Environ. Sci. Technol. **1999**, *33,* 958−964

NMR Investigation of the Interactions between 4′-Fluoro-1′-acetonaphthone and the Suwannee River Fulvic Acid

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The association of 4′-fluoro-1′-acetonaphthone (F-acetonaphthone) with the International Humic Substances Society (I.H.S.S.) Suwannee River fulvic acid (SRFA) standard in 55% CH_3OD/45% D_2O was investigated as a function of SRFA concentration (4.5−13.5 mg/mL) as well as solution $p_\alpha H^*$ (3.88−7.56). ^{19}F NMR measurements of T_1 and T_2 relaxation times were used in conjunction with chemical shift and diffusion coefficient measurements to study the interaction, and the results were interpreted using the current association models reported in the literature (*1*). Analysis of diffusion coefficients measured as a function of SRFA concentration suggests that a small amount of the F-acetonaphthone is associated with SRFA. Unusual behavior of the T_1 and T_2 relaxation time constants, correlation times calculated from these parameters, and diffusion coefficients were observed for F-acetonaphthone in solutions of SRFA in 55% CH_3OD/45% D_2O at $p_\alpha H^*$ 6.10. Analysis of the $p_\alpha H^*$ dependence of the diffusion coefficients suggests two competing mechanisms of association between F-acetonaphthone and SRFA, hydrophobic interactions, and hydrogen bonding. The contribution of the fluorine label to the interaction of F-acetonaphthone with SRFA was investigated using ^{19}F NOE measurements as well as measurements of competitive binding by unfluorinated acetonaphthone and fluorobenzene.

Introduction

Association of organic contaminants and natural humic substances, through entropic effects and by binding through covalent, hydrogen bonding, charge-transfer, and van der Waals interactions, affects the fate and transport of these compounds in the environment (*1*−*3*). A number of structural models of humic and fulvic acids have been devised to account for these interactions. For example, it has been suggested that humic and fulvic acids exist as flexible polymers in solution at concentrations below 3.5 g/L and ionic strengths below 0.05 M (*4*). At higher concentrations, these compounds are thought to have micelle-like behavior in solution, forming nonpolar pockets in their interior (*5*). Detailed studies of the interactions between humic and fulvic acids and organic molecules will lead to a better understanding of the solution chemistry of these natural organic materials and a refinement of models that more effectively describes their effect on the bioavailability and fate of synthetic organic pollutants.

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The study of interactions between aquatic fulvic acid and aromatic organic molecules presents an analytical challenge due to the complex and heterogeneous nature of fulvic acid. Several experimental approaches have been described to investigate the interactions between humic substances and synthetic organic compounds. Solubility studies have demonstrated that the solubility of hydrophobic compounds in water is enhanced in the presence of dissolved humic substances (*6*−*12*). The sorption of hydrophobic compounds such as phenanthrene to soils, sediments, clays, and minerals has also been reported (*13, 14*). Fluorescence polarization and quenching have been used to study the interactions of soluble humic acid with naphthalene and 1-naphthol (*15*); pyrene (*16*); difenzoquat and 1-naphthol (*5*); and fluoranthrene, 1-naphthol, and napropamide (*17*). These studies concluded that a partition-like interaction takes place between hydrophobic regions of humic and fulvic acids and the hydrophobic organic molecules; however, there was disagreement on the nature of the interaction. The ^{13}C NMR measurements in MeOH/D_2O reported by Nanny et al. indicated that three different noncovalent interactions were possible for ^{13}C labeled acenaphthenone (a related compound): a weak sorption to the fulvic acid, enhanced solubilization, and an interaction primarily between acenaphthenone and the solvent. It was concluded that the increased solubility of acenaphthenone in MeOH/D_2O−fulvic acid solutions was due to hydrophobic pockets formed by the fulvic acid, which encapsulated and concentrated both the methanol and the acenaphthenone (*1*).

It has been proposed that the ability of humic substances to solubilize hydrophobic organic compounds is due to the formation of aggregates with hydrophilic exterior surfaces and a hydrophobic interior, similar to micelles. However, a ^{19}F NMR study of CF_3-atrazine in SDS and humic acid micelles showed significant differences in behavior attributed to intermolecular hydrogen-bonding interactions in the interior of the humic micelles. Measurements of ^1H and ^{19}F chemical shift and line width were also used to evaluate the interactions between substituted fluorobenzenes and natural organic matter in aqueous solution (*19*). These authors reported that the aromatic molecules were strongly sorbed to the humic substances and also emphasized the importance of hydrogen bonding between the fluorobenzene solute and charged groups of humic acid.

Many investigators have utilized ^{19}F NMR to study a diverse set of problems including interactions of fluorinated organic compounds with humic and fulvic acid (*18*−*20*). The ^{19}F nucleus has a large chemical shift range, yielding chemical shifts and peak widths that are very sensitive to changes in the local and macroscopic environments. In this research, a ^{19}F labeled aromatic compound, 4′-fluoro-1′acetonaphthone (F-acetonaphthone) is used as a probe of the interactions between Suwannee River fulvic acid (SRFA) and aromatic organic molecules in solution. An additional goal of this research was to examine whether the observations regarding SRFA interactions reported by Nanny et al. (*1*) for a related compound, ^{13}C labeled acenaphthenone, in 55% MeOH/45% D_2O also occur for a probe molecule with an additional contribution to binding from hydrogen-bonding to fluorine. Spin−spin and spin−lattice relaxation time constants and diffusion coefficients have been measured for the ^{19}F nucleus as a function of SRFA concentration and solution acidity and can be used to assess the binding environment and extent of binding. The diffusion coefficients and correlation times of the F-acetonaphthone free in solution and in the presence of excess SRFA are interpreted

10.1021/es980525f CCC: $18.00
Published on Web 02/02/1999

in terms of the current interaction models for fulvic acid with hydrophobic molecules. Nuclear Overhauser enhancement (NOE) and competitive binding studies provide additional information about the mechanism of association between F-acetonaphthone and SRFA.

Methods

Chemicals. F-acetonaphthone, methyl-d_3 alcohol-*d*, and the chemical shift reference, fluorobenzene, were purchased from Aldrich (Milwaukee, WI). Deuterated water (D_2O, 99.8% *d*) was obtained from Fluka (Buch, Switzerland). To accurately measure relaxation rates, oxygen was removed from all solutions and reagents by bubbling with nitrogen gas. All samples were prepared and degassed in an Atmosbag (Aldrich) under a nitrogen atmosphere. Due to the photosensitivity of F-acetonaphthone, all sample preparations were performed in the absence of UV light using a low energy light source. Solutions of 0.9 and 1.8 mM F-acetonaphthone were prepared in 55% deuterated methanol and 45% D_2O. The SRFA solutions were prepared from the SRFA standard, obtained from the International Humic Substances Society (I.H.S.S.), dissolved in the MeOD/D_2O solvent to concentrations ranging from 4.5 mg/mL (5 mM) to 13.5 mg/mL (15mM), assuming an average molecular weight for SRFA near 900 g/mol (*21*). The low concentration sensitivity of ^{19}F NMR restricts our measurements of F-acetonaphthone to 0.9 mM as a practical concentration limit with the available instrumentation. In addition, the relatively weak binding interactions necessitated the use of SRFA concentrations much greater than those normally found in the Suwannee River. The SRFA was obtained in the protonated form and has a low ash content, 0.85%. Concentrated solutions of DCl and NaOD were obtained from Isotec, diluted in D_2O, and used to adjust the acidity of the solutions. The pH measurements were made with a Fisher Scientific Acumet 10 pH meter equipped with a 3 mm Ingold combination pH microelectrode calibrated daily with aqueous pH buffers. All pH measurements were corrected for alcoholic media effects using the relationship $p_aH^* =$ pH meter reading $- \delta$, where $\delta = 0.15$ for solutions of 61.5 wt % MeOH (*22*). No attempt was made to compensate for ionic strength or deuterium isotope effects. At the time these experiments were performed, we were unaware of the lengthy equilibration times required for fulvic acid after addition of the hydroxide ion (*23*). The time between pH adjustment and NMR measurements was not rigorously controlled here; however, the long experiment times and replicate measurements ensured a sufficient time for equilibration.

NMR Chemical Shift and Diffusion Coefficient Measurements. The measurement of ^{19}F diffusion coefficients was carried out using a Bruker AM-360 MHz NMR spectrometer equipped with a 5 mm z-gradient inverse probe for PFG experiments. Detailed information on the instrumentation used for gradient measurements has been reported previously (*24*). All fluorine free induction decays (FIDs) were acquired with a spectral width of 15 151 Hz and 16 384 data points. Chemical shifts are reported relative to an external reference of fluorobenzene (at 0.00 ppm). All NMR spectra were measured at a temperature of 298 K.

The PFG NMR spectra were measured with the BPPLED pulse sequence using a diffusion delay time, Δ, of 0.18 s, an eddy current delay time of 15 ms, and a relaxation delay of 2.0 s (*25*). In these experiments, delay times yielding both optimum signal-to-noise ratios and sufficient reduction of resonance intensity due to diffusion, were determined by trial and error using visual inspection of the resulting spectrum. To achieve adequate signal-to-noise ratios in the spectra of F-acetonaphthone and SRFA solutions, 1008 transients were coadded for each gradient. Eighteen separate BPPLED spectra were acquired for each ^{19}F PFG NMR experiment. The amplitude of the gradients in these experiments ranged from 0.027 to 0.242 T/m in regular intervals. The diffusion coefficient of the SRFA was determined by similar ^1H PFG NMR experiments of the SRFA solution, prior to the addition of F-acetonaphthone, and a linear fit of the resulting data (*26*).

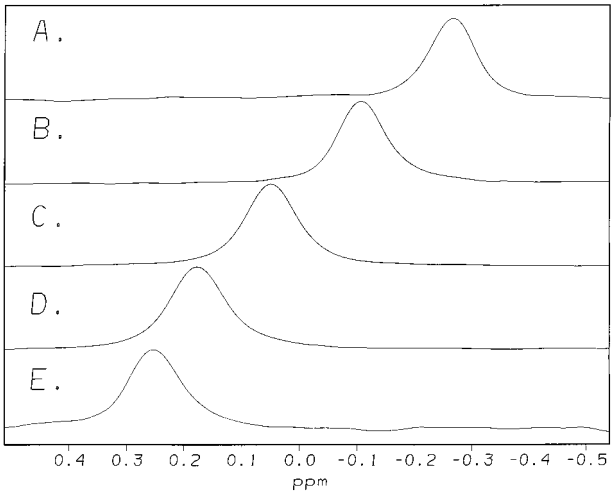

FIGURE 1. ^{19}F chemical shift of 0.9 mM F-acetonaphthone as a function of fulvic acid concentration. The fulvic acid concentrations and corresponding ^{19}F chemical shifts shown are (A) 0 mg/mL (−0.267 ppm), (B) 4.5 mg/mL (−0.105 ppm), (C) 6.75 mg/mL (0.050 ppm), (D) 9 mg/mL (0.179 ppm), and (E) 13.5 mg/mL (0.256 ppm).

The ^{19}F FIDs were processed using FELIX 95.0 (Biosym) Software on a Silicon Graphics Indy workstation. The FIDs were zero filled to 65 536 data points and apodized before Fourier transformation by multiplication with a decaying exponential which corresponded to 20 Hz of line broadening in the transformed spectrum. Correction of the spectral baseline was accomplished by fitting selected baseline points to a fifth order polynomial.

Calculation of Diffusion Coefficients. The intensity of the resonance, I, and the diffusion coefficient, *D*, are related according to the equation (*25*)

$$I = I_o \exp[-D(\Delta - \delta/3 - \tau/2)G^2\gamma^2\delta^2] \qquad (1)$$

where I_o is the signal intensity in the absence of the gradient pulse, Δ is the diffusion delay time in seconds, δ (2.4 ms) is the length of the bipolar gradient pulse pair, γ is the magnetogyric ratio, *G* is the gradient pulse amplitude, and τ (1.1 ms) is the time between the gradient pulse and the following rf pulse. Diffusion coefficients were calculated from the slope of the line obtained by plotting ln *I* vs G^2 using version 3.5 of the Origin software from Microcal as shown in Figure 1. The standard deviation of the slope was also calculated and used to estimate the error in the diffusion coefficients according to the equation.

$$sd_D = D(sd_{slope}/slope) \qquad (2)$$

NMR Chemical Shift and Relaxation Measurements. The ^{19}F NMR relaxation experiments were carried out using a Bruker DRX-400 MHz spectrometer equipped with a 5-mm broad band probe. The ^{19}F FIDs used in the calculation of relaxation times were acquired with a spectral width of 8192 Hz and 16 384 data points. Longitudinal (spin−lattice) relaxation time constants, T_1, were obtained using the inversion−recovery pulse sequence with a recycle time of 7.3 s for ^{19}F. A set of 14 τ values ranging from 0.02 to 14.5 s was used for each T_1 measurement. At each value, 32 transients were coadded in order to achieve an adequate signal-to-noise ratio. Transverse relaxation time constants,

T_2, were measured using the CPMG pulse sequence. A set of 20 τ values ranging from 4 to 152 ms was used for the ^{19}F T_2 measurements, with 32 transients coadded at each value. Both T_1 and T_2 relaxation time constants were calculated using a nonlinear least-squares fit of a single exponential to the resonance intensity since no evidence of biexponential behavior was observed. The reported error was calculated from the difference in the values determined in two independent experimental trials.

Calculation of Molecular Correlation Time. Molecular correlation times, τ_c, were calculated from ^{19}F T_1 and T_2 relaxation times and used to study the interaction of F-acetonaphthone with SRFA (*27*). This method of calculating correlation times is simpler than traditional methods involving the use of NOE data, which can be difficult to measure and interpret quantitatively. T_1 and T_2 relaxation times are measured and converted to their corresponding relaxation rates, R_1 and R_2, respectively. The rates of relaxation are then used in the following equation

$$\tau_c\,(\text{ns}) = a_0 + a_1(R_2/R_1) + a_2(R_2/R_1)^2 + a_3(R_2/R_1)^3 + a_4(R_2/R_1)^4 \quad (3)$$

where $a_0 = -3.1073$, $a_1 = 2.6705$, $a_2 = -0.1397$, $a_3 = 0.00455$, and $a_4 = -0.000\,046$ are constant coefficients which depend only on the nucleus observed, in this case ^{19}F, and the magnitude of the applied field (*28*).

NMR Measurements of the Nuclear Overhauser Effect. The heteronuclear NOE was measured using gated decoupling. Spectra were acquired with 65 536 data points and 640 scans to ensure a sufficiently high signal-to-noise ratio after spectral subtraction. Protons were decoupled from the ^{19}F nuclei prior to acquisition using continuous phase decoupling (CPD), at which point the decoupler was gated off to produce coupled spectra containing the NOE. The NOE was allowed to build up during an initial delay time of 7 s, approximately seven to eight times T_1, before the 90° read pulse was applied. Only regions of the proton spectrum corresponding to SRFA resonances were decoupled, with care taken to avoid decoupling the proton resonances of F-acetonaphthone. Two control experiments were performed in which the decoupler offset was set to −2.0 ppm, for a control upfield of TSP, and 9.3 ppm, for a control downfield of the aromatic proton resonances. To saturate proton regions that consisted only of SRFA resonances, the decoupler offset was placed at 1.2 ppm for the methyl and methylene proton region, 2.5 ppm for the methine and carbohydrate protons, and 7.9 ppm for the aromatic protons. The decoupler was calibrated in order to obtain a field strength, γB_2, of 300 Hz. Spectral subtraction of an unenhanced spectrum from the enhanced spectra was carried out in order to determine the magnitude of the NOE.

NMR Measurement of F-Acetonaphthone Concentration. The concentration of F-acetonaphthone in the stock solution used to prepare the NMR samples was determined by standard addition of 45 μL of a 0.013 M trifluoroacetic acid (TFA) solution according to the standard quantitative NMR method (*29*, *30*). A quantitative ^{19}F NMR spectrum was acquired using a 60° pulse, 32 768 data points, an acquisition time of 1.3 s, and a relaxation delay of 6 s. The resonances were integrated, and the concentration of the F-acetonaphthone stock solution was determined relative to trifluoroethanol. Because the recycle time did not allow for complete relaxation of the TFA resonance, the attenuation of the steady-state resonance intensity of TFA was calculated according to the standard formulas (*31*), and compensation was made when calculating the acetonaphthone concentrations.

Competitive Binding Study. The competition between fluorinated and unfluorinated acetonaphthone for SRFA binding sites was assessed using measurements of ^{19}F chemical shift and diffusion coefficients. Initially, an equimolar concentration of unfluorinated acetonaphthone was added to a solution containing 1.8 mM F-acetonaphthone and 7.5 mM SRFA at $p_\alpha H^*$ 6.60. Subsequent measurements were performed on samples that contained a 3- and a 6-fold molar excess of unfluorinated acetonaphthone. To investigate the influence of ^{19}F hydrogen bonding, experiments were carried out at $p_\alpha H^*$ 3.9 and 10.05 and the competition of fluorobenzene for SRFA binding sites was examined. The experimental parameters of the diffusion experiments remained the same as those discussed previously.

TABLE 1. ^{19}F T_1 and T_2 Relaxation Times, Correlation Times, and Diffusion Coefficients Measured for 0.9 mM F-Acetonaphthone as a Function of Suwannee River Fulvic Acid Concentration in MeOD/D_2O

fulvic acid concn (mg/mL)	T_1 relaxation time (s)	T_2 relaxation time (s)	correlation time (ns)	diffusion coeff ($\times 10^{10}$ m^2 s^{-1})
0.0	1.781 ± 0.016	1.720 ± 0.013	N/A	6.15 ± 0.09
4.5	0.555 ± 0.006	0.083 ± 0.006	9.8	5.48 ± 0.21
6.8	0.396 ± 0.011	0.056 ± 0.002	10.3	5.75 ± 0.35
9.0	0.469 ± 0.005	0.060 ± 0.004	11.0	5.52 ± 0.11
13.5	0.288 ± 0.014	0.036 ± 0.000	11.2	5.26 ± 0.04

Results and Discussion

^{19}F Relaxation Measurements. The ^{19}F T_1 and T_2 relaxation time constants measured as a function of SRFA concentration at $p_\alpha H^*$ 6.25 are reported in Table 1. Upon addition of 4.5 mg/mL SRFA, the ^{19}F T_1 relaxation time of F-acetonaphthone is reduced from 1.781 to 0.555 s. The T_2 relaxation time also shows a sharp decrease from 1.720 to 0.083 s, consistent with the observed broadening of the ^{19}F resonance in solutions containing SRFA. The T_1 and T_2 relaxation times continue to decrease with additional increases in the SRFA concentration, although the decrease is less pronounced. In similar measurements made by Nanny et al. in MeOH/D_2O, the concentration dependence of the ^{13}C T_1 of the organic probe suggested an enhanced mobility in dilute SRFA solutions compared with solutions of the free probe molecule (*1*). This behavior was attributed to the solubilization of the organic probe molecule in methanol-containing hydrophobic pockets of the SRFA. In contrast, the concentration dependent decrease in ^{19}F relaxation times reported here indicates a steady reduction in molecular mobility as a result of association with SRFA, suggesting sorption. The sorption of F-acetonaphthone to SRFA can occur from both weak hydrophobic interactions as well as through hydrogen bond formation between SRFA and the ^{19}F nucleus. Such hydrogen bonding interactions would have a pronounced effect on the ^{19}F relaxation times.

The molecular correlation time, τ_c, is defined as the average time taken to rotate through one radian or the inverse of the rate of tumbling in solution for the corresponding part of the molecule (*32*). Molecular correlation times were calculated from the T_1 and T_2 relaxation times according to a recently reported method (*27*) and are also listed in Table 1. The correlation times calculated from the ^{19}F relaxation data most likely represent the local correlation time for the fluorine and the aromatic ring to which the fluorine is attached and not the correlation time of the molecule as a whole. This correlation time increases upon addition of SRFA and continues to increase upon further addition of fulvic acid. Because the correlation time is an indicator of both the local mobility and effective molecular radius, the dependence of correlation time on SRFA concentration may result from the decreased mobility of the ^{19}F nucleus due to hydrogen bonding as well as from an increased overall molecular radius due to the association of acetonaphthone molecules with SRFA.

In this study, large changes in relaxation and correlation times occur upon initial addition of SRFA, and very small changes are seen upon further additions. The initial change suggests either that a majority of the acetonaphthone molecules are bound, even at the lowest SRFA concentration, or that the relaxation times are weighted by a fraction of the molecules bound very rigidly through noncovalent interactions such as hydrogen bonding. The fact that the values change very little as the fulvic acid concentration is increased suggests that the interaction between F-acetonaphthone and SRFA is favorable. The extent of binding, however, is difficult to ascertain using relaxation measurements, and, therefore, other methods must be employed to answer this question.

^{19}F Diffusion Coefficient Measurements. Diffusion coefficients of the F-acetonaphthone fluorine nucleus were measured as a function of SRFA concentration. Diffusion measurements can provide a unique picture of the extent of binding and therefore provide complementary information to that derived from relaxation measurements. Since diffusion coefficients are indicators of hydrodynamic volume and thus molecular size, they can be used as a measure of molecular association (*24*, *33*−*37*). The diffusion coefficient of free F-acetonaphthone in MeOD/D_2O is 6.15×10^{-10} m^2 s^{-1}, as shown in Table 1. The average diffusion coefficient of the SRFA was measured in MeOD/D_2O by ^{1}H NMR and was found to be $3.6 \times 10^{-10} \pm 2.2 \times 10^{-11}$ m^2 s^{-1}. The SRFA diffusion coefficient provides a reasonable upper boundary for the value of the F-acetonaphthone-SRFA complex. Upon addition of 5 mM SRFA, the F-acetonaphthone diffusion coefficient decreases to 5.48×10^{-10} m^2 s^{-1} and ultimately to 5.26×10^{-10} m^2 s^{-1} at the highest SRFA concentration, 15 mM. This concentration dependent decrease is not as pronounced as those observed for fluorine relaxation times, since the diffusion coefficient is not sensitive to the nature of the molecular interaction. However the observed decrease in diffusion coefficient does indicate association of F-acetonaphthone with the larger, more slowly diffusing SRFA. As with relaxation times, the measured diffusion coefficient is an average of the free and the bound acetonaphthone diffusion coefficients in the limit of fast exchange. Because of the good linear fit of the experimental results, these data can be interpreted as a single average diffusion coefficient supporting the hypothesis that the system is in fast exchange on the time scale of the diffusion measurement. Comparison of the diffusion coefficient measured for F-acetonaphthone to that of SRFA in the same solvent serves as a good estimation of the extent of binding. Even at the highest fulvic acid concentration, the fluorine diffusion coefficient is much closer to that of free F-acetonaphthone than the average diffusion coefficient of SRFA in MeOD/D_2O. Therefore, the diffusion results indicate that only a small fraction of F-acetonaphthone is strongly associated with SRFA.

^{19}F Chemical Shift Measurements. The fluorine chemical shift was also measured as a function of SRFA concentration. The ^{19}F nucleus has a very large chemical shift range and is therefore very sensitive to changes in environment. As shown in Figure 1, the F-acetonaphthone resonance shifts downfield with increasing SRFA concentration indicating decreased electron shielding around the fluorine nucleus. Herbert et al. reports that the electron density around fluorine is affected by hydrogen-bond formation between non-fluorine functional groups and the solvent (*19*). In the study of interactions between nonionic organic compounds and humic material, they report bound fluorine chemical shifts downfield of the free fluorine resonance. The chemical shift changes, like relaxation and correlation times measured in these solutions, suggest that hydrogen bonding as well as hydrophobic interactions contribute to the binding of F-acetonaphthone to SRFA.

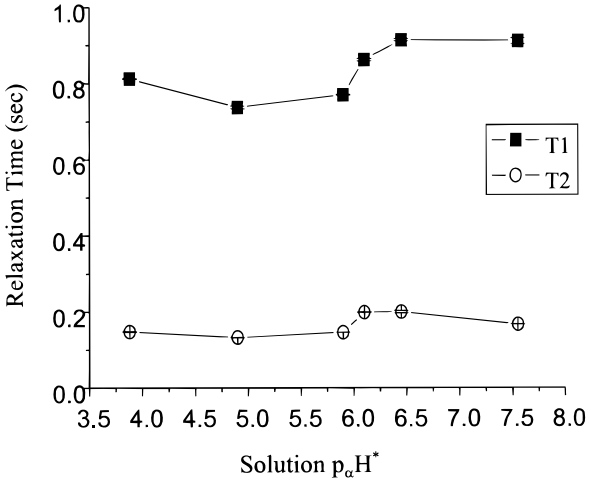

FIGURE 2. The $p_\alpha H^*$ dependence of the ^{19}F T_1 (■) and T_2 (○) relaxation times for 1.8 mM F-acetonaphthone in the presence of 6.75 mg/mL FA in MeOD/D_2O.

The extent of binding between F-acetonaphthone and SRFA was analyzed using a double reciprocal plot of inverse ^{19}F chemical shift versus inverse SRFA concentration to estimate the value of the bound chemical shift, 0.44 ppm. The extent of F-acetonaphthone binding was then estimated using a fast exchange model yielding a value of approximately 70% bound in the 15 mM SRFA solution. This value is double that obtained by similar analysis of the diffusion data. The assumption that the bound chemical shift can be obtained by fitting the data to a linear function falsely assumes that the binding sites of SRFA are homogeneous and all F-acetonaphthone molecules are bound with the same affinity and binding constants. The limited number of data points in this study also contributes to the error in estimating the value of the bound chemical shift. Despite these limitations, this approach has been used by other investigators in this field and can provide useful information (*2*). An advantage of the ^{19}F diffusion measurements is that the accuracy of the chemical shift analysis can be tested in our system since the SRFA diffusion coefficient provides the upper limit of the fully bound ^{19}F diffusion coefficient.

^{19}F NMR Measurements as a Function of Solution $p_\alpha H^*$. It has been suggested that the interactions of SRFA with organic molecules are dependent on the solution pH. Nanny et al. measured the T_1 of ^{13}C-labeled acenaphthenone in the presence of fulvic acid at a variety of pH values (*1*). In these studies, a maximum in T_1 was reported at pH 6.1, suggesting that the acenaphthenone experienced an increased freedom of motion at this pH. From this and other data, Nanny et al. concluded that the interactions between fulvic acid and acenaphthenone molecules differ with solution pH. At pH 6.1, the fulvic acid is believed to encapsulate both methanol and acenaphthenone in its hydrophobic interior. Engbretson et al. also observed such behavior, with the interactions of fulvic acid changing significantly at pH 6.2 (*38*). To further explore this hypothesis and to evaluate whether similar effects can be observed when the association is driven by both hydrogen bonding and hydrophobic interactions, ^{19}F NMR measurements of F-acetonaphthone relaxation, diffusion, and chemical shift in MeOD/D_2O SRFA solutions were performed for $p_\alpha H^*$ values ranging from 3.88 to 7.56. The concentration of SRFA remained constant at 6.75 mg/mL (7.5 mM) in these studies, and a constant concentration of F-acetonaphthone, 1.8 mM, was used throughout.

pH Dependent ^{19}F Relaxation Measurements. The values of the T_1 and T_2 relaxation times measured for 55% MeOD/45% D_2O solutions with $p_\alpha H^*$ values ranging from 3.88 to 7.56 are given in Figure 2. Both T_1 and T_2 follow the same

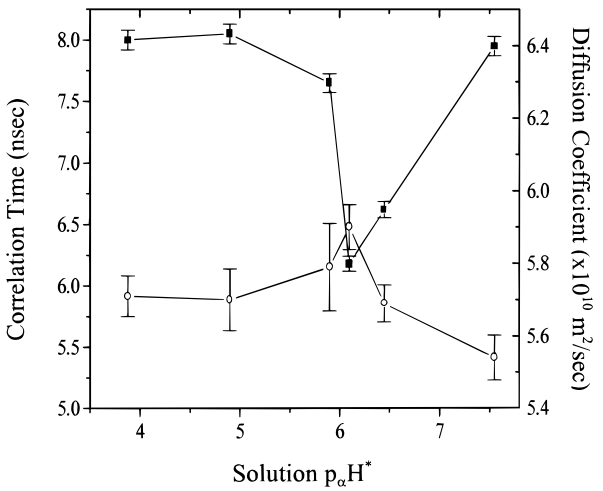

FIGURE 3. The $p_\alpha H^*$ dependence of the ^{19}F correlation times (■) and diffusion coefficients (○) of 1.8 mM F-acetonaphthone in the presence of 6.75 mg/mL FA in MeOD/D_2O.

trend as the $p_\alpha H^*$ is increased, and both show a significant increase at $p_\alpha H^*$ 6.10. This increase supports the idea that changes occur in the noncovalent interactions between the SRFA and F-acetonaphthone, and possibly in the structure of SRFA, as the pH changes. A similar increase in relaxation time at pH 6.10 was reported by Nanny et al. (*1*); however, in their experiments the T_1 decreased at pH values above 6.10. In this research, both the T_1 and T_2 relaxation times remained elevated at $p_\alpha H^*$ values greater than 6.10, indicating a more permanent change in the F-acetonaphthone-SRFA interactions. The relaxation data generally reinforce Nanny's hypothesis that the nonpolar organic molecules are solubilized in a nonpolar region of fulvic acid aggregates. However these results may also simply indicate a decrease in the extent of F-acetonaphthone binding at this $p_\alpha H^*$ value due to reduced hydrogen bonding capacity of the SRFA resulting from deprotonation of the carboxylic acids.

The correlation time of F-acetonaphthone was calculated from the relaxation parameters as a function of solution $p_\alpha H^*$ and is shown in Figure 3. As expected from the T_1 and T_2 relaxation times, the correlation time sharply decreases at $p_\alpha H^*$ 6.10, indicating that the fluorine atom of the acetonaphthone molecule experiences increased molecular mobility in this solution. The correlation times determined at $p_\alpha H^*$ values greater than 6.10 are similar to the results for more acidic solutions. To a large extent, the correlation times reflect the mobility of the ^{19}F nucleus. Therefore the observed minimum in the correlation time at $p_\alpha H^*$ 6.10 could result from two competing pH dependent processes. An increase in pH should reduce the extent of hydrogen bonding of the F-acetonaphthone with specific SRFA functional groups (i.e. COOH), therefore decreasing the τ_c. However, hydrophobic association of F-acetonaphthone in the nonpolar regions of SRFA as suggested by Nanny et al. (*1*) may be relatively more important near neutral pH, causing an increase in the local τ_c.

pH Dependent ^{19}F Diffusion Coefficient Measurements. The diffusion results provide further evidence that the nature of the interactions between F-acetonaphthone molecules and SRFA changes at $p_\alpha H^*$ 6.10. The $p_\alpha H^*$ dependence of the ^{19}F diffusion coefficients of F-acetonaphthone is illustrated in Figure 3. It can be seen from this plot that the diffusion coefficient increases at $p_\alpha H^*$ 6.10, only to decrease again with further increases in $p_\alpha H^*$. Because diffusion coefficients provide information about the global mobility of an analyte and changes in the extent of binding, the observed increase in the diffusion coefficient at $p_\alpha H^*$ 6.10 can be attributed to a decreased hydrodynamic volume resulting from less association of F-acetonaphthone and SRFA. This behavior can also be attributed to two competing pH dependent mechanisms, hydrophobic interactions involving the naphthyl ring and hydrogen bonding of the fluorine nucleus to the SRFA, which should be favored in more acidic solutions. No association of the F-acetonaphthone was detected within experimental error by ^{19}F PFG NMR diffusion measurements in extremely basic solution ($p_\alpha H^*$ 10.05). This observation is not surprising since the hydrogen bond donor ability of SRFA should be greatly reduced at this pH, and the extended conformation adopted under these conditions should also reduce the extent of hydrophobic binding.

Heteronuclear Nuclear Overhauser Enhancement. To further investigate the nature of the association between F-acetonaphthone and SRFA, the heteronuclear NOE from the SRFA protons to the fluorine nucleus was measured. Initially, a one-dimensional, fully relaxed ^{19}F spectrum of the 1.8 mM F-acetonaphthone and 7.5 mM SRFA solution was acquired with no proton decoupling. Next, different regions of the proton spectrum of SRFA were irradiated using gated decoupling, with the decoupler gated off during acquisition. The intensity of the ^{19}F resonance is enhanced by the NOE as a result of the association of F-acetonaphthone with SRFA. The maximum NOE, 53%, which could occur between protons and ^{19}F nuclei can be calculated according to the following equation:

$$\mathrm{NOE}_{\max} = \gamma_{1H}/2\gamma_{19F} \qquad (4)$$

In the control experiments, the decoupler was placed upfield and downfield of the major proton resonances of SRFA at −2.0 and 9.3 ppm yielding NOE's of 2.3% and 2.4%, respectively, relative to the spectrum obtained without decoupling. When the proton regions of SRFA were irradiated at 1.2, 2.5, and 7.9 ppm, NOE's of 5.4%, 5.1%, and 4.8%, respectively, were obtained. These values are significantly higher than those obtained for the controls. The fact that a nuclear Overhauser enhancement is observed when decoupling both the aliphatic and the aromatic regions of the SRFA ^1H NMR spectrum indicates that the SRFA − F-acetonaphthone interactions are not limited to aromatic interactions. Furthermore, the significant and clearly detected NOE indicates that the F-acetonaphthone molecules are strongly associated with SRFA for a period of time sufficient for the NOE to develop.

Competitive Binding Study. Because of the complex and heterogeneous character of natural organic matter, the nature of the interactions between fulvic acid and organic molecules is difficult to ascertain. One may expect that an aromatic molecule in solution would associate with aromatic functional groups of SRFA by weak noncovalent hydrophobic interactions. However, in the case of this research, the fluorine substituent may have a profound effect on the interactions of a molecule such as acetonaphthone. The use of fluorine labeled molecules in conjunction with ^{19}F NMR is common in the investigation of complex systems such as humic substances due to the high abundance of this nucleus and its spectral simplicity. However, the information gained when using a ^{19}F-labeled compound may strongly reflect the behavior of fluorine, which may be very different from the original molecule. Therefore the results of experiments utilizing fluorine labeled compounds should be interpreted with caution. For example, in the study of protein diffusion, Coffman et al. used fluorine labeled albumin as a probe molecule (*39*). Although they reported no significant alteration in the diffusion results, the labeled ovalbumin was considered to be a different probe molecule due to changes in conformation and charge caused by addition of fluorine. The influence of fluorine substituents on aromatic interactions has been studied by Hunter et al., who concluded that

TABLE 2. Competition between Fluorinated and Unfluorinated Acetonaphthone for Binding Sites on Fulvic Acid as Reflected by ^{19}F Measurements of Diffusion Coefficient, Peak Width, and Chemical Shift of F-Acetonaphthone at $p_\alpha H^*$ 6.6[a]

molar ratio of unfluorinated to fluorinated acetonaphthone	^{19}F diffusion coeff ($\times 10^{10}$ m^2 s^{-1})	peak width (Hz)	^{19}F chemical shift (ppm)
0:1	5.69 ± 0.05	20.5	0.04
1:1	5.75 ± 0.06	20.3	0.05
3:1	5.66 ± 0.06	20.9	0.05
6:1	5.79 ± 0.07	20.6	0.05
4′-fluoro-1′-acetonaphthone in MeOD/D_2O solvent with no added fulvic acid	6.15 ± 0.09	18.7	−0.27

[a] Also shown are the corresponding values for 1.8 mM F-acetonaphthone in the MeOD/D_2O solvent in the absence of fulvic acid.

^{19}F has a profound effect on such noncovalent interactions (*40*). In the absence of ^{19}F substituents, the electron clouds of the aromatic rings repel one another, and the rings associate in a perpendicular orientation. The electron-withdrawing fluorine nucleus reduces the repulsion between electron clouds of aromatic systems, causing the rings to stack parallel to one another.

To assess whether the influence of fluorine dominates the interactions of F-acetonaphthone with SRFA, a competitive binding study was performed. Unfluorinated acetonaphthone was titrated into a solution of 1.8 mM F-acetonaphthone and 7.5 mM SRFA at $p_\alpha H^*$ 6.6. To estimate the extent of binding, as discussed previously, the ^{19}F chemical shifts, peak widths, and diffusion coefficients were measured upon each addition of unfluorinated acetonaphthone and are presented in Table 2. Unfluorinated acetonaphthone was added in molar ratios of 1:1, 3:1, and 6:1. Even for a 6-fold excess of unfluorinated acetonaphthone, the ^{19}F chemical shifts, peak widths, and diffusion coefficients of F-acetonaphthone remained unchanged within experimental error. This indicates that unfluorinated acetonaphthone does not effectively compete with the fluorinated analogue for binding sites on SRFA under these conditions. To test the contribution of hydrogen bonding at this pH, a 6:1 molar ratio of fluorobenzene was added to the solution containing both fluorinated and unfluorinated acetonaphthone. The fluorobenzene produced a small shift toward free F-acetonaphthone chemical shifts and diffusion coefficients. However, because only a small amount of F-acetonaphthone was bound initially, it is difficult to say within error whether fluorobenzene competes more effectively than unfluorinated acetonaphthone for binding sites on SRFA at this pH.

The competition experiment was repeated at $p_\alpha H^*$ 3.95 to further investigate the relative contributions of hydrophobic and hydrogen bonding interactions involving the fluorine substituent. Addition of a 6:1 molar ratio of unfluorinated acetonaphthone produced an increase in the ^{19}F diffusion coefficient from 6.01×10^{-6} to 6.36×10^{-6} cm^2 s^{-1}. This is a substantially greater increase than was observed at $p_\alpha H^*$ 6.6, indicating that unfluorinated acetonaphthone competes more effectively for binding sites at low pH. This may reflect a more specific interaction between the SRFA and the acetonaphthone naphthyl ring (for example, specific $\pi-\pi$ stacking rather than solubilization in a hydrophobic pocket) than occurs at $p_\alpha H^*$ 6.6. Addition of a 6:1 molar ratio of fluorobenzene to this solution increased the diffusion coefficient further to 6.58×10^{-6} cm^2 s^{-1}. From these results one may conclude that fluorobenzene can displace F-acetonaphthone from hydrogen bonding sites on SRFA for which unfluorinated acetonaphthone cannot compete. These results, and those presented previously, provide a self-consistent picture for the interactions of F-acetonaphthone with SRFA and suggest that there are two competing mechanisms of association, hydrophobic interactions and hydrogen bonding.

Acknowledgments

This work was supported by an NSF-EPA Waters and Watersheds Grant, NSF CHE-9524514. The support of M.A.M. by NSF REU Site Grant NSF-CHE-9619499 is gratefully acknowledged. The 400 MHz NMR spectrometer used in this research was purchased with funds provided by NSF BIR-9512331. The 360 MHz NMR spectrometer was a generous gift of the Monsanto Company.

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Received for review May 21, 1998. Revised manuscript received October 28, 1998. Accepted December 11, 1998.

ES980525F