NONCOVALENT INTERACTIONS BETWEEN AROMATIC COMPOUNDS AND DISSOLVED HUMIC ACID EXAMINED BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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Abstract—We examined the molecular-level interactions of aromatic compounds with a humic acid that was extensively characterized with one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy. Interactions of target compounds were evaluated by monitoring their NMR relaxation properties. Solid-state NMR revealed that the humic sample contains paraffinic carbon (31%), substituted aliphatic carbon (30%), and aromatic carbon (28%). The liquid-state experiments further identified amino acids, a range of carbohydrates, methoxylated aromatics (likely derived from lignin), and a series of aliphatic chains. The 1H T1 values were also measured and demonstrated that the association with the humic acid was not specific because all the protons acquired the T1 value of the humic acid at the same rate. The lack of a chemical shift change and an increase in signal line broadening indicates that the interaction between these compounds and humic acid is noncovalent. These interactions were detected at low humic concentrations (5 mg C/L) and suggest that low concentrations of humic material, which are prevalent in both aquatic and terrestrial systems, will significantly affect the fate and transport of contaminants in the environment.

Keywords—Spin-lattice relaxation 1H Nuclear magnetic resonance 13C Nuclear magnetic resonance Humic substances Contaminant interactions

INTRODUCTION

Interactions that occur between hydrophobic, aromatic compounds and dissolved organic matter (DOM) may change the biological, chemical, and physical fate of the contaminant. Many studies have examined interactions between a host of environmentally significant nonionic organic contaminants and DOM [1–10]. Collectively, these studies have concluded that organic contaminants and DOM interactions are noncovalent [3,5,11], are dependent on solution conditions such as pH and ionic strength [3,5,11], may increase the mobility of normally insoluble contaminants [4,6,12,13], may reduce the bioavailability of contaminants [12,13], and inhibit sonochemical degradation [14]. Consequently, the impetus for understanding the nature of interactions between DOM and aromatic compound is vital, given that DOM may significantly affect the fate of organic contaminants in aquatic and terrestrial environments.

Several methods have been used to measure interactions of aromatic compounds with dissolved humic material, and these include equilibrium dialysis [4,6,7,15,16], fluorescence quenching [2,5,8,17–19], reverse-phase separation [1,20,21], and nuclear magnetic resonance (NMR) spectroscopy [22–27]. These methods determine binding coefficients as well as extract mechanistic information regarding the type of association. For instance, DOM binding of organic contaminants, such as polycyclic aromatic hydrocarbons, has been attributed to noncovalent, hydrophobic interactions and is dependent on the microorganization of the humic material [5,11]. Attempts to quantify the contaminant–DOM interaction are dependent on several experimental corrections. For example, with fluorescence spectroscopy, dynamic quenching of the probe molecule may occur by non-DOM material, resulting in inaccurate quantification of the DOM–contaminant interaction [28]. Methods such as reverse-phase separation and equilibrium separation may be subject to artifacts arising from disequilibrium between the DOM and the contaminant phase [16].

Molecular-level information has been obtained with NMR spectroscopy in the past to study interactions between dissolved humic material and various organic contaminants [22–26]. If a contaminant becomes covalently bonded to humic material, a change occurs in the chemical shift, which is indicative of the new molecular environment of the bound contaminant [27]. Noncovalent interactions, such as those between DOM and nonionic organic contaminants, are less likely to result in a significant change in the contaminant chemical shift [24,25]. However, by examining the molecular motion and relaxation properties of the contaminant, information regarding the molecular environment and the noncovalent association with DOM can be obtained.

Spin-lattice relaxation time (T1) is characteristic of the overall molecular motion, which includes translational, rotational, and vibrational motion, and is used to examine noncovalent associations between DOM and organic contaminants [22–26]. The value of T1 is dependent on molecular size, solvent properties such as viscosity, magnetic field strength and homogeneity, dipolar interactions, temperature, and chemical shift anisotropy. However, when these variables are equal, T1 values are useful for describing molecular interactions in solution. In general, the T1 value of contaminant molecules is longer than that of dissolved humic material. Consequently, any decrease...
in the contaminant $T_1$ value is attributable to a molecular association with the humic material. Nanny et al. [24] demonstrated that the $^{13}$C $T_1$ value ofacenaphthenone decreased with increasing additions of dissolved fulvic acid. Similarly, the $^{19}$F $T_1$ value of 4′-fluoro-1′-acetonaphthone also was found to decrease with increasing concentration of dissolved fulvic acid [22]. Other investigations with monoaromatic compounds have reported the decline of $^1$H $T_1$ values with both dissolved fulvic and humic acids [25,26]. In all cases, the change in chemical shift of the probe molecule was small and consistent with weak hydrogen bonding and hydrophobic interactions.

In this study, we investigate the $^1$H and $^{13}$C $T_1$ values of three problematic environmental contaminants: naphthalene, 1-naphthol, and quinoline, in the absence and presence of a dissolved humic acid. Because the aqueous solubility would result in a concentration not amenable to observation by $^{13}$C NMR (the natural abundance of $^{13}$C is only 1.108%), we used $^{13}$C-di- and mono-labeled compounds. Furthermore, this would ensure that the $^{13}$C signal of the contaminant would be much greater than that of the dissolved humic material. This approach only provides the $T_1$ information of the specifically labeled $^{13}$C atoms. Consequently, we also measured $^1$H $T_1$ values such that specific interactions between different atoms within the molecule could be investigated. For instance, the hydroxyl group in 1-naphthol or the nitrogen in quinoline may invoke a different association than that occurring with naphthalene. In conjunction with the $T_1$ measurements, the humic acid used in this study was characterized in detail by both solid-state and multidimensional liquid-state NMR spectroscopy.

**MATERIALS AND METHODS**

**Humic material extraction and characterization by NMR**

The humic material was isolated from the A horizon of a forest soil (classified as a Typic Durochrept) from Nittany Ridge (PA, USA) [8]. The humic acid was isolated by the conventional base (NaOH) extraction procedure as described by Swift [29]. The humic acid isolate was treated with 0.3 M hydrofluoric acid–0.1 M HCl to remove clay minerals, especially, paramagnetic minerals that would interfere with NMR experiments. The humic acid was then dialyzed against ionized water until excess salts were removed, and freeze-dried before all analyses. Carbon content was measured on a Carlo-Erba NA 1500 Series 2 elemental analyzer (CE Elantech, Lakewood, NJ, USA). Cross-polarization magic angle spinning $^1$H, $^{13}$C, and $^{15}$N, and CP-MAS $^3$P NMR spectrometer (Bruker Biospin, Rheinstetten, Germany), equipped with a 4-mm H-X MAS probe, and by using the standard ramp–cross-polarization pulse program (Bruker Biospin). Approximately 100 mg of sample was packed into a 4-mm zirconium rotor with a Kel-F cap. The acquisition parameters were as follows: spectral frequency of 75 MHz for $^{13}$C and 300 MHz for $^1$H, spinning rate of 13 kHz, ramp–cross-polarization contact time of 2 ms, 1-s recycle delay, and line broadening of 50 Hz. Chemical shifts were calibrated against that of an external standard (glycine). The spectrum was integrated into the following chemical shift regions: paraffinic carbon (0–50 ppm); substituted aliphatic carbon including alcohols, amines, carbohydrates, ethers, and methoxyl and acetal carbon (50–110 ppm); aromatic and phenolic carbon (110–165 ppm); and carboxyl and carbonyl carbon (165–215 ppm) [30].

Solution NMR data were acquired on a Bruker Avance 400-MHz NMR fitted with a quadruple nucleus probe (tuned to $^1$H, $^{13}$C, $^{15}$N, and $^{31}$P) or a Bruker Avance 600-MHz NMR fitted with a $^1$H, $^{13}$C, $^{15}$N, and TXI probe. Freeze-dried humic acid (50 mg) was further dried over P$_2$O$_5$ for approximately 48 h and then dissolved in 0.75 ml of dimethylsulfoxide-d$_6$ (DMSO-d$_6$). The $^1$H NMR (32 scans) was carried out at 600 MHz with a 2-s recycle delay process and processed with 0.3-Hz line broadening. A heteronuclear single quantum coherence spectrum (32 scans, TD [F1] 1.024, TD [F2] 512, J [{$^1$H–$^{13}$C}] 145 Hz) also was acquired at 400 MHz by using a 45° read pulse. Both dimensions were processed with a sine-squared function, a phase shift of 90°, and a zero filling factor of 2. Correlation spectroscopy ([COSY] 64 scans, TD [F1] 1.024, TD [F2] 512) was acquired at 400 MHz by using a 45° read pulse. Both dimensions were processed with an unshifted sine bell and projected by using a magnitude calculation. Nuclear magnetic resonance data were interpreted based on chemical shift assignments already established for humic substances [31,32].

**Aromatic compound solution preparation**

Synthesis of $^{13}$C-labeled aromatic compounds (naphthalene, 1-naphthol, and quinoline) was performed by Jack E. Richman (University of Minnesota, Minneapolis, MN, USA). Naphthalene and 1-naphthol were diblabeled at the C1 and C4 positions, and the C2 carbon of quinoline was labeled. Conventional naphthalene, 1-naphthol, and quinoline were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and used in $^1$H relaxation studies. Deuterated solvents (water and methanol) were used to prepare aromatic compound solutions. Both D$_2$O and CD$_3$OD were purchased in 1-ml ampoules from Sigma-Aldrich. The low aqueous solubility of naphthalene (30 mg/L) required that all naphthalene solutions be made in a CD$_3$OD: D$_2$O (20:80, v/v) mixture. Quinoline and 1-naphthol solutions were dissolved solely in D$_2$O. Solution preparation involved dissolving 0.5 mg of the $^{13}$C-labeled aromatic compound into 1 ml of NMR solvent. The mixture was then placed in a 5-mm NMR tube (Wilms Glass, Buena, NJ, USA) that had been flushed previously with N$_2$ gas. The humic acid stock solution was made from a known mass of humic material dissolved into D$_2$O adjusted to pH 7 with dilute NaOD. The humic acid solution was filtered through a 0.45-μm filter to remove any particulate matter before addition to aromatic compound solutions in NMR tubes. For aromatic compound–humic acid interaction studies, microliter quantities of the concentrated stock solution were added to the aromatic compound solution. Similar studies demonstrated that the solution conditions used here do not result in significant changes to the overall solution viscosity [25,27]. The pH was measured directly in NMR tubes with a microcombination pH electrode (Lazar Research Laboratories, Los Angeles, CA, USA) and an Accumet 925 pH meter (Fisher Scientific, Pittsburgh, PA, USA). To normalize the pH to 7, microliter quantities of a concentrated NaOD solution were added accordingly.

**Nuclear magnetic resonance spin-lattice relaxation experiments**

Both $^1$H and $^{13}$C $T_1$ measurements were conducted on a Bruker Avance 400-MHz spectrometer equipped with a 5-mm quadrupole nucleus probe. All $T_1$ measurements were conducted at constant temperature of 300 K, 10 to 12 variable delay times ($\tau$) per experiment, a recycle delay of five times the $T_1$ value, and 40 and 8 scans per $\tau$ value for $^{13}$C and $^1$H, respectively.
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Fig. 1. Nuclear magnetic resonance (NMR) characterization of the humic acid used in contaminant interaction studies. (A) Solid-state $^{13}$C NMR spectrum reveals a wide distribution of structural components and the presence of many paraffinic components (0–50 ppm). (B) Liquid-state, one-dimensional $^1$H NMR of the humic acid sample. Several structural components arising from aliphatics, substituted aliphatics, aromatics, amides, and carboxyl are identified; DMSO = dimethylsulfoxide.

The $T_1$ measurements were conducted by using the standard inversion recovery pulse sequence and $^{13}$C measurements were done with power-gated decoupling. The data were processed with 0.5-Hz line broadening and the resulting $T_1$ values were calculated by using XWIN-NMR software (Ver 2.6, Bruker Biospin).

Calculation of humic-associated aromatic compound

The proportion of humic-associated aromatic compound was calculated as outlined in the study of Bortiatynski et al. [23]. The following equation was used to calculated humic-associated aromatic compound from $T_1$ measurements:

$$A = \frac{T_{1\text{obs}} - T_{1\text{free}}}{T_{1\text{assoc}} - T_{1\text{free}}}$$

where $A$ represents the fraction of humic-associated aromatic compound, $T_{1\text{obs}}$ is the measured $T_1$ value that represents an average $T_1$ (in seconds) of both free and humic-associated aromatic compound, $T_{1\text{free}}$ is the measured $T_1$ in the absence of humic acid, and $T_{1\text{assoc}}$ is the $T_1$ value when the aromatic compound is fully associated with the humic acid (the aromatic compound takes on the $T_1$ value of the humic acid and the $T_1$ versus humic acid concentration curve plateaus). The percentage of associated versus free aromatic compound was calculated from Equation 1 and plotted in Figure 3. The relationship described by Equation 1 was fitted with a linear equation by using Origin® software (Ver 6.0, Northampton, MA, USA). Isotherm models were also fitted by using Origin software.

RESULTS AND DISCUSSION

Humic acid characterization by solid- and liquid-state NMR

The organic carbon content of the humic acid was measured to be 55.4% and the integration results from the solid-state NMR data (Fig. 1A) indicated that this carbon is distributed as follows: 31% paraffinic carbon (0–50 ppm), 30% substituted aliphatic carbon (50–110 ppm), 28% aromatic and phenolic carbon (110–165 ppm), and 11% carboxylic carbon (165–200 ppm). The carbon distribution of this humic acid is similar to that reported for other soil humic acids [33] and it is rich in...
paraffinic carbon. This is consistent with the observations made from the liquid-state NMR experiments.

Multidimensional NMR experiments were carried out to aid in the assignments of the major categories of structures in the humic acid. Detailed interpretation of the individual cross-peaks has been reported for other humic acid samples and will not be repeated here [31,32,34]. The COSY experiments are applied to obtain information regarding proton connectivity in the structural elucidation of humic substances [31]. The COSY experiments detect couplings between protons that are directly attached to adjacent carbons. The COSY spectrum for the humic acid studied here is displayed in Figure 2A. The major regions highlighted from the COSY experiment can be assigned to couplings between protons: in aromatic rings (region 1), from carbohydrates (region 2), from amino acids (region 3), adjacent to ester/ether/hydroxyl and protons in aliphatic chains (region 4), adjacent to carboxyl or the carbonyl group of esters and protons in aliphatic chains (region 5, noting that contributions from α-side couplings in amino acids also may contribute to this region), and in methylene and methyl aliphatic chains (region 6, noting that contributions from amino acids side groups also may be present). In addition to the COSY experiment, heteronuclear 1H-13C correlation experiments also were performed. Heteronuclear 1H-13C bond correlation experiments disperse the proton and carbon chemical shift information into two dimensions [31]. This improves the resolution of signals that normally overlap in one-dimensional experiments and is particularly advantageous when examining complex mixtures such as humic substances. We applied heteronuclear single quantum coherence, a single-bond 1H-13C correlation experiment that reveals information on 1H-13C connectivity within the humic acid structure. The heteronuclear single quantum coherence spectrum is plotted in Figure 2B and the highlighted regions are assigned to aromatic rings (region 1), carbohydrates (region 2), methine or methylene adjacent to ester/ether or hydroxyl (region 3), methoxyl groups (region 4), amino acids (region 5), DMSO-d$_6$ (region 6), methine or methylene adjacent to carboxylic acid or the carbonyl group of an ester (region 7), and various aliphatic residues, including some contributions from amino acid side chains (region 8). The increased dispersion and coupling information provided by two-dimensional NMR aids in the interpretation of structural units in humic material. This information can then be used to better understand the less-resolved regions in the one-dimensional spectra (Fig. 1B). The presence of carboxyl and exchangeable amide regions were confirmed through exchange with deuterium (data not shown) [31,35].

13C and 1H Relaxation measurements

In the absence of humic material, the 13C $T_1$ values for the 13C-labeled carbons of naphthalene, 1-naphthol, and quinoline were measured to be 4.99 s, 27.56 s, and 3.44 s, respectively, and are consistent with other reports that have measured the $T_1$ values under similar magnetic field strengths and solution conditions [36]. The $T_1$ values were initially measured in replicate; however, the standard deviation was found to be less than 0.1 s for each compound. Freshly prepared samples with humic acids also were run in duplicate but differences in the measured $T_1$ value between replicates was negligible. The addition of humic acid to the aromatic compound solution resulted in an immediate decline in the $T_1$ values of naphthalene, 1-naphthol, and quinoline and is consistent with other reports that demonstrated a decline in $T_1$ values upon association with humic material [22,24–26]. This decline is depicted in Figure 3, where the $T_1$ value of each aromatic compound is plotted against increasing concentration of humic acid. The $T_1$ values of the C1 and C4 carbon of naphthalene (Fig. 3) diminish rapidly with the addition of humic acid C at only 5 mg/L. This
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Figure 4. (A) $^1$H Relaxation of 1-naphthol protons with increasing humic acid concentration at pH = 7. All protons are observed to relax at a similar rate, suggesting a nonselective interaction between the protons of 1-naphthol and humic acid. (B) $^1$H Relaxation of quinoline protons with increasing humic acid concentration at pH = 7. All protons are observed to relax at a similar rate, suggesting a nonselective interaction between the protons of 1-naphthol and humic acid.

positions in the molecule; however, the lowest addition of humic acid (5 mg C/L) caused a decline of nonequivalent protons to an equivalent associated $T_1$ value. The same trend is observed for higher humic acid additions. The $T_1$ decay of nonequivalent protons to equivalent $T_1$ values after the addition of humic acid in conjunction with a lack of chemical shift confirms that this interaction is strictly noncovalent. Furthermore, this association appears to be nonspecific. For instance, if a specific hydrophobic interaction occurred, such as one with the heterocyclic nitrogen in quinoline interacting with a carboxylic acid group of the humic acid, then the $^1$H $T_1$ values of protons A, B, and G would decline faster than the other protons. Protons A, B, and G of 1-naphthol would also display a sharper decrease than the other protons if a specific interaction occurred between the hydroxyl group and the humic acid. However, this is not what was observed and suggests that the nature of the noncovalent interaction is so strong that all parts of the molecule are associated nonspecifically.

This hypothesis is corroborated by the observed signal broadening with both the $^{13}$C and $^1$H interaction experiments. The $^{13}$C and $^1$H spectra of 1-naphthol are displayed in Figure 5. In the absence of humic acid, the $^{13}$C chemical shifts for...
the dilabeled 1-naphthol are 120.4 ppm for the C4 carbon and 152.4 ppm for the C1 carbon. Both signals are doublets because of long-range carbon–carbon coupling. However, after the addition of humic acid (80 mg C/L), the doublets become singlets and the width of the signal increases. The same is observed in the H experiment. The signal width of the 1-naphthol protons increases and the splittings observed in the absence of humic acid are no longer apparent at higher humic acid concentration. This line broadening is consistent with strong noncovalent interactions and has been documented by other researchers [25,39]. In a study that examined interactions between fulvic acids and monoaromatic compounds, Nanny [25] did not observe changes in signal widths and hypothesized that the association was weak because of the polar nature of fulvic acids. Conversely, Herbert and Bertsch [39] reported signal broadening due to the intensity of the noncovalent interaction between the probe compounds (a series of substituted fluorobenzenes) and humic acid.

This study has several environmental implications. For instance, we demonstrated that humic acids have several functionalities that have the capability of interacting and possibly sequestering environmental contaminants. In NMR interaction studies, the probe compounds (naphthalene, 1-naphthol, and quinoline) readily interacted with a dissolved humic acid, even at low DOM levels. The ubiquity of DOM at low concentrations in streams, lakes, oceans, and soil pore waters will likely alter the fate and transport of environmental contaminants, more so than conventional methods would suggest. For instance, a study by Karthikeyan and Chorover [8] examined the interactions of 1-naphthol with the same humic acid used in this study, by both fluorescence quenching and equilibrium dialysis methods. At a pH of 7 and humic acid concentration of 11 mg C/L, they reported that the percentage of humic acid–bound 1-naphthol was <5% by equilibrium dialysis and between 5 and 12% by fluorescence quenching, depending on the ionic strength. In the current study, the percentage of humic acid–associated 1-naphthol is approximately 55% (Fig. 3). This comparison implies that small changes in the molecular environment that are detected by NMR relaxation studies may go undetected by other popular methods. Consequently, aromatic compounds may associate with humic material to a greater extent than other methods would indicate. Furthermore, the strong, noncovalent attraction of organic compounds with humic materials is nonspecific at neutral pH, and does not appear to be governed by ring substitution, or proton-donating or -accepting groups. Furthermore, the compounds studied here were too hydrophobic to observe specific interactions despite that NMR has been used with other compounds to determine specific interactions between soil organic matter and the nitro group of trifluralin [40]. Consequently, DOM may influence
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the fate and persistence of aromatic compounds in the environment more than is currently recognized. Finally, future T1 studies should address how solution conditions, such as pH and ionic strength, alter the associations that occur between humic materials and aromatic compounds.

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