

Structural environments of carboxyl groups in natural organic molecules from terrestrial systems. Part 2: 2D NMR spectroscopy

Ashish P. Deshmukh ^a, Carlos Pacheco ^b, Michael B. Hay ^c, Satish C.B. Myneni ^{a,*}

^a Department of Geosciences, Princeton University, 151 Guyot Hall, Princeton, NJ 08544, USA

^b Department of Chemistry, Princeton University, Princeton, NJ 08544, USA

^c Department of Civil and Environmental Engineering, Princeton University, Princeton, NJ 08544, USA

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Abstract

Carboxyl groups are abundant in natural organic molecules (NOM) and play a major role in their reactivity. The structural environments of carboxyl groups in IHSS soil and river humic samples were investigated using 2D NMR (heteronuclear and homonuclear correlation) spectroscopy. Based on the ^1H – ^{13}C heteronuclear multiple-bond correlation (HMBC) spectroscopy results, the carboxyl environments in NOM were categorized as Type I (unsubstituted and alkyl-substituted aliphatic/alicyclic), Type II (functionalized carbon substituted), Type IIIa, b (heteroatom and olefin substituted), and Type IVa, b (5-membered heterocyclic aromatic and 6-membered aromatic). The most intense signal in the HMBC spectra comes from the Type I carboxyl groups, including the $^2J_{\text{CH}}$ and $^3J_{\text{CH}}$ couplings of unsubstituted aliphatic and alicyclic acids, though this spectral region also includes the $^3J_{\text{CH}}$ couplings of Type II and III structures. Type II and III carboxyls have small but detectable $^2J_{\text{CH}}$ correlations in all NOM samples except for the Suwannee River humic acid. Signals from carboxyls bonded to 5-membered aromatic heterocyclic fragments (Type IVa) are observed in the soil HA and Suwannee River FA, while correlations to 6-membered aromatics (Type IVb) are only observed in Suwannee River HA. In general, aromatic carboxylic acids may be present at concentrations lower than previously imagined in these samples. Vibrational spectroscopy results for these NOM samples, described in an accompanying paper [Hay M. B. and Myneni S. C. B. (2007) Structural environments of carboxyl groups in natural organic molecules from terrestrial systems. Part 1: Infrared spectroscopy. *Geochim. Cosmochim. Acta* (in press)], suggest that Type II and Type III carboxylic acids with α substituents (e.g., –OH, –OR, or –CO₂H) constitute the majority of carboxyl structures in all humic substances examined. Furoic and salicylic acid structures (Type IV) are also feasible fragments, albeit as minor constituents. The vibrational spectroscopy results also suggest that much of the “Type I” signal observed in the HMBC spectrum is due to carboxylic acid esters and possibly α -substituted alicyclic acids.

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1. INTRODUCTION

Soil organic matter is a heterogeneous mixture of organic debris from soil flora and fauna, which undergoes transformations by soil microorganisms and chemical processes, resulting in the formation of stable humic substances. These

humic substances bear little morphological resemblance to the original debris, but often make up the largest fraction of organic matter in soils (Stevenson, 1994). Under normal soil pH conditions the humic molecules carry a negative charge, predominantly from carboxyl groups (CO₂H) with an average proton dissociation constant ($\text{p}K_{\text{a}}$) centered around 3–5, with a lesser contribution from phenolic and phosphate groups. At pH values below 10, this negative charge is slightly offset by protonated amines, giving zwitterionic properties to humic substances. These negative

* Corresponding author.

E-mail address: smyneni@princeton.edu (S.C.B. Myneni).

charges are distributed throughout the macromolecular matrix and are balanced by positively charged counter-ions, which may include protons, aluminum, iron, trace metals, and radionuclides. Carboxylic acid groups play an important role in environmental processes such as mineral weathering, pH buffering, nutrient availability, and contaminant mobility (Sposito, 1989; Stevenson, 1994).

Although the importance of carboxylic acid groups in humics has been known for several years, very little is known about the structures of carboxyls in these molecules, which is an important factor in determining their behavior in the environment. Presence of functional groups containing heteroatoms in the close vicinity of CO₂H groups affects their ionization efficiency due to inductive effects. Electron-withdrawing substituents on C atoms α to the CO₂H groups not only enhance their acidity, but they may also participate in chelation reactions. Additionally, in macroions such as humic molecules, where multiple ionizable groups are in close proximity, electrostatic effects result in transmission of polarization through the solvent medium. This may affect the carboxylic group pK_a values, which are a measure of their reactivity.

Multi-site models of CO₂H groups based on pH titration data, with “discrete site” pK_a's between ~1.8 and ~5.5, have been formulated for describing the heterogeneity of carboxyl structures in humic substances (Ephraim et al., 1991; Leenheer et al., 1995a,b). Much of the earlier work to determine specific carboxyl structure types (or rather, appropriate analogues) involved comparisons of the metal binding behavior of humics with model compounds (Schnitzer and Skinner, 1965; Schnitzer, 1969; Gamble et al., 1970). While these studies suggested the presence of aromatic carboxyl structures (salicylate and phthalate), later work based on metal binding studies (Gregor et al., 1989a,b; Town and Powell, 1993) highlighted the importance of substituted aliphatic structures similar to malonate, citrate, and amino acids. This dichotomy may be the result of the different procedures employed; the earlier work relied heavily on chemical methods for blocking specific functional group types, such as acetylation, methylation, and saponification (Schnitzer and Skinner, 1965), which were not used in the later work. More detailed structural models for CO₂H groups in humic molecules have been constructed by Leenheer et al. (1995a,b, 1998, 2003) by combining information from elemental composition, average molecular weights, exchangeable acidity, ¹H and ¹³C NMR, and infrared spectroscopy data on isolated sub-fractions of fulvic acid. Using these procedures, they have concluded that some of the most acidic carboxyl groups in humics arise from alicyclic *O*-heterocyclic structures with α-ether (–O–) and α-ester (–O–CO–) substituents (similar to isocitric acid lactone, Fig. 1). This high acidity is believed to originate from stabilization of the carboxylate due to intramolecular H-bonding. A large fraction of the remaining low-acidity carboxyl groups (pK_a > 4) in the fulvic acid are believed to be associated either with aromatic structures, or with the same *O*-heterocyclic structures, which are distant from the O bridge (Leenheer et al., 1995b). Examples of carboxyl structures suggested in the literature are shown in Fig. 1.

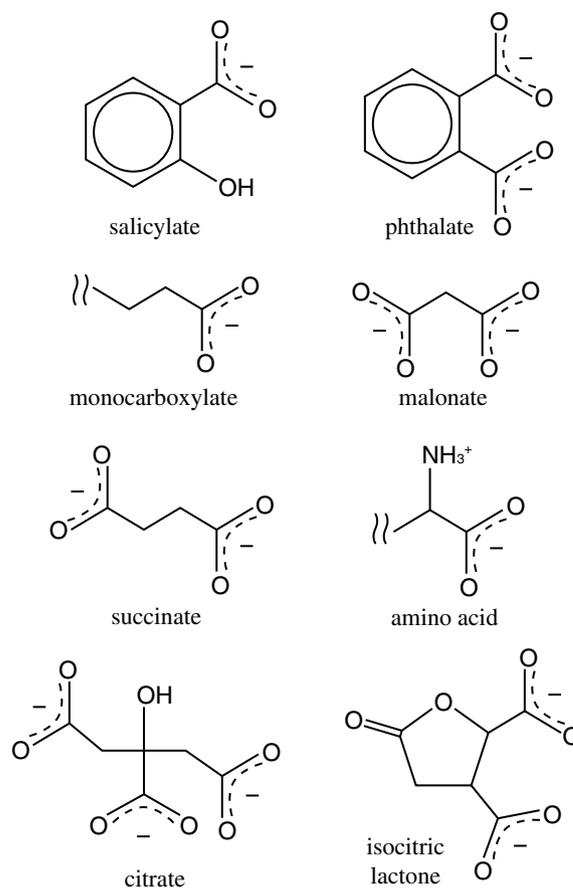


Fig. 1. Carboxyl group structures previously suggested for humic substances. Each of these structures may exist on its own, or covalently bound to a larger organic macromolecule.

1.1. NMR spectroscopy of carboxyl groups

Various spectroscopic techniques have been used to study humic structure, with some having the power to probe carboxyl groups specifically. In Part 1 of this study (Hay and Myneni, 2007), we show how infrared spectroscopy can be used to determine the dominant carboxyl structures in humics. Another important technique available for studying functional groups in NOM is NMR spectroscopy. ¹³C NMR spectra of carboxylic acids exhibit a strong, unambiguous peak between 170 and 185 ppm corresponding to the carboxyl C; in carboxylic esters, this peak occurs slightly upfield between 165 and 180 ppm. Since the carboxyl C atom does not have any directly attached protons, the value of one-dimensional ¹H NMR spectroscopy for studying carboxyl groups is limited. The acidic proton in the free CO₂H group is detectable by NMR and has a chemical shift between 10 and 13 ppm. However, in ionic aprotic solvents such as D₂O, which are commonly used for NMR spectroscopy, this proton is easily replaced by deuterium and thus becomes invisible to ¹H NMR spectroscopy.

NMR spectroscopy of NOM can be performed on both solid and aqueous-phase samples. Although solution-state NMR can be applied to soluble samples including humic

and fulvic acids (Preston and Schnitzer, 1987; Thorn et al., 1989b), the peaks are broad for humic substances. Solution-state NMR is not suitable for insoluble samples such as whole soil or humin, making solid-state NMR the analytical choice for these samples. However, the lack of molecular motion in solid samples gives broader lines for solids when compared to those materials observed in solution (Wershaw and Mikita, 1987). This is also the case with ^{13}C Cross-Polarization Magic Angle Spinning (CPMAS), which aims at turning the solid sample NMR spectrum into a “liquid-like” spectrum.

The chemical shift for a given carboxyl group is a function of (a) the polarity of the NMR solvent used, (b) the protonation state of the carboxyl, and (c) the structural environment of the carboxyl group, which may include the presence of functional groups, unsaturation, or aromaticity neighboring the carboxyl, and esterification of the carboxyl. Polar solvents (e.g., D_2O , DMSO) can shift the carboxyl peak upfield with respect to non-polar solvents (CDCl_3 , benzene), due to varying degrees of solvent H-bonding ability in polar solvents, and dimer formation in non-polar solvents. A shift of 4 ppm has been noted previously for acrylic acid, from 172.1 ppm in benzene to 168.4 ppm in DMSO (Brouwer and Stothers, 1972; Breitmayer and Voelter, 1987). However, a shift of up to 6 ppm (from ~ 180.7 in CDCl_3 to ~ 174.7 in CDCl_3 containing DMSO) may not be uncommon, based on tabulated chemical shifts in the Aldrich reference manuals for long-chain aliphatic mono and dicarboxylic acids (Pouchert and Behnke, 1993). Dissociation of the free carboxyl group can lead to a downfield shift of the carboxyl C peak; for instance, a shift of 4.7 ppm is observed between propanoic acid (180.4 ppm) and propanoate (185.1 ppm) in aqueous solution, a trend that holds for other alkanolic acids as well (Hagen and Roberts, 1969; Stothers, 1972).

As mentioned above, the chemical shift of the carboxyl C is also affected by its structural environment. In general, α -substitutions on an aliphatic acid tend to “shield” the carboxyl C, shifting the carboxyl peak upfield, based on values found in the Aldrich handbook (Pouchert and Behnke, 1993). In CDCl_3 , aliphatic monocarboxylates (chain length greater than three carbons) have a carboxyl chemical shift of 180.7 ppm. Introduction of an α -OH group has a minimal effect, with an upfield shift to 180.4 ppm, while an α -Br shifts this value to 176.4 ppm. Substitution of a carboxylate in the α -position has a similar effect; compare heneicosanoic acid (a long-chain monoacid; 174.7 ppm) with butylmalonic acid (5-C chain, α -carboxylate; 171.09 ppm), collected in a $\text{CDCl}_3/\text{DMSO}-d_6$ mixture. The exception to this trend is alkyl substitution (i.e., branching) at the α -C site, which causes deshielding and produces a downfield shift (solvent-dependent) of 2–3 ppm (Stothers, 1972).

Esterification and unsaturation lead to large upfield shifts in the carboxyl peak relative to the unsubstituted functional group. Esterification of the carboxylic acid may produce an upfield shift as large as 7 ppm (greatest in non-polar solvents) (Stothers, 1972) and may not generally be large enough to resolve acids and esters in humics. Unsaturation in the α -position produces a larger upfield

shift, from 180.7 to 172 ppm (long-chain α -unsaturated aliphatic in CDCl_3), which is similar to the benzoic acid value of 172.6 ppm in CDCl_3 (Pouchert and Behnke, 1993). This large separation between aromatic/olefinic and aliphatic carboxylate peaks is large enough that Thorn (1989a) and Leenheer et al. (1995a) were able to resolve these two components and quantify their relative contributions in Suwannee River fulvic acid.

Despite the sharp peak of the carboxyl C and its dependence on structural environment, differences resulting from substitution are not well resolved in humic substances, making it difficult to distinguish between different types of carboxyl functionalities using one-dimensional ^{13}C spectroscopy alone. However, multidimensional NMR spectroscopic techniques can resolve some of these differences.

1.2. Multidimensional NMR spectroscopy: methods and applications to humics

Multidimensional NMR techniques can be used to resolve contributions that overlap strongly in a 1D NMR experiment. Combinations of these techniques, such as Total Correlation Spectroscopy (TOCSY), Heteronuclear Multiple Quantum Coherence (HMQC), Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple Bond Correlation (HMBC), and Nuclear Overhauser Effect Spectroscopy (NOESY), have been successfully used for identifying the predominant structures in humic substances obtained from diverse sources (Chien and Bleam, 1998; Haiber et al., 1999; Haiber et al., 2001a,b; Simpson, 2001a; Simpson et al., 2001b; Simpson, 2002; Simpson et al., 2002a,b; Cook et al., 2003; Deshmukh et al., 2003; Simpson et al., 2003a,b; Simpson et al., 2004; Deshmukh et al., 2005). In this study, we use the HMBC and TOCSY 2D NMR techniques to infer the structural environments of carboxyl groups in representative humic substances.

The ^1H - ^{13}C HMBC experiment makes use of the 6–10 Hz coupling observed between protons and carbon atoms separated by 2–3 bonds. The experiment is similar to an HMQC experiment, which probes correlations between directly bonded ^1H and ^{13}C , but with a “preparation time” between proton excitation and ^1H - ^{13}C polarization transfer long enough for small (2–3 bonds) couplings to evolve. The phases of the pulses are set such that on each subsequent scan, the single-bond correlations cancel, leaving the multiple-bond correlations unaffected. The HMBC experiment is particularly useful for probing carbons not directly bonded to protons (e.g., carboxyls and carbonyls). In this study, we focus on correlations present between the carboxylic C (170–185 ppm) and protons located on functional groups neighboring the carboxyl (on C atoms α and β to the carboxyl functionality on aliphatic acids, labeled H_α and H_β in Fig. 2, or in the *ortho* position on aromatic acids, labeled H_o). The chemical shifts of these protons are dependent on the local structure, as indicated in Table 1. Correlations between these protons and the carboxyl carbons will therefore yield cross-peaks resolved in the ^1H chemical shift dimension (Fig. 3), thus providing information about the structural environments of carboxyl groups in the sample.

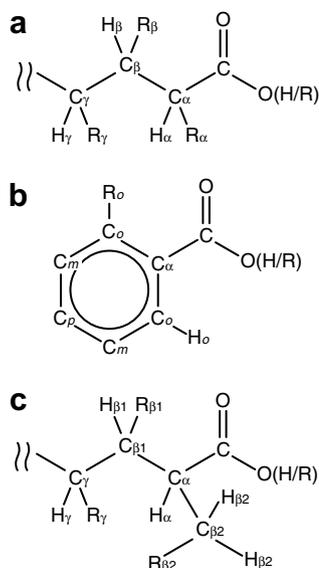


Fig. 2. Schematic of aliphatic (a) and aromatic (b) carboxylates, labeled according to the nomenclature used in the text. Substituents (R values) considered in this study are listed in Table 1. In the case of an alkyl substitution (c), additional protons may be introduced, thereby enhancing the cross-peak signals in the HMBC experiment.

The ^1H – ^1H TOCSY experiment reveals correlations between protons that are in the same spin system (e.g., protons in the same chain), regardless of whether they are coupled to one another. A spin-lock pulse in the pulse sequence transfers coherence between protons, relaying information from proton to proton along the coupled network. Since the carboxylic C does not have any protons attached to it, this method cannot probe the carboxyl group directly. However, information from this method can be used to probe correlations between protons neighboring carboxyls (H_α) and protons further down the chain (H_β , H_γ).

Multidimensional NMR techniques are beginning to be used more broadly in the study of humic substances (e.g., Cook, 2004). To date, however, not many HMBC studies of humic substances exist in the literature, while few studies employing other multidimensional techniques have specifically investigated the local structure around carboxyl groups. Using HMBC, Cook et al. (2003) demonstrated that the majority of the carboxyl groups in Laurentian fulvic acid are aliphatic and also provided possible structures, which included α -OH carboxyls. Though the HMBC results may contain a small-molecule bias, results from 1D ^{13}C methods such as INEPT (insensitive nuclei enhanced by polarization transfer) also suggests that the aliphatic structures in general (not specifically carboxyl structures) are more highly functionalized than the aromatic structures (Cook et al., 2003). Simpson et al. (2001b) performed HMQC-TOCSY (a heteronuclear TOCSY experiment in which correlations are transferred along C chains) on a Podzol fulvic acid fraction, illustrating that a majority of the carboxyl groups were present as long-chain (10-C average) aliphatic mono and dicarboxylic acids, with possible contributions from citric, succinic (ethanedicarboxylic), and adipic (butanedicarboxylic) acid type structures.

Though HMBC experiments were also performed and complemented these results, these data were not shown for the carboxyl region. Their HMBC and COSY data (Simpson et al., 2001b) also suggests that phthalate-type structures (Fig. 1) may also be present in small quantities. On the other hand, using the solid-state through-space ^{13}C – ^1H Heteronuclear Correlation (HETCOR) technique on a peat HA, Mao et al. (2001) found that the majority of carboxyl structures are in the close vicinity of O– CH_n type functionalities. Though these signals could also be obtained from protons on methylene groups attached to the oxygen side of carboxylic esters, the authors suspected that three-bond correlations in such structures may not lead to the strong signals observed in the spectra. They believed that these signals are more likely obtained from carboxyls with O -substitution in the α -position, such as the O -heterocyclic structures proposed by Leenheer et al. (1995b). Differences in these reported results may to some degree be due to differences in sample source.

It is clear that the structural environments of carboxyls can be examined in detail using 2D NMR spectroscopy. However, much work still needs to be done, particularly with different humic substance fractions from a variety of environments. In this work, we present 2D NMR (HMBC, TOCSY) spectroscopic evidence for the predominance of aliphatic carboxyl groups in soil and fluvial humic substances, based on couplings observed (via $^2J_{\text{CH}}$, $^3J_{\text{CH}}$) between aliphatic moieties and COOH groups. The greatest contribution in all samples appears to be from unsubstituted aliphatic carboxyls, though the HMBC evidence for these structures overlaps with signal from aliphatic carboxylic esters and α -substituted alicyclic acids, suggesting that these structures might also be in high abundance. Other aliphatic carboxyls with electron-withdrawing groups in their vicinity were also observed, and the Suwannee River samples also exhibit a significant concentration of aromatic carboxyls. In a companion paper (Hay and Myneni, 2007), we used the infrared spectral features of the carboxylate anion to infer carboxyl structural environments in a wide variety of humic substance isolates and have found that a majority of the carboxyls are aliphatic in nature, but with electron-withdrawing substitutions α to the carboxyl (in addition to salicylate and furan-carboxylate aromatic contributions).

2. MATERIALS AND METHODS

2.1. Samples

The samples used for this study were standards purchased from the International Humic Substances Society (IHSS). Elliott soil humic acid (soil HA) and fulvic acid (soil FA) were isolated by the IHSS using standard procedures from a soil sample obtained from an undisturbed area on the grounds of the Joliet Army Ammunition Plant near Joliet, Illinois. The Suwannee River is a blackwater river that rises in the Okefenokee Swamp in southern Georgia and flows southwest to the Gulf of Mexico, with dissolved organic carbon (DOC) concentrations ranging from 25 to 75 mg/L. Suwannee River humic acid (SR HA) and fulvic acid (SR FA) were isolated by the IHSS

Table 1
¹H chemical shifts of protons neighboring carboxyl groups, calculated using the ACD/Labs software

Substituent	Substituent on α -C (R_α)		Substituent on β -C (R_β)	
	α - ¹ H (H_α) (ppm)	β - ¹ H (H_β) (ppm)	β - ¹ H (H_β) (ppm)	α - ¹ H (H_α) (ppm)
Type I: unsubstituted and alkyl-substituted long-chain aliphatic carboxylic acids (1.3–2.6 ppm)				
H	2.3	1.6	1.6	2.2
CH ₃	2.4	1.5	1.9	2.2
Type II: functionalized C substituted long-chain aliphatic and alicyclic carboxylic acids (2.7–3.6 ppm)				
CO ₂ H	3.0	1.8	2.9	2.5
CO ₂ H (within long-chain)	—	1.9	1.5, 1.7	2.6
CO(CH ₂) _n CH ₃	3.2	1.8	3.1	2.6
COO(CH ₂) _n CH ₃	2.9	1.9	3.0	2.5
CHO	2.7	1.9	2.7	2.6
Ph	3.6	1.9	3.2	2.6
CO ₂ H (on cyclopentane)	—	2.0, 2.4	1.8, 2.2	2.8
CO ₂ H (on cyclohexane)	—	2.0, 2.3	1.8, 2.2	2.8
Type III (a): heteroatom substituted long-chain aliphatic and alicyclic carboxylic acids (3.7–5.0 ppm)				
OH	4.1	1.7	4.0	2.5
OH (within long chain)	—	2.0	1.5, 2.1; 4.0 ^a	2.3
O(CH ₂) _n CH ₃	3.9	1.7	3.9	2.5
OPh	4.8	1.9	4.7	2.6
OCO(CH ₂) _n CH ₃	5.0	1.9	5.3	2.6
NH ₂	3.8	1.8	3.1	2.3
SH	4.6	1.8	3.7	2.6
Cl	4.1	1.8	4.8	2.6
Br	5.1	2.0	4.7	3.0
OH (on cyclopentane)	—	1.6, 2.4	1.8, 2.2; 4.4 ^a	2.6
OH (on cyclohexane)	—	2.0	1.5, 2.1; 4.0 ^a	2.3
Type III (b): unsaturated long-chain aliphatic carboxylic acids (4.0–6.0 ppm)				
β - γ unsaturated	5.6	5.6		
α - β unsaturated	5.9	7.2		
Type IV (a): 5-membered heterocyclic aromatic acids (6.0–7.0 ppm)				
Pyrrole-2-CO ₂ H		6.6		
Pyrrole-3-CO ₂ H			6.4, 7.5 ^a	
Furan-2-CO ₂ H		7.3		
Furan-3-CO ₂ H			6.8, 8.2 ^a	
Type IV (b): 6-membered aromatic acids (7.0 - 9.0 ppm)				
Benzoic acid			7.9	
Salicylic acid (<i>o</i> -OH)			7.8	
Phthalic acid (<i>o</i> -COOH)			7.7	
Pyridine-2-CO ₂ H			8.4	
Pyridine-3-CO ₂ H			8.3, 9.0 ^a	
Pyridine-4-CO ₂ H			7.8	

^a Second value is for the proton on the substituted C, or on the C next to the heteroatom.

from the Suwannee River water by standard procedures (<http://www.ihss.gatech.edu/>).

2.2. NMR spectroscopy

All NMR experiments were performed using a 5 mm triple resonance inverse-probe on a Varian INOVA spectrometer operating at 600 MHz for ¹H and 150 MHz for ¹³C. Solutions were prepared by dissolving 25 mg of the sample in 0.5 mL DMSO-*d*₆ in a 5 mm NMR tube. ¹H and ¹³C data were referenced to the DMSO-*d*₆ chemical shifts (2.50 ppm for ¹H and 39.5 ppm for ¹³C).

The HMBC data were acquired in the phase-sensitive mode using the States method, typically with 192 scans per increment and a recycle delay of 1.0 s, without

decoupling. A 62 ms delay time was used to allow the 2–3 bond correlations to evolve. The datasets were acquired with 7112 and 200 data points for the f_2 (¹H) and f_1 (¹³C) dimensions, with spectral widths of 14998 and 36004 Hz, respectively. (The f_2 dimension refers to the FID signal collected during the detection stage of each scan, while f_1 refers to the FID obtained after processing of each set of scans with varying evolution time t_1 .) The data was zero-filled to form a 4 K × 4 K data set and multiplied by a Gaussian curve in each dimension and linear predicted in f_1 . The delay in the pulse sequence after the initial 90° pulse on the proton was set to 62 ms, which corresponds to a coupling constant of 8 Hz. Two to three-bond coupling constants in aliphatic carboxyls and carboxyls on 5-membered aromatic rings are in the range of 5–7 Hz. A longer delay

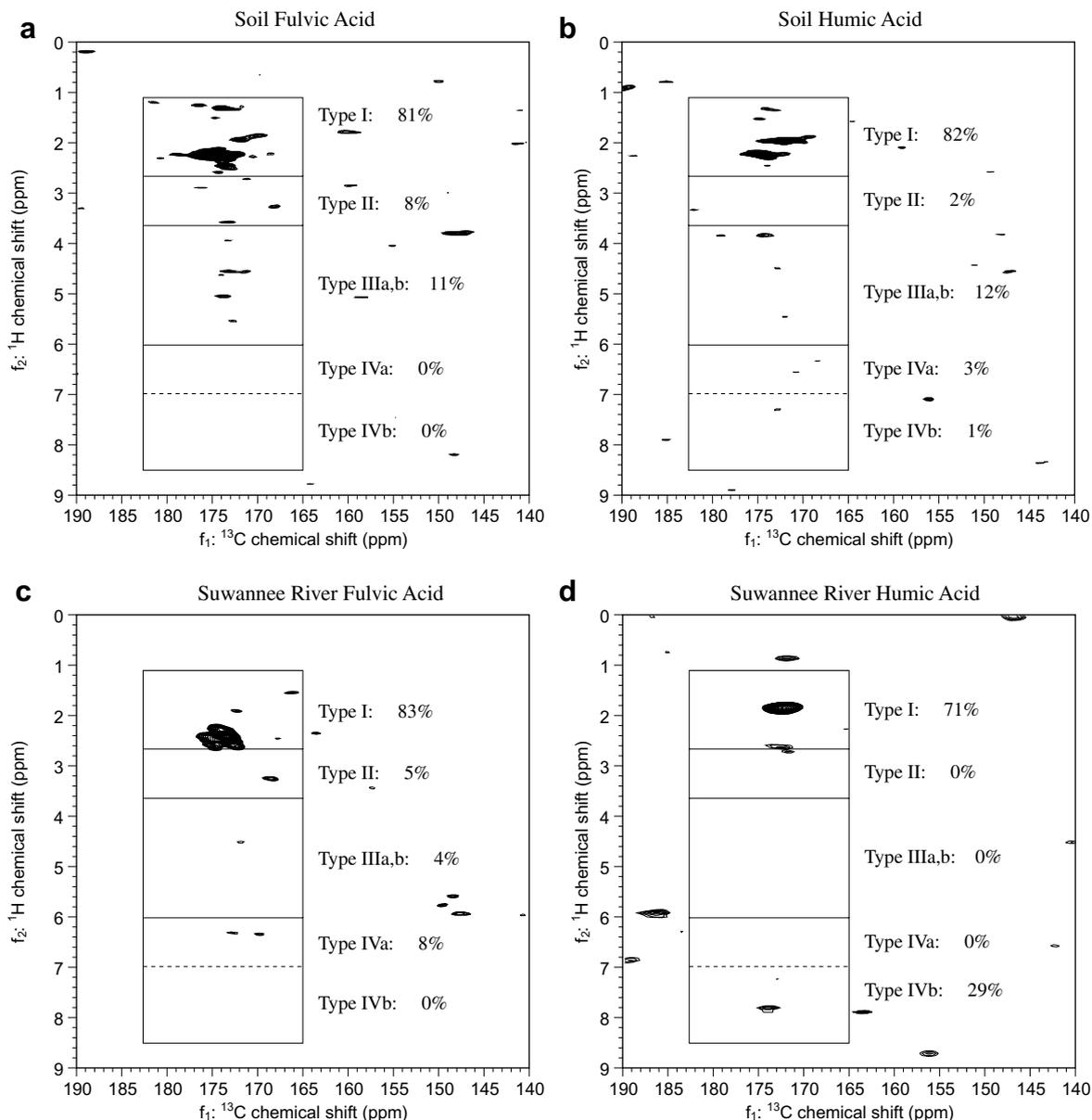


Fig. 3. ^1H – ^{13}C heteronuclear multiple-bond correlation (HMBC) NMR spectra of (a) Elliot soil FA, (b) Elliot soil HA, (c) Suwannee River FA, and (d) Suwannee River HA in d_6 dimethyl sulfoxide (DMSO). Boxes in the figures denote Type I (unsubstituted and alkyl-substituted long-chain aliphatic carboxyls), Type II (functionalized-carbon substituted long-chain aliphatic carboxyls), Type III a, b (heteroatom substituted and unsaturated long-chain aliphatic carboxyls), and Type IV a, b (5-membered ring and 6-membered ring aromatic carboxyls).

may be required to effectively detect carboxyl carbons with coupling constants less than 5 Hz (e.g., on 6-membered aromatic rings). However, we did not observe any enhancement in the signal in the 7.0–8.0 ppm range when the delay was set to 100 ms (5 Hz).

To reduce the appearance of noise in the HMBC spectra (Fig. 3), the minimum threshold was chosen such that tiny correlations are displayed in the contour plots, but peaks occurring below this non-zero baseplane do not appear. Effort was expended to ensure that the peaks present in the plots had heights above baseplane greater than or equal to the peak-to-peak height of the noise oscillations (i.e., the peaks shown have a signal to noise ratio of at least 2–1).

Though all of the peaks in the plots are believed to be significant, this procedure led to some unintended effects. First, since the lowest contours start at the non-zero baseplane, the number of contours on a peak (particularly in the smaller peaks) is not an accurate indicator of the signal to noise level. Second, since the contours only show the “tops” of the smaller peaks, they may appear smaller in volume relative to the larger peaks than what the volume percentages (which are based on integration) may suggest.

Peak volume percentages, listed in Fig. 3 according to type, were calculated from the absolute peak volumes determined using the Varian software. Since a small residual baseline was often present after baseline correction, a

procedure was employed whereby the baseline volume in the immediate vicinity of each peak was determined, normalized according to area, and subtracted from the total volume under the respective peak. Given the low signal to noise level for the smaller peaks, the percentages should only serve as an approximate indicator of spectral contribution. Additionally, peak volume alone does not directly reflect the concentration of the respective component in the sample.

The Total Correlation Spectroscopy (TOCSY) spectra were acquired with 3316 and 400 data points in the f_2 and f_1 dimensions, respectively, with sweep-widths of 9715.8 Hz, and 80 ms of mixing time. The data were acquired in the phase-sensitive mode using the States method, with 16 scans per slice and a recycle delay of 1.0 s. The data was zero-filled to form a 4 K \times 4 K data matrix, multiplied by a Gaussian curve in each dimension, and linear predicted in f_1 .

2.3. Chemical shift predictions

Chemical shift predictions were made with the commercially available software packages “ACD/CNMR” (for ^{13}C shifts) and “ACD/HNMR” (for ^1H shifts) from Advanced Chemistry Development (ACD/Labs). Version 8.0 of ACD/CNMR and ACD/HNMR were used. Structures were entered into ACD/ChemSketch, the structure-drawing interface for the NMR prediction packages. The ACD/HNMR and ACD/CNMR software predicts ^1H and ^{13}C NMR spectra, chemical shifts, and coupling constants for almost any organic chemical structure by utilizing algorithms that are based on more than 1,440,000 assigned ^1H chemical shifts and 2,160,000 assigned ^{13}C chemical shifts for more than 175,000 chemical structures. The standard errors associated with the predictions, according to an unpublished study released on the software developer's website (http://www.acdlabs.com/products/spec_lab/predict_nmr/chemnmr/) are 0.22 ppm for ^1H and 2.33 ppm for ^{13}C , based on literature comparisons for over 54,000 ^1H chemical shifts and over 68,000 ^{13}C chemical shifts.

3. RESULTS AND DISCUSSION

3.1. Heteronuclear multiple-bond correlation (HMBC) spectroscopy

The carboxyl regions of the HMBC spectra of soil FA and HA and Suwannee River FA and HA are presented in Fig. 3. The spectra exhibit correlations of the carboxyl carbons (168–183 ppm) with several aliphatic (1.2–6.0 ppm) and aromatic (6.0–8.0 ppm) protons. This carbon chemical shift range mainly includes carbons in carboxyl (including ester) and amide functionalities. Due to the low N content of the samples relative to O, it is safe to assume that most of the signal is from carboxyl groups, although there could be a small contribution from peptide bonds in proteins. Infrared spectroscopy studies of these samples also indicate that carboxyls are in much greater abundance than amide groups (Hay and Myneni, 2007). Regions of the spectra have been divided into Types I–IV based on the molecular structure of carboxyl groups

(Table 1). Unsubstituted and alkyl-substituted aliphatic/alicyclic acids yield correlations in the Type I region (1.3–2.6 ppm), aliphatic acids with functionalized C substitutions in Type II (2.7–3.6 ppm), aliphatic acids with heteroatom substitutions (O, N, S, halogen) in Type IIIa (3.7–5 ppm), and unsaturated aliphatics in Type IIIb (4–6 ppm). Aromatic acids are denoted as Type IV, with 5-membered heterocyclic acids in the Type IVa region (6–7 ppm), and 6-membered aromatics in the Type IVb region (7–9 ppm). Relative peak volumes, obtained through numerical integration, are also listed in Fig. 3 as a percentage for each type.

3.1.1. Type I carboxyls

Protons on the α -C atoms of aliphatic carboxylic acids exhibit a chemical shift of 2.3 ppm. Substitution of alkyl groups in the R_α and R_β positions does not significantly alter these chemical shifts; a range of 1.3–2.6 ppm for H_α and H_β in alkyl-substituted carboxyls was predicted, using the ACD/HNMR software (Table 1). However, alkyl substitution in the α -position would lead to a greater number of protons in the β position (Fig. 2c), resulting in signal enhancement in the HMBC spectra via $^3J_{\text{CH}}$ couplings. Protons on the α and β -C atoms of alicyclic carboxylic acids (e.g., 5- and 6-membered saturated rings) also exhibit chemical shifts in the 2.3–2.5, and 1.3–2.5 ppm range, respectively.

The strongest correlations in both fluvial and soil fulvic acid spectra (Fig. 3a and c) are observed for carboxyls at 2.3 ppm. The humic acid spectra exhibit a feature at 1.9 ppm, which could be from ^1H – ^{13}C correlations in alkyl-substituted aliphatic carboxyl structures. The 2.3 ppm and 1.9 ppm features are of equal proportion in the soil HA spectrum, while the 1.9 ppm peak dominates in the Suwannee River HA, suggesting that branched aliphatic structures may be particularly important in this sample.

The volume percentages of the spectral regions shown in Fig. 3 suggest that the Type I carboxyls make up the largest fraction of the total in all four samples. However, these percentages may not accurately reflect the true relative proportions of structural types. Type I carboxyls in particular may be overrepresented in the spectra for the following reasons—(i) the delay time chosen for the experiment may be more appropriate for aliphatic carboxyls than aromatic carboxyls (details discussed later), (ii) branched aliphatic acids may also have a greater number of β protons, resulting in a stronger signal, (iii) a significant contribution to this signal could be from carboxyl esters, which cannot be distinguished from carboxylic acids in the NMR spectra, and (iv) signal in the Type I region may also come from β protons on Type II and IIIa structures, in addition to Type I structures. The implications of this latter point are discussed below.

3.1.2. Type II and III carboxyls

The presence of functionalized-carbon groups on the α - and β -C atoms moves the chemical shift of the H_α protons to the 2.5–3.6 ppm range (Type II). Substituents considered for the ACD/HNMR simulations in this study include CO_2H , COR, COOR, CHO, and phenyl groups (Table 1).

The CO₂H substituted structures include malonic and succinic acid structural types, which are believed to be present in humics (Simpson et al., 2001b). Heteroatom substituents in the α -position, such as O, N, S, and halogen functional groups (Type IIIa structures, Table 1), move the H $_{\alpha}$ proton chemical shifts further downfield into the 3.8–5.0 ppm range, and aliphatic carboxyls with unsaturation at the α - or β -C atoms (Type IIIb) exhibit signals between 4.0 and 6.0 ppm.

Clear signals are obtained in the Type II and III areas for both soil FA and HA, indicating the presence of various structures with electron-withdrawing groups α or β to the carboxyls. Suwannee River FA contains a lesser, yet still significant contribution from these types, while Suwannee River HA does not exhibit a significant contribution from either Type II or Type III structures.

As mentioned above, the H $_{\beta}$ correlations in Type II and IIIa structures may significantly contribute to the signal in the Type I region, thereby overestimating the apparent fraction of Type I structures. This effect would be particularly strong for Type II/IIIa structures that lack protons on the α -C (H $_{\alpha}$), including α -substituted alicyclic acids, as they contribute exclusively to signals in the 1.6–2.4 ppm region (Table 1).

3.1.3. Type IV carboxyls

Aromatic carboxyls on 5-membered heterocyclic aromatics (pyrrole, furan) are present as weak signals in the 6.0–7.0 ppm range (Type IVa) in the soil HA and Suwannee River FA HMBC spectra, but these groups appear to be absent in the other two samples. Of the four samples studied, the Suwannee River HA is the only sample that exhibits a significant contribution from benzene- and/or pyridine-type acids (7.0–9.0 ppm; Type IVb), with a spectral contribution near 30%. This is in contrast to the overall aromatic C concentration (estimated based on their 1D ¹³C NMR spectra (Thorn et al., 1989c)), which is actually higher in the Elliot soil HA than in the Suwannee River HA.

For the HMBC results shown, a delay time based on a coupling constant of 8 Hz was used. This delay may not be appropriate for detecting aromatic carboxyls, for which a delay based on a smaller coupling constant (4–5 Hz) might be necessary. However, our efforts at running HMBC experiments with longer delays (~100 ms) did not result in enhancement of signal from aromatic carboxyls, but instead led to a decrease in intensities of cross-peaks from aliphatic carboxyls (data not shown). Efforts could be made to detect different types of carboxyls simultaneously by adopting ACCORDION pulse sequences (Martin et al., 1999), which employ a range of *J* couplings. However, for complex mixtures such as humics, this approach is not expected to provide any significant advantage, since such a pulse sequence would reduce the overall sensitivity. Weak aromatic correlations do not necessarily provide evidence for the absence of such groups in these samples, but may rather be due to a combination of the following: (a) presence of highly substituted aromatic rings with few unsubstituted CH groups (particularly in the *ortho* position), (b) short spin–spin relaxation time constants (*T*₂'s) for rigid aromatic protons, or (c) under-representation due to bias for smaller mole-

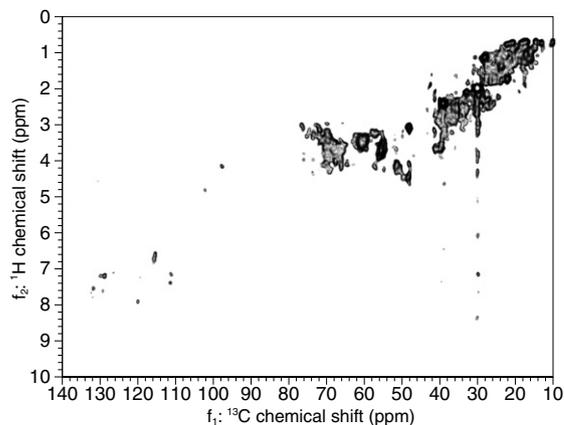


Fig. 4. ¹H–¹³C heteronuclear multiple quantum coherence (HMQC) NMR spectrum of Elliot soil FA. Aliphatic and aromatic ¹H–¹³C couplings can be observed, though the aromatic correlations are comparatively weak.

cules. An HMQC sequence was also performed on the soil FA to probe correlations between directly bonded ¹H and ¹³C (Fig. 4). The comparatively weak signal in the aromatic C–H region (7–9 ppm in ¹H chemical shift, 100–140 ppm in ¹³C chemical shift, Fig. 4) relative to the aliphatic region provides further evidence that a combination of these factors may be at work.

3.2. Total correlation spectroscopy (TOCSY)

The TOCSY spectra of the soil FA and HA (Fig. 5a and b) can be broken down into the characteristic regions A through F (outlined in the figure caption). The spectrum is symmetrical about the diagonal, and so peaks on only one side of the diagonal have been marked. It is not uncommon for peaks to be larger on one side of the diagonal, however, particularly when those peaks are relatively weak. Evidence for the presence of aliphatic carboxyl groups (Type I) in the soil FA comes from the strong TOCSY correlation between protons at 2.3 ppm (α -CH₂ unit) with protons at 1.6 ppm (β) and 1.3 ppm (γ) (Box B in Fig. 4 and Table 1). The TOCSY spectra of soil FA and HA show very well resolved peaks. Aside from the correlation for Type I carboxyl groups, another correlation at 2.3 ppm with protons at 2.0 ppm and 1.8 ppm (Box B) is observed, and is likely from Type I carboxyl groups with α -branched aliphatic chains. The TOCSY correlation at 4.1 ppm with protons at 1.2 ppm (Box D) may be from the CH₂ units on the O-side of aliphatic esters, or from the α -ether or α -ester type carboxyl groups (Type IIIa). This correlation is missing a peak at 1.6 ppm from the β -CH₂ unit, but it is quite common for peaks to be missing in TOCSY correlations if they are relatively weak (Simpson et al., 2001b). Signals obtained in the region assigned to Type II and III carboxyls could in part be derived from proteins in some samples, however it is impossible to quantify the extent to which peptides/proteins contribute to these regions. It is believed that these contributions are very small based on the low nitrogen content of these samples.

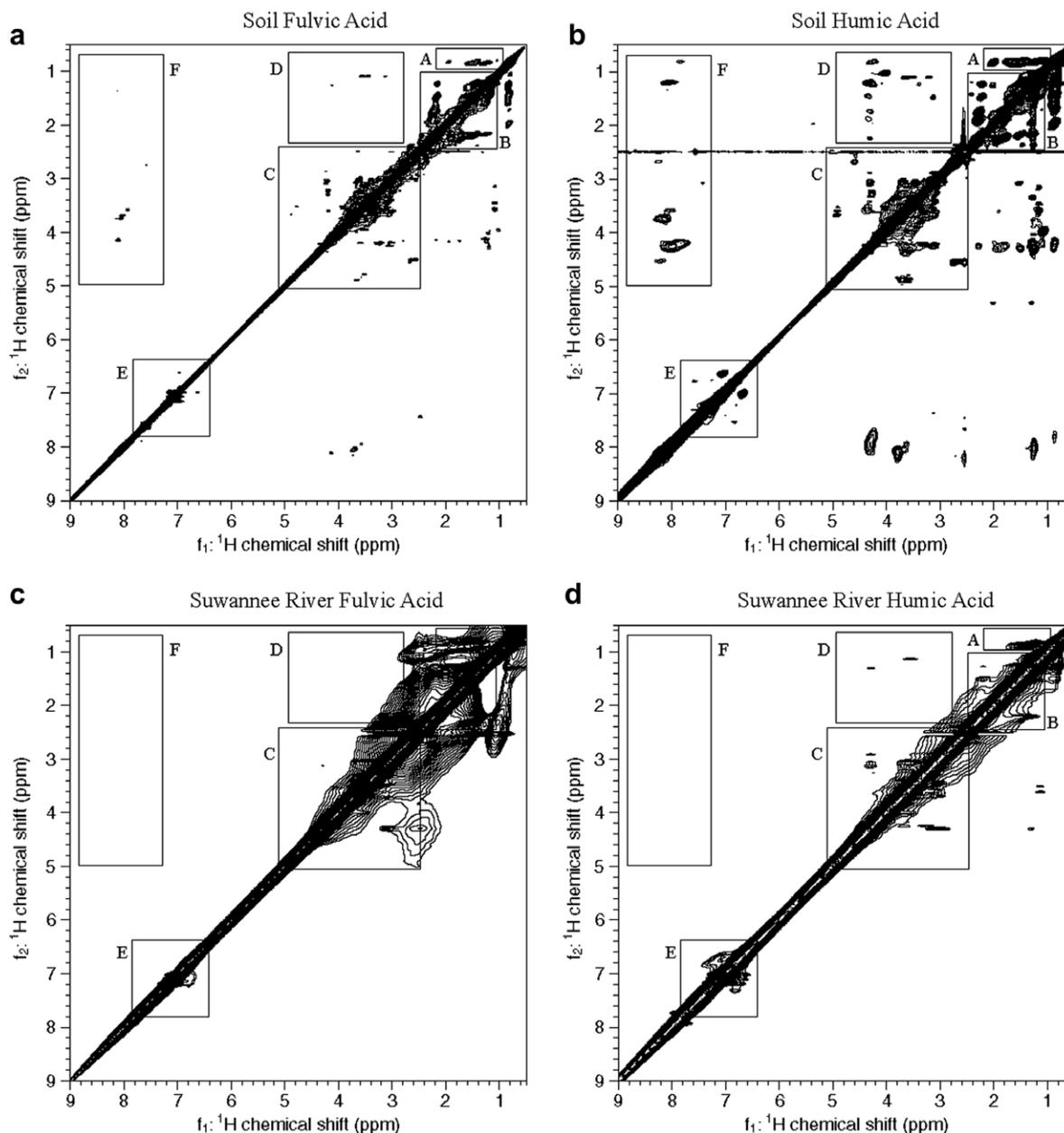


Fig. 5. ^1H – ^1H total correlation spectroscopy (TOCSY) NMR spectrum of (a) Elliot soil FA, (b) Elliot soil HA, (c) Suwannee River FA, and (d) Suwannee River HA in d_6 dimethyl sulfoxide (DMSO). Boxes in the figure highlight the regions for (A) CH_3 units in aliphatic chains and amino-acid side-chains; (B) bulk CH_2 units; (C) carbohydrates, CH units in lignin side-chains, amino-acid side-chains; (D) CH_2 units α -to hydroxyls, α -to O-side of esters and ethers; (E) aromatics; and (F) amide. Note: Couplings between protons on α carbons and side-chain protons in proteins and peptides also resonate in areas covered by boxes C and D.

The TOCSY spectra of the Suwannee River FA and HA (Fig. 5c and d) do not demonstrate the high resolution observed in the spectra of soil FA and HA. The mixing time was varied between 20 and 110 ms to accommodate for possible short spin-lock relaxation time constants ($T_{1\rho}$), but this did not lead to improvement in the resolution. Though they are not well resolved, broad correlations are still visible in the TOCSY spectra. The broad undefined nature of these cross-peaks suggests that the carboxyl groups in the Suwannee River samples may be more heterogeneous than those in the soil derived material. This is logical, considering soil derived ali-

phatic materials are likely to be closer to their parent materials (e.g., plant cuticles or microbial/plant derived fatty acids). On the other hand, material in the aquatic environment will have a diverse array of inputs (terrestrial and aquatic) and may have undergone numerous chemical and biological transformations, in turn leading to a heterogeneous mix of aliphatic species that are much more difficult to detect by TOCSY. While it is difficult to draw definitive conclusions from the TOCSY data, they suggest the aliphatic species in general to be more heterogeneous in the Suwannee River extracts than those in the soil humic extracts.

The Suwannee River FA TOCSY shows a strong correlation from unsubstituted Type I carboxyls (Box B). In the Suwannee River HA TOCSY spectrum, the correlation for unsubstituted Type I carboxyls at 2.3 ppm is not as strong as in the other samples, which is supported by the HMBC data. This indicates that in this sample, aliphatic carboxyl groups may be present in branched chains.

4. CONCLUSIONS

Correlations observed in the HMBC and TOCSY spectra of the humic substances initially suggest that carboxyl groups exist predominantly in the form of unsubstituted and branched aliphatic and alicyclic structures (Type I). The HMBC data also show that a portion of carboxyl groups in soils have electron-withdrawing substituents on neighboring carbon atoms (Types II and III); such groups not only increase the acidity of carboxyl groups, but also improve their ability to bind to metals through chelation. Some samples also exhibit distinct signals for aromatic acids, resulting from carboxyl groups bound to 5-membered heterocyclic rings (Type IVa) and 6-membered heterocyclic and/or benzene-type rings (Type IVb). More specifically, the soil humic and fulvic acids exhibit significant contributions of Type II and III structures, with a small concentration of aromatic structures. The Type II structures are likely similar to the dicarboxylic acid structures observed by Simpson et al. (2001b) in a podzol fulvic acid. In contrast, Suwannee River FA contains a smaller proportion of Type II and III structures, but a higher proportion of Type IVa relative to the soil samples. Suwannee River HA contains no significant contributions from Types II, III, and IVa, but a large contribution from Type IVb.

In the accompanying paper (Hay and Myneni, 2007), the dominant carboxyl structural types were inferred from the vibrational features of the carboxylate anion using infrared spectroscopy. These results indicate that the dominant contribution to free carboxyls in humics is from a mixture of α -substituted aliphatic carboxyls ($-\text{OH}$, $-\text{OR}$, and $-\text{CO}_2\text{H}$ substituents), furan-type aromatic carboxylates, and salicylate-type carboxylates; i.e., Types II–IV, rather than Type I carboxyls. Since unsubstituted aliphatic carboxyl structures were not observed in high abundance using infrared spectroscopy, it is possible that much of the signal observed in the 1.3–2.6 ppm region of the HMBC spectra derives from H_β correlations in Type II and IIIa structures. In particular, a high proportion of the substituted aliphatic structures may be in the form of α -substituted (e.g., $\alpha\text{-OH}$, $\alpha\text{-COOH}$) alicyclic acids, or in some other form similarly lacking $\alpha\text{-C}$ protons. The vibrational energies of α -substituted aliphatic and alicyclic carboxyls are expected to be similar and are indistinguishable in infrared spectroscopy. Cyclohexane carboxylic acids are also found in plant waxes and other biomolecules (Abdel-Mogib et al., 2001; Rogers et al., 1974), and formation of α -substituted alicyclic carboxylic acids from them may not be difficult. Much of the signal in the Type I region may also be coming from unsubstituted aliphatic carboxylic esters, which are not always distinguishable from carboxylic acids in NMR spectroscopy. However, acids and esters are easily resolved in infra-

red spectroscopy, and our data show a significant concentration of esters in the humic samples examined (Hay and Myneni, 2007). Many of these identified aliphatic carboxylic groups in NOM may be similar to the di-carboxylic acids seen by Simpson et al. (2001b), and the acids/esters that are likely to be derived from cuticles (Deshmukh et al., 2005).

Most popular models of humics have significant contributions from aromatic carboxylic structures. In fact, salicylate and phthalate are routinely used for modeling acidity and metal-binding properties of humic molecules (Celi et al., 1997; Dupuy and Douay, 2001; Hadzija and Spoljar, 1995; Kubicki et al., 1997; Stevenson, 1994; Yost et al., 1990). Much of our knowledge of carboxyls in humics is based on structure elucidation methods that include oxidative degradation procedures, which are always associated with artifacts (Reuter et al., 1983; Stevenson, 1994). This work shows that aromatic carboxyls (particularly substituted benzoic acids) may not necessarily be the dominant carboxyl structures in all humics. Though they may often be present in small concentrations, and may dominate in certain humic fractions (such as Suwannee River HA), their concentration appears to be much lower than what has been previously imagined.

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