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Availability of urea to autotrophic ammonia-oxidizing bacteria as related to the fate of ^{14}C- and ^{15}N-labeled urea added to soil

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Abstract Nitrate has been found to accumulate more rapidly in soils fertilized with urea than with inorganic sources of NH_4^+, despite the fact that nitrification must be preceded by hydrolytic decomposition. For acidic conditions, this finding has been attributed to limited uptake of NH_4^+ by ammonium-oxidizing bacteria (also reported herein), suggesting an advantage for direct utilization of a nonionizable N substrate such as urea. If the same advantage applies to urea-C, nitrification of urea-N would also be promoted in neutral or alkaline soils, as reported in numerous studies. To ascertain whether urea-C can be utilized directly by nitrifying organisms, NO_2^- production was measured for *Nitrosomonas europaea* and *Nitrosospira* sp. NPAV in minimal media with urea as the sole source of either C or C and N. Nitrite accumulated only with the latter organism, in which case nearly quantitative recovery was observed for N added as NH_4^+ and/or urea. In a subsequent study, recovery of ^{14}C and ^{15}N in gaseous, extractable, and hydrolyzable forms was determined after incubation with labeled urea for up to 29 days, by using two soils that differed markedly in physiochemical properties affecting nutrient availability. Results obtained in correlating ^{14}C incorporation in the amino acid fraction with ^{15}N accumulation as NO_3^- were consistent with the stoichiometry that would be expected if C fixation were driven by autotrophic nitrification. Our findings demonstrate unequivocally that urea is utilized as a source of C and N by nitrifying microorganisms, which may account for rapid nitrification of urea-N in soils.

Introduction

Ammonium-oxidizing bacteria (AOB) and NO_2^--oxidizing bacteria (NOB) are largely responsible for the net conversion of NH_4^+ to NO_3^- in the environment, and thus collectively contribute to offsite movement of fertilizer N. Both organic and inorganic N fertilizer sources yield NH_4^+ in soils; however, of the organic-N compounds that have been investigated as possible substrates for autotrophic NH_4^+ oxidation, direct utilization was demonstrated only for urea-N (Burton and Prosser 2001). This may account for the finding that urea was much more effective than NH_4^+ in stimulating NO_3^- production in an acidic heath soil, even when alkalization was employed to replicate the pH effect of ureolysis (de Boer et al. 1989). Unlike heath soils, arable land is typically limed to control acidity, yet studies with agricultural soils have repeatedly shown that nitrification proceeds more rapidly with urea than with other N sources, including those that generate alkalinity such as liquid anhydrous NH_3, $(NH_4)_2HPO_4$, or $(NH_4)_2CO_3$ (Hauck and Stephenson 1965; McInnes and Fillery 1989; Mulvaney et al. 1997a). This distinctive difference has important implications for the loss of NO_3^- by leaching and denitrification, and thereby becomes a source of economic and environmental concern, particularly since urea is the leading N fertilizer in the world market.

If the stimulating effect of urea on nitrification is not related to alkalization, then this effect presumably originates because of a nutritional advantage (C or N) to autotrophic ammonia-oxidizing bacteria. To date, attention has focused on urea as a hydrolyzable source of NH_4^+, which is believed to be of limited bioavailability under acidic conditions (de Boer and Kowalchuk 2001). Recent work with *Nitrosospira* sp. showed that at pH<5, growth occurs on urea but not on NH_4^+ (de Boer and Laanbroek 1989; Burton and Prosser 2001), which was attributed to diffusive uptake of the neutral urea molecule throughout the physiological pH range and subsequent intracellular ureolysis (Burton and Prosser 2001). This interpretation provides a plausible mechanism for urea-stimulated autotrophic nitrification under acidic conditions, in which case ammoniacal N occurs largely as NH_4^+, but is less applicable to neutral or alkaline soils that contain appreciable NH_3.

Besides providing 2 mol of oxidizable N, intracellular hydrolysis of urea liberates 1 mol of HCO_3^-, which could easily satisfy the C requirement for autotrophic NH_3 oxi-

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dation, estimated as 1 mol of C fixed per 35 mol of NH_3 oxidized (Alexander 1965; Prosser 1989). This superfluous source of cytoplasmic HCO_3^- provides an obvious advantage for growth on urea in acidic environments where carbonate solubility is limited; moreover, significant utilization would be expected under neutral or alkaline conditions owing to ureolytic production of nondiffusible HCO_3^- (Mobley and Hausinger 1989) in close proximity to the site of CO_2 fixation (Smith and Ferry 2000).

The primary purpose of our work was to test the hypothesis that urea is utilized by nitrifying organisms as a source of C. This was accomplished through (1) culture studies to assess the growth response of *Nitrosomonas europaea* and *Nitrosospira* sp. NPAV when urea supplied the only C in the media; and (2) a laboratory incubation experiment to trace ^{15}N and ^{14}C applied as urea to soils that differ widely in biological productivity. In addition, a study was conducted to evaluate the effect of pH on membrane permeability during ammoniacal N uptake by these organisms.

Materials and methods

Pure culture studies

Microorganisms

Culture studies were performed with two autotrophic ammonia-oxidizing bacteria, a urease-negative *N. europaea* (ATCC 25978) and a urease-positive *Nitrosospira* sp. NPAV supplied by J. I. Prosser, University of Aberdeen, UK.

Media

For N-uptake experiments, organisms were maintained and cultured on a minimal medium described by Sato et al. (1985), but with the following modifications. To study growth response to urea as a source of C or C and N, the medium was modified by replacing the carbonate buffer, or both the carbonate buffer and $(NH_4)_2SO_4$, with urea. As a negative control, Sato medium was used without a source of C.

Nitrogen-15 uptake

The effect of solution pH on permeation of bacterial cells at 25°C by ^{15}N added as labeled NH_4Cl (99 at.% ^{15}N; ICN Pharmaceuticals, Irvine, CA, USA) was examined using a standard assay for instantaneous uptake in a thick cell suspension (Marquis 1994). Cultures of *N. europaea* or *Nitrosospira* sp. NPAV were grown to log phase in 12 l Sato medium, after which cells were harvested by centrifugation (14,750 *g*, 15 min), washed three times with 2 mM phosphate (pH 8), and resuspended in an ammonium assay mixture (7.8 mM $^{15}NH_4Cl$ and 2 mM potassium phosphate buffered at pH 4, 5, 7, or 8) to achieve a concentration of 250 g cells (wet weight) l^{-1} in a 1.5-ml microfuge tube. After incubation for 10 min, cells were removed by centrifugation at 8,975 *g* for 2 min, and a 90- (*Nitrosospira*) or 175-µl (*Nitrosomonas*) aliquot of the supernatant was transferred to a 473-ml (1 pint) wide-mouth Mason jar containing 8 ml of deionized water and 1 ml of 14.3 mM unenriched $(NH_4)_2SO_4$. Accelerated diffusions to recover $^{15}NH_4$-N were performed on the aforementioned supernatant, and also on an identical aliquot of the ammonium assay mixture prior to addition of cells, using the technique described by Khan et al. (1997).

The permeability index, S_w, was calculated according to Marquis (1994) as

$$S_w = [V_a/V_p]/(C_a/C_f) - 1], \quad (1)$$

where V_a is the volume of ammonium assay mixture added to the cell pellet, V_p is the pellet volume (measured as wet weight assuming a density of 1.02 g ml^{-1}), C_a is the ^{15}N concentration of the ammonium assay mixture, and C_f is the final ^{15}N concentration of the supernatant. Correction for dilution by interstitial water in the cell pellet was based on incubation of cells with a nonpermeating substrate (dextran, M_W >2×10^6 Da; Sigma, St. Louis, MO, USA). The corrected uptake value, R_w, describes the fraction of the cell volume penetrated by the solute, and was obtained in our work using the equation of Marquis (1994), as

$$R_w = (S_{sol} - S_{dex})/(1 - S_{dex}), \quad (2)$$

where S is the permeability index for the ^{15}N solute (sol) or for dextran (dex).

Growth response to urea-C

After preliminary work revealed appreciable scavenging of CO_2 from ambient air by urease-negative *N. europaea*, subsequent studies with *Nitrosospira* sp. NPAV investigated the direct utilization of urea-C by measuring NO_2^- accumulation in the absence of atmospheric CO_2. This was accomplished by adding 20 ml modified Sato medium through the septum of a sealed 120-ml serum bottle that had been purged of ambient air with a 0.2-µm filter sterilized mixture of N_2 and O_2 (approximately 79% N_2 containing less than 1 µl l^{-1} CO_2; Praxair, Danbury, CT, USA). After introducing 1 ml inoculum in log phase, cultures were incubated for 39 days at 25°C in the absence of light, during which 100-µl aliquots were withdrawn with a sterile syringe at various intervals. Colorimetric analyses for NO_2^--N were performed by the Griess–Ilosvay method (Mulvaney 1996) using a microplate reader (Spectromax Model 190; Molecular Devices, Sunnyvale, CA, USA) for absorbance measurements, with or without dilution to ensure that all absorbance values were within the range for calibration.

Table 1 Selected properties of soils

Soil	pH[a]	Organic C ($g\ kg^{-1}$)	Total N ($g\ kg^{-1}$)	Sand ($g\ kg^{-1}$)	Silt ($g\ kg^{-1}$)	Clay ($g\ kg^{-1}$)	WHC ($l\ kg^{-1}$)	CO_3^{2-}-C ($g\ kg^{-1}$)	CEC ($cmol\ kg^{-1}$)	Urease activity ($g\ urea\ kg^{-1}\ h^{-1}$)
Bloomfield	7.8	3.0	0.57	830	80	90	0.33	0.2	1.4	8.5
Drummer	7.1	31.2	2.14	170	530	300	0.58	0.3	22.4	89.5

WHC Water holding capacity
[a]Soil/water ratio 1:1

Soil study

Soil

The soils used (Table 1) were surface (0–15 cm) samples of two Illinois soils chosen for their contrasting properties. These soils included an uncropped sandy soil under coniferous vegetation (Bloomfield), and a poorly drained soil from a grass border at the Crop Sciences Research and Education Center at Urbana, IL (Drummer). Before use, the soils were air-dried and crushed to pass through a 2-mm screen. Cation exchange capacity (CEC) was estimated using a rapid saturation–diffusion technique (Mulvaney et al. 2004). The other analyses reported in Table 1 were performed as described by Marsh et al. (2004a).

Experimental approach

To ascertain whether nitrification of urea-N is affected by the associated carbonyl moiety, an incubation experiment was conducted that examined the fate of ^{14}C and ^{15}N applied to soil as doubly labeled urea. In this experiment, a 5-g sample of air-dried soil was placed in a 57-mm-diameter Al weighing dish and treated with 0.5 ml (Bloomfield) or 1 ml (Drummer) of deionized water containing sufficient ^{14}C- and ^{15}N-labeled urea [^{14}C (60,500–113,200 Bq ml^{-1}), 1.459–1.504 at.% ^{15}N] to achieve a concentration of 100, 500, or 1,000 mg N kg^{-1} for the Drummer soil, and 1,000 mg N kg^{-1} for the Bloomfield soil.[1] After adjusting the soil to 60% of water-holding capacity and mixing the urea-treated soil, the dish was placed in a 473-ml Mason jar. The jar was then sealed by attaching a lid that had been fitted with a rubber sleeve stopper and modified as described by Khan et al. (1997) to support a 60-mm-diameter Petri dish containing 5 ml H_3BO_3-indicator solution (40 g H_3BO_3 l^{-1}).

Incubation of urea-treated soil was performed in the absence of light at 25°C for 1, 3, 7, 14, 21, or 29 days, after which a 50-ml sample of headspace atmosphere was withdrawn through the sleeve stopper with a gas-tight syringe and subsequently injected into a sealed Mason jar containing 1 ml 0.2 M NaOH in a 20-ml liquid scintillation vial. After removing the Petri dish from the incubation jar, a spatula was used to quantitatively transfer the soil sample from the weighing dish to a 125-ml screw-cap polyethylene bottle, and the sample was shaken for 1 h with 50 ml 2 M KCl. The soil extract was obtained by filtering the resulting suspension under vacuum through Whatman QM-A quartz filter material in a 5.5-cm (diam.) Büchner funnel, prior to rinsing the extraction bottle twice with 10 ml deionized water to ensure complete transfer of the incubated soil sample to the funnel. After evacuation to remove residual KCl, the soil sample was dried at room temperature for 2 days, transferred to a 125-ml Erlenmeyer flask fitted with a 24/40 standard-taper joint, and hydrolyzed by heating under reflux for 12 h at 110–115°C with 20 ml 6 M HCl and two drops of octanol. The hydrolysis mixture was filtered under vacuum to obtain the soil hydrolysate, followed by neutralization (pH 6.5–6.8) and dilution to 100 ml with deionized water (Mulvaney et al. 2001).

Samples incubated for 7, 14, 21, or 29 days were aerated at 72-h intervals after atmospheric sampling as described previously. Aeration was carried out by allowing the jar to stand open for 20 min to ensure complete replacement of the headspace atmosphere. During this period, soil moisture content was determined gravimetrically, and deionized water was added as required to replace evaporative loss. Incubations were resumed after sealing the jar to utilize the original H_3BO_3 solution.

To ensure complete absorption of CO_2, the alkaline trapping solution was allowed to equilibrate for 2 days after injecting the atmospheric sample, prior to radioisotope analysis by liquid scintillation spectrometry. The latter technique was employed to analyze an aliquot of soil extract (1 ml), C recovered by combusting 0.1 ml soil hydrolysate with a biological oxidizer (R. J. Harvey, OX500, Hillsdale, NJ, USA), and carboxyl-C liberated from 10 ml hydrolysate during ninhydrin (triketohydridene hydrate) oxidation of amino acids (Marsh et al. 2004a).

Gaseous NH_3 collected during incubation was determined by titrating the H_3BO_3-indicator solution with 0.01 M H_2SO_4 using an automatic titrator (719 S Titrino; Metrohm, Herisau, Switzerland), followed by N-isotope analysis of the resulting $(NH_4)_2SO_4$ samples. Accelerated diffusion techniques were employed to recover (1) extractable (urea + NH_4^+)-N (Marsh et al. 2004b); (2) extractable inorganic N as NH_4^+, $NH_4^+ + NO_3^-$, or $NH_4^+ + NO_3^- + NO_2^-$ (Khan et al. 1997); (3) hydrolyzable NH_4^+-N, or (NH_4^+ + amino sugar)-N (Mulvaney and Khan 2001); and (4) hydrolyzable amino acid-N (Marsh et al. 2004a). In each case, quantitative determinations were performed by acidimetric titration, prior to processing for isotope-ratio analysis.

[1]The urea-N rates employed were selected to represent the concentration range expected for broadcast or band fertilization.

Isotopic analyses

Carbon

A liquid scintillation spectrometer (Tri-Carb Model 1900TR, Packard, Meriden, CT, USA) was employed for radioactive analyses to determine ^{14}C activity through internal calibration with corrections for background luminescence and chemiluminescence, following addition of 15 ml Scintiverse BD scintillation cocktail (Fisher Scientific, Pittsburgh, PA, USA). Percentage recovery of ^{14}C was calculated as $100AV/S$, where A is the measured activity per milliliter in the aliquot under analysis, V is the total volume from which the aliquot was obtained, and S is the quantity of radioactivity added.

Nitrogen

Following titration, samples were processed as previously described (Khan et al. 1997; Mulvaney et al. 1997c), for N-isotope analysis using a mass spectrometer equipped with an automated Rittenberg system (Mulvaney et al. 1990, 1997b; Mulvaney and Liu 1991). The isotope dilution equation,

$$A_sN_s + 0.3663N_a = (N_s + N_a)A_m, \quad (3)$$

was used to calculate for the sample under analysis, either atom % ^{15}N (A_s) or µg of N (N_s), from the measured value of atom % ^{15}N (A_m) and the µg of unlabeled NH_4^+-N added (N_a). The concentration of ^{15}N (C) used in Eq. (1) was calculated as A_sN_s/V, where V is the aliquot volume.

Whenever the titration indicated <50 µg of diffused N, 100 µg of N was added to the sample as unlabeled $(NH_4)_2SO_4$, and A_m was subsequently corrected for the resulting isotopic dilution. Percentage recovery of the labeled N (R) was calculated as

$$R = N_s(A - B)/50(U - B), \quad (4)$$

where N_s is the amount of N (µg) determined by titration, A is the amount of ^{15}N (at.%) obtained by direct measurement (A_m) or with isotopic dilution (A_s), and the remaining variables represent the amount of ^{15}N (at.%) measured for the labeled urea-N added (U) and the background concentration of ^{15}N (B) in the fraction under analysis. For extractable or hydrolyzable forms of N, the latter value was obtained using soil that had neither been treated with urea nor incubated. Because this approach precluded volatilization, the conventional value for natural ^{15}N abundance (0.3663 at.%) was used as a background concentration (B) to calculate recoveries reported for NH_3. Percentage recovery of urea-^{15}N in four of the eight fractions studied was calculated by difference, namely urea-N [as (urea + NH_4^+)-N - NH_4^+-N], NO_3^--N [as (NH_4^+ + NO_3^-)-N - NH_4^+-N], NO_2^--N [as (NH_4^+ + NO_3^- + NO_2^-)-N - (NH_4^+ + NO_3^-)-N], and amino sugar-N [as hydrolyzable (NH_4^+ + amino sugar)-N - NH_4^+-N].

Data analysis

All experiments reported were performed in triplicate. Data obtained for replicate samples were characterized by computing means and standard deviations. In the ^{15}N-uptake study, mean values for each organism were compared on the basis of a least significant difference (LSD) at the 0.05 probability level. Simple regression analyses were employed to correlate extractable ^{14}C with urea-^{15}N, amino acid-^{14}C with $^{15}NO_3^-$, amino acid-^{14}C with amino acid-^{15}N, and $^{14}CO_2$ with gaseous $^{15}NH_3$.

Results and discussion

Recent research suggests that the stimulating effect of urea on nitrification under acidic conditions may be related to the limited bioavailability of NH_4^+ (Burton and Prosser 2001; de Boer and Kowalchuk 2001). This possibility was evaluated by conducting a tracer study to estimate N uptake by two cultures of ammonia-oxidizing bacteria that were incubated at pH 4–5 or 7–8, so as to effect at least a hundred-fold difference in speciation of ammoniacal N. The results in Table 2 reveal significantly less uptake of NH_4^+ at pH 4 or 5 than at pH 7 or 8 for *Nitrosospira* sp. NPAV. This finding is consistent with previous reports that growth of this organism occurs over a more extensive pH range with urea than with inorganic N (Burton and Prosser 2001), suggesting a serious limitation in membrane permeability to the NH_4^+ ion. No pH effect on N uptake was observed for the urease-negative *N. europaea*, indicating that this type of limitation does not apply to all ammonia-oxidizing bacteria. The finding that substantial uptake of ammoniacal N occurred at pH 7 or 8 with both organisms provides conclusive evidence for the bioavailability of NH_3, and leaves no doubt that permeability limitations due

Table 2 Effect of pH on N uptake by autotrophic NH_3-oxidizing bacteria[a]

pH	Fraction of the cell volume penetrated, R_w[b]			
	N. europaea		*Nitrosospira* sp. NPAV	
	Mean	SD	Mean	SD
4	0.62	0.344	0.28	0.262
5	0.72	0.123	0.12	0.176
7	0.81	0.065	0.68	0.139
8	0.88	0.010	0.81	0.068
LSD (0.05)	0.35		0.33	

SD Standard deviation

[a] Cells harvested at log phase were incubated (25°C, 10 min) in 8 mM NH_4Cl (99 at.% ^{15}N) dissolved in 2 mM phosphate buffer (cell/solution ratio 1:4) at the pH specified

[b] Four analyses

to differences in NH_4^+ speciation alone cannot account for the rapid nitrification of urea-N in neutral or alkaline soils, as compared to inorganic-N fertilizers.

The HCO_3^- liberated through ureolysis has obvious implications for promoting autotrophic growth, so a study was conducted to examine the ability of *Nitrosospira* sp. NPAV to utilize urea-C in the absence of atmospheric CO_2. As expected, growth was negligible when the Sato medium was modified to eliminate the C source (Fig. 1). Introduction of urea as a source of both C and N resulted in 67% of maximal NO_2^- production if urea were utilized only as an energy source. As shown by Fig. 1, doubling the ammoniacal N concentration through addition of $(NH_4)_2SO_4$ led to a nearly stoichiometric increase in NO_2^- production, demonstrating that urea provided superfluous C to support complete oxidation of the N liberated through ureolysis.

If urea-C promotes autotrophic nitrification in soil, then some of this C should be incorporated into a biochemical fraction during production of NO_2^- and NO_3^-. Owing to the limited C requirement by nitrifying microorganisms, only trace incorporation would be expected, requiring the use of highly sensitive radiocarbon techniques for detection. In our work, these techniques were employed in studying the fate of ^{14}C and ^{15}N applied as doubly labeled urea to either a sandy soil (Bloomfield) exhibiting very limited ureolytic activity or a fine-textured soil typically used for intensive crop production (Drummer). Three application rates were employed with the latter soil, so as to simulate the concentration range obtained with normal fertilization practices. Incubations were carried out using a closed system that facilitated the recovery of C and N in mineralized and assimilated forms. Mineralization of C was determined by headspace analysis to avoid depleting the system of CO_2, which can lead to serious inhibition of autotrophic ammonia oxidation (Kinsbursky and Saltzman 1990; Saltzman and Brates 1990). A technique recently developed to detect ^{14}C incorporation into amino acids (Marsh et al. 2004a) was utilized in the present project as a means for direct measurement of autotrophic assimilation of urea-C.

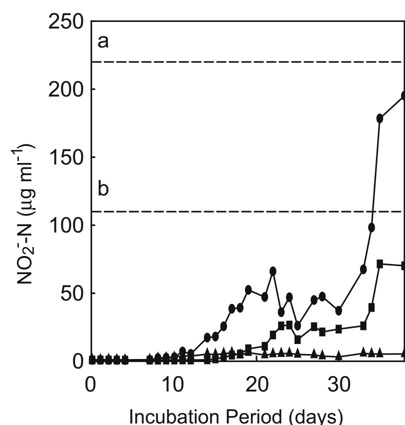

Fig. 1 Nitrite production by *Nitrosospira* sp. strain NPAV during incubation in Sato medium modified by replacement/deletion of $(NH_4)_2SO_4$ or carbonate buffer. The treatments were designed to eliminate the C source (▲), or provide urea as the sole source of C (●) or C and N (■). The theoretical maximum for NO_2^- production is represented by *broken line* **a** when urea was the sole source of C, and by *broken line* **b** for the remaining two treatments

Urea hydrolysis

As expected from the difference in urease activities for the two soils used (Table 1), ureolysis was almost ten times more rapid for the Drummer than for the Bloomfield soil. This is apparent from the decreases that occurred in urea concentration during the first 3 days when incubations were performed with 1,000 mg N kg^{-1} (Fig. 2a). In the case of the Bloomfield soil, hydrolysis was only 65% complete when incubation was terminated (after 29 days), whereas only with the highest application rate was urea detected after incubating the Drummer soil for 3 days.

Volatilization loss

Owing to a much lower CEC (Table 1) and buffering capacity, gaseous loss of urea-N as NH_3 was expected to be much more extensive with the Bloomfield than with the Drummer soil. At the end of the experiment, volatilization losses ranged from 6 to 50% of the applied N (Fig. 2b), which is typical for laboratory incubations in a closed vessel containing an acidic absorbent (Hargrove 1988). When expressed as a percentage of urea-N hydrolyzed, volatilization losses averaged 80% for the Bloomfield soil, compared to 35% for the Drummer soil, which decreased to 8% as the N application rate was reduced from 1,000 to 100 mg kg^{-1}.

Nitrification

If not eliminated through volatilization upon hydrolysis, urea-N was subject to assimilation (immobilization) or nitrification. The course of the latter process is shown in Fig. 2 as recoveries of extractable NH_4^+ (C), NO_2^- (D), and NO_3^- (E). In the case of the Drummer soil, a transient accumulation of exchangeable NH_4^+ preceded a pulse of NO_2^-, which was consumed as NO_3^- accumulated under the aerobic conditions employed in our work. Owing to limited hydrolysis and extensive volatilization, these trends were not observed for the Bloomfield soil.

Assimilation

Microbial assimilation of urea-^{15}N would lead to incorporation of label into a variety of nitrogenous forms that can be released by acid hydrolysis, so this process was employed in our work to recover hydrolyzable NH_4^+, amino sugar-N, and amino acid-N. Concentrations of hydrolyzable NH_4^+ remained relatively constant regardless of incubation period (Fig. 2f), which is consistent with more

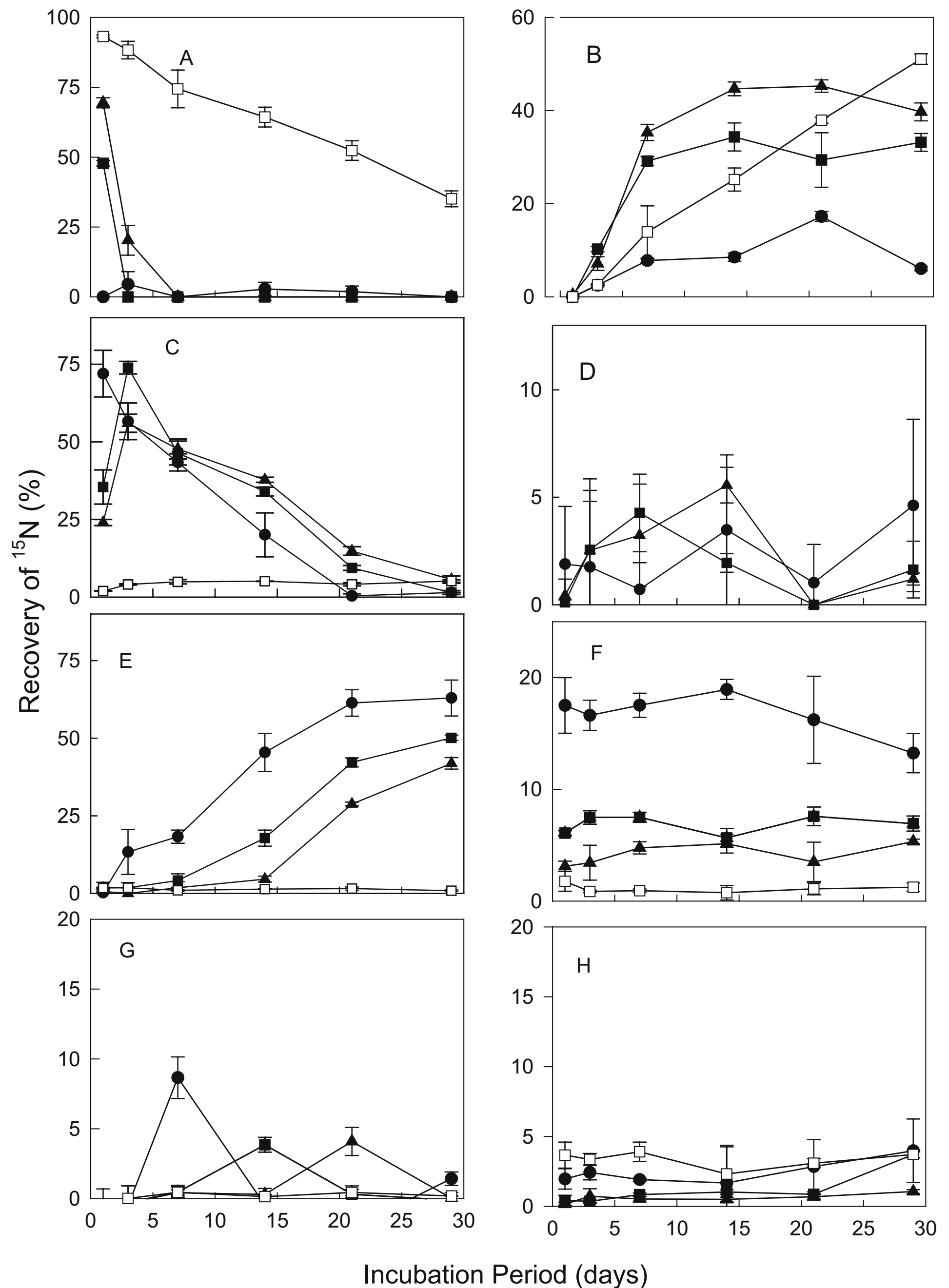

Fig. 2 Fate of ^{15}N applied as urea to the Bloomfield (1,000 mg N kg^{-1}) or Drummer (100, 500, or 1,000 mg N kg^{-1}) soil. Following incubation, recovery of ^{15}N was determined as urea (**a**), gaseous NH_3 (**b**), 2 M KCl extractable NH_4^+ (**c**), NO_2^- (**d**), and NO_3^- (**e**); as well as 6 M HCl hydrolyzable NH_4^+ (**f**), amino sugars (**g**), and amino acids (**h**). In each panel, data for the Bloomfield soil are marked with *open squares*, and the three application rates used with the Drummer soil are represented as follows: 100 mg kg^{-1} (●), 500 mg kg^{-1} (■), and 1,000 mg kg^{-1} (▲). Data are reported with *error bars* representing one standard deviation above and below the mean of three replicates

extensive tracer recovery in the abiotic portion of this fraction (nonexchangeable NH_4^+), as compared to assimilatory N that was deaminated during acid hydrolysis.

Comparison of Fig. 2g, h reveals a very different pattern in recovery of urea-^{15}N for hydrolyzable amino sugars and amino acids. In contrast to the limited ^{15}N incorporation observed for these fractions with the Bloomfield soil, data in Fig. 2g for the Drummer soil show labeling of amino sugars to be a transient occurrence that was progressively delayed by increasing application of urea from 100 to 1,000 mg N kg^{-1}. This sort of temporal trend, which was observed only recently owing to improvements in methodology for determination of hydrolyzable soil N (Mulvaney and Khan 2001; Mulvaney et al. 2001), is probably attributable to heterotrophic incorporation of N into cell wall constituents and subsequent release during decay of microbial biomass. Assuming the pulses in recovery of amino sugar-^{15}N coincided with maximal microbial growth, a longer period would have been required to complete a growth cycle as substrate concentration increased. Although some variation occurred in recovery of α-^{15}N from amino acids liberated by hydrolyzing the Drummer soil, very little evidence was observed of a clearly defined temporal trend. This finding supports recent evidence (Mulvaney et al. 2001) that amino acids are much less labile than amino sugars.

Recovery of ^{14}C and ^{15}N

Although ureolysis generates 1 mol of CO_2 for every mole of urea hydrolyzed, a stoichiometric relationship would not be expected between the recovery of gaseous ^{14}C and the extent of hydrolysis without also accounting for precipitation of labeled carbonates, which would accumulate as alkalinity is generated through urea hydrolysis. As the incubations in our work were performed without alkaline trapping to collect CO_2, none of the C fractions studied would have included carbonate, and hence total recoveries were lower for ^{14}C (58–94%) than for ^{15}N (88–102%). After incubation for 29 days, radiocarbon recoveries as CO_2 ranged from 45 to 85% (Fig. 3a), which represented 75–85% of the urea hydrolyzed. The latter deficits were virtually eliminated by an equilibrium adjustment of $^{14}CO_2$ recoveries to account for the magnitude by which total ^{14}C recovery was underestimated, implicating carbonate precipitation as the key factor that prevented stoichiometric measurement of the C liberated by ureolysis.

Recoveries of ^{14}C extractable with 2 M KCl (Fig. 3b) followed the same trend observed for urea-^{15}N (Fig. 2a), and, as shown by Table 3, these two parameters were correlated very highly significantly (r=0.98–0.99) with a slope of approximately 1.0. Based on this evidence, there is little doubt that extractable ^{14}C occurred entirely as urea.

Following extraction of inorganic N and urea with 2 M KCl, the residual soil would have contained ^{14}C in the form of carbonates or biosynthesized organic compounds. Any carbonates present would have been liberated as CO_2 during subsequent acid hydrolysis, such that total radiocarbon analysis of the hydrolysate (Fig. 3c) would provide an indication of chemoautotrophic assimilation of urea-C. Regardless of urea application rate or soil type, recovery of hydrolyzable ^{14}C was maximized within the first 2 weeks of incubation and tended to decrease thereafter. Maximal recovery was higher for the Drummer (0.8–1.3%) than for the Bloomfield soil (0.2%), which is consistent with the difference observed between these soils in assimilation of urea-^{15}N. The transient nature of the recoveries reported in Fig. 3c can be attributed to heterotrophic utilization of fixed C, which would have led to a gradual loss of total hydrolyzable ^{14}C through selective decomposition of readily mineralizable substrates (e.g., amino sugars).

The ninhydrin reaction with amino acids affords a more specific means of detecting chemoautotrophic C assimilation, although the carboxyl-C would represent only a fraction of total amino acid-C. Despite this limitation, incorporation of ^{14}C was often detected in hydrolyzable amino acids, and recovery tended to increase over time (Fig. 3d), suggesting greater stability toward degradation than for other hydrolyzable constituents. Without further

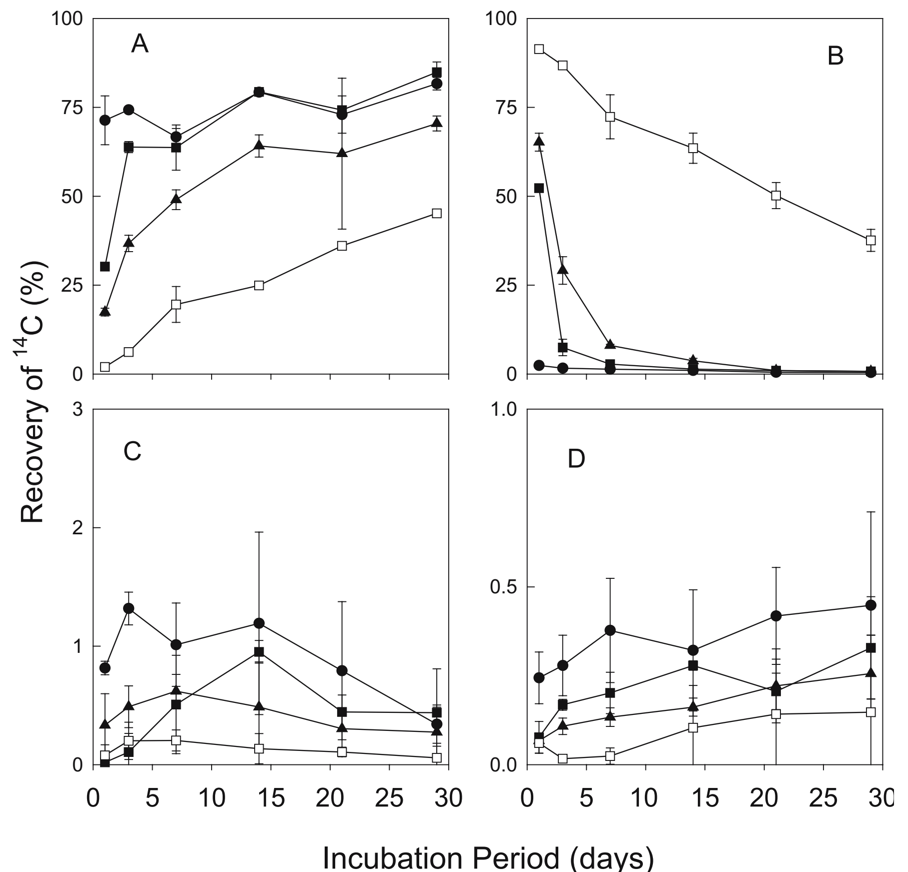

Fig. 3 Fate of ^{14}C applied as urea to the Bloomfield (1,000 mg N kg^{-1}) or Drummer (100, 500, or 1,000 mg N kg^{-1}) soil. Following incubation, recovery of ^{14}C was determined as headspace CO_2 (**a**), by radiocarbon analysis of 2 M KCl soil extracts (**b**) or 6 M HCl soil hydrolysates (**c**), and as amino acid-C released upon ninhydrin oxidation of hydrolysates (**d**). In each panel, data for the Bloomfield soil are marked with *open squares*, and the three application rates used with the Drummer soil are represented as follows: 100 mg kg^{-1} (●), 500 mg kg^{-1} (■), and 1,000 mg kg^{-1} (▲). Data are reported with error bars representing one standard deviation above and below the mean of three replicates

Table 3 Relationship between the distribution of ^{14}C and ^{15}N recovered in different Drummer soil fractions

Fraction of interest		N applied	Regression parameter[a]	
^{14}C	^{15}N	(mg kg^{-1})	Slope[b]	R^2
Extractable	urea	100	NR[c]	NR[c]
		500	0.95	0.98***
		1,000	1.08	0.97***
		100–1,000	1.00	0.96***
Amino acid	NO_3^-	100	69.37	0.18
		500	115.91	0.33**
		1,000	140.88	0.51***
		100–1,000	98.18	0.38***
Amino acid	amino acid	100	2.22	0.05
		500	10.20	0.63***
		1,000	1.65	0.19
		100–1,000	2.60	0.09
Gaseous[d]	gaseous[d]	100	4.92	0.42**
		500	21.73	0.63***
		1,000	21.72	0.40**
		100–1,000	19.01	0.57***

**Significant at $P<0.01$
***Significant at $P<0.001$
[a]Regression followed the form $y=mx+b$, where y is the % recovery in the ^{15}N fraction, x is the % recovery in the ^{14}C fraction, m is the slope, and b is the intercept (not reported)
[b]Mole ratios would be obtained by multiplying the values reported by 2.
[c]Not reported (urea-N undetectable)
[d]Correlated as $1/CO_2$ with NH_3

information concerning the amino acid composition, the same limitation would preclude an exact calculation to determine the mole ratio of urea-N oxidized to urea-C fixed, which would be useful in estimating the relative importance of urea for providing C to autotrophic nitrifying organisms. This ratio can, however, be ascertained for the carboxyl-C from the regression of percentage recoveries for amino acid-C and NO_3^--N (Table 3), provided the slope is doubled to account for the relative proportions of N and C in urea.

For the Drummer soil, significant correlations were obtained between the percentage of label recovered as amino acid-C and NO_3^--N, but only with 500 or 1,000 mg urea-N kg^{-1}. In these cases, an average of 257 mol of urea N was oxidized per mole of urea C fixed, which would decrease to one fifth of this value after estimating carboxyl-C as 20% of total amino acid-C, based on the carboxyl-C content of the six most abundant amino acids obtained from soil hydrolysates (Hayashi and Harada 1969; Kai et al. 1973). The resulting ratio (51 mol urea-N oxidized per mol urea-C) compares favorably with values of approximately 35 mol N oxidized per mol C fixed as commonly reported for *Nitrosomonas* or *Nitrosospira* species (Alexander 1965; Prosser 1989). These organisms provide a more conservative reference for this comparison than do NO_2^--oxidizing bacteria, owing to the greater energy yield associated with ammonia oxidation, which promotes C assimilation. There is thus reasonable evidence to conclude that urea applied to soil can contribute substantially in supplying the C required for autotrophic nitrification.

Although significant for one of the urea application rates, Table 3 shows that the correlation between amino acid-^{14}C and -^{15}N was weaker than for amino acid-^{14}C and $^{15}NO_3^-$, suggesting that different microbial populations were responsible for the assimilation of C and N. As also shown by Table 3, highly significant correlations were achieved in relating gaseous ^{14}C and ^{15}N, but only when regressions were performed using the reciprocal of $^{14}CO_2$ recoveries. The latter finding can be attributed to the opposing effects of pH on liberation of NH_3 and CO_2.

Conclusion

This work complements previous evidence that urea is directly utilized in acidic environments, and suggests that, even in neutral or alkaline soils, autotrophic NH_3-oxidizing organisms are capable of utilizing urea-C through ureolytic activity. Intracellular ureolysis by these organisms would enhance the uptake of urea while diminishing the uptake of NH_3 and CO_2, owing to elevated internal concentrations of HCO_3^- and NH_4^+. The stimulatory effect of urea would be less pronounced for NO_2^- oxidizers, which do not utilize NH_3 as an energy source, and would thereby contribute to the tendency that exists for NO_2^- accumulation in urea-treated soils. There are obvious environmental implications when a soil amendment supplies both C and N that can be utilized by autotrophic nitrifying organisms, particularly if this amendment is a common fertilizer material that also occurs as a major constituent of animal wastes.

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