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THE OCCURRENCE AND POSSIBLE SOURCES OF NITRITE IN A GRAZED, FERTILIZED, GRASSLAND SOIL

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Summary—Concentrations of NO_2^--N in land drainage and river waters in Northern Ireland in recent years have frequently exceeded EEC guide values. Very little information exists to indicate if and when NO_2^- accumulates in soil solution, and whether NO_2^- from the soil profile is the source of NO_2^- in drainage and river waters. The occurrence of NO_2^- in the field was studied and laboratory incubation experiments carried out to determine the possible sources of NO_2^- in grassland soil. Field studies were carried out to determine the occurrence and spatial variability of NO_2^- in a grazed, grassland soil. Plots receiving either 100 or 500 kg N ha^{-1} yr^{-1} were systematically sampled in May and October 1992. Concentrations of NO_2^- in soil were highly variable and ranged from 0 to 2.747 μg N g^{-1}, the data being significantly skewed to the right. Correlation matrices and stepwise multiple regression analyses showed relationships between NO_2^- and a number of soil variables. Nitrite appeared to be related to variables which indicated its occurrence as a result of nitrification of either fertilizer- or urine-derived NH_4^+. Nitrate was repeatedly correlated to NO_2^- concentrations, suggesting that both nitrification *and* nitrate reduction may be responsible for NO_2^- formation. Spatially, nitrite occurred at random, basic geostatics producing only one variogram, showing an increase in NO_2^- concentrations with an increase in distance between sampling points. There was no pattern to the distribution of NO_2^- with depth, indicating differences in the ratios of the rates of NO_2^- production and consumption. Numbers of NH_3-oxidizers were consistently higher than numbers of NO_2^--oxidizers, with some degree of variation between samples. The microbial aspects of NO_2^- formation are discussed, including partial recycling of NO_2^- via the NO_3^- pool, and possible causes of NO_2^- accumulation due to the inhibition of NO_2^--oxidizing bacteria. Laboratory incubation studies were carried out in which measurable NO_2^- flushes were induced. Increasing soil pH and NH_4^+ concentrations produced large NO_2^- flushes, which peaked after about 17 days of incubation, then rapidly declined. Soil incubated with urea produced NO_2^--N concentrations equivalent to those encountered in the field, suggesting that NH_4^+ oxidation accounts for a significant proportion of NO_2^- formed in this soil.

INTRODUCTION

At the Agricultural Research Institute (ARI), Hillsborough, Northern Ireland, the drainage waters from a grazed, fertilized grassland receiving different rates of fertilizer nitrogen are analysed continuously for a wide range of variables. A cause for concern has been the high nitrite (NO_2^-) concentrations periodically observed in these samples. During 1991, average observed concentrations in drainage water from plots receiving 100 and 500 kg N ha^{-1} yr^{-1} were 17 and 28 μg NO_2^--N l^{-1}, respectively. The guide values for rivers for salmonids and coarse fish are 3 and 9 μg NO_2^--N l^{-1} (European Economic Community, 1978), respectively. Nitrite concentrations in the six major rivers of the Lough Neagh catchment area during 1991 were between 4 and 172 μg NO_2^--N l^{-1}. The causal factors for the occurrence of NO_2^- in the land drainage and river water are not known. If NO_2^- was formed in soil solution, it could be leached down the soil profile to the sub-soil and drainage system and eventually into stream and river water. However, very little information exists to indicate if and when NO_2^- will accumulate in soil solution. Jones and Schwab (1993) measured NO_2^- concentrations in soil solution samples from ceramic cups, and found significant concentrations of NO_2^-: up to 7 mg N l^{-1}. The appearance of NO_2^- in soil solution from the fertilized, grassland soil was sporadic and unpredictable and followed no particular pattern.

The two main processes for NO_2^- formation in soil are ammonium (NH_4^+) oxidation and nitrate (NO_3^-) reduction. Morrill and Dawson (1967), Passioura and Wetselaar (1972), Wetselaar *et al.* (1972) and Elliot (1989) have shown that NO_2^- can accumulate in soil as a result of NH_4^+ oxidation, particularly when soil pH is elevated. In grazed, grassland soils, high pH and high NH_4^+ concentrations can occur as a result of the application of artificial fertilizers or in the region of urine spots. Monaghan and Barraclough (1992) detected large amounts of NO_2^- in soil which received urine of high N concentration.

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The accumulation of NO_2^- by NO_3^- reduction has been reported in aquatic systems. In freshwater lakes, it is thought that NO_2^- may originate from the reduction of NO_3^- through the activity of phytoplankton (EIFAC, 1984), although Heathwaite (1993) reported that NO_2^- is rarely present in appreciable concentrations in freshwaters, since the oxidation of NH_4^+ to NO_2^- by *Nitrosomonas* is the rate limiting step in nitrification in surface waters. In batch reactor systems, NO_2^- is thought to accumulate due to NO_3^- reduction occurring at a greater rate than subsequent NO_2^- reduction (e.g. Betlach and Tiedje, 1981; Wilderer *et al.*, 1987). Although numerous studies have been carried out on denitrification in soils, there is little evidence to suggest that NO_2^- in soils is derived from the reduction of NO_3^-. This may be due, however, to the monitoring of the end products of denitrification, i.e. N_2O and N_2, rather than the intermediate species, NO_2^-.

Our aims were firstly, to obtain information about NO_2^- in the field by measuring the occurrence and spatial variability of NO_2^- (and a number of other soil variables) in a grazed, fertilized, grassland soil; by determining whether relationships existed between concentrations of NO_2^- and other soil properties; by studying the variation in numbers of nitrifying bacteria and by examining changes in NO_2^- concentrations with soil depth. Secondly, to determine whether measurable NO_2^- flushes could be induced in soil derived from the experimental site during laboratory incubations with different rates and forms of fertilizer.

MATERIALS AND METHODS

Field Study

Site

The site is situated at the ARI, Hillsborough, Co. Down, Northern Ireland, and consists of six hydraulically isolated plots, 0.2 ha each (16 × 150 m). The soil is an acid brown earth (48% sand, 31% silt, 20% clay), of pH 6.0, containing 11.6% organic matter (on a dry weight basis). The sub-soil is of a similar texture, but is less humose and with more stones. All plots have slopes, the upper and lower halves having slopes of 6 and 14%, respectively. The average annual rainfall is 904 mm. Five plots have perennial rye-grass swards receiving 100 up to 500 kg N ha^{-1} yr^{-1} as ammonium nitrate–calcium carbonate (CAN, 27% N) in six split applications (the first application in 1992 was on 5 May and the final on 25 August). All plots were grazed with 6–9 month old steers during the growing season to maintain a sward height of 7 cm.

Spatial variation of nitrite

Plots receiving 100 and 500 kg N ha^{-1} yr^{-1} were selected for this study. They were marked into a grid 12 m wide × 132 m long, providing three rows of 33 points, the points being equidistant at 4 m apart. This arrangement left at least 2 m strips around the perimeter of the grid, thereby avoiding edge effects. Duplicate cores (3 cm dia. × 7.5 cm deep) were taken at each point of the grid, giving 198 cores per plot. Cores were sampled on 12 May 1992, 1 week after the first fertilizer application, and on 8 October 1992, after all split applications had been made. The cores were transferred, in the field, to 300 ml sterile jars. One of the duplicate cores from each grid was air-dried for moisture content (MC). The dried soil also was used for the determination of soil pH, extractable K, exchangeable Mn (May sampling) and Mg (October sampling). The second core was used for determination of mineral N (NO_2^-, NO_3^- and NH_4^+) and Fe(II) concentrations. Potassium and Mg were measured because concentrations of these nutrients are often concentrated in the urine of ruminants (Monaghan and Barraclough, 1992; Haynes and Williams, 1992) and their association with NO_2^- might indicate urine patches as a source of NO_2^- in a grazed soil. Association of NO_2^- with Mn or Fe(II) would indicate its occurrence in sites where reducing conditions prevail.

Variation of nitrite concentrations with depth

In December 1992, 10 cores (4 cm dia × 50 cm deep) were taken at random from the plot receiving 500 kg N ha^{-1} yr^{-1}. The cores were divided into 10 cm sections and extracted with 2 M KCl for mineral N analyses.

Variability in numbers of nitrifying bacteria

The spatial variability of NH_3-oxidizer and NO_2^--oxidizer numbers was studied to see whether differences in nitrifier numbers could be related to mineral N concentrations. Twenty soil cores (3 cm dia × 7.5 cm deep) were taken separately, aseptically, and at random from the plot receiving 500 kg N ha^{-1} yr^{-1} in May 1992. Nitrifier numbers and mineral N concentrations were determined in each sample. In addition, composite cores were taken from the 100, 300 and 500 kg N ha^{-1} yr^{-1} plots. These consisted of 20 cores (3 cm dia × 7.5 cm deep), bulked to give one sample. From the bulked samples, three replicate samples were taken for determination of nitrifier numbers and mineral N concentrations.

Numbers of nitrifiers in each sample were determined by the most-probable-number (MPN) technique. NH_3-oxidizers and NO_2^--oxidizers were enumerated separately using the media described by Alexander and Clark (1965). Both media were dispensed in 3 ml aliquots in 15 × 1.5 cm test-tubes and autoclaved at 121°C for 15 min. Tubes of media (five per dilution) were inoculated with 1 ml aliquots from a 10-fold dilutions series prepared from a "stock solution" of the fresh soil (50 g) in sterile 1/4 strength Ringer's solution (450 ml). Tubes were kept at 28°C for 5 weeks. The presence or absence of NO_2^- in the culture tubes after this period was determined as

described by Cooper (1975). The numbers of organisms in the original samples were determined by reference to a table for use with 10-fold dilutions and five tubes per dilution from Cochran (1950).

Laboratory Incubation Studies

Soil

The soil used in the incubation experiments was taken to a depth of 7.5 cm from the plot receiving 100 kg N ha^{-1} yr^{-1} in the field study. The soil was sieved (< 5 mm), and mixed thoroughly before use.

The effect of elevated ammonium concentrations and soil pH on soil nitrite formation

The equivalent of 50 g of dry soil at an MC of ca. 30% were weighed into sterile, 300 ml jars. There were three rates of NH_4^+ application with or without $Ca(OH)_2$. Calcium hydroxide was added at two rates to raise the soil pH to ca. 8 (500 mg per jar) and 11 (1000 mg per jar). Ammonium applications were made 2 days after $Ca(OH)_2$ addition. Sufficient $NH_4(SO_4)_2$ was dissolved in distilled water to give final concentrations in the incubation jars of 0, 125 or 250 μg NH_4^+-N g^{-1} of dry soil, with a resulting MC of 50%. Jars were sealed and then aerated every day by unscrewing the airtight lids and exposing the soil to laboratory air for 5 min prior to re-sealing (water loss was assumed to be negligible). Jars were kept at 20°C (in the dark) for 7, 17 and 28 days. At each harvest, three replicates per treatment were destructively sampled. An aliquot of soil was removed for pH determination, the remaining soil was then extracted with 2 M KCl for determination of mineral N concentrations.

Time–course incubation

In order to establish the pattern of NO_2^- accumulation and subsequent assimilation, the procedure described for the first laboratory incubation study was repeated at the highest NH_4^+ and $Ca(OH)_2$ rates, with frequent harvests over a 23 day period.

The effect of N form on nitrite formation

This study involved the surface application of different forms of N fertilizer commonly used in the field. The three N fertilizers used were: CAN, ammonium nitrate (AN) and urea. The experimental design was similar to the first incubation experiment, but only one rate of N was applied (equivalent to 100 kg N ha^{-1}) to the surface of soil previously adjusted to 50% MC. Sampling times were at 0, 5, 10, 15 and 20 days after N application. At harvest, soil pH and mineral N concentration were determined.

Chemical Analyses

Determination of soil pH, extractable K, exchangeable Mn and Mg were according to MAFF (1985). Determination of mineral N concentrations was carried out by extraction of fresh soil with 2 M KCl (1:1, soil–solution). Soils were shaken for 1 h on an orbital shaker, the extracts were then filtered (Whatman GF/D) and stored at 4°C until analysis within 1 week. Concentrations of NO_2^--N, NO_3^--N and NH_4^+-N were determined by flow-injection-analysis (Tecator Ltd, 1982, 1983, 1984, respectively). Fe(II) concentrations in the same KCl extracts were determined spectrophotometrically (Krishnamurti and Huang, 1990).

All concentrations are expressed on a soil oven-dried basis, unless otherwise stated.

Statistical Analyses

The statistical analysis of the data from the study of spatial variability of NO_2^- and other soil properties measured was carried out in three stages [stages (i) and (ii) were performed using STATGRAPHICS Version 4.0, and stage (iii) on GENSTAT Version 5.5-1].

(i) Generation of descriptive statistics. This allowed assessment of the nature of data distribution for each variable.
(ii) Examination of the relationships between soil NO_2^- and other soil variables by correlation and stepwise multiple regression analyses of the data.
(iii) Estimation of variability of NO_2^- concentrations with distance between samples using basic geostatistics to generate mean semi-variograms where possible for each soil variable studied. The validity of the variograms was determined by establishing whether trends existed in the data with respect to slope of the plots.

Correlation coefficients were calculated and multiple regression analysis was performed on the data from the depth study and on the data from the study of nitrifier numbers to determine whether relationships existed with depth and nitrifier numbers, respectively. Analysis of variance (ANOVA) was performed on the data from the incubation experiments.

RESULTS

Field Study

Spatial variation of nitrite

Descriptive statistics. Figure 1 shows the frequency distribution of NO_2^- in the soil cores taken in May 1992. The distribution for both plots is highly positively skewed. Over 90% of the cores from the 100 kg ha^{-1} yr^{-1} plot contained < 0.1 μg NO_2^--N g^{-1} soil, with no cores having NO_2^- concentrations above 0.4 μg NO_2^--N g^{-1} soil. Cores from the 500 kg N ha^{-1} yr^{-1} plot generally had higher NO_2^- concentrations, the highest being nearly 3 μg NO_2^--N g^{-1} soil. However, the majority of cores (70%) contained < 0.5 μg NO_2^--N g^{-1} soil. Descriptive

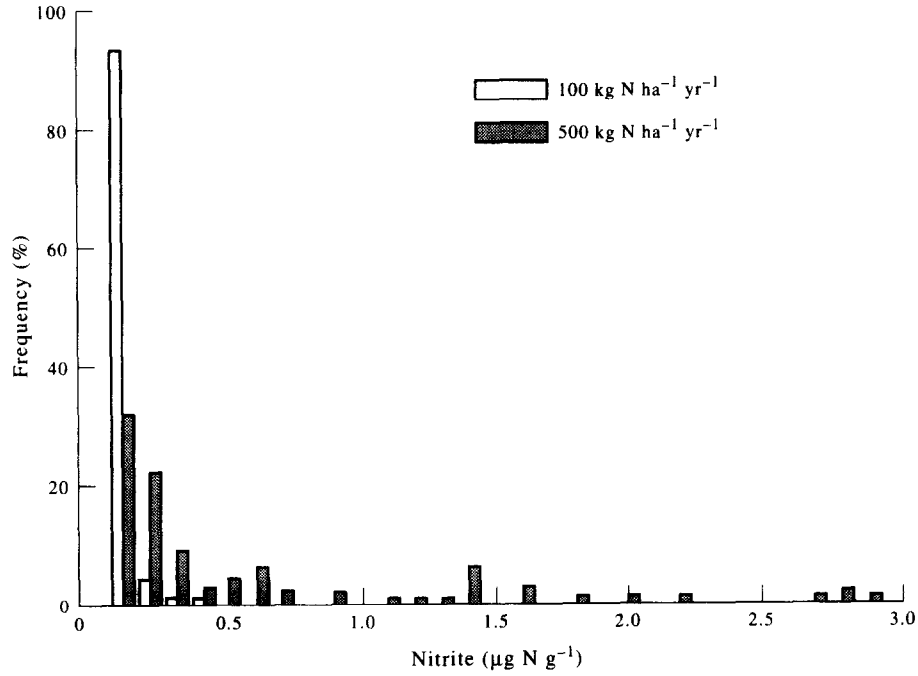

Fig. 1. Frequency distribution of nitrite concentrations in cores sampled from grazed grassland receiving 100 or 500 kg N ha^{-1} yr^{-1} and sampled in May 1992.

statistics for mineral N species in both plots measured at both sampling times are presented in Table 1. Mean NO_2^- concentrations were greater at both sampling dates in the 500 kg N ha^{-1} yr^{-1} plot, with concentrations in each plot being higher at the beginning, compared with the end, of the growing season. The distribution of the data for the three species of mineral N measured was highly positively skewed, the degree of skew and coefficients of variance (CV) increasing between May and October, indicating an increase in the "patchiness" of the distribution of mineral N within the plots. Generally, NO_2^- exhibited the highest degree of variation and skew. The concentrations of NO_3^- and NH_4^+ were very high in the 500 kg N ha^{-1} yr^{-1} plot in October, which may have been due to an accumulation of N from fertilizer applications and urine deposits.

Relationships between soil nitrite and other soil variables. Correlation matrices for each plot at both sampling times were generated. The correlation coefficients (r^2) and their levels of significance for NO_2^--N are presented in Table 2. Included in the matrix is the position of the core as a variable, to give an indication as to whether any of the soil variables exhibited trends in the plots with respect to slope. Concentrations of NO_2^- showed a significant relationship with NH_4^+ in May 1992 in the 100 kg N ha^{-1} yr^{-1} plot. There were significant relationships between NO_2^- and NO_3^- concentrations in both plots on both sampling occasions, with pH being significantly correlated with NO_2^- in May only. Only one other variable, K concentration in cores from the 500 kg N ha^{-1} yr^{-1} plot in October, showed a significant correlation with soil NO_2^-.

Table 1. Summary statistics for mineral N concentrations (μg N g^{-1} soil) in cores taken from plots receiving 100 or 500 kg N ha^{-1} yr^{-1} and sampled in May and October 1992

N rate (kg ha^{-1} yr^{-1})	Sampling time	Variable	Mean ($n = 99$)	Minimum	Maximum	Standard deviation	Coefficient of variance	Skewness*
100	May	NO_2^--N	0.073	0.041	0.299	0.032	43.325	4.397
100	Oct	NO_2^--N	0.022	0.000	0.590	0.060	267.423	8.724
500	May	NO_2^--N	0.429	0.042	2.747	0.587	136.961	1.895
500	Oct	NO_2^--N	0.053	0.000	0.800	0.122	229.958	4.286
100	May	NH_4^+-N	5.472	1.914	38.396	4.170	76.212	5.589
100	Oct	NH_4^+-N	4.498	1.050	78.220	9.144	203.295	6.273
500	May	NH_4^+-N	25.544	3.479	108.513	23.942	93.727	1.618
500	Oct	NH_4^+-N	8.717	1.960	175.550	19.998	229.407	6.521
100	May	NO_3^--N	0.316	0.000	1.023	0.237	74.895	0.774
100	Oct	NO_3^--N	0.369	0.020	2.410	0.407	110.293	3.129
500	May	NO_3^--N	23.587	0.477	102.784	20.841	88.359	1.237
500	Oct	NO_3^--N	8.826	0.520	110.300	15.636	177.156	3.893

*All data are significantly ($P < 0.001$) skewed.

Table 2. Correlation coefficients (r^2) for the relationships between NO_2^- and other soil variables in cores from grazed, fertilized, grassland receiving 100 or 500 kg N ha^{-1} yr^{-1} and sampled in May and October 1992

	May		October	
Variable	100	500	100	500
NH_4^+	0.35**	0.16	0.15	0.13
NO_3^-	0.43**	0.21*	0.45**	0.25*
MC	0.13	0.19	0.03	−0.16
pH	0.23*	0.25*	0.15	0.09
K	0.00	−0.09	−0.03	0.23*
Position†	0.01	0.05	0.03	−0.12
Mn	−0.19	−0.19	ND	ND
Mg	ND	ND	0.12	0.00
Fe(II)	ND	−0.17	ND	−0.06

*,**Indicate significant correlations at the 0.05 and 0.001 levels of probability, respectively ($n = 99$).

†Position refers to the point on the slope of the plot from which the cores were sampled, the position number increasing from top to bottom of the slope.

The final models from stepwise multiple regression analyses for variables related to NO_2^- are shown in Table 3. Nitrate-N was selected in all four models, with pH featuring in three. Ammonium and K were selected in models for the 100 kg N ha^{-1} yr^{-1} plot sampled in October, and Fe(II) for the 500 kg N ha^{-1} yr^{-1} plot sampled in May.

Assessment of pattern of spatial distribution of nitrite and other soil variables by basic geostatistics. The semi-variogram can provide a concise and unbiased description of the scale and pattern of spatial variation (Oliver and Webster, 1992), although the data must be trend-free for variograms to be valid. Prior to the generation of mean semi-variograms, ANOVA was performed on the data to determine whether trends existed with respect to slope, since the correlation matrices indicated that a number of variables exhibited significant relationships with position in the plot (data not shown), thus invalidating the generation of a semi-variogram. Each of the four NO_2^- data sets was trend-free and the semi-variograms calculated are shown in Fig. 2(a)–(d). Of these four semi-variograms, only one showed a relationship with distance between points, i.e. a model was fitted to the data [Fig. 2(c)—NO_2^- concentrations in cores from the plot receiving 100 kg N ha^{-1} yr^{-1} sampled in October). The model fitted was linear with an r^2 value of 0.95.

Variation of nitrite concentrations with depth

Table 4 shows the concentrations of soil mineral N with depth. Concentrations of NO_2^- seemed to decrease with depth. The mean at the 0–10 cm depth was high due to one core which had nearly 2 μg NO_2^--N g^{-1} soil, and when removed, leaves an average NO_2^- concentration of 0.05 μg N g^{-1} soil. Concentrations of NO_3^- and NH_4^+ were 10–100 times higher than those of NO_2^-. A correlation matrix for the variables measured in our study showed that NO_2^- did not correlate significantly with depth. There was, however, a significant ($P < 0.05$) positive relationship between NO_3^- and depth, and a significant ($P < 0.001$) negative relationship between NH_4^+ and depth.

Variability in numbers of nitrifying bacteria

Figure 3 shows the $\log_{10}$ MPN of NH_3-oxidizers and NO_2^--oxidizers in 20 random cores from the 500 kg N ha^{-1} yr^{-1} plot. In every sample, the numbers of NH_3-oxidizing bacteria were greater than NO_2^--oxidizing bacteria, and with the exception of two cores, the confidence intervals indicate significant ($P < 0.05$) differences between the two populations. Numbers of NO_2^--oxidizers correlated significantly with both NO_3^- ($r^2 = 0.56$, $P = 0.014$) and NH_4^+ ($r^2 = 0.59$, $P = < 0.001$) concentrations. Multiple regression analyses and stepwise selection for variables related to NO_2^- gave no further information regarding relationships between nitrifier counts and mineral N concentrations.

Results from the composite cores from plots receiving different rates of fertilizer N are shown in Table 5. As with the individual random sampling, numbers of NH_3-oxidizers were consistently and significantly ($P < 0.05$) higher than those of NO_2^--oxidizers. The numbers of both nitrifier groups appear to be independent of soil N additions.

Table 3. Final models from stepwise selection for variables related to NO_2^- concentrations (dependent variable) for soil data ($n = 99$) from grazed, fertilized, grassland soil receiving 100 or 500 kg N ha^{-1} yr^{-1} and sampled in May and October 1992

	N application rate	Sampling time	Independent variable	t-value	Level of significance (P)	R^2
Model 1	100	May	NO_3^-	4.88	0.000	0.23
			pH	2.76	0.007	
Model 2	500	May	NO_3^-	2.28	0.025	0.12
			Fe(II)	−2.00	0.048	
			pH	3.02	0.003	
Model 3	100	October	NH_4^+	2.04	0.044	0.28
			NO_3^-	6.08	0.000	
			K	−3.49	0.001	
Model 4	500	October	NO_3^-	3.67	0.000	0.11
			pH	2.74	0.007	

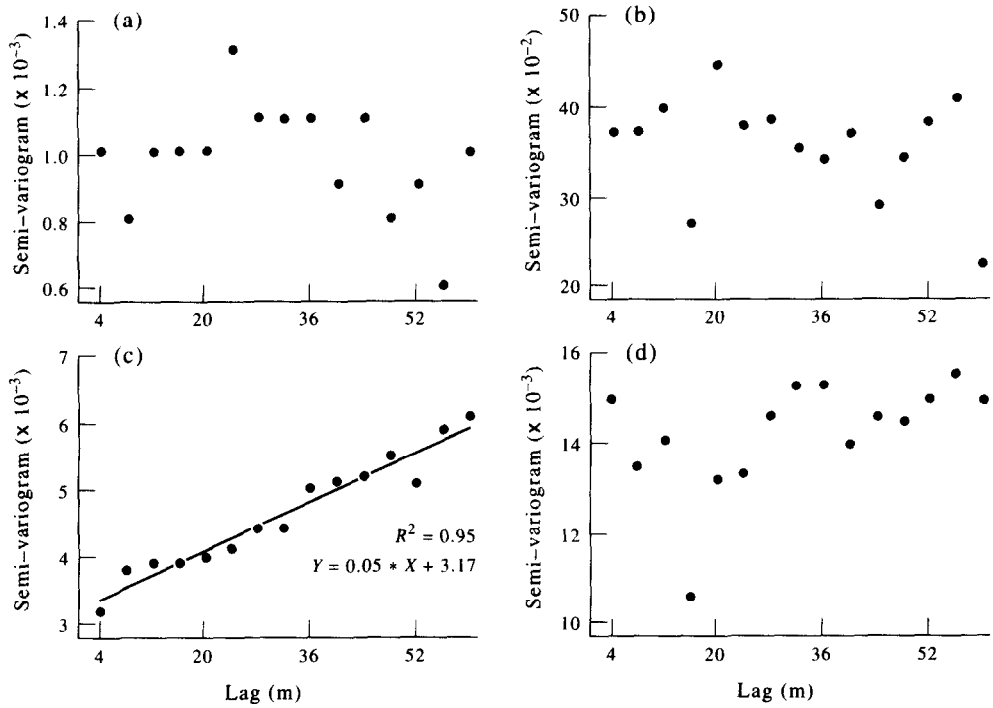

Fig. 2. Mean semi-variograms for nitrite concentrations in cores from a grazed, grassland soil sampled in May 1992 and receiving 100 (a) and 500 (b) kg N ha^{-1} yr^{-1} and sampled in October 1992 and receiving 100 (c) and 500 (d) kg N ha^{-1} yr^{-1}.

Laboratory incubation studies

Nitrite flush at differing rates of ammonium sulphate and calcium hydroxide application

Table 6 shows the concentrations of mineral N and soil pH at three rates of $NH_4(SO_4)_2$ and $Ca(OH)_2$. Soil from jars receiving no $Ca(OH)_2$ contained negligible NO_2^-, irrespective of initial NH_4^+ concentrations. During the experiment, NH_4^+ concentrations declined, NO_3^- concentrations increased and soil pH declined as nitrification occurred.

When 10 mg $Ca(OH)_2$ g^{-1} dry soil was applied, there was a large flush of NO_2^- in soil where NH_4^+ concentrations were also elevated. Concentrations of NO_2^- in these two treatments were significantly ($P < 0.001$) higher than those in soil treated with $Ca(OH)_2$ alone. The highest NO_2^- concentration was 26.36 μg N g^{-1} soil, at day 7. The NO_2^- flush had disappeared by day 18 when concentrations returned to amounts not significantly different from those receiving no NH_4^+. By day 18, NH_4^+ concentrations had declined significantly ($P < 0.001$) to near background amounts, with NO_3^- concentrations increasing due to nitrification.

Table 4. Mean concentrations of mineral N (μg g^{-1} soil) with depth for a grazed, fertilized, grassland soil receiving 500 kg N ha^{-1} yr^{-1} and sampled in December 1992

Depth (cm)	NO_2-N	NO_3-N	NH_4^+-N
0–10	0.24* (0.61)‡ 0.05† (0.03)	2.81 (1.55)	3.46 (1.11)
10–20	0.02 (0.01)	2.29 (0.83)	1.70 (0.79)
20–30	0.01 (0.00)	1.98 (0.66)	1.07 (0.35)
30–40	0.01 (0.01)	3.50 (3.30)	0.80 (0.38)
40–50	0.02 (0.01)	9.94 (1.91)	0.71 (0.46)

*Values are means with $n = 10$.
†Mean without the value for core 7, NO_2^- = 1.9 μg N g^{-1} soil.
‡Numbers in parentheses are standard deviations.

Application of $Ca(OH)_2$ at a rate of 20 mg g^{-1} soil tended to inhibit nitrification. Nitrite concentrations in soil from this treatment were near to background amounts. Concentrations of NO_3^- were significantly ($P < 0.01$) less when 20 mg g^{-1} $Ca(OH)_2$ were applied than with only 10 mg g^{-1} or no $Ca(OH)_2$. The soil pH in this treatment was always >8, and where NH_4^+ was applied in addition to the highest rate of $Ca(OH)_2$, the pH remained >10 throughout the 28 days of incubation. Where 20 mg g^{-1} $Ca(OH)_2$ were applied alone, concentrations of NH_4^+ increased during the incubation. This was probably due to enhanced mineralization due to solubilization of soil organics by the increase in soil pH. Where both NH_4^+ and $Ca(OH)_2$ were applied, the decline in NH_4^+ concentrations was delayed due to the inhibition of NH_4^+ oxidation by the high soil pH, the effect being more pronounced where concentrations of NH_4^+ were also high.

Time–course incubation

The changes in mineral N concentrations in soil incubated with NH_4^+ and $Ca(OH)_2$ over the course of 23 days are shown in Table 7. Ammonium concentrations at time zero were less than the applied 250 μg N g^{-1} dry soil. This may have been due to

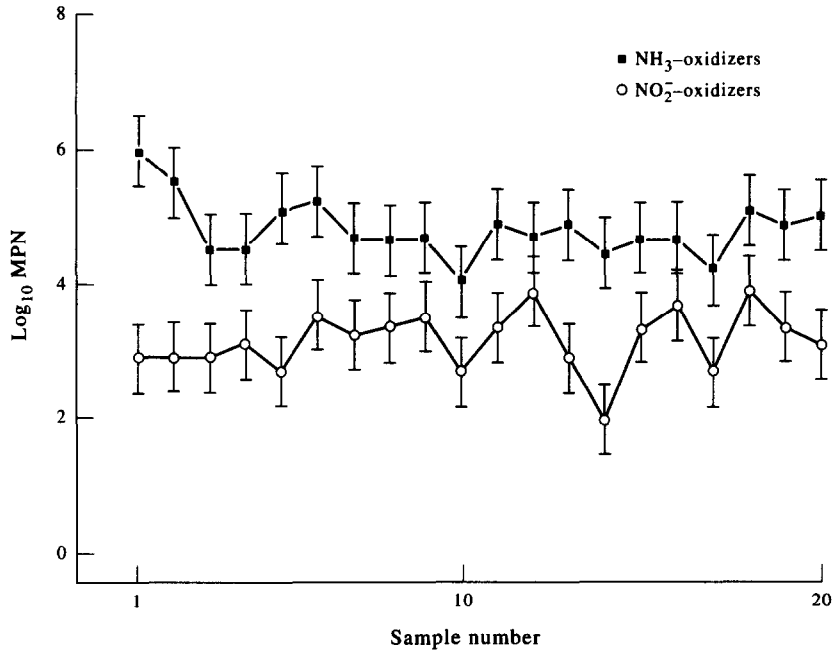

Fig. 3. Log_{10} MPN of nitrite- and ammonium-oxidizing nitrifying bacteria in 20 random cores sampled in May 1992 from a grazed grassland soil receiving 100 kg N ha^{-1} yr^{-1}.

rapid immobilization of NH_4^+ by the soil biomass or loss of NH_4^+ from soil as NH_3 via volatilization at the time of $NH_4^+(SO_4)_2$ solution application or during extraction with KCl. Between 0 and 14 days, NH_4^+ concentrations fluctuated, with an increase prior to the marked decrease after day 14. The initial increase, although not significant, may have been caused by increased mineralization of native N due to a pH increase in the soil as a result of $Ca(OH)_2$ application. The flush of soil NO_2^- during this incubation continued to increase rapidly until day 17 when mean concentrations of NO_2^--N were 32.8 μg N g^{-1} soil. The average NO_2^- concentrations fail to highlight the variability of the NO_2^- concentrations in soil from separate jars (see standard deviations, Table 7). The CV of NO_2^- concentrations at any time between days 7 and 20 was ca. 50%. Nitrate concentrations had an inverse relationship to soil NH_4^+, and fluctuated initially, followed by an increase which became significant ($P < 0.01$) after day 14, i.e. as a result of nitrification of NH_4^+.

Effect of N form on nitrite accumulation

Concentrations of NO_2^- in soils incubated with CAN or AN were generally low and not significantly greater than the control. Ammonium concentrations from these two treatments remained relatively stable throughout the incubation, with NO_3^- concentrations increasing over the 20 day period. The increase in NO_3^- concentrations may have been due to nitrification of applied NH_4^+, but this was not reflected by a decrease in NH_4^+ concentrations.

Nitrite only accumulated in soil incubated with urea (Table 8). The highest measured NO_2^- concentration was 2.64 μg N g^{-1} at day 5, significantly ($P < 0.001$) higher than NO_2^- concentrations in control incubations. This concentration is of the same order of magnitude as the highest concentrations recorded in the field study of spatial variation of NO_2^- (Table 1). The flush of NO_2^- in soil incubated with urea coincided with a significant ($P < 0.001$) increase in NH_4^+ concentrations and pH, both consequences of the hydrolysis of urea. Urea was the only treatment which raised the soil pH over 7. During the incubation, concentrations of NH_4^+ declined in urea-treated soils, and NO_3^- concentrations increased significantly ($P < 0.001$) due to nitrification.

Table 5. Log_{10} MPN of NH_3-oxidizing and NO_2^--oxidizing bacteria in composite cores from grazed, fertilized, grassland receiving 100, 300 or 500 kg N ha^{-1} yr^{-1} and sampled in May 1992

N application rate (kg N ha^{-1} yr^{-1})	Log_{10} MPN NO_2^--oxidizers	Log_{10} MPN NH_3-oxidizers
100	2.95 (2.44–3.48)	4.39 (3.88–4.92)
300	3.56 (3.03–4.08)	4.78 (4.26–5.30)
500	3.29 (2.77–3.80)	5.09 (4.57–5.61)

Values are means ($n = 3$) with 95% confidence intervals in parentheses.

DISCUSSION

From the field study, it is apparent that the occurrence of NO_2^- in the grazed, grassland soil is a complex issue. The plots are not only receiving precursors for NO_2^- formation in the form of artificial fertilizers (NH_4^+ and NO_3^-), but also in the form of cattle excreta (NH_4^+ via urea hydrolysis). In grazed, grassland soils, urine patches can represent a significant source of NH_3 and NO_3^- (Monaghan and Barraclough, 1992). The uneven return of excretal N

Table 6. Mineral N concentrations and pH in soil incubated with three rates of NH_4^+-N and $Ca(OH)_2$

NH_4^+ application rate (μg N g^{-1} soil)	$Ca(OH)_2$ application rate (mg g^{-1} soil) 0			10			20			SE*
	7 days	18 days	28 days	7 days	18 days	28 days	7 days	18 days	28 days	
				NO_2^- (μg N g^{-1} soil)						
0	0.05	0.09	0.07	1.34	0.20	0.15	0.88	0.98	0.41	
125	0.04	0.10	0.08	24.60	0.24	0.18	0.70	0.71	1.05	0.37
250	0.05	0.07	0.10	26.36	0.62	0.22	0.80	0.58	0.78	
				NO_3^- (μg N g^{-1} soil)						
0	31.0	20.9	41.8	55.0	123.1	154.3	7.5	3.2	3.1	
125	93.3	163.5	174.9	80.8	228.5	250.7	6.3	5.8	5.9	9.96
250	90.1	174.2	308.5	63.6	332.1	358.3	7.5	5.1	3.4	
				NH_4^+ (μg N g^{-1} soil)						
0	2.0	1.4	1.8	5.0	2.7	1.0	29.1	27.4	74.2	
125	48.7	10.5	8.8	101.3	3.4	2.0	65.2	52.7	46.7	5.21
250	157.0	101.7	75.8	227.5	5.4	3.7	104.1	108.4	48.8	
				pH						
0	5.63	5.26	5.21	7.79	7.70	7.81	11.23	8.88	8.02	
125	5.08	4.68	4.69	7.89	7.65	7.73	11.19	10.67	10.05	0.15
250	5.17	4.59	4.50	8.01	7.42	7.61	11.09	10.73	10.20	

*SE = standard errors of means ($n = 3$) for time and application rate.

to these plots will enhance the natural soil heterogeneity of NO_2^- precursors and a number of other soil properties. For example, K and Mg are concentrated in the urine of ruminants (Monaghan and Barraclough, 1992; Haynes and Williams, 1992) and amounts of these elements will be elevated in soil urine patches. High soil pH also develops due to hydrolysis of the elevated urea concentrations. There was a high degree of heterogeneity in soil NO_2^- concentrations with depth and space. The spatial study showed the highly positive skew of mineral N data. The CV and skew of the NO_2^- data were generally higher than those for NO_2^- and NH_4^+, the factors controlling NO_2^- formation being more complex than those controlling NO_3^- and NH_4^+ occurrence.

Results from the correlation matrices and regression analyses show that concentrations of NO_2^- are often closely related to the concentrations of its potential precursors and end-products. In the spatial study, NO_2^- correlated with NH_4^+ in one plot in May, and with NO_3^- in both plots on both sampling dates,

Table 7. Concentrations of mineral N in soil incubated with 250 μg NH_4^+-N g^{-1} and 10 mg $Ca(OH)_2$ g^{-1} soil

Incubation time (days)	Mineral N (μg N g^{-1} soil) NH_4^+	NO_2^-	NO_3^-
0	216.5* (14.9)†	0.5 (0.0)	18.5 (3.1)
1	195.3 (20.1)	1.7 (0.4)	19.0 (0.7)
2	227.3 (6.5)	3.2 (1.1)	18.8 (0.8)
3	219.2 (18.0)	4.8 (0.4)	20.2 (0.6)
4	237.4 (0.8)	3.5 (0.8)	25.5 (4.1)
5	241.6 (2.6)	4.5 (1.6)	31.2 (6.1)
6	248.7 (24.0)	7.7 (1.6)	28.1 (6.1)
7	240.2 (21.5)	10.8 (5.0)	42.4 (15.8)
10	218.3 (27.8)	20.0 (11.1)	67.8 (30.6)
12	187.5 (23.1)	28.6 (15.6)	107.3 (21.7)
14	186.5 (21.1)	29.6 (15.1)	113.7 (34.4)
17	88.4 (92.9)	32.8 (27.6)	177.7 (66.5)
20	3.4 (3.0)	9.0 (14.7)	286.8 (47.5)
23	1.9 (1.7)	0.2 (0.1)	329.3 (9.7)

*Values are means ($n = 3$).
†Numbers in parentheses are standard deviation.

so high NO_2^- concentrations were found in soil cores where NO_3^- or NH_4^+ concentrations were high.

Nitrite was the only variable from the "depth study" not to correlate with depth. As expected, NH_4^+ concentrations decreased from 0 to 50 cm, concentrations being higher in the top layers due to fertilization and urine deposition, and also to its retention in the soil. The decrease in NO_3^- concentration down the profile is presumably due to transport from the surface layers. Nitrite would be expected to behave in a similar way to NO_3^- with respect to movement within the soil profile, but its occurrence with depth was completely without pattern and exhibited a high degree of variation between cores. Jones and Schwab (1993) found no particular pattern in the appearance of NO_2^- in soil solution at two depths. Detection of NO_2^- throughout the soil profile indicates that the processes producing NO_2^- are operating at a greater rate than those consuming NO_2^-. The formation of NO_2^- could be due to a number of processes, as could its consumption. For example, NO_2^- might be produced in the surface layers by NH_3-oxidizing bacteria and consumed by NO_2^--oxidizing or denitrifying bacteria following transport down the soil profile. If NO_2^- is occurring down the soil profile irrespective of depth, then it is present as a result of varying rates of a number of processes responsible for its production and consumption. These rates will be influenced by changes in soil conditions (e.g. aeration, moisture status, C supply) with an increase in depth.

Basic geostatistics produced only one variogram to which a model could be fitted (for NO_2^- in cores sampled in October from the plot receiving 100 kg N ha^{-1} yr^{-1}). The semi-variance increases with an increase in distance, or lag, and could be interpreted as an increase in the source of variation of NO_2^- with an increase in the area of interest. In addition, the variogram exhibits semi-variances which tend towards some positive value as the lag approaches zero, even though, by definition, the

Table 8. Concentration of mineral N and pH in soil incubated with ammonium nitrate (AN), CAN or urea at a rate equivalent to 100 kg N ha^{-1}

Treatment	Incubation time (days) 0	5	10	15	20	SE*
			NO_2^- (μg N g^{-1} soil)			
Control	0.000	0.000	0.000	0.000	0.000	
CAN	0.047	0.006	0.017	0.060	0.048	
AN	0.024	0.011	0.008	0.017	0.043	0.042
Urea	0.000	2.639	0.032	0.006	0.009	
			NO_3^- (μg N g^{-1} soil)			
Control	2.1	7.5	10.5	5.4	16.8	
CAN	378.0	412.0	366.3	464.0	470.0	
AN	394.0	385.0	436.0	463.0	467.0	13.3
Urea	4.2	73.4	240.4	412.0	440.0	
			NH_4^+ (μg N g^{-1} soil)			
Control	1.2	1.0	1.0	0.8	1.0	
CAN	378.0	374.0	391.3	392.0	386.0	
AN	381.0	384.0	381.0	391.0	379.0	7.1
Urea	34.6	542.0	502.0	429.0	391.0	
			pH			
Control	5.17	5.28	6.02	5.41	6.35	
CAN	4.97	5.32	5.44	5.13	5.55	
AN	4.91	5.10	5.30	5.05	5.44	0.04
Urea	5.46	7.05	5.20	5.83	5.35	

*SE = standard error of means ($n = 3$) for time and N treatment.

semi-variance at lag zero is itself zero. Webster and Oliver (1990) suggested that the principal cause for this "nugget variance" at lag zero in soil surveys, is usually the spatially dependent variation that occurs over distances smaller than the shortest sampling interval. The true shape of the variogram in this range would be identified by sampling at shorter intervals. Nevertheless, Fig. 2(c) shows an increase in the variance of NO_2^- concentration with an increase in distance between sampling points, as well as indicating variation on a much smaller scale (< 4 m) with the positive interception of the y-axis.

An increase in NO_2^- concentration variability with distance could be due to the lower fertilizer application rates and grazing densities on the 100 kg N ha^{-1} yr^{-1} plot compared with those for the 500 kg N ha^{-1} yr^{-1} plot, where a very weak linear variogram ($r^2 = 0.25$) was produced. At the higher fertilizer rates and grazing densities, areas of high NO_2^- precursor concentration from CAN and urine spots will be more common, and sites of potential NO_2^- formation will be closer together. Where the application rate and grazing intensities are lower, the frequency of sites of high NO_2^- precursor will be further spaced, hence the increase in variability of NO_2^- concentrations with distance between cores sampled. No valid models were produced for NO_2^- concentrations measured in May. A possible explanation is that grazing commenced the day following the May sampling date and, as a result, potential sites of NO_2^- formation were not as distance-dependent as those measured in October, following 6 months grazing.

Study of numbers of nitrifiers revealed some degree of variation in both NH_3- and NO_2^--oxidizers and confirmed their presence in this soil. More interestingly, numbers of NH_3-oxidizers were nearly always significantly ($P < 0.05$) higher than numbers of NO_2^--oxidizers (Fig. 4), in both individual and composite cores. In a previous study of nitrifier numbers in this soil, Cooper (1975) also found higher MPN counts for NH_3-oxidizers than NO_2^--oxidizers. Both *et al.* (1992a), Berg and Rosswall (1987) and Rosswall *et al.* (1990) and others have reported numbers of NO_2^--oxidizers to be higher than those of NH_3-oxidizers in soil.

During active nitrification, numbers of NH_3 and NO_2^--oxidizing bacteria should be of the same order of magnitude if the maximum oxidation capacities per cell of both are taken into account (Focht and Verstraete, 1977; Schmidt and Belser, 1982; Prosser, 1989). The large numbers of NH_3-oxidizers compared to NO_2^--oxidizers found by us could be a possible explanation for the presence of NO_2^- in this soil. Monreal *et al.* (1986) found that NH_3-oxidizers outnumbered NO_2^--oxidizers in soil incubated with nested urea-N, with measurable NO_2^- flushes occurring. Where the urea was mixed uniformly throughout the soil, numbers of NO_2^--oxidizers were greater than NH_3-oxidizers and no NO_2^- flush was detected. So NO_2^- flushes could be due to an imbalance in the numbers of NO_2^- and NH_3-oxidizing bacteria. Alternatively, the low numbers of NO_2^--oxidizers compared with NH_3-oxidizers could have been due to methodology. Underestimation of NO_2^--oxidizer numbers can occur due to the sensitivity of the MPN technique to the duration of the incubation (Both and Laanbroek, 1991) and the concentration of the NO_2^- used in the incubation medium (Both *et al.*, 1990). An incubation of only 5 weeks might have been too short for the NO_2^--oxidizing bacteria to consume all the NO_2^- offered in the enumeration medium. Both and

Laanbroek (1991) recommend the use of a range of incubation medium concentrations and analysis of the results after a maximal incubation period as the most reliable approach to establishing the population size of chemolithotrophic NO_2^--oxidizers.

Analysis of the data from the study of nitrifier numbers produced very few relationships between the variables measured. There was no relationship between the numbers of NH_3-oxidizers and NO_2^--oxidizers ($r = -0.10$, $P = 0.67$). A lack of correlation between the numbers of the two nitrifier types has been observed by Belser (1979), Both *et al.* (1992a, b), Blacquière (1986) and Klemedtsson *et al.* (1987) and others. This could indicate spatial and temporal separation of the two populations. The complete temporal separation of NH_3- and NO_2^--oxidation under conditions of low aeration and high temperature has been reported (Belser, 1979). Nitrifier populations have also been reported to be present in soil in the form of microcolonies or zoogloea (Keen and Prosser, 1988), and under certain conditions, the differences in sensitivity between NH_3-oxidizers and NO_2^--oxidizers to a number of environmental factors (Belser, 1979; Prosser, 1989) could result in their spatial separation.

The significant correlation between NO_2^--oxidizers and both NO_3^- and NH_4^+ concentrations may be a consequence of NO_2^--oxidizer numbers being indirectly dependent on NH_4^+ concentrations for a source of substrate (NO_2^-), with the product of their nitrification activity (NO_3^-) being correlated to their numbers for two reasons: (a) oxidation of NO_2^- to NO_3^-, and (b) reduction of NO_3^- to NO_2^- by NO_2^--oxidizers. Nitrifier denitrification has been reported by Tanaka *et al.* (1983), Poth and Focht (1985), Sundermeyer-Klinger *et al.* (1985), Freitag *et al.* (1987) and Bock *et al.* (1988, 1990), that is, partial recycling of NO_3^- to the NO_2^- pool, thereby providing their own substrate for nitrification.

Laanbroek and Gerards (1993) have also shown that oxygen limitation not only stimulates partial denitrification, but also nitrification. Under conditions of limited O_2 supply, some NO_2^--oxidizers are repressed, while the NH_3-oxidizers are still able to consume O_2, a potential cause of NO_2^- accumulation.

The correlation of mineral N concentrations with nitrifier numbers may, however, be misleading. Concentrations represent net values of production and consumption only, and it is the fluxes themselves that are more important with respect to bacterial activity. In addition, numbers of bacteria are not necessarily related to their activity and a correlation between nitrifier numbers and mineral N concentrations may be due to other variables related to both bacterial numbers and NO_2^- concentrations.

Conditions inhibitory to NO_2^--oxidizers are relatively well known (Belser, 1979; Prosser, 1989), NO_2^--oxidizers being more sensitive to high soil pH, NH_3 and light than NH_3-oxidizers. NO_2^--oxidizers may also be inhibited in the presence of low molecular weight soil organics. This has been attributed to the inhibitory effect of hydroxylamine released by NH_3-oxidizers in the presence of additional electron donors in the form of simple organic compounds (Stüven *et al.*, 1992). Although this has not been proven in the field, the availability of easily oxidizable organic compounds in a grassland soil might stimulate the local accumulation of NO_2^-. Previous studies of NO_2^- formation in soils have focused on the occurrence of NO_2^- as a consequence of nitrification (e.g. Chapman and Leibig, 1952; Wetselaar *et al.*, 1972; Chalk *et al.*, 1975). The persistence of measurable NO_2^- flushes in soil has been attributed to inhibition of NO_2^--oxidizers by high soil pH and NH_4^+ concentrations. These conditions will occur in the region of urine spots and from the application of artificial fertilizers, especially urea.

The three laboratory incubation studies provided further evidence that NO_2^- flushes occur in soil when NH_4^+ concentrations and pH are elevated. When NH_4^+-N (500 μg ml^{-1} soil solution) and $Ca(OH)_2$ (10 mg g^{-1} soil) were applied to soil, the second stage of nitrification (NO_2^--oxidation) was temporarily inhibited until NH_4^+ concentrations and soil pH declined (Table 6). However, pH appears to have a greater inhibitory effect on NO_2^--oxidizers than elevated NH_4^+ concentrations, since a small NO_2^- flush occurred when the soil pH was elevated at background NH_4^+ concentrations, whereas no NO_2^- flush occurred when NH_4^+ concentrations were elevated with no $Ca(OH)_2$ additions. The time–course incubation gave further information regarding the extent and duration of the flush of NO_2^- in this soil. Other reported NO_2^- flushes are of differing magnitude and length, due to use of different soil types and concentrations of N fertilizer (e.g. Pang *et al.*, 1973, 1975a, b).

In the first two of the three laboratory experiments relatively large concentrations of NO_2^- were induced. Although the conditions used to bring about the flush were not truly representative of the bulk of the soil in the field environment, localized soil NH_4^+ concentrations and pH may be similar to those used in the incubations, especially in the region of urea pellets and urine patches. The highest rate of $Ca(OH)_2$ application not only inhibited NO_2^--oxidation, but also NH_4^+-oxidation. As a result, very little NO_3^- was formed in these treatments, and NH_4^+ concentrations remained comparatively high throughout the incubation. The third laboratory experiment used the surface application of forms and concentrations of N commonly encountered in the field. The flush of NO_2^- which occurred with the incubation of soil with urea, was comparable to the highest concentration of NO_2^- measured in the field study, i.e. ca. 3 μg NO_2^--N g^{-1} soil, indicating urine-derived N depositions as potentially important sites for NO_2^- accumulation in the field.

The accumulation of NO_2^- in soil treated with urea in both the field and laboratory incubations has

been well-documented (Chapman and Leibig, 1952; Wetselaar *et al.*, 1972; Passioura and Wetselaar, 1972; Pang *et al.*, 1973, 1975a b; Chalk *et al.*, 1975; Christianson *et al.*, 1979; Yadvinder-Singh and Beauchamp, 1986, 1988; Magalhaes *et al.*, 1987). Although the plots in our field study were not treated with urea fertilizer, a source of urea would be urine patches from grazing steers. The correlation of NO_2^- with properties associated with urine patches could indicate its occurrence as a result of nitrification of urea-derived NH_4^+. From the spatial study, significant and positive correlations of NO_2^- with NH_4^+ and pH emerged several times, with K being positively correlated with NO_2^- once. That is, NO_2^- concentrations were higher where concentrations of NH_4^+ and K were high, and where soil pH was elevated—conditions which will prevail in or around urine patches. These results suggest that NO_2^- in the field is occurring, at least in part, as a result of nitrification of urine-derived N. Monaghan and Barraclough (1992) attributed the occurrence of large amounts of NO_2^- in urine-treated soils to the inhibition of NO_2^--oxidizer activity during nitrification due to unfavourable pH and NH_3 concentrations.

Although nitrification appears to be a common source of NO_2^- in field and laboratory studies, it is probably not the sole process responsible for NO_2^- formation. Results from the field study repeatedly selected NO_3^- as a variable correlated with NO_2^-. This close and recurring association of NO_2^- with NO_3^- suggests two possibilities. Firstly, that NO_2^- is related to NO_3^- due to nitrification. Secondly, that NO_3^- reduction could also be partly responsible for NO_2^- formation in soil. One process involving the reduction of NO_3^- to NO_2^- is biological denitrification. In the field, the rate of this process is governed by a range of environmental factors, and has been described as the most difficult of all biogeochemical processes to study, due to its high variability (Tiedje *et al.*, 1989). Parkin (1987), Robertson and Tiedje (1988), Groffman and Tiedje (1989), Christensen *et al.* (1990) and Pennock *et al.* (1992) and others have studied the spatial variability of denitrification and the rates of this process often display a highly positively skewed distribution. This same skewed distribution was found for NO_2^- concentrations, most being in the lower concentration ranges, with a few nitrite "hotspots".

If denitrification was responsible for the accumulation of NO_2^-, then NO_2^- concentrations may be expected to correlate with factors associated with anaerobic conditions, since denitrification has traditionally been viewed as a primarily or even exclusively anaerobic process. NO_2^- was found to correlate positively with MC (Table 3), a variable indicative of cores where O_2 concentrations would tend to be low. Even where NO_2^- concentrations did not correlate with variables associated with anaerobic conditions [or correlated negatively with such variables, e.g. negative correlation with Fe(II), Table 3], partial denitrification could still play a major role in NO_2^- formation. Aerobic denitrification may be environmentally widespread (Lloyd, 1993) and NO_2^- concentrations need not necessarily correlate with conditions indicative of anaerobism for its occurrence to be due to NO_3^- reduction. The presence of anaerobic microsites within soil aggregates (Parkin, 1987; Christensen *et al.*, 1990), within a population of cells (Poth and Focht, 1985) or even on a subcellular level (Focht, 1992), means that NO_3^- can become reduced in an apparently aerobic soil.

To conclude, the formation of NO_2^- in soil appears to be as a result of a combination of several processes, its persistence being a result of differences in the rates of processes contributing to its production and consumption. Several environmental factors will influence these processes differentially, and may result in production rates of NO_2^- being in excess of the rates of NO_2^- consumption.

The occurrence of NO_2^- is highly variable spatially and with depth, and even where variables were selected as being highly correlated with NO_2^- concentrations, very little of the variation in the NO_2^- data was accounted for (see r^2 values, Table 3). There is evidence that NO_2^- can accumulate during nitrification or denitrification (complete and/or partial), although the relative contribution of these processes to NO_2^- occurrence is not known. Laboratory incubations with urea produced concentrations in the soil equivalent to those encountered in the field, suggesting that NH_4^+ oxidation accounts for a significant proportion of NO_2^- formed in this soil.

In order to determine the relative contribution of these processes to NO_2^- formation, mechanistic studies involving ^{15}N-labelled precursors and end-products are essential. This would allow studies on the fate of NO_2^- within the soil, its movement down the profile and its contribution to NO_2^- found in soil drainage and river waters.

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