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INCORPORATION AND EXTRACTABILITY OF RESIDUAL ^{15}N IN A CONIFEROUS FOREST SOIL

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Summary—The effect of ^{15}N labelling duration (24 h, 7 and 31 months) on the incorporation and extractability of residual ^{15}N in the humus (H) horizon of a Humo-Ferric Podzol was investigated. Extractability of residual ^{15}N was studied using 2 M KCl, 0.5 M K_2SO_4, autoclaving with 10 mM $CaCl_2$, and acidic permanganate and fumigation-extraction. Incorporation of ^{15}N into the classical fulvic acid (FA), humic acid (HA) and humin fractions was also studied. Greater amounts of total and applied (fertilizer) N were extracted from the 24 h than from the 7 and 31 months treatments ($P < 0.05$), with the difference between the last two non-significant. The $KMnO_4$ strength had no effect on the amount of N extracted. The extracted fractions were always enriched with ^{15}N relative to the bulk soil regardless of treatment. Among the extraction methods used, the autoclaving method extracted the greatest amount of total and applied N, but had the lowest extractability ratio (ER) for each treatment. The fumigation- and KCl-extraction methods were more desirable in obtaining biologically meaningful N fractions. Nitrogen-15 enrichment in microbial biomass was found similar to that in the inorganic N fractions extracted by KCl and K_2SO_4 for each labelling treatment. For the FA fraction, a greater percentage of applied N was recovered in the 24 h (52%) than in the 7 and 31 months treatments (3 and 2%, respectively), while less applied N was recovered in the 24 h (47%) than in the other two treatments (96 and 96%, respectively) for the humin fraction. ER values increased with shorter labelling duration and were always greater than 1 for the FA fraction, and less than 1 for the humin fraction. These results show that the extractability of residual ^{15}N was quickly reduced due to its incorporation into stable soil humin fraction. However, residual ^{15}N was more extractable than the bulk soil N regardless of the labelling duration used in this study. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The incorporation of fertilizer nitrogen into different soil organic matter (SOM) fractions is one of the major controls on the fate of applied fertilizer N (Schimel and Firestone, 1989a,b; Kelley and Stevenson, 1995). The longer the fertilizer N is applied, the more the N is immobilized by and stabilized in the soil through the repeated mineralization-immobilization process (Jansson and Persson, 1982). However, the mechanisms for applied N stabilization are largely unclear. The nature of low availabilities of residual fertilizer N one growing season after being applied, which is affected by the immobilization and stabilization of applied N by SOM, is a question that needs to be addressed (Chang *et al.*, 1997). Knowledge about factors affecting variations in the availability and extractability of applied N immobilized by SOM may improve our understanding of the N immobilization-mineralization processes (Legg *et al.*, 1971).

The availability of soil N can be evaluated by chemical extraction methods to overcome the tedious and time consuming nature of methods employing lengthy incubations or methods measuring actual plant N uptake (Kelley and Stevenson, 1985). In trying to find the best extraction method to measure the availability of N in soils, researchers have developed various procedures; for example, extraction with HF (Stevenson *et al.*, 1967), HCl hydrolysis (Stewart *et al.*, 1963; Porter *et al.*, 1964), extraction with salts, i.e., 10 mM $NaHCO_3$ (MacLean, 1964), 0.5 M K_2SO_4 (Brookes *et al.*, 1985), and 2 M KCl (Bremner, 1965), autoclaving in water or 10 mM $CaCl_2$ (Stanford and DeMar, 1969), and organic matter fractionation (He *et al.*, 1988; Azam *et al.*, 1989a). Extractions with salts (2 M KCl and 0.5 M K_2SO_4) and autoclaving with 10 mM $CaCl_2$ have been the most widely used methods that give a measure of the available N (Keeney, 1982). Fumigation with chloroform has been shown to release a biologically meaningful N fraction (Juma and Paul, 1984). Others (Stanford and Smith, 1978) suggested that NH_4^+ released by partial oxidation of soil organic N with dilute per-

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manganate solutions comes from a soil fraction readily susceptible to biological mineralization. For the objectives stated below, we used extraction with 2 M KCl and 0.5 M K_2SO_4, autoclave, and fumigation-extraction to study soil N extractibility.

While studies on N incorporation with labelled N applied for a prolonged period (McGill and Paul, 1976; Smith and Power, 1985) and for a very short period, e.g., from a few hours to a few days (He *et al.*, 1988; Schimel and Firestone, 1989a) are available, studies are needed where N is applied for both short and long terms. A study on the sequential changes in residual N extractability in samples with N applied at different times may yield useful information particularly on the dynamic aspects of N incorporation into different SOM fractions. Information from the extraction study, where the different methods extract N from different pools, and from humus fractionation, may indicate the movement of applied N between different pools with time and may improve our understanding of the N mineralization-immobilization-turnover (MIT) process.

As part of the Salal-Cedar-Hemlock integrated research program (Prescott and Weetman, 1994), we have been studying the fate of fertilizer N applied to western redcedar (*Thuja plicata* Donn ex D. Don) — western hemlock (*Tsuga heterophylla* (Raf.) Sarg) (CH) forest ecosystems (Chang *et al.*, 1996). The experimental set-up provided plots with ^{15}N-labelled fertilizer applied at different times, thus allowing us to examine the effect of labelling duration on the characteristics of residual fertilizer N in forest soils. The objectives of this study were (1) to determine the effect of labelling duration on the extractability of both total and applied N using various extraction methods; and (2) to investigate the effect of labelling duration on the stabilization of applied N into the recalcitrant fractions of soil organic matter.

MATERIALS AND METHODS

The site

The study site is in the very wet maritime subzone of the Coastal Western Hemlock (CWH) biogeoclimatic zone (Pojar *et al.*, 1991), located near Port McNeill (50° 36'N, 127° 15'W) on northern Vancouver Island, British Columbia, Canada. The site receives 1700 mm of precipitation annually, of which about 65% is received between October and February. The driest months are May, June, July and August. Mean daily temperatures vary from 3.0°C in January–February to 13.7°C in July–August (Lewis, 1982).

The site was clearcut and slash-burnt in 1985. The original vegetation cover before the clearcut was a very old western redcedar–western hemlock forest. The site occupies the middle to upper slopes in the topography. The mineral soil is an orthic Humo-Ferric Podzol (Agriculture Canada Expert Committee on Soil Survey, 1987), formed on unconsolidated glacial moraine and fluvial outwash (Lewis, 1982). A typical soil profile has the following horizons: LF layer, usually 10–25 cm in thickness (in the burned cutover sites, this horizon is often reduced to less than 5 cm because of burning); a thick humus layer mostly greater than 45 cm; a thin Ae layer; Bfh; Bf; and a BC or C horizon (A. Germain, 1985). The plots used for this study were located within an area of about 50 × 100 m and thus had relatively uniform conditions, especially as the sampling was confined to the H horizon.

Field labelling

Application of ^{15}N labelled fertilizer $(NH_4)_2SO_4$ (200 kg N ha^{-1}) to microplots (1 m radius) was conducted on 16 April, 1991, 24 April, 1993, and 1 December, 1993. When samples were collected from those microplots on 2 December, 1993, H horizon material was obtained with ^{15}N residence times of 24 h, 7 months, and 31 months. Nitrogen-15 enrichment in the fertilizer solution applied was 3.38044% for the 31 months and 2.37753% for the 7 months and 24 h treatments. In all situations, fertilizer solutions (in 2 L water) for each plot were applied using a watering can after removing the thin L/F layer. Details of the field labelling and sampling procedures can be found in Chang *et al.* (1997).

The top 10 cm of the H horizon material was collected from the whole plot in the field. Humus material collected from each plot form one sample (about 12 kg each, fresh weight). After the samples were brought into the laboratory, they were picked free of visible roots, sieved (8 mm), thoroughly mixed using a Monarch (Winnipeg, Canada) cement mixer, and subsampled (approx. 1 kg) for chemical extraction and organic matter fractionation studies in the laboratory.

In this study, because samples with ^{15}N labelled in the field were used, we were unable to avoid the potential effect of different weather conditions (^{15}N applied in April for the 31 and 7 months treatments and in December for the 24 h treatment) on N incorporation; however, the most we can predict of the effect of weather conditions on N transformation is the rate of N incorporation and N cycling

Laboratory and statistical analyses

Fresh samples of humus were used for the extraction studies. For the extraction with KCl, approximately 50 g (wet weight) sample was weighed into a 200 ml plastic bottle and about 50 ml of 2 M KCl was added. The mixture was shaken for 1 h and filtered through Whatman No. 42 filters on a vacuum filtration system. The extracts were kept in a freezer until analysis for total N and ^{15}N. A similar procedure was used for extraction with 0.5 M K_2SO_4,

except that the mixture was only shaken for 30 min. The extraction using 0.5 M K_2SO_4 is identical to that used in the fumigation-extraction method for quantifying microbial N (Brookes *et al.*, 1985).

The autoclaving procedure used was as follows: 3 g (air-dry basis) of H material was weighed into a 50 ml polyethylene test tube which can resist the heat and pressure of the autoclaving. Samples in the test tubes were amended with 25 ml 10 mM $CaCl_2$ and autoclaved for 16 h (121°C and 104 kPa). After the samples were cooled, they were centrifuged and filtered. Approximately 25 ml of 10 mM $CaCl_2$ was used to wash the residue, which was again centrifuged and filtered. The combined extracts were made to 100 ml and frozen until further chemical analysis.

For the fumigation-extraction experiment, fresh samples of approximately 25 g (wet weight) were fumigated with ethanol-free chloroform for 24 h at room temperature (20°C). After removal of the chloroform, the fumigated samples were extracted with 0.5 M K_2SO_4 on an end-over-end shaker (150 rev min^{-1}) for 0.5 h, followed by vacuum filtration through Whatman No. 42 filters (Brookes *et al.*, 1985; Chang *et al.*, 1995).

Samples (5 g air-dry) were extracted with 50 ml 0.5 M H_2SO_4, or with 10 mM, 20 mM, or 50 mM $KMnO_4$ (in 0.5 M H_2SO_4) for 1 h. The oxidative release of NH_4–N (data reported in Table 2) from SOM was obtained by subtracting NH_4–N extracted by 0.5 M H_2SO_4 from that extracted by the acidic permanganate.

Organic matter fractionation followed the method of Schnitzer (1982). Briefly, 15 g of air-dried sample was weighed into a centrifuge bottle, amended with 100 ml of 0.5 M K_2SO_4 and shaken for 0.5 h. After centrifugation, the supernatant was discarded by decantation. This step was used to remove the K_2SO_4 extractable N in the samples. The residue was then amended with 150 ml 100 mM NaOH (1:10 soil:NaOH solution) and shaken for 24 h at low speed. After centrifugation (at 1000 *g* for 30 min), the supernatant was recovered by decantation. The residue (the humin fraction) from this step was washed twice with about 30 ml of deionized water. The recovered supernatant solutions were combined and acidified to pH 1 using 3 M H_2SO_4. After being left to settle for 24 h, the mixture was centrifuged and separated into fulvic (FA, in the solution) and humic (HA, the precipitate) acids. The HA was washed with deionized water twice and the solution recovered after centrifugation was added to the FA fraction. The FA fraction was made to 200 ml and the HA fraction was redissolved in 100 mM NaOH and made up to 100 ml. The humin fraction was transferred to a paper bag and air-dried.

Extracts from the extractions and the autoclaving experiment were analyzed for NH_4^+–N by steam distillation with MgO and titration (Keeney and Nelson, 1982). The FA and HA solutions were measured for total N by the Kjeldahl digestion and steam distillation method (Bremner and Mulvaney, 1982). The distillates from the above inorganic or total N measurements, collected in boric acid–ethanol, were dried at 70°C and analyzed for ^{15}N abundance as described in Preston *et al.* (1990). Spiking with a standard was used to raise the total N content in each sample for some of the samples before ^{15}N analysis.

According to Azam *et al.* (1989b), the extractability ratio proposed by Legg *et al.* (1971) can be simplified as follows:

$$\text{Extractability ratio} = \frac{\text{Atom\% } ^{15}\text{N of the extracted N}}{\text{Atom\% } ^{15}\text{N of the total soil N}}$$

If the incorporated fertilizer N has the same chemical extractability as that of the total soil, the extractability ratio will be one (Legg *et al.*, 1971). The calculation of applied N in each extracted fraction followed He *et al.* (1988). Therefore, "total N" in this paper refers to the sum of native and applied N in any analysis.

Statistical analysis was performed using the commercial statistical analysis system (SAS) software (SAS Institute Inc., 1989). Group means of independent variables were compared between treatments for each extraction method (or permanganate strength) or between methods (or concentrations) of each treatment using Scheffe's multiple range test if interactions were significant. Otherwise, multiple comparisons were performed for treatment and method (or concentration) means using Scheffe's multiple range test.

RESULTS

Effect of labelling duration on N extractability

Analysis of variance results showed that there were non-significant treatment × extraction method interactions for the total N extracted (ANOVA not shown). Multiple comparison analysis showed that there was no significant difference for the total amount of N extracted between the 7 and 31 months treatments; however, a greater amount was extracted from the 24 h treatment than from the other two treatments ($P < 0.05$, Table 1). The total N extracted represented from 8.45 to 13.38%, 0.03 to 3.54%, and 0.15 to 4.51% of the total soil N, for the 24 h, 7 and 31 months treatments, respectively, depending on the extraction methods used. There were significant treatment × extraction method interactions (ANOVA not shown) for the percentage of total soil N extracted, the applied N extracted, the applied N extracted as a percentage of total N extracted, and the extractability ratios. Therefore, multiple comparison was performed on treatments of each extraction method, or on extrac-

Table 1. Extractability ratios and total and applied N extracted by various extraction methods in samples with ^{15}N labeled for different durations

Treatment	Extraction method				
	2 M KCl	0.5 M K_2SO_4	Autoclave	Fumigation	Average
			Total N extracted ($\mu g\ g^{-1}$ soil)		
31 months	20.66	22.87	634.19	191.57	217.32 a*
7 months	4.93	18.31	605.39	243.72	218.09 a
24 h	1086.09	1032.06	1616.44	1632.84	1304.36 b
Average	370.56 A†	357.75 A	952.00 B	639.38 C	
			Total N extracted as a % of total soil N		
31 months	0.15 a A	0.16 a A	4.51 a B	1.37 a A	
7 months	0.03 a A	0.11 a A	3.54 a B	1.43 a AB	
24 h	8.89 b A	8.45 b A	13.26 b B	13.38 b B	
			Applied N extracted ($\mu g\ g^{-1}$ soil)		
31 months	2.52 a A	2.21 a A	29.76 a A	22.24 a A	
7 months	1.01 a A	1.96 a A	32.74 a A	45.86 a A	
24 h	949.25 b A	907.83 b A	866.14 b A	1298.84 b B	
			Applied N extracted as a % of total N extracted		
31 months	11.98 a A	9.09 a A	4.67 a A	11.48 a A	
7 months	22.06 a A	10.70 a AB	5.43 a B	18.74 a A	
24 h	87.35 b A	87.68 b A	53.45 b B	79.36 b A	
			Extractability ratios		
31 months	3.29 a A	2.52 a AB	1.16 a B	3.16 a A	
7 months	5.09 ab A	2.48 a BC	1.17 a C	4.30 ab AB	
24 h	5.99 b A	6.00 b A	3.55 b B	5.43 b AB	

*The same lower case letters indicate that there was no significant ($P = 0.05$) difference between the treatments for each extraction method.

†The same upper case letters indicate that there was no significant ($P = 0.05$) difference between the extraction methods at each treatment level.

tion methods of each treatment. It was clear that, regardless of the extraction method used, a greater percentage of the total soil N was extracted from the 24 h than from the 7 and 31 months treatments ($P < 0.05$). However, there was no difference in the percent of total soil N extracted between the 7 and 31 months treatments (Table 1).

The amount of applied N extracted followed fairly closely that of the total N extracted. A greater amount of applied N extracted in the 31 than in the 7 months treatment by the KCl and K_2SO_4 methods vs a greater amount of applied N extracted in the 7 than in the 31 months treatment by the autoclaving and fumigation methods resulted in a significant treatment × extraction method interaction. However, regardless of the extraction method used, the only significant difference was between the 24 h and the other two treatments ($P < 0.05$, Table 1). There was consistently a greater percentage of applied N in the total N extracted for the 7 than for the 31 months treatment, although the difference was non-significant. The greatest percentage of applied N extracted was in the 24 h treatment.

The extractability ratio increased as the labelling duration decreased. Extractability ratios were all greater than 1, indicating that the extracted fractions were always more enriched with ^{15}N than the bulk soil. In other words, the amended N was always more extractable than total N in this study. The significant treatment × extraction method interaction for the extractability ratio was caused by the outstandingly high value in the 7 months treatment of the KCl extraction and the apparently low value in the 24 h treatment of the autoclaving method.

Effect of extraction method on N extractability

On average, of the four extraction methods used, the autoclaving method extracted the most total N and the KCl and K_2SO_4 methods extracted the least (Table 1). There were no significant differences between the 2 M KCl and 0.5 M K_2SO_4 extraction methods, except for the extractability ratio of the 7 months treatment. The autoclaving method extracted a greater percentage of the total soil N than the KCl and K_2SO_4 methods ($P < 0.05$), regardless of the treatment (Table 1). Fumigation of the samples resulted in an intermediate percentage of the total N being extracted for the 7 and 31 months treatments, while a percentage similar to the autoclaving method was found for the 24 h treatment.

For the applied N extracted, the only significant difference among the extraction methods was between fumigation-extraction and the other three methods for the 24 h treatment. The greater amount of applied N recovered in the fumigation-extraction than in the other three methods was due to the release of the extra N immobilized in the microbial biomass. The amounts of applied N recovered by the autoclaving and fumigation methods were higher than that recovered by the KCl and K_2SO_4 extraction methods for the 7 and 31 months treatments. However, the differences were not statistically different due to high variations in the data set. Comparing the applied N and the total N extracted, it was obvious that the autoclaving method extracted a lower percent of applied N in the total N extracted than the other three methods.

Table 2. Extractability ratios and total and applied N extracted by $KMnO_4$ in samples with ^{15}N labeled for different durations

Treatment	$KMnO_4$ concentration			Multiple comparison
	10 mM	20 mM	50 mM	
		Total N extracted ($\mu g\ g^{-1}$ soil)		
31 months	31.24	44.14	52.18	a
7 months	20.52	32.34	39.43	a
24 h	1480.28	1520.27	1505.54	b
M-C[1]	A	A	A	
		Total N extracted as a % of total soil N		
31 months	0.22	0.31	0.37	a
7 months	0.12	0.19	0.23	a
24 h	12.12	12.45	12.33	b
M-C	A	A	A	
		Applied N extracted ($\mu g\ g^{-1}$ soil)		
31 months	2.82	3.66	4.26	a
7 months	3.59	3.74	4.18	a
24 h	1295.42	1302.28	1219.89	b
M-C	A	A	A	
		Applied N extracted as a % of total N extracted		
31 months	9.39	8.03	7.90	a
7 months	17.82	11.67	10.62	b
24 h	87.41	85.40	80.75	c
M-C	A	AB	B	
		Extractability ratio		
31 months	2.67	2.21	2.15	a
7 months	4.07	2.66	2.49	a
24 h	5.97	5.83	5.52	b
M-C	A	AB	B	

*M-C: multiple comparison. The same lower case (upper case) letters indicate that there was no significant ($P = 0.05$) difference between the treatments (concentrations).

The extractability ratios, which combine information on the ^{15}N abundance both in the extracted fractions and the bulk soil, were far more diverse than the other measurements. The main difference was a smaller ER value for the autoclaving than for the other three methods. The similarity in ER values among the fumigation, KCl and K_2SO_4 extraction methods reveals that there was similar ^{15}N enrichment in the microbial biomass and in the extractable inorganic N, for each labelling duration.

Extraction with acidic permanganate

There were no treatment × concentration (of acidic permanganate) interactions for any of the parameters examined (ANOVA not shown), therefore, multiple comparison analysis was performed for treatment or concentration means (Table 2).

Acid permanganate solution concentration had no effect on total N extracted, applied N extracted and total N extracted as a percentage of total soil N. None of those variables were different between the 7 and 31 months treatments. However, the amount of total N extracted, applied N extracted and total N extracted as a percentage of total soil N were greater in the 24 h treatment than in the other two treatments ($P < 0.05$). The percentage of applied N recovered in the total N extracted increased from the 31 to the 7 months, and from the 7 months to 24 h treatment ($P < 0.05$). The percentage decreased as the strength of $KMnO_4$ increased, with the difference between the 10 mM and 50 mM $KMnO_4$ extraction significantly different (Table 2). The ER values also decreased as the strength of $KMnO_4$ increased, and were significantly greater in the 24 h than in the other two treatments.

Humin, humic and fulvic acids

The percentage of applied N recovered in humic (HA) and fulvic acids (FA) and humin is presented in Fig. 1. The humus fraction by treatment interaction was significant ($P < 0.05$, ANOVA not shown) due to changes in the relative ^{15}N distribution among the humic fractions. In the FA fraction, a significantly greater ($P < 0.05$) percentage of the total recovered ^{15}N was in the 24 h treatment than in the other two treatments. However, there was no difference in ^{15}N distribution among the

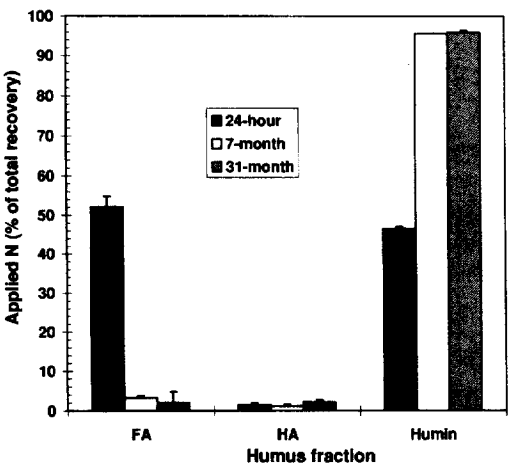

Fig. 1. Percentage of applied N recovered in humus fractions. FA – fulvic acid; HA – humic acid. Error bars are standard error of the means.

Table 3. The amount of applied N ($\mu g\ g^{-1}$ soil) flushed after chloroform fumigation and recovered in the humus fractions. This Table shows that fertilizer N flushed after fumigation was greater than that recovered in the FA fraction in the 24 h treatment and the trend was reversed for the 7 and 31 months treatments. This implies that the distribution of applied N in microbial biomass among the humus fractions is affected by fertilizer N residence time in the soil. Numbers in the parentheses are standard errors

Treatment	Humus fractions			
	N flush	FA	HA	humin
31 months	20.02 (3.24)	10.48 (1.28)	11.17 (2.26)	502.29 (83.28)
7 months	29.26 (4.09)	24.64 (3.97)	8.86 (2.85)	720.66 (16.07)
24 h	260.69 (16.65)	441.98 (60.95)	12.16 (2.38)	394.73 (43.64)

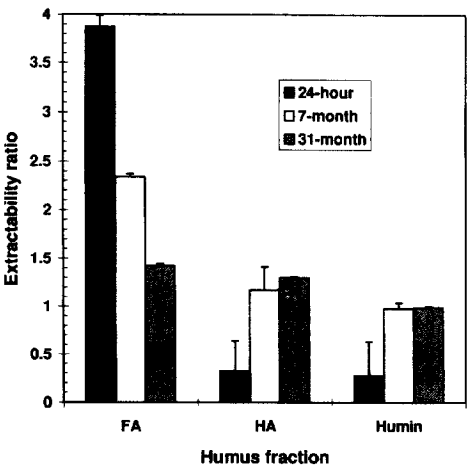

Fig. 2. Extractability ratios of humus fractions. FA – fulvic acid; HA – humic acid. Error bars are standard error of the means.

treatments for HA. In the humin fraction, a significantly lower ($P < 0.05$) percentage of applied N was recovered in the 24 h treatment than in the other two treatments which was opposite to the ^{15}N distribution in the FA fraction. Most of the recovered ^{15}N was in the humin fractions for the 7 and 31 months treatments, while for the 24 h treatment, most of the recovered ^{15}N was evenly distributed between FA and humin fractions, with little in the HA fraction (Table 3).

The ER values decreased with increased labelling duration for the FA fraction, while the opposite was observed for the HA and humin fractions (Fig. 2). The ER values also showed that nitrogen contained in the FA fraction was always enriched with ^{15}N relative to the bulk soil for each treatment, while the opposite is true for the humin fraction. In the HA fraction, relative to the bulk soil, ^{15}N was enriched for the 7 and 31 months treatment while depleted for the 24 h treatment.

DISCUSSION

It was quite clear that samples with ^{15}N labelled for one and three growing seasons had very similar properties in terms of N extractability and incorporation in SOM, and that applied N became more difficult to extract and thus much less available for tree uptake just one growing season after application. However, in the samples with ^{15}N labelled for 24 h, applied N was more readily extractable. The results were consistent with N mineralization studies of the same samples in which immobilized N in the 7 and 31 months treatments had lower mineralization potentials than that in the 24 h treatment (Chang *et al.*, 1997). Clay and Malzer (1993) suggested that those differences may mean compositional changes in soil organic N pools, which influence organic N extractability and mineralization. They (Clay and Malzer, 1993) showed that the efficiency of the extraction (by phosphate borate and hot KCl) of mineralisable residual N was a function of the length of time that the fertilizer was in the soil; the amount of mineralisable N for fertilizer N labelled in the soil for 2 y was less than that with fertilizer N labelled for 1 y, while total fertilizer N for both treatments was similar. The differences were proposed to be caused by changes in the composition of ^{15}N-labelled organic compounds with time.

Extractability ratios were greater than unity, even 3 y after fertilizer application, regardless of treatments and extraction methods used (Table 1), indicating that the residual ^{15}N was more extractable than the native soil N (He *et al.*, 1988). However, the ratios decreased with increased labelling duration. This indicates that the longer the ^{15}N residence time, the less extractable the immobilized N became relative to the native soil N. The percentage of applied N relative to the total N extracted also confirmed this trend which showed gradually decreasing values from the samples with increasing ^{15}N residence times. These results could partly explain field observations that indicate little additional N being taken up by trees 1 y after fertilizer N application (Preston and Mead, 1994), i.e., the low uptake rate of residual fertilizer N is caused by the low extractability or availability of residual fertilizer N.

The lowest extractability ratios were obtained by the autoclaving method for each treatment. Other authors have concluded that the autoclaving method gives low ER values and has poor selectivity for immobilized ^{15}N (Legg *et al.*, 1971; Juma and Paul, 1984; Kelley and Stevenson, 1985; Azam *et al.*, 1989b). Legg *et al.* (1971) argued that this could be due to the dispersion of protective soil aggregates by the autoclaving treatment which

increases soil N accessibility during extraction. In our study, total applied N extracted by the autoclaving method was less than that of the KCl extractable applied N in inorganic form, with higher total N extracted by the autoclaving method. This shows that (1) the autoclaving process had more even access to both immobilized ^{15}N and native N as suggested by Legg *et al.* (1971); (2) there was chemical mineralization-immobilization going on during autoclaving which exchanged native soil N in the structural organic matter for ^{15}N, because microbial mineralization-immobilization would be impossible under the autoclaving condition; and (3) autoclaving is a rigorous extraction method which may attack and decompose the humin fraction, because for the 7 and 31 months treatments, only the humin fraction had ER values less than 1. Only if large amounts of N enter into the extracts from the humin fraction will the ER values for the autoclaving extraction become closer to 1.

Both total and applied N extracted by 0.5 M K_2SO_4 after the samples were fumigated with chloroform increased from the non-fumigated samples which gave ER values of the fumigation-extraction similar to the 2 M KCl extraction method. The difference between the N extracted before and after the fumigation represents the size of N stored in the soil microbial biomass (Brookes *et al.*, 1985). The fumigation-extraction and 2 M KCl extraction methods were highly selective for extracting newly immobilized N at each treatment level and were sensitive to treatment effects. Thus they are more desirable extraction methods in obtaining more biologically meaningful N fractions. Differences in the amount of total and applied N extracted and in extractability ratios seemed to demonstrate that these two extraction methods and the autoclaving method rendered N from different pools, e.g., from microbial biomass pool, active non-biomass pool, or stabilized non-biomass pool (Stockdale and Rees, 1994). Stockdale and Rees (1994) suggested that if two extraction methods are extracting N from only one pool the actual amounts extracted would be very well correlated.

The ER values and the amount of total and ^{15}N extracted using acidic permanganate for the 24 h treatment resembled that of the fumigation-extraction method, regardless of the permanganate strength used. However, for the 7 and 31 months treatments, the result was similar to that of the 0.5 M K_2SO_4 or 2 M KCl extraction. In studying the oxidative release of potentially-mineralisable soil nitrogen by acidic permanganate extraction, Stanford and Smith (1978) found that the extracted NH_4^+–N was derived from oxidation of the soil organic N fraction most readily susceptible to decomposition by microorganisms. Perhaps the most recently immobilized ^{15}N (24 h treatment) was more susceptible to oxidative reaction with the acidic permanganate than that which had been immobilized for a longer period. The recently immobilized ^{15}N can be converted to highly stable humin forms as was indicated by the higher ER values for the HA and humin fractions in the 7 and 31 months treatments than in the 24 h treatment.

Although both total and applied N extracted by the acidic permanganate increased with increasing permanganate strength (except for the 24 h treatment using 50 mM $KMnO_4$), the increase of extracted applied N lagged behind that of the total N, thus giving decreasing ER values with increased permanganate strength at each treatment level. This shows that for the best selectivity of extracting immobilized ^{15}N, 10 mM is the optimal $KMnO_4$ concentration for this soil type and the range of $KMnO_4$ concentrations tested.

Extraction and humus form fractionation studies may also provide quantitative data on the immobilization of applied N by the soil and the movement of applied N between different N pools. There are studies that have reported that labelled N applied to soils is quickly immobilized by soil microbial biomass and organic matter. For example, Azam *et al.* (1989b) found that the time required for complete immobilization of applied N ranged from as little as 24 h with low N application rates (66 and 133 $\mu g\ g^{-1}$ soil) to 106 h with the highest rate used (333 $\mu g\ g^{-1}$ soil) when the soil was incubated at 30°C with glucose added as a highly-available C source. However, in field situations under natural condition the time required for complete immobilization might be quite variable. Smith and Power (1985) reported that complete immobilization of added nitrogen did not occur even after five growing seasons in a silt loam soil with an established crested wheatgrass (*Agropyron desertorum* var Mandan) stand. In our experiment, 24 h after ^{15}N application, 47.70% of the recovered applied N was found in the insoluble fraction of the SOM; after 7 and 31 months, the corresponding numbers were 98.50 and 98.87%, respectively. This confirms that immobilization of added N by organic material was relatively fast under our field conditions. The relatively high immobilization rate in this study may be related to the humus content of the samples. The soil samples used by Azam *et al.* (1989b) have a C content of 0.69%; Smith and Power (1985) also worked with agricultural soils with low organic C content; the organic C content of the humus material used in this study is around 50% (data not shown).

We removed the 0.5 M K_2SO_4 extractable N before fractionating the organic matter into the classical fractions. Therefore, the applied N recovered in the three humus fractions included ^{15}N in the microbial biomass, but not the inorganic ^{15}N in the soil. Thus, a comparison of applied N recovery in the microbial biomass and in the humus fractions

may clarify the relationship between N immobilization by microbial populations and applied N distribution in humus fractions. Table 3 shows that applied N recovered in the (FA + HA) fraction was only slightly greater than applied N flushed after fumigation-extraction in the 7 and 31 months treatments. Since there must have been some ^{15}N chemically immobilized in the FA and HA fractions (cf. Hart *et al.*, 1993, in a laboratory incubation study of sterilized soil samples, as much as 50% of the ^{15}N recovered in the SOM pool may have been abiotically fixed), the only reasonable explanation is that most of the microbially immobilized ^{15}N was in the recalcitrant humin fractions in the 7 and 31 months treatments. However, in the 24 h treatment, a greater percentage of the newly immobilized microbial ^{15}N is presumably in the FA fraction as indicated by the apparently large amount of ^{15}N recovered in that fraction.

The fact that 24 h after ^{15}N-labelled fertilizer was applied to the humus a quantity of the applied N larger than that flushed from fumigation was recovered in the humus (primarily FA and humin) fractions can be explained by (1) the fast turnover of microbial biomass in the soil. Microorganisms may be able to immobilize ^{15}N in a quantity greater than the microbial biomass N in a short period because of microbial biomass turnover. He *et al.* (1988) found that much of the newly immobilized N was in the insoluble components of microbial cells; and (2) the immobilization of some of the recovered ^{15}N by soil organic matter through chemical fixation (Hart *et al.*, 1993); although the study by Clinton *et al.* (1995), using ^{15}N NMR techniques, supported the view that biological processes dominate over direct chemical fixation for incorporation of fertilizer N in soil. Clinton *et al.* (1995) also found that almost all of the ^{15}N incorporated in humified organic matter was accounted for by substances with structures resemble proteins and nucleic acids.

The decrease in the amount of applied N recovered in the FA fraction from the 24 h treatment to the 7 and 31 months treatment and a generally reversed trend in the humin fraction indicates that fertilizer N immobilization by SOM is firstly by the FA fraction and then transformed into and stabilized in the humin fraction. It has been proposed that, in some cases, the dominate humification mechanism may involve abiotic polymerization reactions of small organic molecules producing fulvic acids as precursors to humic acids (Kelley and Stevenson, 1995). However, an explanation of the mechanism for the ^{15}N movement from the FA to the humin fraction can not be provided for this study.

Labelled nitrogen immobilized into the humin fraction is highly insoluble (He *et al.*, 1988). He *et al.* (1988) found that after a 7 d incubation, much of the ^{15}N in the humin fraction could not be solubilized by sequential extraction with a variety of inorganic and organic extractants. In our study more ^{15}N was incorporated into the humin fraction in the 7 and 31 months treatments than in the 24 h treatment, indicating that more immobilized ^{15}N became recalcitrant due to the transformation process as time progressed. Azam *et al.* (1989a) also demonstrated that the percentage of ^{15}N recovered in the humin fraction increased with time (from 0 to 112 d of laboratory incubation) for two Pakistani soils and that recovery of ^{15}N in the humin fraction was very high (to 89%) for each of the three soils studied.

In summary, our study confirmed that the extractability of applied N relative to native N was reduced with increasing residence time, because much immobilized N was incorporated into the stable humus fractions. One growing season after ^{15}N application, a large percentage of the recovered applied N in the SOM had been immobilized by the humin fraction, leading to non-significant differences in applied N extractabilities from those with fertilizer N present for three growing seasons. Different extraction methods may attack different N pools and thus yield a range of applied and native N being extracted. The fumigation-extraction and KCl extraction methods extracted the most biologically meaningful N fractions and are recommended for studying residual fertilizer N availabilities in field experiments. The immobilization of ^{15}N into the FA fraction is apparently associated with microbial activities; whether the stabilization of ^{15}N (from being primarily in the FA fraction to being primarily in the humin fraction) is through microbial turnover or through other mechanisms is not clear.

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