite (Burford and Bremner, 1975; Myrold and Tiedje, 1985). Vermes and Myrold (1992) found that in situ denitrification rates in soil of pine and Douglas-fir were better correlated with soil NO$_3^-$ concentrations than with the availability of C, measured by soil respiration rates, or with soil moisture. This is in contrast to conditions in agricultural soils (Myrold and Tiedje, 1985; Drury et al., 1991) and...
is possibly a consequence of a lack of organic C for denitrifying microorganisms in agricultural soils (McCarty and Bremner, 1992). The negative correlation between extractable NO$_3^-$ concentrations and denitrification in the Stråsan soils and the inverse relationship between soil organic C concentrations and denitrification suggests that other factors, such as low pH, that tend to depress denitrification (Müller et al., 1980; Nägeli and Conrad, 1990) are more important in controlling rates. It is also possible that the NO$_3^-$ concentrations are too low at all four sites to allow anything but marginal variations in the denitrification rates.

Another possibility is that denitrification rates are underestimated. A potential source of error may be the reduced efficiency of the acetylene blockage technique in soils with low concentrations of NO$_3^-$ (Slater and Capone, 1989; Rudolph et al., 1991). The presence of acetylene in bioassays of denitrification can also reduce the production of NO and potentially cause an underestimate of N$_2$O production (Davidson, 1992; McKenney et al., 1996). However, in acid soils such as those at Stråsan, where N$_2$O tends to be the dominating end product of denitrification, acetylene has not been observed to have any inhibitory effect on the N$_2$O production (Knowles, 1982; Nielsen et al., 1994).

The larger activity of ammonifiers compared with denitrifiers coincides with differences in frequency of the two groups of soil bacteria visible on selective media after incubation (Fig. 4), possibly suggesting that, on average, activity and abundance are related to each other. In contrast to the general correspondence between differences in activity and in abundance of ammonifiers and denitrifiers, little agreement was observed between variations in abundance of ammonifiers or denitrifiers on one hand and the corresponding ammonification and denitrification on the other. The frequency of nitrate reducers was correlated with the fertilizer dosage (Fig. 4) and, consequently, unrelated to the variations in nitrate reduction rates at the sites. The same kind of lack of correlation between denitrification rates and denitrifier counts were observed by Volz et al. (1975), suggesting that the abundance of denitrifiers, many of which may be capable of using other electron acceptors than NO$_3^-$ and probably a variety of electron donors, may be underestimated by the lack of appropriate nutritional media for their counting. Parsons et al. (1991) also found weak correlations between denitrification rate and denitrifier biomass ($r^2 = 0.1–0.3$) when all observations, reflecting spatial and temporal variations, were considered. The suggested explanation was that denitrifier growth is primarily controlled by the availability of soil carbon under aerobic conditions and that the cells start denitrifying under anaerobic conditions by activating or synthesizing denitrifying enzymes.

Denitrifiers are not necessarily limited in population sizes by the concentration of O$_2$ and NO$_3^-$, both denitrifiers and ammonifiers are probably capable of aerobic respiration and may prevail in high numbers and also metabolize carbon under oxygenated conditions. This would obviously make attempts to correlate abundance or frequency of denitrifiers, identified by their production of N$_2$ on selective media, with in situ denitrification rates difficult in soils with transient O$_2$ conditions, such as well drained forest soils. However, a large number of strains capable of aerobic denitrification have been observed (Robertson and Kuenen, 1984; Anderson and Levine, 1986; Lloyd et al., 1987; Bengtsson and Annadotter, 1989; Robertson et al., 1995) and some aerobic denitrifiers might also be heterotrophic nitrifiers (Robertson et al., 1989). Oxygen can be used simultaneously with nitrate as an electron acceptor, thus promoting the growth rate of the strain and potentially stimulating the activity of synthesized nitrate reductases (Bonin and Gilewicz, 1991; Patureau et al., 1994). How common aerobic denitrifiers are in soils compared with denitrifiers that respire O$_2$ is unknown.

Acknowledgements

Dr. Dan Berggren at Swedish University of Agricultural Sciences kindly provided the soil samples and the pH data from Stråsan. Dr. Tryggve Persson and Mr. Michael Sjöberg at the same university are acknowledged for sharing their unpublished data on C and N concentrations in the Stråsan soils. The work was supported by the National Swedish Environment Protection Board and Crafoordska Stiftelsen.

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