Understanding and Managing Organic Matter in Soils, Sediments, and Waters..
R.S. Swift and K.M. Spark (Eds)

Two dimensional NMR spectroscopy of humic substances

N. Hertkorn[1], Ph. Schmitt-Kopplin[1], I. V. Perminova[2], D. Kovalevski[2] and A. Kettrup[1]

[1]*GSF-Research Center for Environment and Health, Institute of Ecological Chemistry, Ingolstädter Landstrasse 1, D-85764 Neuherberg, Germany*
[2]*Lomonosov State University of Moscow, Department of Chemistry, Vorob´ovy Gory, 119899 Moscow, Russia*

Abstract

A great variety of homo- and heteronuclear two-dimensional NMR experiments wer made on humic substances. These provide detailed insight into the structure of humic substances including an identification of exchangeable protons as well as an unprecedented in detail characterisation of the carbon skeleton.

Key words: Humic substances, two-dimensional NMR

Introduction

NMR spectroscopy is an established tool for the structural analysis of humic substances. Typically, one dimensional ^{1}H and ^{13}C NMR spectra are integrated and divided into several distinct ranges of chemical shift, each corresponding to a specified set of chemical environments such as aliphatic, heteroatom substituted or aromatic positions. Analogous procedures have been applied to ^{15}N and ^{31}P NMR spectroscopy of humic substances.

In one dimensional NMR spectra, chemical shifts are measured that characterise the chemical environment of the different nuclei. However, no information can be obtained on the spatial relationships between the observed nuclei in this manner. The introduction of a second frequency variable offers opportunities to relate spins with respect to their pair interactions. There are two important pair interactions in nuclear spin systems, a)the scalar through-bond electron-mediated spin-spin interaction (J coupling) and b) the through-space magnetic dipole-dipole interaction. Both interactions can be used to transfer coherence between spins, this transfer being the key requisite for obtaining meaningful 2D NMR spectra.

Two dimensional NMR spectroscopy is a general concept providing more detailed information and dispersion of resonances by introducing a second independent frequency variable. The second frequency domain available in 2D NMR spectroscopy provides information concerning spatial or bonding interactions between pairs of atoms. The main objective of 2D NMR is the elucidation of *connectivity patterns*. Interactions between bond electrons provide information about atom connectivities; giving coupled pairs of atoms or extended coupling networks. Dipole-dipole coupling between spatially proximate nuclei gives rise to magnetisation transfer by the nuclear Overhauser effect (Croasmun *et al*., 1996).

Two dimensional spectra are very important in aiding the assignment of complex one dimensional NMR spectra. The quantitative information obtained from cross peak integrals in 2D NMR spectra is strongly affected by the time dependence of the transfer amplitude of the respective experiment and by the relaxation characteristics of the material. In humic substances, where we find not only a distribution of functional groups and larger chemical substructures, but also one of relaxation times, this poses an obvious challenge.

There is an enormous variety in the experimental pulse schemes employed in the two dimensional NMR spectroscopy. This has expanded the applicability of NMR to the characterisation of complex molecules including natural products, peptides, proteins and other biopolymers. At present, almost all NMR studies of biological macromolecules depend heavily upon 2D NMR methods. So far only sporadic attempts appear in the literature using 2D NMR for the structural analysis of humic substances (Schmitt-Kopplin *et al*., 1998; Buddrus *et al*., 1989, Randall *et al*., 1997; Simpson, *et al*., 1997)); however, systematic studies utilising two dimensional NMR spectroscopy for the structural analysis of humic substances are yet lacking (Bortiatynski, 1996; Preston, 1996).

In these, the application bandwidth of homonuclear 2D NMR spectroscopy ranges from the characterisation of *exchangeable protons* to a description of *extended spin systems*, while heteronuclear 2D NMR spectra are very powerful tools for a detailed structural analysis of the *carbon skeleton* of humic substances.

In this paper we will present homo- and heteronuclear two dimensional NMR spectra of terrestrial and aquatic humic substances, which will demonstrate the great importance of this method allowing a more precise characterisation of a variety of refractory organic substances. The great value of 2D NMR spectroscopy for the structural analysis of humic substances resides in the combination of information from two (identical or different) 1D NMR spectra producing significantly more detailed information concerning the chemical environment of individual spins. While 2D NMR spectra produce connectivity maps necessary for spectral assignment, quantitative resonance intensities, corresponding to certain chemical environments, are available from the respective 1D spectra.

Classification of 2D NMR experiments

Most of the 2D NMR experiments described are of the correlated type. The chemical shift of one nucleus is correlated with the chemical shift of other nuclei based on an interaction between them. In J-resolved 2D NMR spectra the chemical shifts are defined on one axis while the coupling constants occur along the second axis. The coupling may occur between the same nuclear species (homonuclear 2D J-resolved spectra) or between different nuclear species (heteronuclear 2D J-resolved spectra) of humic substances (Buddrus *et al*., 1989). The principal 2D NMR experiments can be classified as follows:

Scalar J coupling experiments (2D correlation spectroscopy)

homonuclear ^{n}J	COSY, INADEQUATE, TOCSY
heteronuclear ^{1}J (direct detection of X-nucleus)	HETCOR
heteronuclear ^{n}J (direct detection of X-nucleus)	COLOC
heteronuclear ^{1}J (indirect detection by ^{1}H)	HMQC, HSQC
heteronuclear ^{n}J (indirect detection by ^{1}H)	HMBC
homonuclear J-resolved	JRES
heteronuclear J-resolved	JRES

Internuclear cross relaxation experiments (2D nuclear Overhauser effect spectroscopy)

homonuclear	NOESY
heteronuclear	HOESY

Chemical exchange (2D exchange spectroscopy)

homonuclear	EXSY

2D NMR spectroscopy in the form of *homonuclear correlation spectroscopy* offers a way to identify spin-coupled pairs or even spin-coupled networks in a molecule. 2D NMR in the form of *heteronuclear shift correlation spectroscopy* offers a variety of ways to identify directly bonded and long range pairs of different atoms, such as ^{1}H and ^{13}C. Far-reaching structural constraints are deduced and information from one spectrum is helpful in the assignment of the other spectrum. The heteronuclear one-bond coupling constants ($^{1}J(CH)$: 125-165 Hz, $^{1}J(NH)$: 75-100 Hz) are much larger than geminal and vicinal ones ($^{2,3}J(HH)$: < 15 Hz) and therefore more magnetisation can be transferred between the coupled heteronuclei. However, without isotopic enrichment the heteronuclear 2D NMR spectra are scaled by the low natural abundance of ^{13}C (1,1 per cent) and ^{15}N (0.37 per cent) resulting in an inherently low sensitivity.

Examples of 2D NMR experiments and its potential for the structural analysis of humic substances

Homonuclear correlation spectra

COSY COSY experiments were the first 2D NMR experiments to be devised and still are among the most popular 2D NMR experiments in the NMR analysis of small molecules. COSY cross peaks indicate coupled spins of protons typically separated by two and three bonds. They require proton couplings to be not much smaller than the ^{1}H resonance linewidth. This line width is approximately proportional to the inverse of the molecular tumbling rate and therefore increases with the size of the aggregate. In humic substances HH-couplings are frequently smaller than the natural linewidth, causing partial cancellation of COSY cross peaks and tending to make this particular experiment ineffective.

COSY cross peaks indicate vicinal and geminal coupled spin pairs rather than (partial) coupling networks to be determined from TOCSY spectra. So, especially in the heavily overlapping “carbohydrate region“ of humic substances COSY spectra frequently provide better resolved cross peaks than do TOCSY spectra. COSY spectra are very useful in describing vicinal couplings in aromatic rings allowing detailed conclusions concerning the substitution pattern. In our experience of 2D NMR experiments with humic substances, COSY spectra are the only ones where the absolute value display is frequently superior to phase sensitive calculation and acquisition.

TOCSY The TOSCY sequence provides relayed connectivities by utilising isotropic mixing during a spin lock period to transfer in-phase magnetisation between spins. It offers considerable advantage with regard to COSY experiments, namely a higher sensitivity for larger molecules, near absorption mode lineshapes for diagonal and cross peaks and the opportunity to observe multiple relay cross peaks by adjusting the mixing time τ_m. The spin-locking field induces an oscillatory exchange of spin-locked magnetisation between two spins, provided the effective local RF fields experienced by the two spins are identical. This causes the spins to become temporarily equivalent.

The spin-lock sequences are termed “isotropic mixing“ (no effective field); complete exchange of magnetisation occurs for contact times (spin-lock mixing times) of 1/2J. This contrasts favourably with COSY type experiments where coherence transfer is achieved by scalar coupling only after 1/J without net magnetisation transfer resulting in antiphase peaks.

The magnetisation can be transferred through several couplings during the course of mixing. Relaxation permitting cross peaks can be generated between all resonances within a spin system. So, the observation of a cross peak between two spins in the TOCSY experiment does not necessarily indicate directly coupled spins. A proper adjustment of the spin lock mixing time helps in tracing coupling networks with multiple relay cross peaks.

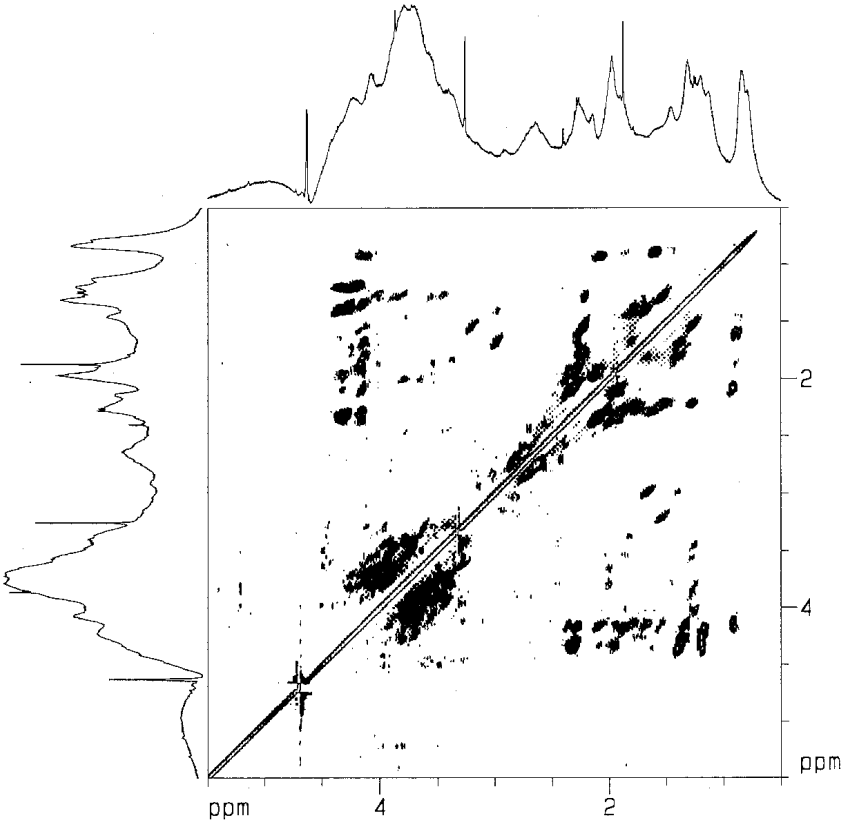

Figure 1 ^{1}H-^{1}H-TOCSY spectrum of a soil humic acid (τ_{mix} = 35 msec; upfield section)

NOESY / EXSY NOESY spectra rely on the transfer of longitudinal magnetisation to establish connectivities between the spins either by the nuclear Overhauser effect or by chemical exchange. The value of NOESY spectra for the characterisation of humic substances lies in the identification of exchangeable protons and the determination of their exchange rates, which can be deduced from the build-up of the cross peak integrals at different mixing times. With careful exclusion of moisture, carboxyl, phenolic and aliphatic hydroxy groups appear in the EXSY spectrum together with amide and unusual highly shielded resonances. The location of the cross peaks indicates selective chemical exchange between different labile proton species.

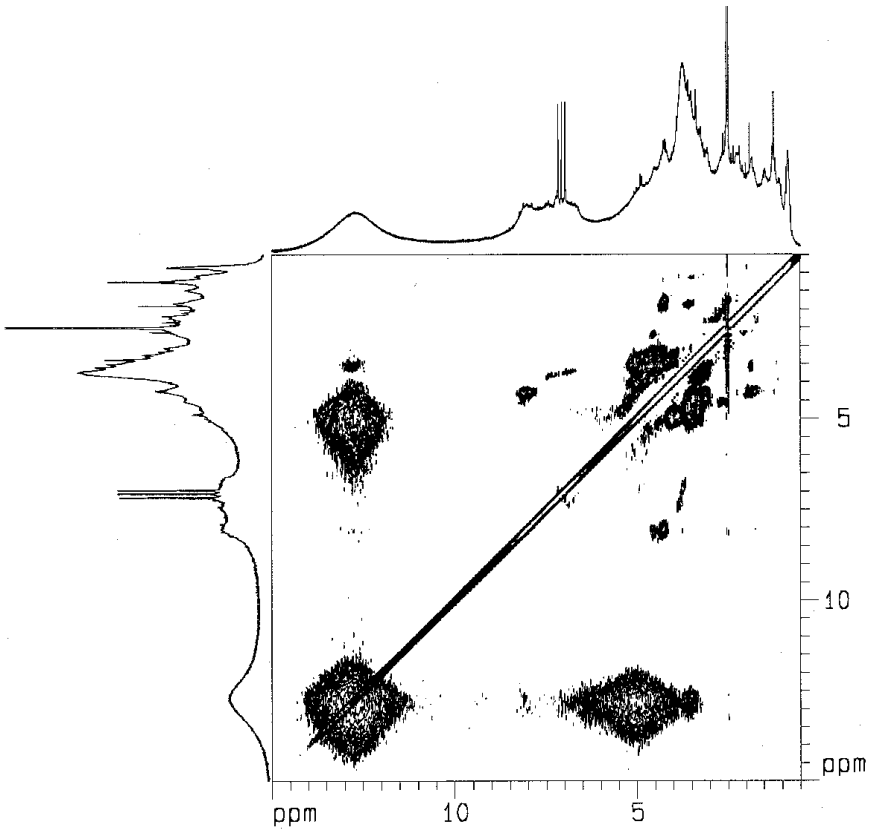

Figure 2 ^{1}H-^{1}H-EXSY spectrum of an aquatic fulvic acid in DMSO-d_6 (τ_{mix} = 250 msec)

In macromolecules both types of magnetisation transfer (caused by cross relaxation and by chemical exchange) result in NOESY cross peaks of the same sign as the diagonal.

Heteronuclear correlation spectra

Typical heteronuclear NMR experiments correlate a heteronuclear resonance with a proton resonance and allow assignments already proposed for one nuclear species to be transferred to the other. Heteronuclear correlation spectra provide enhanced signal dispersion by spreading the signal intensity into two dimensions; the frequently heavily overlapping proton resonances are spread out according to the shifts of the heteronuclei to which they are coupled.

A principal distinction between different methods lies in which nucleus is detected directly and which indirectly. Traditionally the term ´inverse' is used for heteronuclear techiques that detect the nucleus with the higher magnetiogyric ratio (usually ^{1}H), whereas detection of the low γ spin is referred to as direct. Inverse detection offers a significant advantage in sensitivity for most applications.

Proton observe methods show typically significant enhancement in sensitivity, but severe T_1 noise may occur which is caused by protons not coupled to this heteronucleus and by a large residual HDO resonance for measurements in water or sodium hydroxide solution.

Methods that rely on the detection of the less sensitive X-nucleus may have certain advantages in the structural analysis of humic substances: the T_1 noise of the

large water resonance is avoided and the shortest single bond heteronuclear pulse sequence available is a CH-INEPT experiment, providing information about fast relaxing fractions of the humic substances. The spectral dispersion of carbon exceeds that of the proton by a factor of 5:1 in Hertz. Therefore putting ^{13}C into the indirect dimension requires a considerable number of increments to make full use of the carbon-inherent good resolution. This makes inverse experiments somewhat less attractive in comparison with the theoretical sensitivity values. However, extensive re-laxation during the larger values of t_1 (i.e. at higher increments) may contribute mainly to noise, effectively degrading the S/N ratio of the 2D NMR matrix. In humic substances, paramagnetic impurities, aggregate size and aggregation, viscosity and temperature severely affect the relaxation behaviour, so optimum acquisition parameters for 2D NMR spectroscopy of humic substances have to be carefully adjusted for every sample. The dependence of electrophoretic mobilities on the composition of ionisable functional groups and aggregate size of humic substances in capillary electrophoresis (CE) indicates that NMR relaxation of humic substances is not solely governed by its molecular mass but also affected by its functional group distribution.

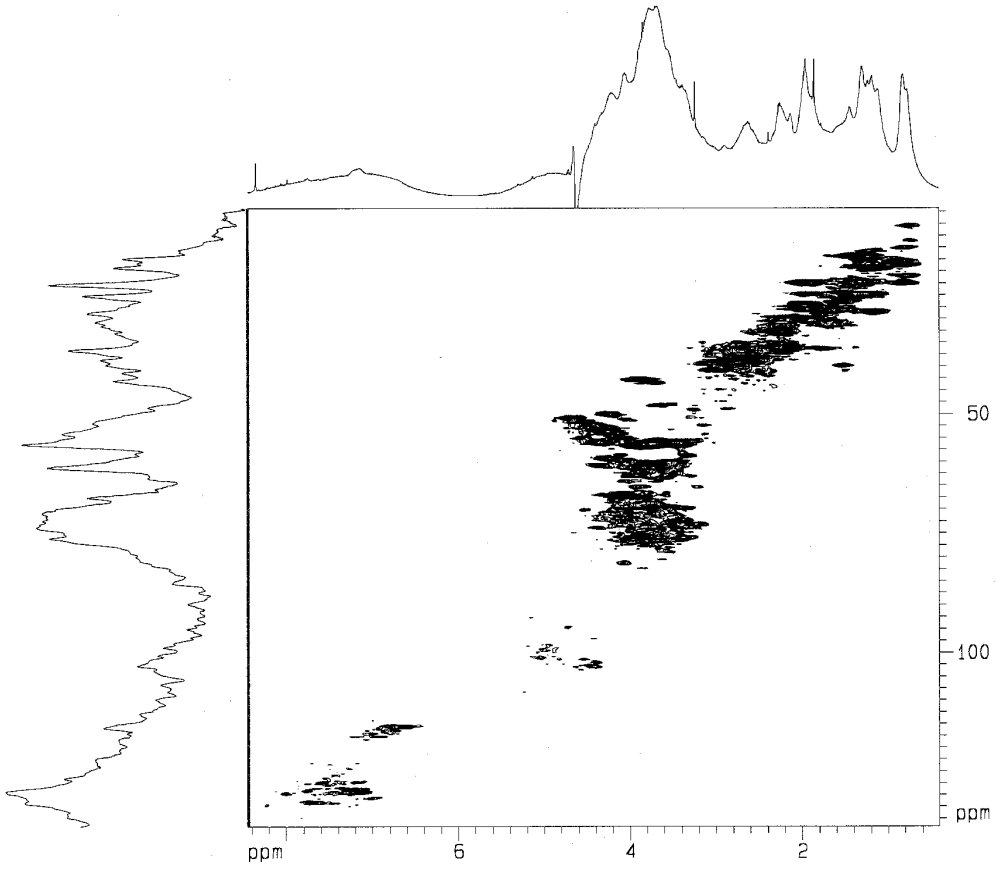

Figure 3 gs-^{1}H-^{13}C-HSQC spectrum of a soil humic acid

For HMQC and HSQC type experiments, gradient-enhanced spectroscopy generally provides results which are superior to those obtained with presaturation and solvent non-excitation methods (Schmitt-Kopplin *et al*., 1998). HSQC and HMQC experiments yield essentially the same information, in particular, a one bond correlation between protons and carbons. Yet they differ in the way the coherence transfers from ^{1}H to ^{13}C and back to ^{1}H are accomplished (Croasmun and Carlson,

1996). The HMQC cross peaks are broadened in the ^{13}C dimension by ^{1}H-^{1}H coupling yielding multiplets, thus limiting the achievable resolution in this dimension. Another difference is the relaxation behaviour during t_1, which is different for spin S antiphase coherence (HSQC) or I-S multiquantum coherence (HMQC) (Cavanagh *et al*., 1996).

Edited heteronuclear correlation spectra

DEPT spectra are valuable tools to edit 1D ^{13}C NMR spectra according to their CH_n multiplicity (n = 0-3) (Buddrus *et al*., 1989). The DEPT sequence can be incorporated into heteronuclear 2D correlation spectroscopy yielding 2D NMR spectra of humic substances (relaxation permitting) which are edited according to the CH_n multiplicity. An example is shown in Fig. 4; these resonances represent somewhat extended substructures characterised by their chemical shift in ^{1}H and ^{13}C as well as by their multiplicity.

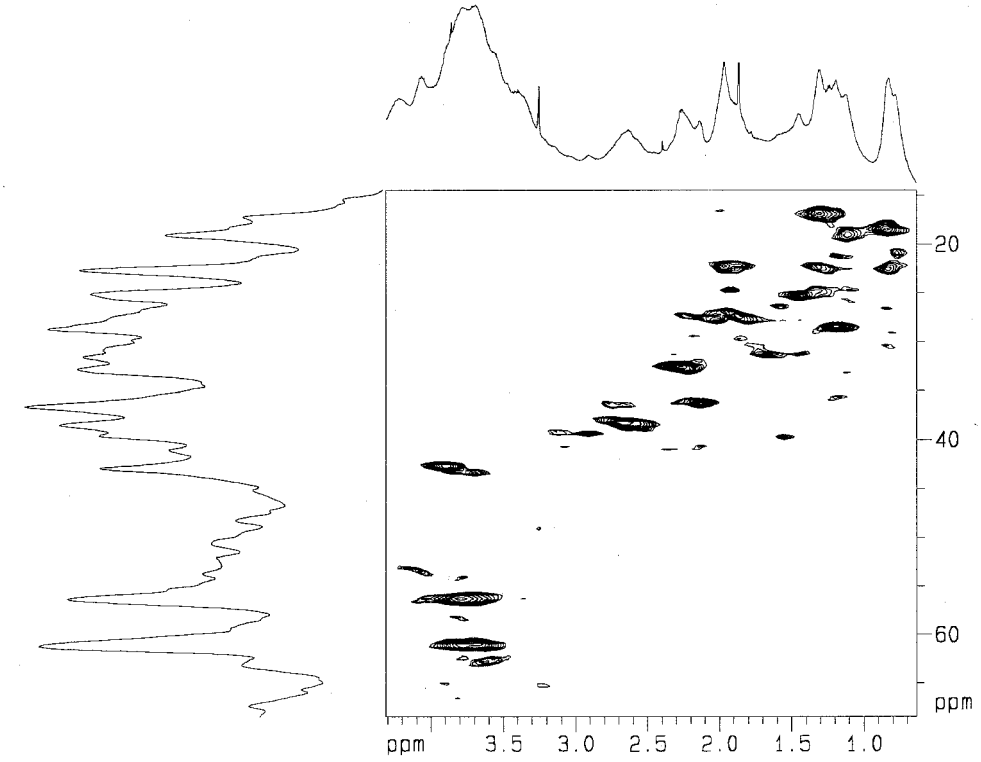

Figure 4 ^{1}H,^{13}C-DEPT-HSQC spectrum of the aliphatic and carbohydrate region of a soil humic acid showing CH_2 only (F1 projection: CH_2 only, calculated from ^{13}C-DEPT-spectra)

HMBC This is a sensitive technique for the determination of long range (geminal and vicinal) heteronuclear connectivities. These would be of substantial value to assign correlations of carbonyl-C atoms with remote hydrogen atoms. However, combined ^{13}C editing with 1.1 per cent natural abundance and loss of magnetisation due to relaxation makes detection of meaningful HMBC spectra of humic substances a difficult task.

Consequences of 2D NMR spectra on the view of humic substance substructures

Many humic materials of terrestrial and aquatic origin yield 2D NMR spectra closely resembling the NMR spectra shown in this paper. Remarkably, the most intense cross peaks show up at almost identical positions for a wide range of materials. However a close inspection reveals significant differences preferentially in the heteroatom substituted and aromatic region of cross peaks which give extensive clues about the origin and the reactions that have lead to the refractory material. For the sake of brevity the discussion is limited to the most prominent cross peaks.

The increased signal dispersion in ^{1}H and ^{13}C dimensions provides meaningful insight into the composition of the aliphatic part of humic substances. The upfield section is dominated by a clearly non-continuous distribution of methyl and methylene groups indicating a rather restricted variety of main structural elements. The long methylene chains show up as a cross peak but are not a very prominent substructure (1.2/28.5). The occurence of strongly shielded ^{1}H ($\delta < 1$ ppm) and ^{13}C ($\delta < 25$ ppm) methylene resonances indicates highly substituted, probably at least in part cyclic alkane substructures. These constitute a prominent fraction of the aliphatic part of humic substances. Polymethylated cyclic structures exhibit a rather rigid conformation, giving rise to γ-increments on $\delta(^{13}C)$ causing substantial upfield shifts, not easily attainable in flexible open chain structures. Terpenoid derived aliphatics such as hopanoids were proposed by Buddrus *et al*. from NMR data of humic substances (Ourisson, 1979; Buddrus, 1996), but several classes of higher terpenoids also show the same NMR characteristics (Charlwood, 1991). The distribution of methyl groups indicates the absence of methylketones and -aromatics, whereas the cross peak representing branched ethyl derivatives (0.8/10) is a prominent one. Branched aliphatic side chains, which are supposedly more resistant to microbial degradation, are indicated by cross peaks deshielded in the ^{13}C dimension and shielded in the ^{1}H (e. g. methylene: C-$\mathbf{CH_2}(CH_3)_2$ at 1.5/40) dimension.

Homonuclear 2D NMR spectra show a group of methyl resonances coupled to different types of C-substituted aliphatics indicating a distribution of rather short side chains or an array of similar cyclic substituents. Various classes of H_3C-CH-O- and amino acid side chain substructures form a prominent class of cross peaks within TOCSY and COSY spectra. In the heteroatom substituted region the CH_2-C(=O)-NH- resonance (3.9/42) indicates peptide bonds from Glycine to be a prominent fraction of the visible peptide bonds in the spectrum. Six membered ring carbohydrates are revealed by a significant cross peak (3.7/62). The anomeric region shows several resonances, the most prominent ones centered around 96 and 103 ppm similar to glucose and cellulose, respectively (Nehls *et al*., 1994). The ^{13}C resonance at 105 ppm does not show a cross peak, supporting the assignment of these resonance as tannin-derived alkylated carbon (Preston, 1996). The aromatic region is dominated by cross peaks representing doubly ortho- or ortho/para oxygen and di-ortho carbon substituted rings. Any carbonyl substitution causes substantial downfield shift to ortho hydrogen positions ($\delta > 7.5$ ppm,), while double ortho keto and ester substitution causes downfield shifts in excess of 8 ppm.

Experimental: All spectra shown have been recorded from samples dissolved in 0.1 M NaOD, unless otherwise specified, at 500 MHz proton frequency with an 5 mm inverse geometry broadband probehead equipped with an actively shielded z-gradient coil (^{1}H,^{13}C-HMQC and -HSQC: $^{1}J(CH) = 150$ Hz). Chemical shifts are referred to

residual HDO (^{1}H: 4.63 ppm), DMSO-d_6 (^{1}H: 2.49 ppm) and external methanol in D_2O: (^{13}C: 49.00 ppm).

Abbreviations: CE (Capillary Electrophoresis), COLOC (Correlation Spectroscopy for Long-Range Couplings), COSY (Correlated Spectroscopy), EXSY (Exchange Spectroscopy), gs (gradient enhanced), HETCOR (Heteronuclear Correlated Spectroscopy), HMBC (Heteronuclear Multiple Bond Correlation), HMQC (Heteronuclear Multiple Quantum Correlation), HOESY (Heteronuclear Overhauser Enhancement Spectroscopy), HSQC (Heteronuclear Single Quantum Coherence), INADEQUATE (Incredible Natural Abundance Double Quantum Transfer Experiment), NOESY (Nuclear Overhauser and Exchange Spectroscopy), ROESY (Rotating Frame Overhauser Enhancement Spectroscopy), TOCSY (Total Correlation Spectroscopy).

Acknowledgements: The authors wish to thank Eva Schindlbeck and Heidi Neumaier for skillful technical assistance with NMR and HPCE techniques. Part of the presented research was supported by the Deutsche Forschungsgemeinschaft within the ROSIG Programme (He 2422/1-3) and by the German Ministry of Research WTZ: Wissenschaftlich-Technische Zusammenarbeit: RUS-143-97).

References

Bortiatynski, J. M., Hatcher, P. G., and Knicker, H. 1996. NMR Techniques (C, N, and H) in Studies of Humic Substances. In: Gaffney J. S., Marley N. A., Clark S. B. (Eds.). *Humic and Fulvic Acids*. pp. 57-77. (American Chemical Society Symposium Series 651, American Chemical Society, Washington.

Buddrus, J., Burba, P., Herzog, H., Lambert, J. 1989. Quantification of Partial Structures of Aquatic Humic Substacnes by one- and Two-Dimensional Solution ^{13}C Nuclear Magnetic Resonance Spectroscopy. *Analytical Chemistry*, 628-631.

Buddrus, J. and Lambert, J. 1995. Isolated Paraffinic Methyl Groups in Humic Substances. *Organic Geochemistry*, **23**, 269-271.

Charlwood, B. V., and Banthorpe, D. V. 1991. (Eds.). Terpenoids. In: Dey, P. M. and Harborne, J. B.: *Methods in Plant Biochemistry*. **Vol. 7**. Academic Press, London.

Cavanagh, J., Fairbrother, W. J., Palmer, A. G., Skelton, N. J. 1996. (Eds.). *Protein NMR Spectroscopy, Principles and Practice.* Academic Press, London.

Croasmun, W. R., and Carlson, R. M. K. 1996. (Eds.) *Two-Dimensional NMR Spectroscopy.* Wiley-VCH: Weinheim.

Lambert, J., and Buddrus, J. 1996. Quantification of Isolated Methyl Groups in Aquatic Humic Substances by Means of ^{1}H and ^{13}C NMR Spectroscopy, Magn. *Resonance Chemistry*, **34**, 276-282.

Nehls, I., Wagenknecht, W., Philipp, B., and Stscherbina, D. 1994. Characterisation of Cellulose and Cellulose Derivatives in Solution by High Resolution ^{13}C-NMR Spectroscopy. *Progress in Polymer Science*, **19**, 29-78.

Preston, C. M. 1996. Applications of NMR to Soil Organic Matter Analysis: History and Prospects. *Soil Science*, **161**, 144-166.

Ourisson, G., Albrecht, P., and Rohmer, M. 1979. The Hopanoids. *Pure and Applied Chemistry*, **51**, 709-729.

Randall, E. W., Mahieu, N., Ivanova, G. I. 1997. NMR studies of soil, soil organic matter and nutrients: Spectroscopy and imaging. *Geoderma*, **80**, 307-325.

Schmitt-Kopplin, P., Hertkorn, N:, Schulten, H.-R., Kettrup, A. 1998. Structural changes in a Dissolved Soil Humic Acid during Photochemical Degradation Processes under O_2 and N_2 Atmosphere. *Environmental Science and Technology*, **32**, 2531-2541.

Simpson, A. J., Boersma, R. E., Kingery, W. L., Hicks, R. P., Hayes, M. H. B. 1997. Applications of NMR Spectroscopy for Studies of the Molecular Compositions of Humic Substances. In: *Humic Substances, Peats and Sludges* Eds M. H. B. Hayes, W. S. Wilson pp. 46-62. Royal Society Of Chemistry, Cambridge.