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Nitrate in soil humic acids revealed by ^{14}N nuclear magnetic resonance spectroscopy

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Abstract

Ecosystem management such as plant residue retention and prescribed burning can significantly affect soil organic matter (SOM) composition and, thereby, the closely associated carbon (C) and nitrogen (N) cycling processes, which underpin terrestrial ecosystem productivity and sustainability. Humic acid (HA) is an important SOM component and its chemical composition has attracted much attention. Here we report the first application of ^{14}N nuclear magnetic resonance (NMR) spectroscopy to soil HA study, revealing the surprising existence of nitrate-N and ammonia-N in the HAs. This newly discovered HA nitrate-N, though in a relatively low concentrations, is closely related to soil N availability and responsive to plant residue management regimes in contrasting forest ecosystems. The HA nitrate-N may be a useful and sensitive biochemical indicator of SOM quality in response to different ecosystem management regimes.

Additional keywords: humic acid, nitrate-N, ^{14}N -NMR, plant residue management, forest ecosystems.

Introduction

Both amount and composition of soil organic matter (SOM) can significantly influence important carbon (C) and (N) cycling processes in terrestrial ecosystems (Schnitzer 1991; Vitousek *et al.* 1997; Schulten and Schnitzer 1998; Guinto *et al.* 1999). The humic acid (HA) is an important SOM component (Schnitzer 1991; Preston 1996; Schulten and Schnitzer 1997; Mathers *et al.* 2000). Applications of ^{13}C NMR have advanced our understanding of soil HA composition (Wilson *et al.* 1978; Preston 1996; Mahieu *et al.* 1999; Mathers *et al.* 2000). Recent applications of solid state ^{15}N NMR with cross polarisation (CP)/magic angle spinning (MAS) have revealed rich information about organic N composition, particularly the evidence of amide-N as a major N component in SOM (Zhuo *et al.* 1992; Knicker *et al.* 1993, 1997; McCarthy *et al.* 1997). However, solid state CP/MAS ^{15}N NMR has difficulty in detecting the naturally abundant, low concentration ^{15}N not attached to protons (Engelke 1996). Even for liquids, ^{15}N NMR has its intrinsic limitation. Solution ^{14}N NMR is much more sensitive than solution ^{15}N NMR. The high abundance of ^{14}N balances its quadrupolar nature (Laszlo 1996). Therefore, small molecules like nitrate and ammonia can be easily detected by solution ^{14}N NMR (Simeral 1997).

Solution ^{14}N NMR can be traced back to the discovery of the chemical shift phenomenon in early 1950s (Proctor and Yu 1950). Since the development of FT NMR in

1960s, ^{14}N NMR has been widely applied to structural chemistry studies (Witanowski *et al.* 1986). With spin $I = 1$ and a relatively small quadrupole ($eQ = 0.0193 \times 10^{-24} \text{ cm}^2$), ^{14}N is an easily NMR-accessible nucleus. The problem of ^{14}N NMR is that the line width of the ^{14}N NMR signal increases dramatically with the molecular weight. The physical mechanism of the serious line broadening is the fast quadrupolar relaxation (Abragam 1961), which is due on the one hand to the asymmetric nature of the chemical structure of most N-containing compounds and on the other hand to the fact that the reorientation correlation time increases with the molecular weight (Abragam 1961). However, small N species such as NO_3^- , NH_3 , or NH_4^+ , which we are interested in, have high symmetry and consequently rather narrow line shapes. A preliminary test showed us that 2 mg NH_4NO_3 gave sharp and strong ^{14}N signals for both NH_4^+ and NO_3^- in <10 min. Therefore, we were confident of performing this study. Here we present the results of the first solution ^{14}N NMR application to soil HA study, particularly in relation to plant residue management regimes in contrasting forest ecosystems of subtropical Australia.

Materials and methods

Experimental sites and soil sampling

Soil samples were collected from 4 different sites. (a) Site 1 ($26^\circ 00' \text{ S}$, $152^\circ 49' \text{ E}$) was planted in May 1996 with seedlings of an F_1 hybrid between slash pine (*Pinus elliottii*) and Caribbean pine (*P. caribaea*) after clearfall harvesting of the first-rotation slash pine plantation at age 29.4 years. Soil (FAO: Gleyic Acrisol) samples (0–10 cm) were collected in December 1997 (20 months after the residue management regimes applied), by using an auger of 7.5 cm diameter to take 5 such cores and make them a composite sample from each of the 2 treatments: residue removal—the first-rotation harvesting residues removed; and double residues—the residues retained plus the residues from residue removal plots with a total dry matter (DM) of the residues at 141 t/ha in a large residue management experiment. (b) Site 2 ($26^\circ 32' \text{ S}$, $152^\circ 37' \text{ E}$) was established on a second-rotation site planted with seedlings of hoop pine (*Araucaria cunninghamii*) growing between 2.5-m-wide windrows (522 t DM/ha) of harvesting residues from the first-rotation hoop pine plantation. Soil (FAO: Lithosol) samples (0–10 cm) were collected in April 1998 from the 2 residue management areas: 1 year between windrows—areas with the first-rotation hoop pine residues removed between the windrows (15 m apart) about 1 year after their establishment; and 1 year under windrows—areas just under the windrows. (c) Site 3 ($26^\circ 31' \text{ S}$, $152^\circ 38' \text{ E}$) was also established on a second-rotation site planted with hoop pine seedlings between the windrows (348 t DM/ha) of harvesting residues from the first-rotation hoop pine plantation. Soil (FAO: Lithosol) samples (0–10 cm) were collected in April 1998 from the 2 residue management areas: 3 years between windrows—areas with the first-rotation hoop pine residues removed between the windrows (10 m apart) about 3 years after their establishment; and 3 years under windrows—areas just under the windrows. (d) Site 4 ($26^\circ 50' \text{ S}$, $152^\circ 53' \text{ E}$) was a natural wet sclerophyll forest dominated by blackbutt (*Eucalyptus pilularis*). Soil (FAO: Ferric Luvisol) samples (0–10 cm) were collected in August 1994, about 25 years after establishment of the prescribed burning experiment (Guinto *et al.* 1999, 2000), from the 3 treatments: biennial burning—forest floor burnt every 2 years with a total of 10 burns at the time of soil sampling; quadrennial burning—forest floor burnt every 4 years with a total of 7 burns; and no burning—forest floor remained unburnt for the last 25 years.

Sample preparation and chemical and statistical analyses

All the soil samples were air-dried immediately after soil sampling in the field, passed through 2-mm sieve, and subsampled before storing in sealed plastic containers. Soil samples were ground and passed through 0.14-mm sieve prior to soil organic C and total N analysis reported by Xu *et al.* (1993). Soil potentially mineralisable N (PMN) derived from an anaerobic incubation method was determined as reported by Waring and Bremner (1964).

Soil HAs were extracted using the International Humic Substances Society method (Swift 1996). Briefly, in Step 1, roots were removed from air-dried soil samples and then soil samples sieved to pass through a 2.0-mm sieve; the samples were equilibrated with 1 M HCl to pH 1–2 at room temperature; the solution volumes were adjusted with 0.1 M HCl to provide a final ratio of 10 mL liquid per gram dry soil

samples (in our case, 4 g air-dried soil with 40 mL liquid in 50 mL Falcon tube); the liquid suspension samples were shaken for 1 h and then the supernatant liquids were separated from the residues by low speed centrifugation; and the supernatant liquids were saved as fulvic acids (FAs) for other related studies. In Step 2, the residue samples were neutralised with 1 M NaOH to pH 7; 40 mL 0.1 M NaOH was added to each of the residue samples under an atmosphere of N_2 to give a final extractant to soil ratio of 10:1; the samples were shaken for a minimum of 4 h; and the alkaline suspension samples were allowed to settle overnight and then centrifuged at 2000 rpm for 20 min to collect the supernatant samples. In Step 3, the supernatant samples were filtered through 0.2- μm polyethersulfone membrane filter (Pall Gelman Supor 200) to remove clays and ash without HF; the filtrate samples were acidified with 6 M HCl and constantly stirred to pH 1.0; the suspension samples were allowed to stand overnight (12–16 h); and then the suspension samples were centrifuged at 2000 rpm for 20 min to separate the HAs (precipitated) from the FAs (supernatant—FA extract 2 for other related studies). In Step 4, distilled water was added to the HA samples and the HA samples were transferred to Visking dialysis tubing (ID = 14.3 mm, MWCO = 12000–14000 Dalton); the HA samples were dialysed against distilled water until the dialysis water gave a negative Cl^- test with silver nitrate (AgNO_3); and then the HA samples were transferred to sample jars and freeze-dried.

The HA total N and total C were determined on an Europa Scientific 20-20 ANCA SL mass spectrometer in the laboratory at CSIRO Land and Water, Adelaide, Australia. All the HA extractions were replicated 2 times for each soil sample. All the chemical analyses for the soil and HA samples were repeated at least twice. The HA extraction for the soil samples was repeated at different times (minimum 2-week intervals between two repeated HA extractions for the same soil samples) to ensure that there would be no cross contaminations and no apparent laboratory operational errors and that all the results of chemical analyses including ^{14}N NMR were accurate and reproducible in the relevant laboratories of Australia and China. In addition, a series of the related laboratory and ^{14}N NMR studies were performed to verify the experimental results reported here, but detailed results of these related studies would only be referred to in the text since they would be reported elsewhere. Statistical analyses and graphing for the data were performed with the STATISTICA software (StatSoft 1999).

^{14}N -NMR analyses

The ^{14}N NMR experiments were performed on a Bruker ARX-500 NMR machine with a 5-mm inverse probe tuned at 36.12 MHz for ^{14}N without proton decoupling. Spectral width was kept to 500 ppm, although the desired signals were all within a range of 350 ppm. For data acquisition, 90° pulse (30 ms) was used and the interval between 2 consecutive pulses was 99 ms (79 ms acquisition time and 20 ms relaxation delay) by including the acquisition time. The ^{14}N NMR samples were first prepared by dissolving the maximum amounts of the HAs and the reference substance CH_3NO_2 in 0.5 mL 0.1 M NaOH solutions, where the nitrate and ammonia signals were detected (Fig. 1) with sufficient accumulations of transients and their integration ratios of nitrate to ammonia signal were obtained for the HAs. Then the samples were air-dried at room temperature and 25 mg dried HAs was dissolved in 0.5 mL 0.2 M NaOH solutions, followed by ^{14}N NMR measurements with equal number (50000) of scans (measurement time 1.5 h for each sample). The HA nitrate signal was quantitatively integrated compared with an external reference of NaNO_3 (0.94 mg in 5 mL 0.2 M NaOH, corresponding to 2.2 mM NO_3^-). Ammonia did not show signal because it had been volatilised into the air during the drying process.

Results and discussion

In ^{14}N NMR spectra (Fig. 1) of soil HAs prepared according to the International Humic Substances Society method (Swift 1996), 3 signals can be observed. According to the chemical shift data referenced to neat nitromethane (Witanowski *et al.* 1986), the strong and sharp signal at -7 ppm is assigned to nitrate-N, the weak and broad signal at -380 ppm is assigned to ammonia-N, and the phase-twisted signal at -77 ppm is assigned to dinitrogen (N_2) absorbed in the HA solution (McIntyre *et al.* 1989). The phase twist of the N_2 signal could be due to the relatively fast diffusion of the N_2 molecule in the solution. The N atoms in the HA components such as protein-N and heterocyclic-N cannot be observed by ^{14}N NMR because their relaxation times are too short. However, small molecules like nitrate, ammonia, and dinitrogen have much shorter reorientation correlation times and relatively long relaxation times, typically 1 to 20 ms (McIntyre *et al.* 1989). As a result, they give

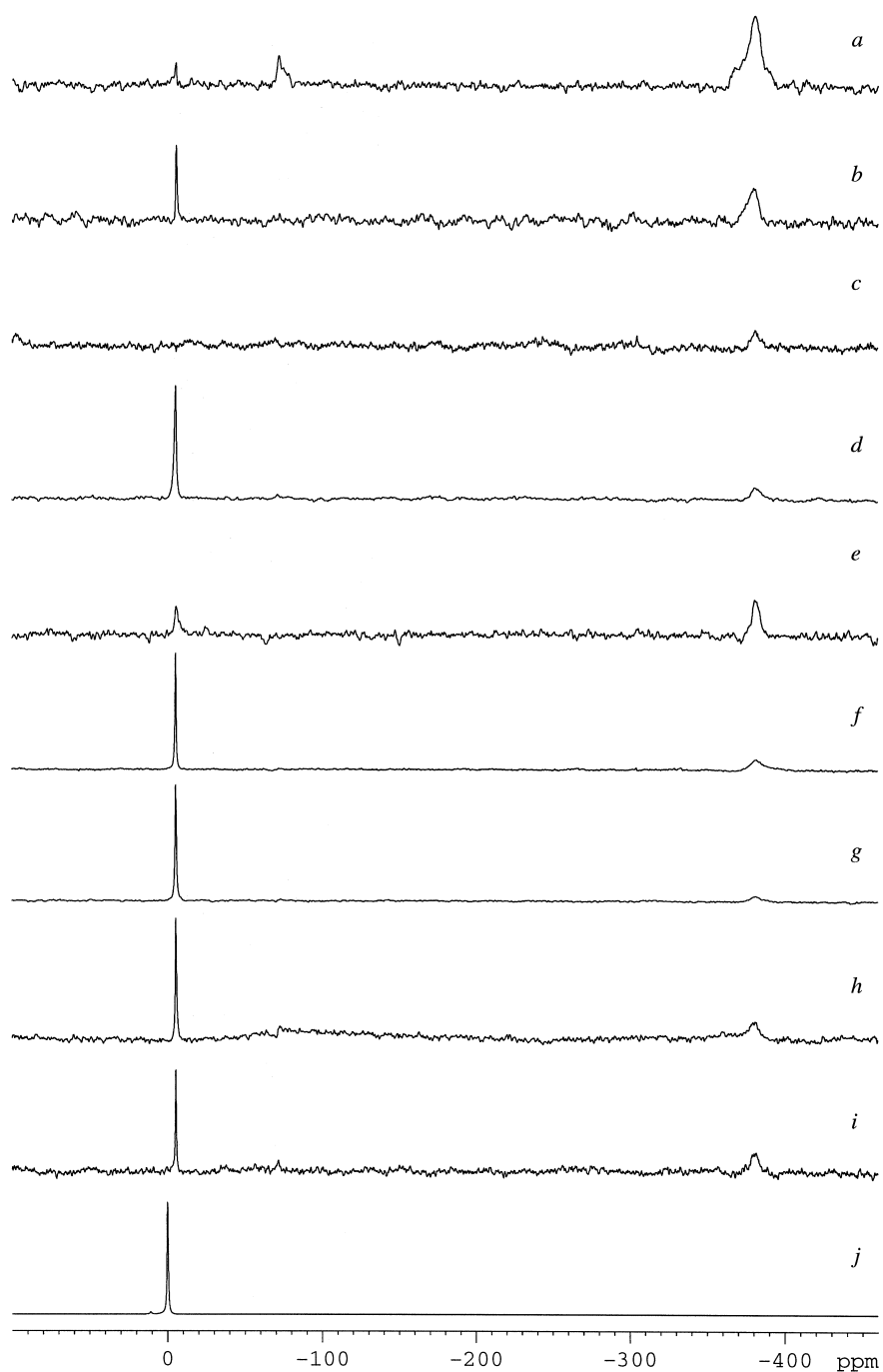


Fig. 1. ^{14}N NMR spectra of saturated soil humic acids in 0.1 M NaOH solutions with sample codes of *a*–*i* described in Table 1 and the spectrum of the reference substance CH_3NO_2 (*j*) recorded on a Bruker ARX-500 NMR spectrometer. The number of scans was between 25 000 and 250 000, corresponding to measurement time 40 min to 7 h. In order to avoid the baseline roll, 120 ms pre-acquisition delay was applied.

signals in the ^{14}N NMR spectra. The signal line width alone can indicate that these signals are not organic N compounds such as nitrophenols and oximes, which would have much broadened line shapes. Indeed, in related studies (unpublished results), we have tested a series of organic samples for ^{14}N NMR spectra, none showing signals as sharp as nitrate and ammonia. We have also tested if the HA nitrate-N signal could be overlapped by small organic N molecules such as CH_3NO_2 by adding known amounts of CH_3NO_2 and NaNO_3 to a number of the HA samples, and the ^{14}N NMR spectra indicate that it is nitrate-N instead of the CH_3NO_2 signal at the -7 ppm. While signal overlapping is quite common, it is not true in this case.

By using a reference sample (0.94 mg NaNO_3 in 5 mL 0.2 M NaOH solution) in the ^{14}N NMR analysis, nitrate-N and ammonia-N in the HAs can be quantitatively determined. The HA nitrate-N and ammonia-N are presented in Table 1, together with other soil properties under different plant residue management regimes in 4 forest ecosystems of subtropical Australia. In plantation forest ecosystems (Sites 1–3), soil PMN (a soil N availability index) is more responsive to the plant residue management regimes than soil organic C and total N, with the PMN values under residue retention treatments being 32–113% higher than those under residue removal treatments. In the natural forest ecosystem (Site 4), PMN, organic C, and total N in soils under fuel-reduction burning of forest floor every 2 years in the last 25 years are significantly lower than those of the control without fuel-reduction burning. It is interesting to note that the HA nitrate-N and, to a lesser extent, ammonia-N mirror the trends of the PMN in the plantation forest ecosystems, with the HA nitrate-N under residue retention treatments (2.6–4.4% of total N in the HAs) being 3.6–21.5 times that under residue removal treatments. In the natural forest ecosystem, however, the HA nitrate-N and ammonia-N under the fuel-reduction burning (39.4% and 11.7% of total N in the HA) are 14.2 and 3.7 times, respectively, those under no fuel-reduction burning, which is opposite to the trend of the PMN. In all cases, the HA nitrate-N and ammonia-N are much more responsive to the plant residue management regimes than the other soil characteristics in Table 1.

The HA nitrate-N is closely related to the HA ammonia-N (Fig. 2a) and the PMN (Fig. 2b), indicating that the HA nitrate-N may be biologically active and depend on microbial nitrification of ammonium-N derived from soil N mineralisation. It should be noted that the HA nitrate-N signals inherently appear in the ^{14}N NMR spectra (Fig. 1) irrespective of pre-treatments of the soils by (a) anaerobic incubation (Waring and Bremner 1964) for a week and then extraction with 25 mL 2 M KCl solution; and (b) extraction with the KCl solution and then rinsing with 25 mL distilled water 3 times before the HA extraction (unpublished results). Therefore, the HA nitrate-N could not be removed from the HAs by the anaerobic incubation, the KCl and water extraction, and the HA extraction procedures. However, it is not yet known how the nitrate ion is bound to the HA. The existence of metal elements such as manganese, magnesium, iron, and aluminium in HAs has been well known (Shindo and Huang 1984; Schnitzer 1991; Schulten and Schnitzer 1997), but attempts to correlate the HA nitrate-N to these metal elements have failed (unpublished results). This implies that the structure of HAs is more complicated than the existing models (Schnitzer 1991; Schulten and Schnitzer 1997), where inorganic ions such as the HA nitrate reported here are not fully considered.

Our finding that the PMN is more responsive to the plant residue management regimes than soil organic C and total N in the forest ecosystems, is consistent with the results reported elsewhere (Bubb *et al.* 1998; Guinto *et al.* 1999; Piatek and Allen 1999). While ^{14}N NMR has been applied to examine mechanisms of uptake of nitrate-N and

Table 1. Soil C and N pools under different plant residue management regimes in subtropical forest ecosystems of Australia

Detailed descriptions of experimental sites and the relevant plant residue management regimes are presented in the **Materials and methods**. PMN, potentially mineralisable N. Means within a column followed by the same letter are not significantly different at $P = 0.05$ by Duncan's multiple range test (DMRT); values in the Table are means of 4 replications for Site 1, 3 replications for Site 2, 9 replications for Site 3, and 6 replications for Site 4, except for values followed by no letter, which are the analytical results of individual samples where DMRT cannot be performed

Sample code	Plant residue management	Whole soils		Soil humic acids			Total C (mg/g)
		PMN (mg N/kg)	Organic C (g C/kg)	Total N (g N/kg)	NO ₃ -N (mg/g)	NH ₃ -N (mg/g)	
<i>Site 1. Exotic pine plantation</i>							
a	Residue removal	3.47b	12.5a	0.376a	0.013	0.22	257
b	Double residues	7.38a	16.6a	0.492a	0.280	0.82	279
<i>Site 2. Native hoop pine plantation</i>							
c	1 year between windrows	10.1a	50.1a	4.10a	0.28	0.29	246
d	1 year under windrows	13.3a	63.1a	3.90a	1.00	3.30	273
<i>Site 3. Native hoop pine plantation</i>							
e	3 years between windrows	7.53b	36.2b	2.30b	0.033	0.067	248
f	3 years under windrows	11.20a	44.3a	2.50a	0.70	0.57	396
<i>Site 4. Natural forest</i>							
g	Biennial burning	12.2b	23.7b	1.00b	5.40	1.60	227
h	Quadrennial burning	16.2ab	38.7ab	1.60ab	0.27	0.31	371
i	No burning	20.2a	43.0a	1.83a	0.38	0.43	412

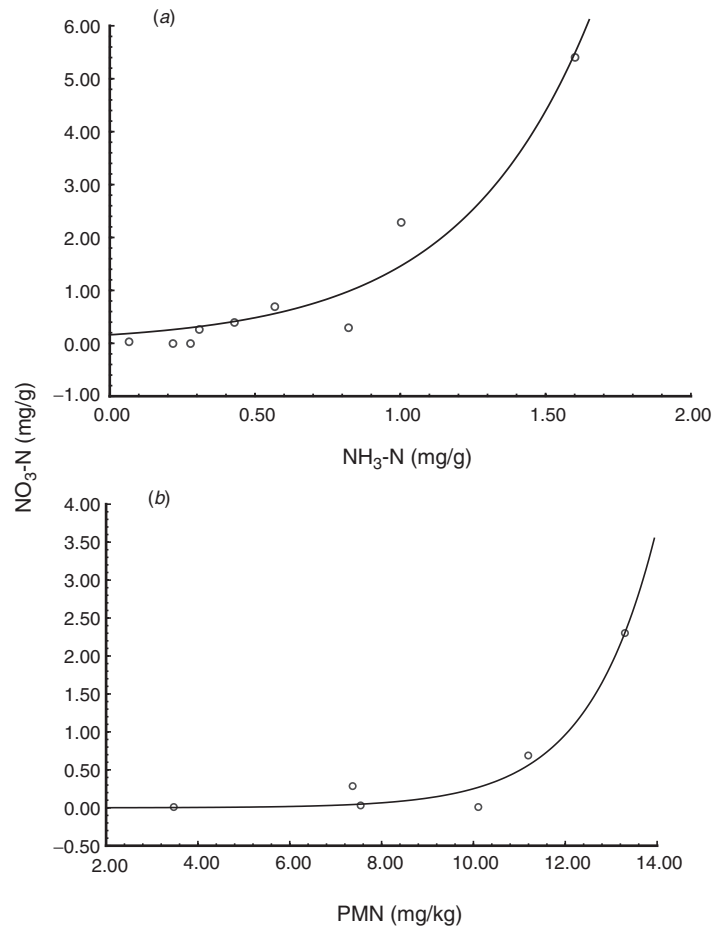


Fig. 2. Relationships between (a) NO₃-N and NH₃-N in soil humic acids estimated from the ¹⁴N-NMR spectra (Fig. 1): $y = 0.160 e^{2.21x}$ ($R^2 = 0.945$, $n = 9$, $P < 0.01$); and (b) NO₃-N in soil humic acids from plantation forest ecosystems (Sites 1–3 in Table 1) and soil potentially mineralisable N (PMN): $y = 0.000301 e^{0.672x}$ ($R^2 = 0.964$, $n = 6$, $P < 0.01$).

ammonium-N by plants (Lee *et al.* 1992; Gerendas *et al.* 1995), we believe that this study represents the first attempt to apply ¹⁴N NMR to soil HA study. Gross nitrification rates in forest soils can be high and soil microbial communities have the capacities to assimilate almost all of the nitrate-N produced (Stark and Hart 1997). Our findings of the close relationships of the HA nitrate-N with the HA ammonia-N and the PMN lend strong support to the notion that soil microbial communities may play an important role in the formation of the HA nitrate-N and in the internal N cycle of forest ecosystems. It is interesting to note that while the PMN is significantly lower in the soil under fuel-reduction burning of forest floor every 2 years than that of the control without fuel-reduction burning in the natural forest ecosystem, the HA nitrate-N under the fuel-reduction burning appears to be much higher than that of the control. This suggests that soil microbial composition might be changed substantially in favour of soil bacteria with low C-to-N ratios against soil fungi due to the fuel-reduction burning (Ajwa *et al.* 1999) although the fuel-reduction

burning significantly reduced soil total N and possibly total microbial biomass pool, leading to the lower PMN. This lends further support to the notion that the HA nitrate-N is biologically active and its formation could be closely mediated by soil microbial communities, which are sensitive to both plant residue management regimes and environmental changes.

The possible mechanisms for the existence of HA nitrate-N may include: (1) the HA nitrate-N was the product of microbiological activities in the stored samples; (2) the HA nitrate-N was produced by the HA extraction procedure; (3) the nitrate-N was physically trapped in the HA, as the host-guest interactions in humic substances, reported by Smeulders *et al.* (2000, 2001) and Wilson *et al.* (2000); and (4) the nitrate-N was trapped in biological materials such as non-decomposed microbial cell bodies. The first mechanism could be ruled out since the soil samples in the contrasting residue management regimes would have experienced similar microbiological activities and the differences in soil mineral N would be minimal or not detectable by conventional mineral N analyses. The large differences (ranging from 260% to 2050%) in the HA nitrate-N between the residue management treatments could not be explained by such mechanism. Again, the same HA extraction procedure would be applied to all the soil samples, and indeed no mineral N could be detected in the soil HA by conventional soil mineral N analyses after the HA extraction procedure. The large differences in the HA nitrate-N could not be attributed to the HA extraction procedure. The host-guest interaction theory proposed by Smeulders *et al.* (2000, 2001) and Wilson *et al.* (2000) could be a plausible mechanism, but the differences in the HA nitrate-N (2.6–20.5-fold) between the residue treatments are much greater than those in total HA dry weights (1–2-fold). This would tend to discount the third mechanism, of the host-guest interaction hypothesis, since in theory the proportion of the HA nitrate-N would be in the same order as the total HA dry weight and this is not the case. In terms of the last mechanism of nitrate-N present in soil HAs, we advance, based on our results and the above discussion, the hypothesis that soil microbial population, particularly certain low C-to-N ratio groups of soil bacteria, would have the capacity of assimilating and storing nitrate-N in their bodies, rendering such nitrate-N stored in the microbial bodies resistant to the anaerobic incubation, KCl and water extraction, and the HA extraction procedures. Our research findings reported here have highlighted the potential of using ^{14}N NMR together with ^{13}C and ^{15}N NMR as well as relevant microbiological methods to advance the understanding of the chemical composition of soil HAs and SOM (Mathers *et al.* 2000). In addition, the HA nitrate-N can be a useful biochemical indicator of SOM quality, which is rather responsive to ecosystem management, particularly plant residue management regimes in the terrestrial ecosystems. However, further research is required to (a) examine the utility of ^{14}N NMR to soil HAs in diversified terrestrial ecosystems under different environmental conditions and management practices; (b) understand the mechanisms of how the HA nitrate-N is formed, particularly testing the hypotheses of both host-guest interactions in humic substances and microbial entrapping model; and (c) evaluate the role of the HA nitrate-N in terrestrial C and N cycling processes in response to land management practices and global climate changes (Matson *et al.* 1998; Nasholm *et al.* 1998; Nadelhoffer *et al.* 1999).

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