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N-15 NMR spectra of naturally abundant nitrogen in soil and aquatic natural organic matter samples of the International Humic Substances Society

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ABSTRACT

The naturally abundant nitrogen in soil and aquatic NOM samples from the International Humic Substances Society has been characterized by solid state CP/MAS ^{15}N NMR. Soil samples include humic and fulvic acids from the Elliot soil, Minnesota Waskish peat and Florida Pahokee peat, as well as the Summit Hill soil humic acid and the Leonardite humic acid. Aquatic samples include Suwannee River humic, fulvic and reverse osmosis isolates, Nordic humic and fulvic acids and Pony Lake fulvic acid. Additionally, Nordic and Suwannee River XAD-4 acids and Suwannee River hydrophobic neutral fractions were analyzed. Similar to literature reports, amide/aminoquinone nitrogens comprised the major peaks in the solid state spectra of the soil humic and fulvic acids, along with heterocyclic and amino sugar/terminal amino acid nitrogens. Spectra of aquatic samples, including the XAD-4 acids, contain resolved heterocyclic nitrogen peaks in addition to the amide nitrogens. The spectrum of the nitrogen enriched, microbially derived Pony Lake, Antarctica fulvic acid, appeared to contain resonances in the region of pyrazine, imine and/or pyridine nitrogens, which have not been observed previously in soil or aquatic humic substances by ^{15}N NMR. Liquid state ^{15}N NMR experiments were also recorded on the Elliot soil humic acid and Pony Lake fulvic acid, both to examine the feasibility of the techniques, and to determine whether improvements in resolution over the solid state could be realized. For both samples, polarization transfer (DEPT) and indirect detection (^{1}H–^{15}N gHSQC) spectra revealed greater resolution among nitrogens directly bonded to protons. The amide/aminoquinone nitrogens could also be observed by direct detection experiments.

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1. Introduction

Humic substances are the predominant form of natural organic matter (NOM) in soil and water. Because nitrogen in humic substances comprises the major form of biologically refractive organic nitrogen in these compartments, knowledge of the formation and mineralization pathways of nitrogen in NOM is critical for an understanding of the biogeochemical cycling of nitrogen, which is linked to the cycling of carbon. The nitrogen content of soil organic matter is a major factor controlling soil fertility. NOM is a source of nitrogen to heterotrophic microorganisms in aquatic systems and therefore a factor in the biological productivity of natural waters. Photochemical release of ammonia and amino acids has been documented as one mechanism through which humic nitrogen is made available to aquatic microorganisms (Bushaw et al., 1996; Tarr et al., 2001). Dissolved organic nitrogen delivered into coastal waters through riverine transport is considered a contributing factor to hypoxia (Scott et al., 2007). Formation of nitrogen containing disinfection byproducts, such as haloacetonitriles, haloacetamides, trichloronitromethane and N-nitrosodimethylamine, upon chlorination and chloramination of NOM during water treatment, is of current interest to health officials (Chen and Valentine, 2007; Lee et al., 2007; Plewa et al., 2008). For these reasons, an understanding of the structural forms of nitrogen in humic substances is of long term interest.

Since the early application of solid state CP/MAS ^{15}N NMR for detection of naturally abundant nitrogen in soil humic substances, discrepancies between the NMR results and previously held conceptions on the origin of nitrogen in humic substances, based partly on wet chemical analyses, have been apparent (Knicker et al., 1993). For example, whereas NMR indicated that amide (peptide) nitrogen is the predominant form of nitrogen in humic substances, amino acid analyses have only accounted for 30–50% of total nitrogen in soil materials (Andersen et al., 1989; Schulten and Schnitzer, 1998; Stevenson, 1994). In classical theories of soil humus formation outlined by Stevenson (Stevenson, 1994), including the Maillard pathways, condensation of ammonia and amino acids with quinones and reducing sugars was thought to result in formation of heterocyclic nitrogen structures, which were not detected in significant quantities in first reports of natural abundance ^{15}N NMR spectra (Knicker et al., 1993). Subsequent reports indicated the presence of heterocyclic nitrogen in humic acids from highly humified soil organic matter (Mahieu et al., 2000; Maie

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et al., 2006a). Condensation of ammonia with the carbonyl groups of humic substances was also thought to be one of the mechanisms for abiotic incorporation of nitrogen into soils impacted by anthropogenic inputs of nitrogen. Tracer studies indicated that a fraction of the liquid ammonia applied to agricultural soils as fertilizer reacted with organic matter in what was termed ammonia fixation (Stevenson, 1994). Another mechanism for abiotic incorporation of nitrogen into organic matter in fertilized soils, that received considerable attention in the older literature, was reaction with nitrite. Under conditions of heavy fertilization, accumulation of nitrite from nitrification of ammonia was thought to result in nitrosation of organic matter and accompanying chemodenitrification reactions, i.e. release of NO, N_2O and N_2 (Stevenson, 1994). More recently, in the case of acid forest soils subjected to atmospheric deposition, nitrosation of organic matter has been proposed to account for retention of nitrate at rates too high to be explained by biological mechanisms (Davidson et al., 2003, 2008; Colman et al., 2007; Morier et al., 2008). Incorporation of nitrite into NOM has been documented in stable isotope studies (Fitzhugh et al., 2003a,b). The structural forms of nitrogen resulting from incorporation of ^{15}N labeled ammonia and nitrite into soil and aquatic humic substances have been determined (Thorn and Mikita, 1992, 2000). Indole, pyrrole, pyridine, pyrazine, primary amide and primary aminohydroquinone nitrogens were among the structures observed from incorporation of ammonia into NOM while nitrosophenols, oximes and N-nitrosamides and/or N-nitrosamines formed from reaction with nitrite (Thorn and Mikita, 1992, 2000). Pyridine, pyrazine, nitrosophenol, oxime, N-nitrosamide and N-nitrosamine nitrogens have not been observed in natural abundance spectra of NOM thus far.

The potential shortcomings of the solid state CP/MAS experiment in detecting certain types of nitrogens and in accurately quantifying the distribution of nitrogens detected have been documented in both NOM and fossil fuel studies (Solum et al., 1997; Kelemen et al., 2002; Smernik and Baldock, 2005). In the CP/MAS experiment, because the cross polarization time constants (T_{HN}) and proton rotating frame relaxation times ($T_{1\rho H}$) vary with the type of nitrogen, no single value of contact time may achieve a quantitative distribution of nitrogens. In particular, slowly cross polarizing nitrogens with no directly attached proton may be underestimated or undetected with the 1 ms contact time commonly used to acquire solid state spectra of fossil fuel and NOM samples. This has been demonstrated in the case of model compounds such as azaindole and caffeine (Kelemen et al., 2002; Smernik and Baldock, 2005). With azaindole, the pyridine nitrogen attained 94% of the intensity of the pyrrole nitrogen at a 5 ms contact time but only 42% at a 1 ms contact time, on a 200 MHz spectrometer (Kelemen et al., 2002). An effect of field strength was also noted, with the pyridine attaining 36% and 17% of the pyrrole intensities at 5 ms and 1 ms contact times, respectively, on a 400 MHz spectrometer. The large chemical shift anisotropy is an additional complicating factor in the detection of pyridine type nitrogens. Protonation with p-toluenesulfonic acid enabled detection of pyridine nitrogen in coals (Solum et al., 1997). From spin counting experiments, Smernik and Baldock (2005) concluded that up to half the organic nitrogen in HF treated soil clay fractions could be in the form of non-amide nitrogen that was undetectable at a 1 ms contact time on a 400 MHz spectrometer. Additionally, problems of chemical exchange or molecular motion at room temperature may hamper detection of nitrogens in enamino-imino systems such as porphyrins and phthalocyanins, with the result that low temperature experiments may be necessary for observation (Earl, 1987).

Although recent nitrogen K-edge XANES (X-ray Absorption Near Edge Structure) and XPS (X-ray photoelectron spectroscopy) studies have revealed amide nitrogen as a major form of nitrogen in humic substances, XANES data was interpreted to indicate significant concentrations of pyridine and oxidized pyridine nitrogen in sediments and humic substances, including the IHSS Suwannee River and Pahokee peat fulvic and humic acids and the Elliot soil humic acid (Vairavamurthy and Wang, 2002), while X-ray photoelectron spectroscopic data was interpreted to indicate the occurrence of pyridine, imine and/or aromatic aniline derivatives in soil humic acids (Abe and Watanabe, 2004). Pyridine, oxidized pyridine and imine nitrogens thus far have not been detected in published natural abundance NMR spectra of soil or aquatic humic substances.

The universal availability of the IHSS samples to researchers provides an opportunity for reconciling the data from the various spectroscopic techniques, including mass spectrometric investigations (e.g., Berwick et al., 2007), and from specific chemical analyses of nitrogen in humic substances, such as determinations of amino acids, amino sugars and nucleic acid derivatives. In this context, and to provide further information that may help to explain the reactivity of the nitrogen functionality in the humic and fulvic acids, we present solid state CP/MAS ^{15}N NMR spectra of the IHSS soil and aquatic samples. Furthermore, although spectra of marine and estuarine NOM samples isolated via ultrafiltration have been published (McCarthy et al., 1997; Aluwihare et al., 2005; Maie et al., 2006b), spectra of dissolved aquatic NOM samples isolated on XAD-8 and XAD-4 resins, a procedure commonly used for freshwater samples and especially in water treatment studies, have not been widely reported. For Suwannee River water, therefore, we report spectra of the XAD-4 acids and the XAD-8 hydrophobic neutral fraction in addition to the humic and fulvic acids, and compare the Nordic humic, fulvic and XAD-4 acids. We also examine the utility of liquid state ^{15}N NMR (^1H–^{15}N gHSQC (gradient selected heteronuclear single quantum coherence), DEPT (distortionless enhancement by polarization transfer), and direct detection experiments) for detection of naturally abundant nitrogen in the Elliot soil humic and Pony Lake fulvic acids. Objectives of the latter effort were to determine whether liquid state affords improved resolution over solid state analyses, as has been demonstrated in ^{13}C NMR studies of humic substances, and, in the case of direct detection, to assess whether liquid state ^{15}N NMR can be applied to the quantitation problem.

2. Experimental

2.1. Samples

Standard and reference samples were obtained from the International Humic Substances Society. Descriptions of the sources, isolation procedures and chemical properties of the samples are available from the IHSS (http://ihss.gatech.edu/ihss2/). In brief, the Elliot is an Illinois prairie soil, Summit Hill soil underlies tussock grass south of Christchurch, New Zealand, the Pahokee is a peat soil of the Florida Everglades, originating in organic deposits of freshwater marshes, and the Waskish is a sphagnum bog peat from Minnesota. The Leonardite formed from natural oxidation of exposed lignite coal in North Dakota. The Nordic samples were isolated from the Hellrudmyra tarn, outside Oslo, Norway. Pony Lake, Antarctica (Brown et al., 2004) fulvic acid was isolated between December, 2005 and February, 2006. Detailed information on the Suwannee River fulvic and humic acid samples, including further ^{13}C and ^1H NMR characterization, can be found in Averett et al. (1994).

Water was also collected on May 4, 1995, near the sampling location for the IHSS Suwannee River materials (DOC = 51.0 mg C/l), filtered through Balston DH (25 μm) and AH (0.3 μm) glass fiber filters, acidified to pH 2.0 with 1.0 N HCl and processed through XAD-8 and XAD-4 resins to obtain the ‘‘hydrophobic neutral

fraction" of acids (acetonitrile eluate of XAD-8 resin, performed after base elution of fulvic and humic acid fractions and column neutralization) and XAD-4 acids (base eluate of XAD-4 resin) (Aiken et al., 1992). The base eluate of the XAD-4 resin has alternately been referred to as the hydrophilic or transphilic acid fraction in the literature. To avoid confusion, this fraction is hereby referred to as the XAD-4 acids. Analytical DOC fractionation of the water sample was performed by Huffman Laboratories, Golden, Colorado. XAD-4 acids were also isolated along with the Nordic fulvic and humic acids at the same time as the preparation of these samples from the Hellrudmyra tarn.

2.2. Solid state NMR spectra

Solid state CP/MAS (cross polarization/magic angle spinning) ^{15}N NMR spectra were recorded on a Chemagnetics CMX-200 NMR spectrometer at a nitrogen resonant frequency of 20.3 MHz, using a 7.5 mm ceramic probe (zirconium pencil rotors). Acquisition parameters included a 30,000 Hz spectral window, 17.051 ms acquisition time, 2.0 or 5.0 ms contact time, 0.2–0.5 s pulse delay and spinning rate of 5 KHz, collected at $1–10 \times 10^6$ transients. Nitrogen-15 chemical shifts were referenced to glycine, taken as 32.6 ppm, and reported downfield of ammonia, taken as 0.0 ppm.

2.3. Liquid state spectra

Liquid state ^{1}H–^{15}N gHSQC spectra of the standard Elliot soil humic and reference Pony Lake fulvic acids were recorded on VARIAN INOVA 750 and 500 MHz spectrometers, respectively (5 mm Z-gradient HCN probes; ~80 mg in 0.75 ml dmso-d_6, 100% D). For the Elliot soil humic acid, 1100 transients were collected over 64 increments with a 0.1 s acquisition time, 1.0 s pulse delay, $^1J_{NH}$ of 93.0 Hz, 20,000 Hz (26.7 ppm) spectral window in the ^{1}H dimension and 12,500 Hz (164.6 ppm) spectral window in the ^{15}N dimension. For the Pony Lake fulvic acid, 512 transients were collected over 128 increments with a 0.064 s acquisition time, 1.5 s pulse delay, $^1J_{NH}$ of 93.0 Hz, 12,001.2 Hz (24.0 ppm) spectral window in the ^{1}H dimension and 5571.3 Hz (110.0 ppm) spectral window in the ^{15}N dimension. A one dimensional proton spectrum of the Pony Lake FA was also recorded with presaturation to remove the water signal. DEPT ^{15}N spectra were recorded on a VARIAN 300 MHz spectrometer, using a 10 mm broadband probe (~300 mg in 2.25 ml dmso-d_6). Acquisition parameters included a 0.2 s acquisition time, 0.3 s pulse delay for proton relaxation, $^1J_{NH}$ of 90 Hz, 7890.4 Hz (259.5 ppm) spectral window for the Elliot soil HA and 26,000 Hz (854.95 ppm) spectral window for the Pony Lake FA and 3.6–3.8 million transients. The ACOUSTIC spectrum of the Pony Lake fulvic acid was also recorded using a 10 mm broadband probe on the 300 MHz spectrometer; acquisition parameters included a 35,111.7 Hz (1154.3 ppm) spectral window, 0.2 s acquisition time, 0.25 s pulse delay and tau delay of 0.1 ms. The ^{15}N chemical shifts are reported in ppm downfield of ammonia, taken as 0.0 ppm.

Quantitative liquid state ^{13}C NMR spectra of the Nordic XAD-4 and Suwannee River XAD-4 acids, Suwannee River FA Std. II, Suwannee River hydrophobic neutral fraction and Pony Lake FA were recorded using a 10 mm broadband probe on the 300 MHz spectrometer with 50,000 Hz spectral window, 0.2 s acquisition time, 90° pulse angle, 12 s pulse delay and inverse gated decoupling, as described previously (Thorn et al., 1989). Sodium salts of the samples were dissolved in 75% D_2O; the $^+$H saturated

Table 1
Chemical properties of IHSS NOM samples.

Sample	%N	%N as amino acid	%C	C:N Ratio	^{13}C f_a (carbon aromaticity)	δ^{15}N
Elliot soil FA Std. (1S102F)	2.72	39.2	50.6	18.6	0.30	3.89
Elliot soil HA Std. (1S102H)	4.14	39.3	58.1	14.0	0.50	5.34
Summit Hill HA ref (1R106H)	5.13	59.6	54.0	10.5	0.30	2.99
Florida Pahokee Peat FA Std. (1S103F)	2.56	13.3	50.4	19.7	0.34	1.42
Florida Pahokee Peat HA Std. (1S103H)	3.69	20.3	56.4	15.3	0.47	1.29
Florida Pahokee Bulk Peat (2BS103P)	3.08	nd	45.7	14.8	nd	nd
Minnesota Waskish Peat FA ref (1R107F)	1.07	nd	53.6	50.1	0.36	nd
Minnesota Waskish Peat HA ref (1R107H)	1.47	nd	54.7	37.2	0.43	nd
Leonardite HA Std. (1S104H)	1.23	1.64	63.8	51.9	0.58	2.13
Suwannee River FA Std. I (1S101F)	0.72	6.83	52.4	72.8	0.24	−1.85
Suwannee River FA Std. II (2S101F)	0.67	nd	52.3	78.1	0.30	nd
Suwannee River HA Ref (1R101H)	1.17	14.2	52.9	45.2	0.37	−2.42
Suwannee River XAD-4 Acids	1.15	nd	50.2	43.6	0.15	nd
Suwannee River hydrophobic neutral	nd	nd	nd	nd	0.22	nd
Suwannee River NOM (1R101N)	1.10	nd	52.5	47.7	0.23	nd
Nordic FA ref (1R105F)	0.68	10.4	52.3	76.9	0.31	−1.55
Nordic HA ref (1R105H)	1.16	19.4	53.3	46.0	0.38	nd
Nordic XAD-4 Acids	nd	nd	nd	nd	0.21	nd
Pony Lake FA Ref (1R109F)	6.51	nd	52.5	8.06	0.13	nd

%N as AA and C/N ratios calculated from elemental analyses and amino acid analyses provided by IHSS. δ^{15}N's provided by IHSS. Numbers in parentheses refer to IHSS sample number. nd = Not determined. Carbon aromaticities taken from Thorn et al. (1989), or determined by authors.

Suwannee River hydrophobic neutral fraction was dissolved in dimethyl-^{12}C, d_6 sulfoxide. The carbon aromaticity, f_a, is defined as the spectrum area from 165 to 110 ppm, divided by the total spectrum area. Quantitation is achieved in the liquid state by employing pulse delays 3–5 times the longest spin lattice relaxation times (T_1's) present in the samples to eliminate differential saturation effects by allowing complete relaxation of all ^{13}C nuclei between pulses, and inverse gated decoupling to eliminate nuclear Overhauser enhancement (NOE) effects. The longest T_1 measured for these samples was 2.4 s (Thorn et al., 1989). Carbon nuclei with very short spin-spin relaxation times, T_2's, such as those in close proximity to paramagnetic metal ions or stable organic free radicals, or nuclei in very slowly tumbling molecules, that could result from aggregation, may have linewidths that are too broad to be observed.

2.4. Reaction of samples with glycine ^{15}N

Five hundred milligrams of Elliot Soil HA was added to 500 ml deionized water, adjusted to pH 7.0 with 1 N NaOH, charged with 150 mg of glycine ^{15}N (ISOTEC, 99 atom% ^{15}N), stirred for 7 days, ^{+}H saturated on an MSC-1 cation exchange column, and freeze dried. Suwannee River FA Std. I (190 mg) and glycine ^{15}N (58 mg) were similarly reacted.

3. Results and discussion

3.1. Properties of samples

Nitrogen contents of the samples range from 0.67 % N for the Suwannee FA Std. II to 6.51% for the Pony Lake FA (Table 1). Carbon

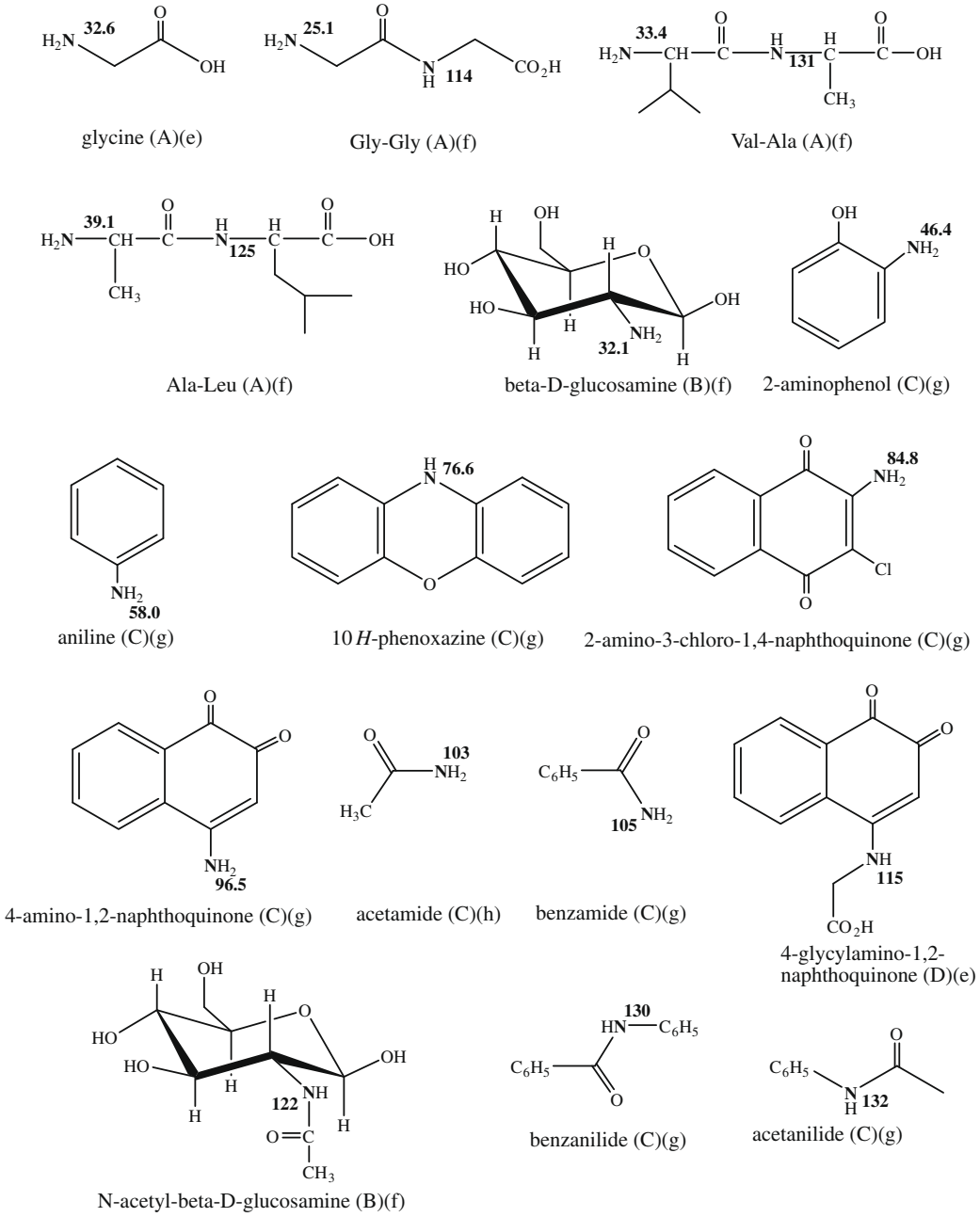


Fig. 1. N-15 NMR chemical shifts on the ammonia scale in ppm for selected structures. Data taken from (A) Berger et al., 1997; (B) Witanowski et al., 1986; (C) Thorn and Mikita, 1992; Thorn et al., 1996; (D) Determined in this lab. e = solid state, f = H_2O, g = dimethyl sulfoxide, h = chloroform, i = acetone, g = ether, k = neat.

to nitrogen ratios range from 8.06 for the Pony Lake FA to 78.1 for the Suwannee FA Std. II. Quantitative liquid state ^{13}C NMR spectra of the samples have been reported (IHSS; Thorn et al., 1989) or are presented further on. Carbon aromaticities determined from these spectra range from 0.13 for the Pony Lake FA to 0.58 for the Leonardite HA (Table 1). Amino acid analyses are available from the IHSS for ten of the samples that were analyzed by ^{15}N NMR in this study. The percent of the total nitrogen content that can be accounted for by the detected amino acids ranges from 1.64% for the Leonardite HA to 59.6% for the Summit Hill soil HA (Table 1). The Summit Hill HA appears to be an outlier, as percentages for the remaining samples are all under 40%, and literature values for soil materials are generally under 50% (Stevenson, 1994). The percent nitrogen accounted for by amino acids is higher in the humic acid than in the corresponding fulvic acid for the Pahokee peat, Suwannee River and Nordic samples, also consistent with the literature (Thurman, 1985; Stevenson, 1994). Among those standards for which δ ^{15}N values are available, the aquatic humic and fulvic acids are depleted in ^{15}N with respect to the soil, peat and Leonardite samples (Table 1).

3.2. Solid state ^{15}N NMR spectra of soil, peat and Leonardite samples

Nitrogen-15 NMR chemical shifts of representative structures are shown in Fig. 1. The solid state CP/MAS ^{15}N NMR spectra of the Elliot soil, Pahokee peat, Waskish peat, Summit Hill and Leonardite humic and fulvic acids share major features in common with spectra reported in the literature (Figs. 2 and 3). With the exception of the Leonardite (Fig. 2F), the major peak in each spectrum has a maximum in the range from 117 to 123 ppm, with most close to 119–121 ppm. These peaks are comprised of amides in the form of peptide (2° amide) nitrogens and possibly N-acetyl nitrogens (2° amides). Secondary aminoquinone nitrogen in the form of amino acids bonded to a quinone group as a Michael adduct also

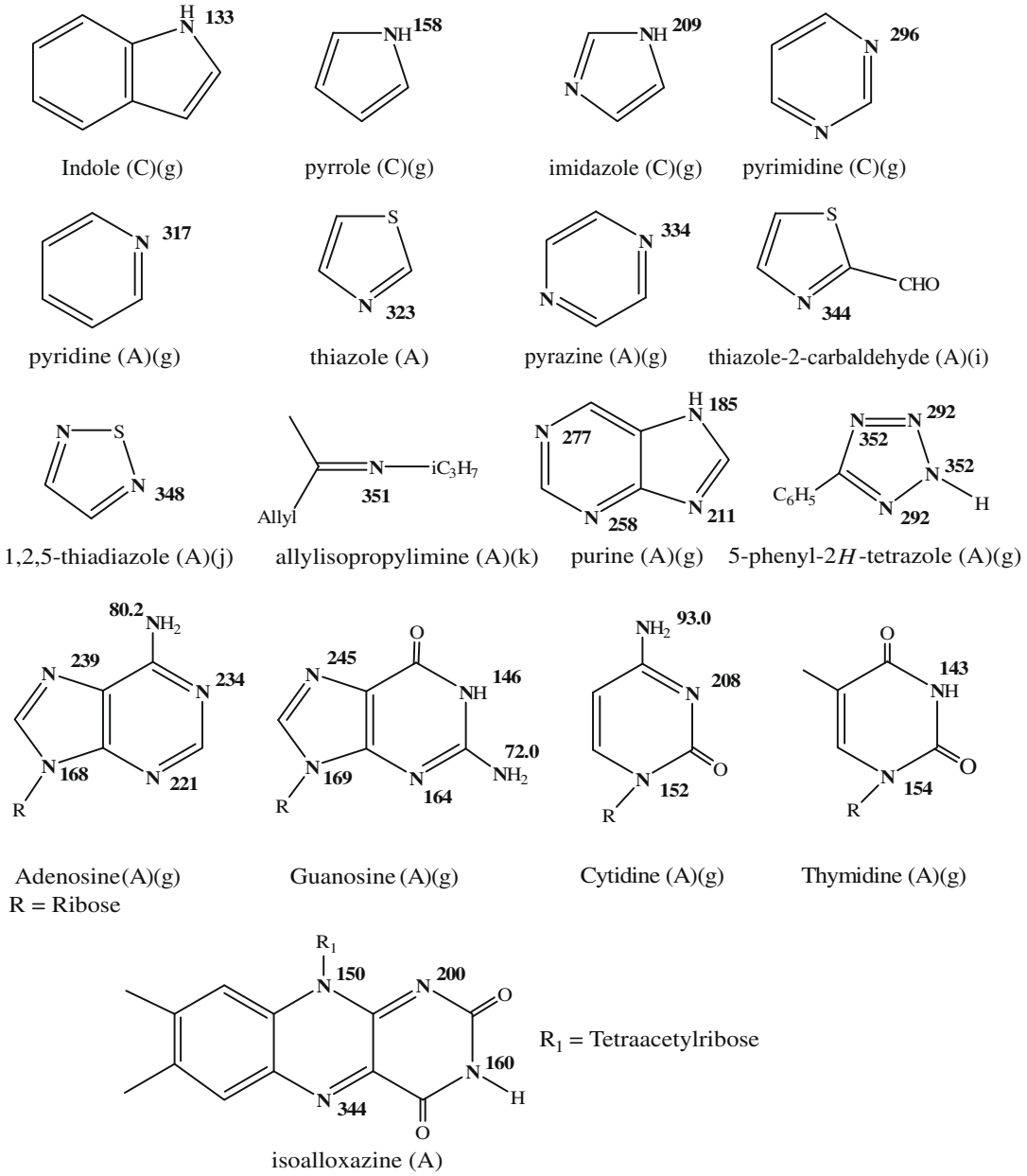


Fig. 1 (*continued*)

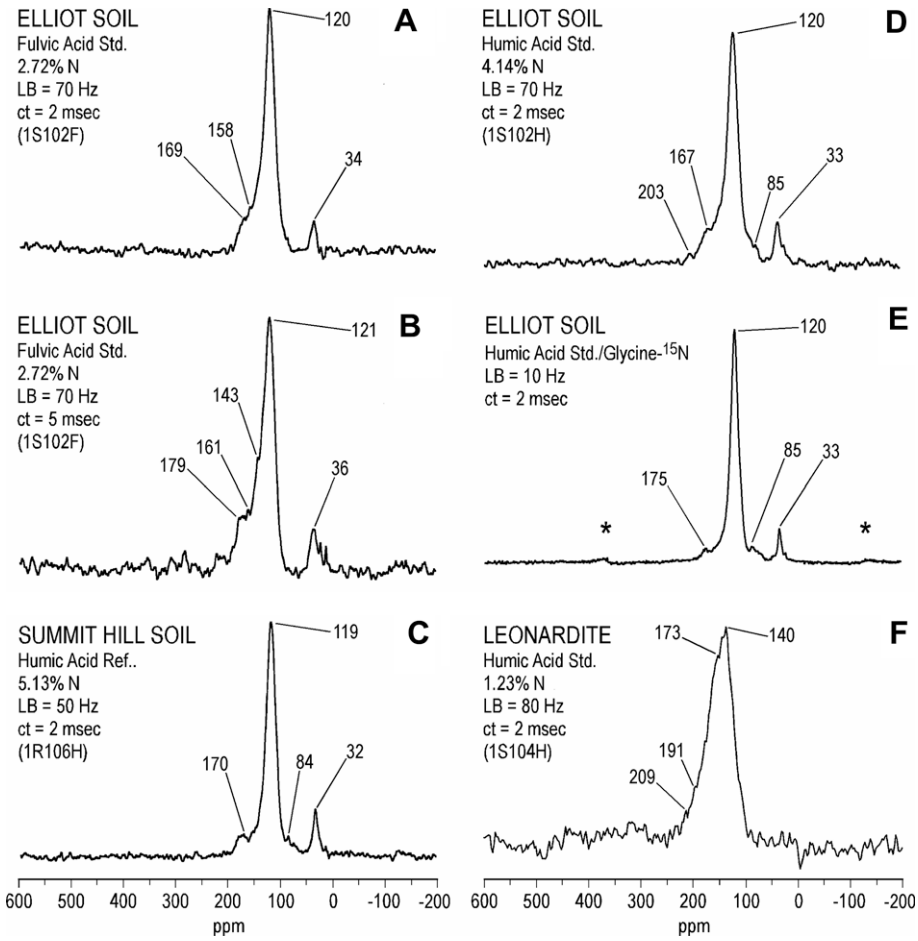


Fig. 2. Solid state CP/MAS ^{15}N NMR spectra of Elliot Soil fulvic and humic acids, Elliot soil humic acid reacted with glycine ^{15}N, Summit Hill soil and Leonardite humic acids. LB = line broadening in Hertz; ct = contact time in milliseconds. Asterisks denote spinning sidebands. Numbers in parentheses refer to IHSS sample number.

occur in this chemical shift region, and may contribute to the major peaks (Scheme 1). Anilide nitrogens have also been inferred from analyses of soil humic acids (Schmidt-Rohr et al., 2004). Peptide nitrogen was differentiated from N-acetyl amino polysaccharide nitrogen, in the case of marine DON isolated by ultrafiltration, by following the conversion of amide nitrogen into amine nitrogen upon mild acid hydrolysis and quantifying the amount of acetic acid (from the N-acetyl N) vs. amino acid nitrogen (from peptide) released (Aluwihare et al., 2005).

With the exception again of the Leonardite, all spectra of the soil and peat samples exhibit peaks at 31–36 ppm, attributable to amino sugars and the free amine groups of terminal amino acids. Several of the spectra (Elliot, Summit Hill and Pahokee humic acids; Figs. 2D,C and 3B, respectively) show low intensity shoulders upfield of the amide/aminoquinone peaks in the range from about 73 to 84 ppm. Possible assignments would include the primary amines of purine and pyrimidine bases such as adenine, guanine and cytidine, primary aminoquinone and other aromatic amines, and N-glycosyl nitrogens (Fig. 1). All spectra exhibit shoulders, if not well defined peaks, downfield of the amide/aminoquinone nitrogens extending to about 200 ppm, attributable to heterocyclic nitrogens. Resolution of the heterocyclic nitrogen peaks or shoulders tends to vary with the relative signal to noise ratios achieved for the spectra (Table 2), in general a function of the nitrogen contents. Spectra with well resolved heterocyclic nitrogen peaks include the Elliot soil HA (167 ppm; Fig. 2D), Waskish peat humic (172 ppm; Fig. 3E) and fulvic acid (173 ppm; Fig. 3D), Pahokee Peat FA (174 ppm; Fig. 3A) and Summit Hill soil HA (170 ppm; Fig. 2C). The types of heterocyclic nitrogens with chemical shifts in the region from approximately 130 to 200 ppm include indoles, pyrroles, the imide or lactam nitrogens of nucleotide bases and the side chains of amino acids such as tryptophan and histidine. Peak areas for the spectra, which can be interpreted only semi-quantitatively because of limitations in the CP experiment discussed above, are listed in Table 2. Two sets of intervals were integrated electronically: 0–60 ppm (amino sugar/terminal amino acid), 60–160 ppm (amide/aminoquinone), 160–250 ppm (heterocyclic) and 0–60 ppm, 60–140 ppm and 160–250 ppm. The interval of 160–250 ppm was chosen in the first set because the natural trough in several of the spectra occurs close to 160 ppm; this interval corresponds to a minimum estimate of heterocyclic nitrogens. The interval of 250–140 ppm was chosen in the second set because 140 ppm corresponds to the downfield limit of peptide nitrogens, and from the liquid state DEPT spectrum of the Elliot soil HA (vide infra), 140 ppm is a conservative chemical shift cut off position for amide nitrogens; this interval corresponds to an intermediate estimate of heterocyclic nitrogens, but may be more susceptible to overlap problems. By the minimum estimates, heterocyclic nitrogens range from 10.3% for the Summit Hill HA to 33.4% for the Leonardite HA; by the intermediate estimates, the range is from 17.8% to 60.2% for the two samples, respectively. The relatively low heterocyclic nitrogen content of the Summit Hill HA is consistent with its having the highest percentage of nitrogen accounted for by amino acids. Conversely, the Leonardite HA has the highest concentration of heterocyclic nitrogens and the lowest percentage nitrogen accounted for by amino acids. It is also

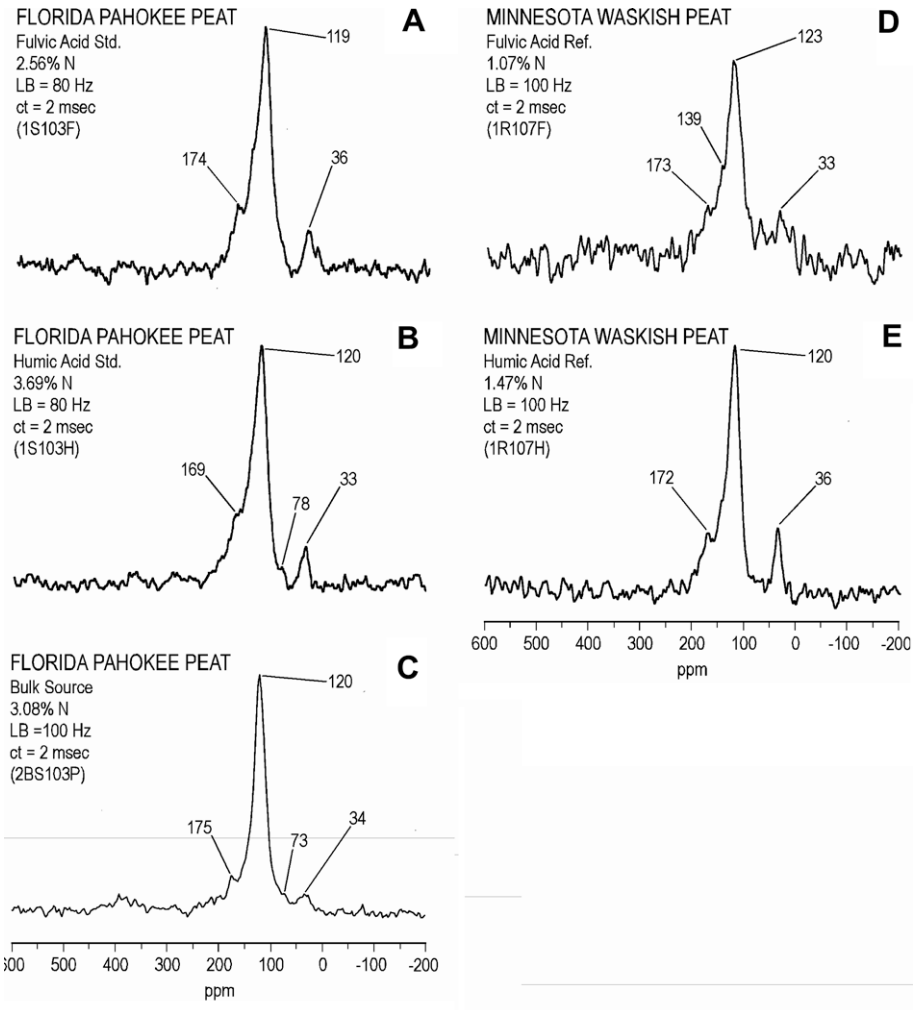
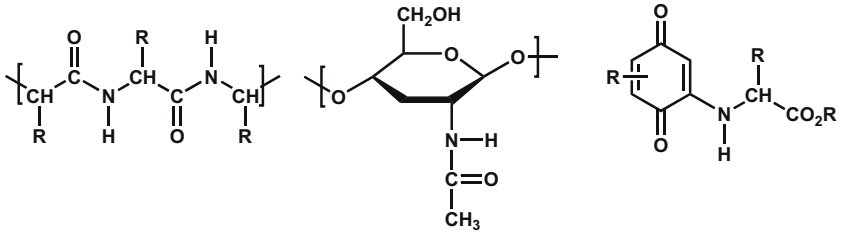


Fig. 3. Solid state CP/MAS ^{15}N NMR spectra of Florida Pahokee Peat fulvic acid, humic acid, bulk peat and Minnesota Waskish Peat fulvic and humic acids. LB = line broadening in hertz; ct = contact time in milliseconds. Numbers in parentheses refer to IHSS sample number.

noteworthy that the chemical shift position of 140 ppm for the peak maximum of the Leonardite (Fig. 2F) is shifted downfield toward heterocyclic and away from amide nitrogens.

The fact that the percentages of heterocyclic nitrogens listed in Table 2 may be underestimated is substantiated from a comparison of the Elliot soil FA recorded at 2 and 5 ms contact times (Fig. 2A–B). An increase of approximately 6–8% in the proportion of heterocyclic nitrogen is apparent in going from 2 to 5 ms, and reflected in the integration values (Table 2), although the overall signal to noise ratio of the spectrum at 5 ms is diminished.

The spectra of the Elliot soil fulvic and humic acids (Fig. 2A and D) and bulk Pahokee peat (Fig. 3C) correspond to longer accumulation times than the spectra of these samples we reported previously (Thorn et al., 1996; Thorn and Mikita, 2000). The signal to noise ratios of the heterocyclic nitrogen peaks or shoulders and the amino sugar/terminal amino acid peaks are significantly improved with the additional number of transients. In the case of the bulk Pahokee peat, the heterocyclic nitrogen peak at 175 ppm was not detected in the original spectrum recorded at approximately one third the number of transients (Thorn and



Scheme 1.

Table 2
Peak areas as percent of total nitrogen for solid state CP/MAS ^{15}N NMR spectra of NOM samples.[a]

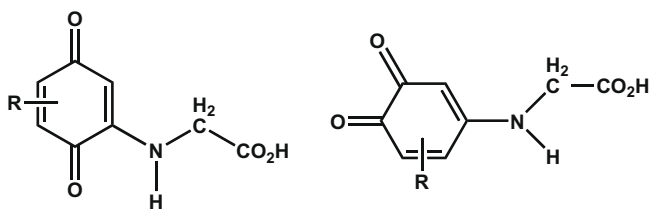
Sample	Heterocyclic 250–160 ppm	Amide/aminoquinone 160–60 ppm	Amino sugar/terminal AA 60–0 ppm	Heterocyclic 250–140 ppm	Amide/aminoquinone 140–60 ppm	Amino sugar/terminal AA 60–0 ppm	S/N ratio [b]
Elliot soil FA Std.	10.4	84.2	5.5	21.6	73.0	5.5	60.0[b]
Elliot soil FA Std. ct = 5ms	16.6	74.8	8.6	29.4	62.1	8.6	33.7[b]
Elliot soil HA Std.	12.7	79.8	8.4	23.5	68.8	8.4	56.0[c]
Summit Hill HA ref	10.3	81.6	8.9	17.8	74.2	8.9	91.7[b]
Florida Pahokee Peat FA Std.	15.0	76.4	8.5	26.9	64.5	8.6	26.2[b]
Florida Pahokee Peat HA Std.	19.7	73.3	6.9	31.9	61.2	6.9	30.6[b]
Florida Pahokee Bulk Peat	12.1	84.0	6.5	21.4	74.7	6.5	32.5[c]
Minnesota Waskish Peat FA ref	14.0	71.1	14.9	26.3	58.8	14.9	11.4[c]
Minnesota Waskish Peat HA ref	16.2	72.3	11.1	26.9	61.6	11.1	23.1[b]
Leonardite HA Std.	33.4	65.0	1.6	60.2	38.2	1.6	17.5[c]
Suwannee River FA Std. I	19.8	78.9	2.7	36.6	62.2	2.7	22.8[b]
Suwannee River FA Std. II	21.9	73.3	4.9	39.2	56.0	4.9	14.5
Suwannee River HA Ref	21.1	70.9	8.0	36.8	55.2	8.0	10.7[b]
Suwannee River XAD-4 Acids	24.6	74.7	1.5	41.3	58.0	1.5	19.1[b]
Suwannee River Hydrophobic Neutral	9.9	86.7	3.4	17.6	78.8	3.4	22.8[b]
Suwannee River NOM	17.9	79.0	2.9	32.9	64.0	2.9	10.7[b]
Nordic FA ref	24.9	64.6	10.5	41.3	48.2	10.5	11.6[c]
Nordic HA ref	16.0	72.0	13.0	27.4	60.6	13.0	15.3[b]
Nordic XAD-4 acids	17.9	71.2	10.9	35.1	54.0	10.9	9.6[c]
Pony Lake FA ref	12.7	83.4	3.9	29.0	67.1	3.9	49.2[c]
Pony Lake	13.7 [d]	82.8	3.5	32.7	63.8	3.5	38.6[c]
FA ref ct = 5	(11.8)	(71.4)	(3.0)	(28.2)	(55.0)	(3.0)	

[a] Values are semi quantitative. Error estimated at ±4%.
[b] S/N ratio calculated from signal intensity of tallest peak and noise level from −200 ppm to −600 ppm at a line broadening of 100 Hz.
[c] S/N ratio calculated from signal intensity of tallest peak and noise level from 500 ppm to 900 ppm at a line broadening of 100 Hz.
[d] Numbers in parentheses adjusted to include peak centered at 350 ppm, which comprises 13.8% of the total spectrum.

Mikita, 2000). An obvious point is that sufficient spectrometer time needs to be invested in order to detect the low intensity heterocyclic nitrogens, which may in turn be underestimated in the CP experiment. There is a natural tendency to cease acquisition once the major amide peaks have attained an adequate signal to noise ratio.

3.3. Elliot soil HA reacted with glycine-15

The structural configuration of amino acids in humic and fulvic acid molecules is incompletely understood. Amino acids and peptides are known to undergo condensation reactions with quinones and reducing sugars and so are plausibly linked to NOM molecules through aminoquinone and glycosidic bonds. Whether amino acids are bonded to the NOM molecules as monomers, dipeptides, tripeptides or greater, also is not known. Because the chemical shifts of 2° aminoquinone (2° amino acid-quinone) and peptide bonds overlap with one another, these structures cannot necessarily be resolved from one another by ^{15}N NMR. The spectrum of the Elliot soil HA reacted with ^{15}N labeled glycine illustrates this point (Fig. 2E). Here the spectrum can be assigned entirely to the ^{15}N label, as the spectrum developed fully with a number of transients less than required for the naturally abundant nitrogens to appear. The main peak at 120 ppm is comprised of 2° aminoquinone nitrogens, adducts from Michael addition (1,4-addition) of the glycine to quinone groups in the humic acid (Scheme 2). (The aminoquinone nitrogen peak correlates to protons at 7.9 ppm by liquid state ^{1}H–^{15}N gHSQC analysis, not shown.)



Scheme 2.

The ^{15}N NMR chemical shift position of the aminoquinone peak coincides with the peak maximum of the naturally abundant nitrogens of the soil humic acid at 120 ppm. This observation confirms the argument that the major peaks in the naturally abundant spectra of the samples can plausibly be assigned in part to aminoquinone nitrogens in addition to amide nitrogens. The spectrum of the humic acid reacted with glycine-^{15}N also shows a low intensity, broad peak from about 50 to 93 ppm that may be comprised of aminohydroquinone and N-glycosyl nitrogens. Heterocyclic nitrogens also occur downfield of the aminoquinone peak out to about 190 ppm, with a distinct peak at 175 ppm. The unreacted glycine occurs at 33 ppm. The spectrum reveals that heterocyclic nitrogen structures may form from uncatalyzed nucleophilic addition reactions of glycine with NOM. These observations are replicated in the spectrum of Suwannee River FA reacted with glycine (Fig. 4C), which exhibits heterocyclic nitrogen peaks at 149, 173 and 202 ppm.

Potentially supportive evidence for the occurrence of aminoquinone nitrogens in NOM samples is the detection in soil humic acids of nitrogens bonded to aromatic carbons by the SPIDER pulse se-

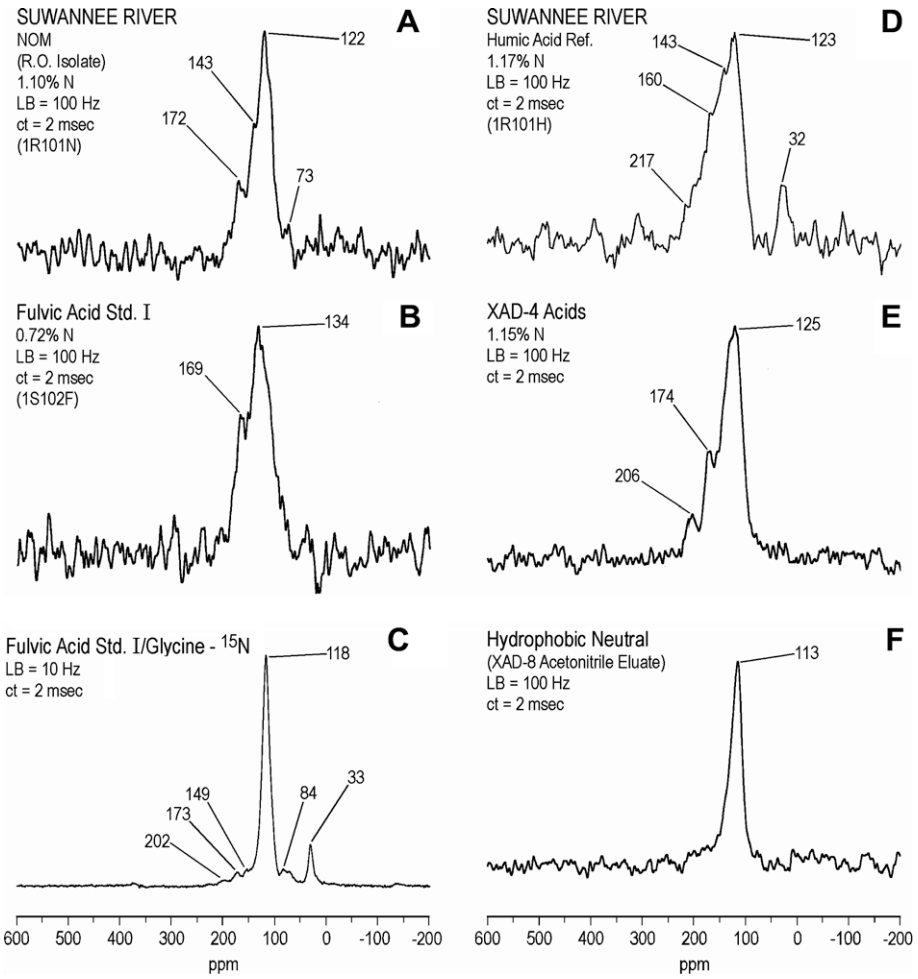


Fig. 4. Solid state CP/MAS ^{15}N NMR spectra of Suwannee River NOM, fulvic acid, humic acid, XAD-4 Acids, Hydrophobic neutral fraction, NOM and fulvic acid reacted with glycine-^{15}N. LB = line broadening in Hertz; ct = contact time in milliseconds. Numbers in parentheses refer to IHSS sample number.

quence (Schmidt-Rohr et al., 2004). Model compound studies have indicated that the bonds formed between amino acids and quinones are partially resistant to acid hydrolysis (Flaig et al., 1975; Zhuo and Wen, 1993; Stevenson, 1994); aminoquinone bonds in NOM could partly account for what have been termed nonhydrolysable amide bonds (Abe et al., 2005). The conditions for the reaction in this study were designed to effect the nucleophilic addition of glycine to the humic acid. These conditions should be differentiated from the long term incubation of glycine in unsterilized peat in the original study of Benzing-Purdie, where, in addition to direct condensation of the glycine with peat, ammonification of glycine followed by uptake and resynthesis of the labeled ammonia into peptide nitrogen by microorganisms is possible (Benzing-Purdie et al., 1986).

3.4. Solid state ^{15}N NMR spectra of aquatic samples

The set of Suwannee River fulvic acid, humic acid, XAD-4 acids, hydrophobic neutral fraction and reverse osmosis isolate (SRNOM) spectra comprises a unique compilation (Figs. 4 and 5). A comparison of these fractions from the same water source by solid state CP/MAS ^{15}N NMR has not been available before. The fractions were collected at different times however: the fulvic and humic acids from 1982–1983, the XAD-4 acids and hydrophobic neutral fraction in 1995 and the R.O. isolate in 1999. The FA Std. II sample was isolated in 2003. Comparison of the spectra therefore is made with the caveat that properties of the individual fractions, and relative proportions of the fractions within the total DOC, can change over time. Over the two month sampling period in 1982–1983, the fulvic (Std. I) and humic acids constituted *by recovery* 65.4% and

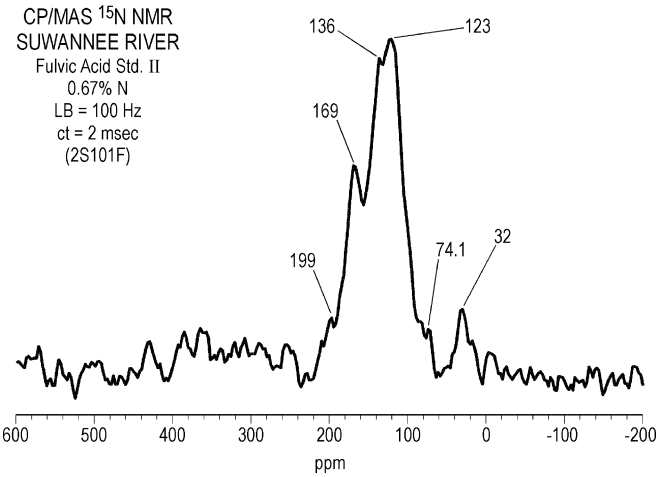


Fig. 5. Solid state CP/MAS ^{15}N NMR spectrum of Suwannee River Fulvic Acid Std. II. LB = line broadening in Hertz; ct = contact time in milliseconds. Number in parentheses refers to IHSS sample number.

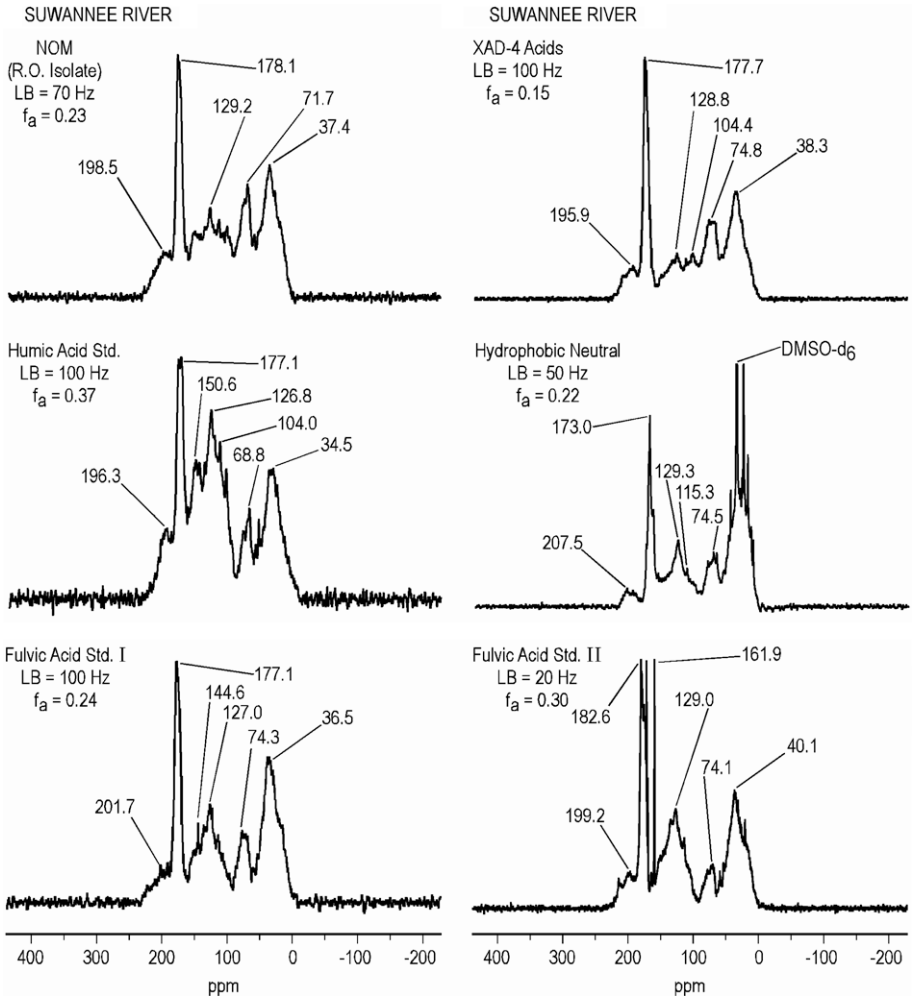


Fig. 6. Quantitative liquid state ^{13}C NMR Spectra of Suwannee River NOM, humic acid, fulvic acid, XAD-4 Acids and hydrophobic neutral fraction. LB = line broadening in Hertz; f_a = ^{13}C aromaticity.

9.6%, respectively, of the total DOC, which varied from 35 to 50 mg C/l (Averett et al., 1994). For the 1995 water sample, the XAD-4 acids, hydrophobic neutral and combined fulvic and humic acid fractions comprised 35%, 33% and 27% of the total DOC (51.0 mg C/l), respectively, *by analytical fractionation*. Quantitative liquid state ^{13}C NMR spectra of the Suwannee River humic, fulvic and XAD-4 acids illustrate a structural relationship that is consistent for these three fractions from other water sources (including Hellrudmyra tarn, discussed next): from humic to fulvic to XAD-4 acids, one observes an increase in the concentration of carboxylic acid/amide carbons, decrease in aromatic carbons and increase in O-alkyl carbons (Fig. 6; Table 3). The hydrophobic neutral fraction has the lowest concentration of carboxylic acid carbons among the four fractions. The SRNOM R.O. isolate corresponds to a 92.9% recovery of organic carbon from Suwannee River water over the period of isolation. The SRNOM should correspond approximately to the bulk dissolved organic nitrogen therefore. In its ^{15}N spectrum, the major amide/aminoquinone peak occurs at 122 ppm; a well resolved heterocyclic nitrogen peak occurs at 172 ppm and heterocyclic shoulder at 143 ppm (Fig. 4A). The heterocyclic nitro-

Table 3
Peak areas as percent of total carbon for quantitative liquid state ^{13}C NMR spectra of NOM samples.[a]

Sample	Ketone/quinone 230–190 ppm	Carboxyl/amide/ quinone 190–165 ppm	160–90 ppm	Aromatic/Olefinic 165–110 ppm	Acetal/Ketal 110–90 ppm	O-Alkyl 90–60 ppm	Aliphatic 60–0 ppm	Aromaticity, f_a
SRNOM	8	20	30	23	7	15	27	0.23
Suwannee River HA	8	19	45	37	9	7	21	0.37
Suwannee River FA Std. I	7	20	28	24	5	11	33	0.24
Suwannee River FA Std. II	7	23	34	30	4	7	30	0.30
Suwanee River XAD-4	7	24	22	15	7	17	30	0.15
Suwannee River HPO-N	4	16	26	22	4	12	42	0.22
Nordic HA	10	19	45	38	7	11	15	0.38
Nordic FA	10	24	37	31	7	12	18	0.31
Nordic XAD-4	8	24	30	21	9	16	23	0.21
Pony Lake	6	19	14	13	0.3	8	53	0.13

[a] From Thorn et al. (1989) or determined by the authors.

gens occur downfield to approximately 205 ppm. No amino sugar/terminal amino acid peak is visible. Amide/aminoquinone nitrogens are the peaks of major intensity in the fulvic acid (Figs. 4B and 5), humic acid (Fig. 4D), XAD-4 acids (Fig. 4E) and hydrophobic neutral fractions (Fig. 4F). The fulvic and humic acids contain heterocyclic nitrogens downfield to 195 ppm and 222 ppm, respectively. The spectrum of the XAD-4 acids contains the most well resolved heterocyclic nitrogen peaks, at 174 ppm and 206 ppm. Heterocyclic nitrogen contents range from 17.9% to 24.6% by minimum estimate and 32.9–41.3% by intermediate estimate for the NOM, fulvic, humic and XAD-4 fractions. The spectrum of the hydrophobic neutral fraction consists mainly of a narrow amide peak with no well resolved heterocyclic nitrogen peak apparent, at the signal to noise ratio achieved. This fraction appears to have the least heterogeneous distribution of nitrogens. Among the SRNOM and four fractions, only the humic acid and FA Std. II exhibit an amino sugar/terminal amino acid peak, at 32 ppm, again, at the S/N ratios attained.

Comparison of the data for the Suwannee River FA Std. I (1982–1983) and Std. II (2003) samples offers some insight into the question of how the characteristics of this fraction can change over time, or alternatively, the reproducibility of the isolation procedure. The elemental analyses are consistent (IHSS), including the carbon and nitrogen contents (52.4% and 0.72% vs. 52.3% and 0.67% for Std. I and Std. II, respectively; Table 1). The ^{13}C NMR spectra indicate a higher aromaticity for the Std. II FA (f_a = 0.30 vs. 0.24; Table 3); the carbonate indicated by the peak at 161.9 ppm was present in the Std. II sample as received (Fig. 6). The heterocyclic nitrogen peak at 169 ppm is more clearly resolved in the ^{15}N spectrum of the Std. II sample, as is the amide peak, with maximum at 123 ppm and a shoulder at 136 ppm (Fig. 5). As mentioned above, an amino sugar/terminal amino acid peak is observed in the Std. II but not the Std. I sample.

Like the Suwannee River, the Hellrudmyra tarn, source of the Nordic humic, fulvic and XAD-4 acids, may be considered a blackwater body because of its high DOC content (range 10–25 mg/l) and pH (range 4.0–4.9). The Nordic samples are individually the most highly aromatic aquatic humic, fulvic and XAD-4 acids that we have encountered, based upon quantitative liquid state ^{13}C NMR analyses (Fig. 7; Table 3). The humic, fulvic and XAD-4 acids contain a substantial amino sugar/terminal amino acid peak (34, 36 and 35 ppm, respectively) in the ^{15}N NMR spectra (Fig. 8). The fulvic and XAD-4 acids have well resolved heterocyclic nitrogen peaks at 168 ppm and 173 ppm, respectively. Heterocyclic nitrogens extend downfield to 220, 207 and 220 ppm, constituting at minimum 16.0%, 24.9% and 17.9% of the total nitrogen, in the humic, fulvic and XAD-4 acids, respectively.

Pony Lake FA, with the highest nitrogen content (6.51%) and lowest carbon aromaticity of all the samples (Fig. 9; Table 3), is considered to be derived autochthonously from microbial biomass; higher plants, including lignin, are excluded as source materials (Brown et al., 2004). Similar to the other aquatic fulvic acids, it contains a peak of major intensity in the amide/aminoquinone region (121 ppm), as well as resolved heterocyclic nitrogen peaks at 144 ppm and 172 ppm (Fig. 8D). No clearly resolved amino sugar/terminal amino acid peak is visible, although a shoulder upfield of the amide peak occurs at 43 ppm. A unique feature of the sample that becomes apparent in the spectrum recorded at a contact time of 5 ms (Fig. 8E–F) is the low intensity, broad peak from approximately 235 to 450 ppm, with maximum at 351 ppm, which constitutes approximately 13.8% of the spectrum. There may be a minor spinning side band component to this peak, as determined from varying the rotor speed, although an upfield component is not visible. At the spinning rate of 5 KHz used to record the spectrum, the first order spinning sidebands to the main amide/aminoquinone peak at 121 ppm should be centered at approximately 375 and

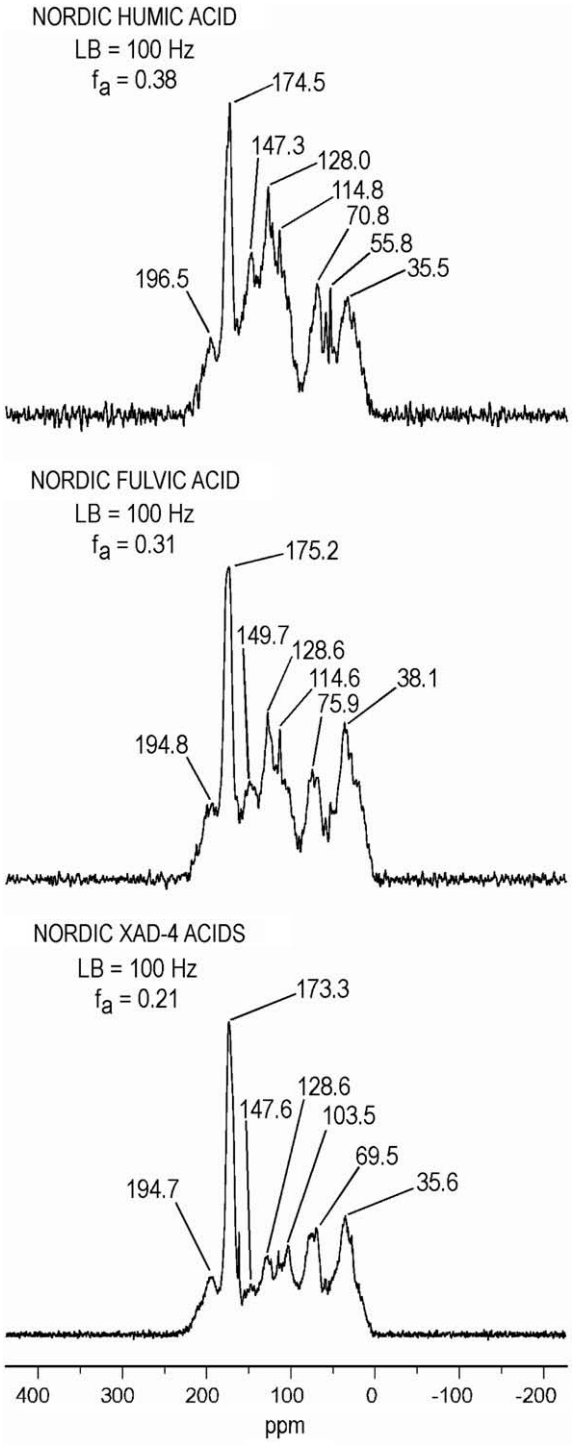


Fig. 7. Quantitative liquid state ^{13}C NMR spectra of Nordic humic, fulvic and XAD-4 acids. LB = line broadening in Hertz; f_a = ^{13}C aromaticity.

−132 ppm. Thus there is some uncertainty in defining the downfield endpoint of the broad peak. From blank control runs on rotors packed with alumina, the peak does not appear to be a background signal from the probe or spectrometer. The broad peak encompasses pyridine (~317 ppm), pyrazine (~334 ppm) and imine (~330 ppm) nitrogens. Nitrogens with chemical shifts in the range from 340 to 350 ppm include flavin (e.g., the 1,4 diazine unit in the isoalloxazine ring of flavins), tetrazole, thiazole and 1,2,5-thiadiazole (Fig. 1). We are currently seeking to reproduce the spectrum at higher field, to completely rule out spinning side bands and

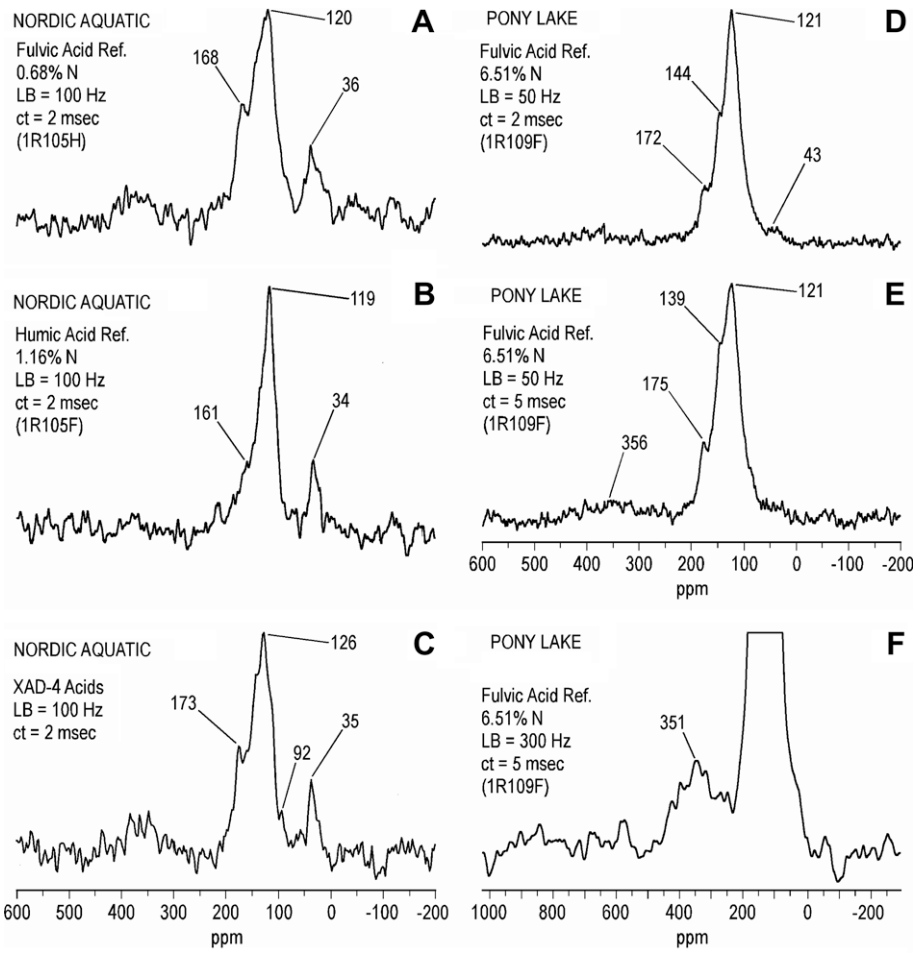


Fig. 8. Solid state CP/MAS ^{15}N NMR spectra of Nordic fulvic, humic, and XAD-4 acids and Pony Lake fulvic acid. LB = line broadening in Hertz; ct = contact time in milliseconds. Numbers in parentheses refer to IHSS sample number. Fig. 8F is a horizontal and vertical expansion of 8E.

background signals. If the peak is confirmed as real, then this would be the first observation of these types of nitrogens in an NOM sample by NMR. Likewise, attempts to confirm as real the peaks at approximately 330–430 ppm in the spectra of the Nordic fulvic and XAD-4 acids are underway.

The solid state ^{15}N NMR spectra of the IHSS Pony Lake FA differ from the previously reported spectrum of the fulvic acid isolated in 1997 (Mao et al., 2007). Differences may be attributable both to a varying set of NMR acquisition parameters and genuine compositional changes in the sample resulting from alternating limnological conditions, such as conversion from ice cover to ice free conditions (Brown et al., 2004). The 1997 spectrum exhibited a more intense and well resolved heterocyclic nitrogen peak at 140 ppm. The broad peak at 351 ppm in the IHSS sample was not observed in the 1997 sample. However, these nitrogens, if real, would not necessarily be observed at the shorter contact time of 1msec used to record the 1997 sample.

3.5. Liquid state ^{15}N NMR spectra of Elliot Soil HA and Pony Lake FA

In theory, the liquid state polarization transfer experiment (DEPT) and indirect detection experiment (gHSQC) can provide up to approximately 10 and 300 times the signal intensity, respectively, relative to direct observation of ^{15}N in the absence of nuclear Overhauser enhancement (NOE) (Claridge, 1999). The ^{1}H–^{15}N gHSQC experiment has received very limited application to naturally abundant nitrogen in humic substances (Cook et al., 2003). The DEPT and gHSQC spectra of the Elliot Soil HA and Pony Lake FA (Fig. 10) show only nitrogens directly bonded to protons. In general, the set of DEPT and gHSQC spectra are consistent with one another for each of the two samples and thus provide a cross check on the reproducibility of the NMR experiments. Nitrogens bonded to protons occur from approximately 100 ppm to 130 ppm in the Elliot Soil HA. The peak maximum for the amide/aminoquinone nitrogens occurs at 117.0 ppm in the DEPT spectrum, with a shoulder at 104.3 ppm. These nitrogens are resolved into separate peaks at 117.0 ppm and 104.9 ppm in the gHSQC spectrum; both nitrogen peaks correlate to a band of protons over approximately 2 ppm centered at 7.6 ppm. The possibility that the peak at 104.9 ppm may correlate to primary amides needs to be explored. The amino sugar/terminal amino acid peak present at 33 ppm in the solid state spectrum of the Elliot Soil HA (Fig. 2D) is not observed in the DEPT or gHSQC spectra, possibly because these nitrogens are undergoing rapid exchange with protons or, alternatively, are simply of insufficient concentration for detection in the liquid state experiments. Similar to the Elliot soil HA, a DEPT spectrum of the Pahokee Peat HA, not shown, exhibited a shoulder at 106.5 ppm upfield of the amide/aminoquinone peak maximum at 117.4 ppm.

The DEPT spectrum of the Pony Lake FA exhibits an ammonia peak at 22.6 ppm and enhanced resolution among nitrogens bonded to protons not apparent in the solid state spectrum. Resolved peaks occur at 86.3 ppm, 108.2 ppm and 115.2 ppm. The peak at 86.3 ppm is upfield of the range for peptide nitrogens, and as discussed previously, may be assigned possibly as primary amine, primary aminoquinone or N-glycosyl nitrogens. These

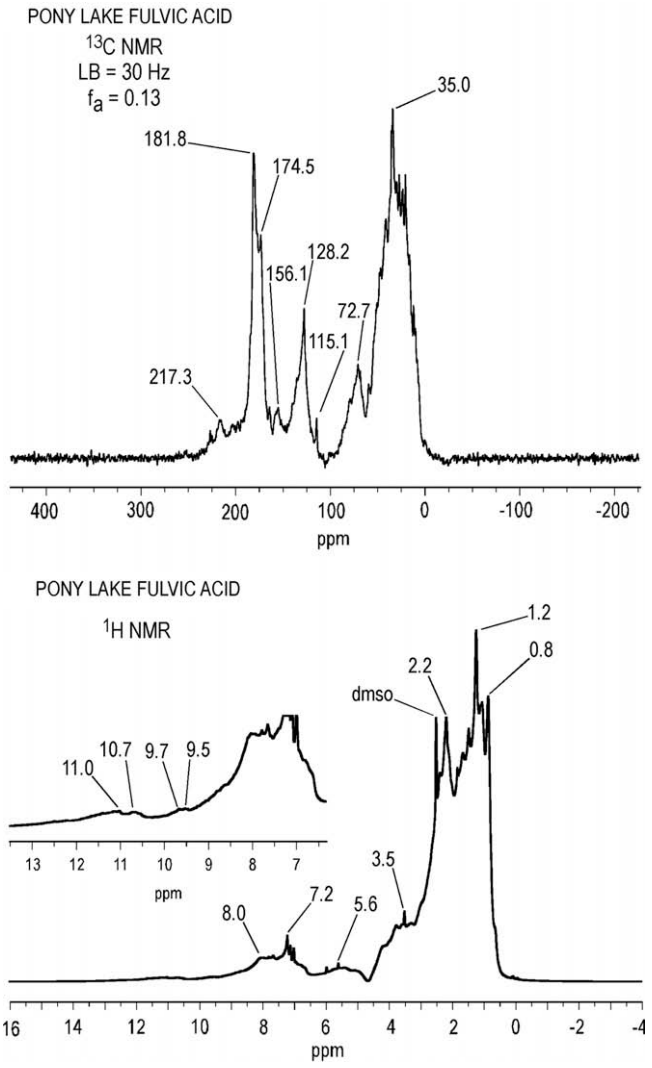


Fig. 9. Quantitative liquid state ^{13}C NMR spectrum and liquid state ^{1}H NMR spectrum of Pony Lake fulvic acid. LB = line broadening in hertz; f_a = ^{13}C aromaticity. ^{13}C spectrum recorded in 75% D_2O. ^{1}H spectrum recorded in dmso-d_6 with presaturation of water peak.

peaks are also evident in the ^{15}N projection of the gHSQC spectrum, which also shows peaks at 103.5 and 120.4 ppm. The nitrogens correlate to protons over an approximately 3 ppm chemical shift range at 6.8, 7.3 and 7.9 ppm. The full proton spectrum is shown in Fig. 9. More specific assignments for the individual nitrogen peaks are beyond the scope of this study. The DEPT spectrum also shows low intensity peaks from approximately 135 to 174 ppm, which would correlate to heterocyclic nitrogens bonded to protons such as pyrroles and imides. The spectral window employed for the gHSQC spectrum excluded the ammonia peak observed in the DEPT spectrum.

Liquid state spectra were also obtained by direct observation; a single pulse experiment with paramagnetic relaxation reagent (chromium III acetylacetonate) in the case of the Elliot soil HA (500 MHz) and the ACOUSTIC pulse sequence without paramagnetic reagent in the case of the Pony Lake FA (300 MHz). The spectrum of the Pony Lake FA is shown in Fig. 11; it exhibits the major amide peak at 112.4 ppm and a heterocyclic nitrogen peak at 153.4 ppm, both inverted due to negative NOE factors. The peak at 315.9 ppm is a background signal from the probe. The ammonia peak observed in the DEPT spectrum was not evident in the ACOUSTIC spectrum when a narrow line broadening was applied. The spectrum of the soil humic acid also exhibited the major amide peak at 121.2 ppm; it is not shown because of uncertainties over the presence of aliased peaks.

These results demonstrate the feasibility of acquiring liquid state spectra of naturally abundant nitrogen in NOM, at least with samples of relatively high nitrogen content ($\geqslant$4%). The ^{1}H–^{15}N gHSQC experiments are in the realm of practicality, as these spectra were obtained in approximately one day accumulation times. (DEPT spectra required 2–3 weeks of spectrometer time on the 300 MHz instrument.) Acquisition of indirect detection spectra of samples from more typical aquatic environments where the nitrogen contents are closer to 1% will require longer blocks of spectrometer time, but the increasing availability of cryogenic probes and higher field spectrometers mitigates in favor of this. The ^{1}H–^{15}N gHSQC experiment has potential application as a complement to the CP/MAS experiment in studies on nitrogen photochemistry and reaction of nitrogen with disinfectants in aquatic NOM. For example, limitations in resolution restricted interpretation of the CP/MAS ^{15}N spectrum of SRNOM subjected to UV irradiation (Thorn, 2002). Further investigation of acquisition parameters for direct detection experiments is worth pursuing, where spectrometer time is available, as these experiments may ultimately shed light on the unresolved questions of quantitation and detection of heterocyclic nitrogens, and other nitrogens not directly bonded to protons.

4. Conclusions

Solid state CP/MAS ^{15}N NMR spectra of naturally abundant nitrogen in humic substances have been appearing in the literature for about 15 years by now, however, there is still much opportunity for further investigation of nitrogen in natural organic matter by both solid and liquid state ^{15}N NMR. In the solid state, CP/MAS at low temperature, Dynamic Nuclear Polarization (DNP) (Solum et al., 1997; Hu et al., 2000) and at higher fields CP/MAS with longer contact times and DP/MAS (direct polarization) experiments have not been examined in detail. Together with more specific chemical analyses of the IHSS samples such as amino sugars and nucleic acid derivatives, these additional NMR analyses have the potential to narrow the current gap with information provided by XPS and XANES, notably again the detection by XANES but not NMR of pyridine and oxidized pyridine nitrogens in the Suwannee River and Pahokee peat humic and fulvic acids, and the Elliot soil HA (Vairavamurthy and Wang, 2002). The most accurate and comprehensive nitrogen accounting possible, through identification of trace nitrogen compounds that can be released from the humic materials through hydrolytic or other degradative techniques, would advance a resolution of the problem.

Although limited, the set of solid state spectra of aquatic samples reported here is the most extensive of materials isolated on XAD resins compiled thus far. The spectra provide unequivocal evidence for the occurrence of heterocyclic nitrogens in humic, fulvic and XAD-4 acids, of both microbial and mixed microbial-higher plant origins. The spectra also reveal differences among samples for which an explanation is not readily apparent, e.g. the prominent occurrence of the amino sugar/terminal amino acid peaks in the blackwater Nordic vs. their variable occurrence in the Suwannee River samples. Within the set of IHSS standards, the fact that the aquatic samples may or may not contain amino sugar/terminal amino acid nitrogens may be a characteristic that distinguishes them from the soil and peat samples. With the aquatic data, modest attempts at correlating structural features of the samples to properties such as metal binding and production of nitrogen containing photochemical and disinfection degradation products may now be possible. Heterocyclic nitrogens may derive both from biochemical constituents of plants and microorganisms (purine and pyrimidine bases, N containing side chains of amino acids,

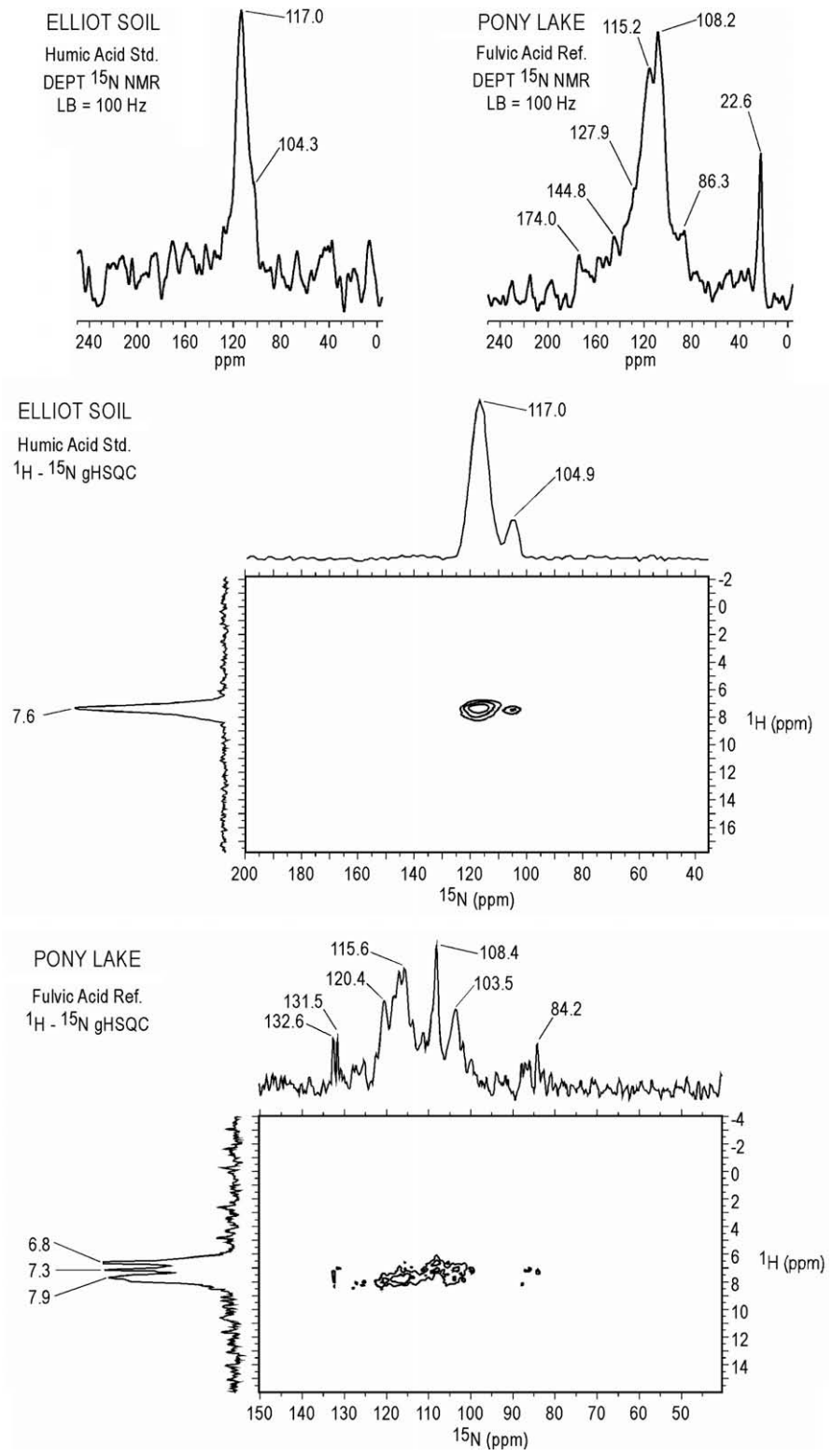


Fig. 10. Liquid state DEPT ^{15}N and ^{1}H–^{15}N gHSQC NMR spectra of Elliot soil humic acid and Pony Lake fulvic acid. LB = line broadening in hertz. A line broadening of 128.39 and sine bell function of 0.003 were applied to the F2 (^{1}H, directly detected) and F1 (^{15}N, indirectly detected) dimensions, respectively, of the Elliot soil HA gHSQC. A line broadening of 74 and sine bell function of 0.023 were applied to the F2 (^{1}H, directly detected) and F1 (^{15}N, indirectly detected) dimensions, respectively, of the Pony Lake FA gHSQC. Positive contours only were plotted for the Pony Lake ^{1}H–^{15}N gHSQC spectrum to eliminate the aliased ammonia peak.

alkaloids, etc.) and through condensation reactions of amines with carbonyl compounds. Until these formation pathways are elucidated, both sources must be considered valid in aquatic environments. Here again, analyses of nucleosides in the aquatic samples would be helpful.

The general characteristics of the solid state spectra of aquatic samples shown here – major peak intensity in the amide/amino-quinone region, heterocyclic nitrogens in resolved peaks and extending downfield to about 215 ppm – are not limited to the particular aquatic environments of the IHSS samples. Spectra of other

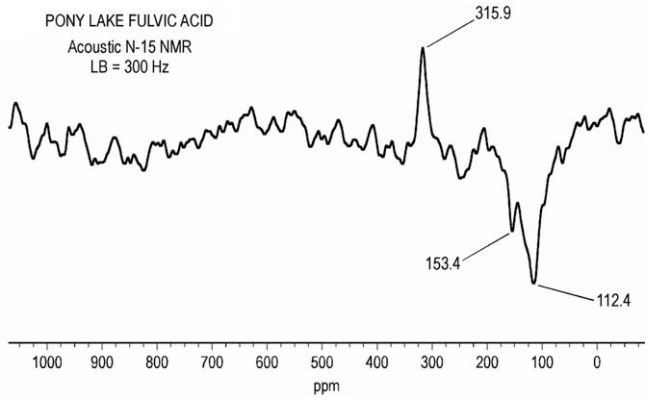


Fig. 11. Liquid state ACOUSTIC ^{15}N NMR spectrum of Pony Lake fulvic acid. LB = line broadening in Hertz. Solvent = dmso-d_6.

samples that we have examined, including a major river fulvic acid from the Mississippi River at St. Francisville, Louisiana, have similar characteristics. Methods of filtration for water samples prior to isolation of NOM on XAD resins are not consistent. In particular, an ultrafiltration step to remove colloids after water is passed through glass fiber filters is not always employed. In that organic matter in the colloid fraction is likely to contain high molecular weight proteins, it is important to remove colloids from truly dissolved NOM so that characterization of nitrogen in dissolved fractions is not distorted by the presence of colloidal material. Adaptation of standardized filtration procedures should be considered in future work comparing XAD isolates.

Acknowledgments

The efforts of the many individuals responsible for the collection and isolation of the IHSS samples made this study possible. A portion of the research was performed using EMSL, a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research located at Pacific Northwest National Laboratory. We thank Drs. Joseph Ford of EMSL and David Jones of University of Colorado Health Sciences Center for ^{1}H-^{15}N gHSQC spectra. (Use of trade names in this report is for identification purposes only and does not constitute endorsement by the U.S. Geological Survey.)

Associate Editor—**Jim Rice**

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