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Humic Substances in Soils: Are They Really Chemically Distinct?

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Humic substances (HS) are an operationally defined fraction of soil organic matter, and they represent the largest pool of recalcitrant organic carbon in the terrestrial environment. It has traditionally been thought that extractable HS consist of novel categories of cross-linked macromolecular structures. In this study, advanced nuclear magnetic resonance approaches were used to study the major components (proteins, carbohydrates, aliphatic biopolymers, and lignin) that are known to be present in HS, and to identify their fingerprints in humic mixtures. Theoretically, once all known components have been identified, the remaining signals should be from materials with novel structures, themselves forming a distinct chemical category of humic materials. Surprisingly, nearly all of the NMR signals in traditional HS fractions could be assigned to intact and degrading biopolymers. We therefore suggest that the vast majority of operationally defined humic material in soils is a very complex mixture of microbial and plant biopolymers and their degradation products but not a distinct chemical category. It is important to note this work in no way rules out the existence of a distinct category of humic macromolecules, either at low abundance in the soluble fraction from young soils, in diagenetically evolved samples (for example lignites, etc.), or in the nonextractable humin fraction.

Introduction

The global soil carbon pool is 3.3 times the size of the atmospheric pool and 4.5 times that of the biotic pool (*1*). Organic carbon represents approximately 62% of global soil carbon while at least 50% of this carbon can be categorized as the chemically resistant component known as humic substances (HS) (*1*−*3*). Extractable HS are ubiquitous in nature and play essential roles in sustainable agriculture (*4*), water quality (*5*), and immobilization and transport of nutrients and anthropogenic chemicals (*6*−*8*) while also potentially offering exciting opportunities for the discovery of novel compounds for use in industry and medicine (*9*). They have largely remained uncharacterized at the molecular level and have necessarily been defined operationally in terms of the methods used to extract or isolate them. Formation processes of HS have been debated for decades (*10*, *11*). It has traditionally been thought that extractable HS consist of novel categories of structures formed through varying biotransformation processes (*12*−*14*). Today, the predicted future and modeling of the soil carbon stock relies heavily on the temperature sensitivity of this carbon component (*15*, *16*). The difficult task of predicting the impact of a warmer climate on soil carbon is exemplified by disagreements on the sensitivity of the nonlabile fraction to temperature variation (*15*−*18*). It has been suggested that the anomalous response of soil organic carbon to temperature variation (*16*, *19*) experiments is due to the heterogeneity of soil carbon (*20*), and there is currently no explicit model that can facilitate such complexities (*17*). Therefore, our understanding of the chemical composition of HS or the resistant and nonlabile components of soil organic carbon (SOC) is vital to our predictions of the influence of climate change on soil carbon. To this end, we apply 2D NMR techniques to describe the vast majority of extractable HS in terms of components that are representatives of the major chemical classes found in plants and organisms.

The primary source for organic matter formation in soil is plant and microbial biomass, the composition and properties of which are important controlling factors for humification processes. When considering structural aspects of HS, we must therefore examine the contribution of the various compound classes that form such tissues. These compound categories include intracellular storage materials (for example, proteins) and structural materials (for example, polysaccharides, lignin, and aliphatic components including membrane lipids and plant cuticles) that form membranes and cell walls (*21*). Various biopolymers are known to be present in SOM; however, it is generally considered that these biopolymers exist alongside humic materials, and it is this humic fraction that is considered to be highly recalcitrant and not well defined structurally. In an excellent review, Hedges et al. pointed out the analytical challenges associated with the molecularly uncharacterized component of nonliving organic matter (*22*).

Here, we consider the contribution to HS structure of four representatives of the principal compound classes in plants and microbes, namely, protein, lignin, cutin (cutin is an aliphatic biopolymer, chosen to represent aliphatic inputs in general, including lipids, waxes, etc.), and carbohydrates. Modern multidimensional NMR approaches were applied with the goal of assigning all that is known to be in soil, and thus by a process of elimination find the additional signals that are expected if humic materials exist as a distinct chemical category, as has been suggested for decades (*23*). This is by no means the first application of solution state 2D NMR to the study of soil organic matter [see reviews by Cardoza and Simpson (*24*, *25*) and references within]. In summary, contributions thus far have demonstrated the applicability of 2D NMR as a powerful tool to study humic materials, and have identified some contributions from various biopolymers (namely, lignin, proteins, carbohydrates, and aliphatic biopolymers), as well as some specific biomarkers (including various cuticular and lignin derived structures). Here we attempt to correlate the detailed 2D NMR patterns of soil biopolymers to those observed in humic materials with the ambition to assess their contributions and unravel some of the mystery surrounding humic material.

Experimental Section

Biopolymers, protein (Albumin Bovine Serum), Kraft Lignin, and Amylopectin were used as purchased. Cutin was isolated from tomatoes (*26*). The Florida peat HA standard that represents 70% of the total SOC was purchased from the International Humic Substances Society (IHSS). Soil HS were

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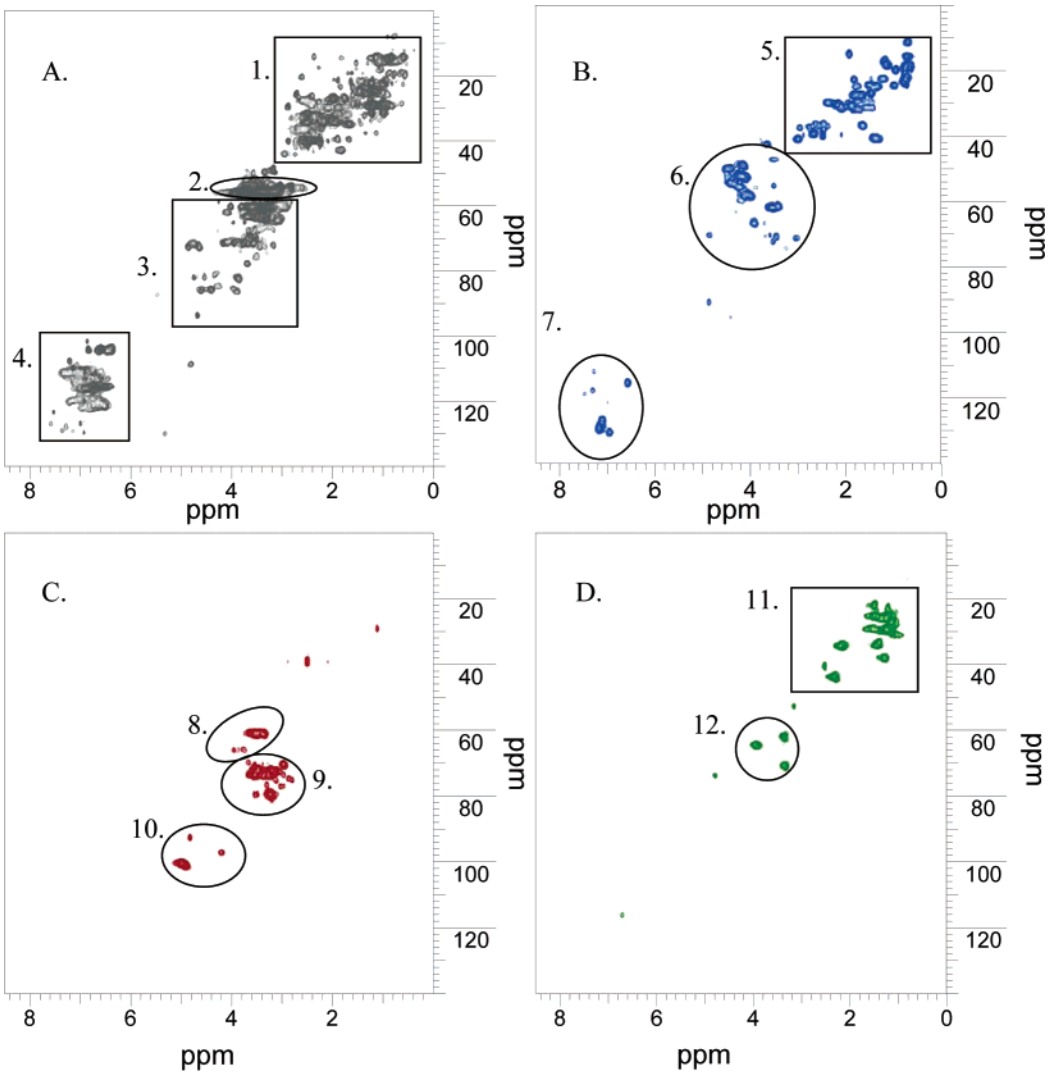

FIGURE 1. HSQC spectra of biopolymer representatives: (A) lignin (aromatic biopolymer), (B) albumin bovine serum (protein), (C) amylopectin (carbohydrate), and (D) tomato cuticle (aliphatic biopolymer). The highlighted regions outlined in the four spectra represent general assignments of the major structural classes as follows: (1) aliphatic linkers (and aliphatic coproducts extracted from wood in lignin isolation), (2) methoxyl, (3) linkers between aromatic rings, (4) aromatic rings, (5) aliphatic side chain residues, (6) amino acid α-protons in peptide chains, (7) aromatic side chain residue, (8) CH_2 in carbohydrate, (9) CH in carbohydrate, (10) anomeric units, (11) residues in main aromatic chain, and (12) esters and ethers linkers.

isolated from the surface A_h horizon of soils under Aspen and Grass (*27*) and represent 52% and 79% of the total soil organic carbon, respectively. The HS extracts were prepared by exhaustive extraction using 0.1 M NaOH, filtered through 0.2 μm Teflon filters, cation exchanged to remove the Na^+, and freeze-dried. Samples (100 mg) were thoroughly dried under vacuum and over P_2O_5, dissolved in DMSO-d_6 (1 mL, in a dry atmosphere), and transferred to a 5 mm NMR tube for analysis (it is essential to dry samples thoroughly and use only ampules of DMSO-d_6 to prevent a large water peak often centered at ~3.3 ppm that can obscure many of the real humic signals). Note that DMSO is an excellent solvent for HA and FA acids, but only when the material is in the fully protonated form (HA−H not HA−Na form). This is easily achieved by ion exchange using Amberlite 1200H plus resin and is actually the final step in the IHSS standard isolation procedure for aquatic HA and FA, and soil FA. On the other hand, soil HA is dialyzed by the IHSS as this has proved to be time- and cost-effective. Dialysis, however, does produce samples that still have some sodium content, which can, in turn, lower their solubility in DMSO. For complete solubility in DMSO, the IHSS peat HA was redissolved in 0.1 M NaOH (same as the original extraction solvent used by the IHSS) and then passed over Amberlite 1200H plus resin. After being freeze-dried, the cation exchanged humic acid is completely soluble in DMSO-d_6, as were all the soil fractions considered in this paper. In Figure 4B, D_2SO_4 (deuterated sulfuric acid) was added to the NMR tube to reduce the contribution from exchangeable protons. As DMSO is an excellent solvent for cations (note that DMSO has a partial negative charge on its oxygen), the addition of acid actually amplifies the overall positive charge on the humic molecules which in turn increases their solubility in DMSO. Thus, as expected, no precipitate was observed with the addition of acid.

In the case of cutin, which is not completely soluble, two-dimensional high-resolution magic angle spinning (HR-MAS) NMR was carried out using DMSO as the swelling solvent and a sample spinning speed of 10 kHz (*26*). NMR experiments were carried out on a Bruker Avance 500 MHz instrument. Total correlation spectroscopy (TOCSY) spectra were acquired in the phase sensitive mode, using time proportional phase incrementation (TPPI), 1024 scans, and a mixing time of 60 ms. Heteronuclear single quantum coherence (HSQC) spectra were collected in the phase sensitive mode using Echo/Antiecho-TPPI gradient selection, without sensitivity enhancement. Scans (2048) were collected

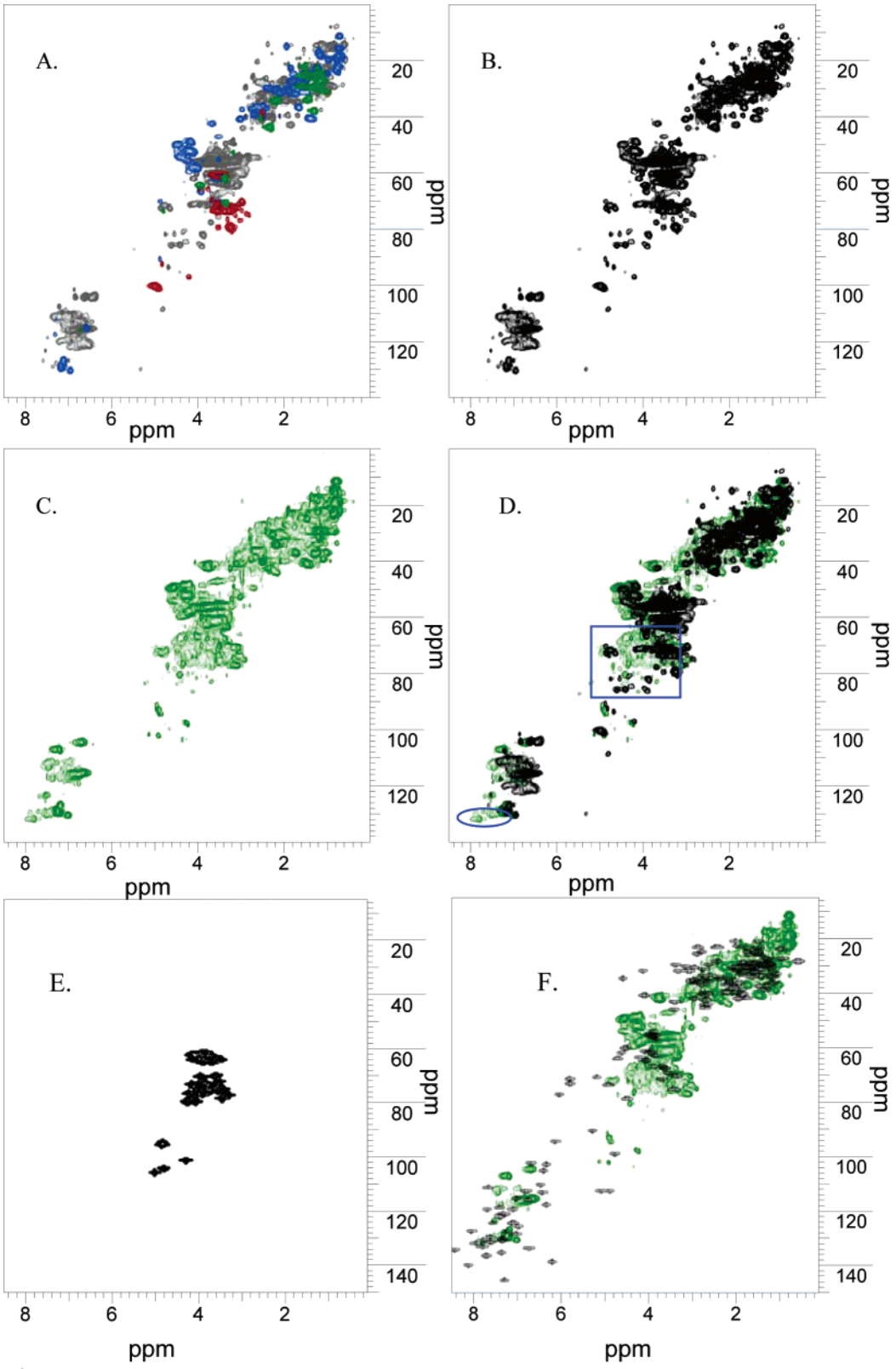

FIGURE 2. Overlaid HSQC spectra of biopolymers on IHSS peat: (A) biopolymers, lignin (grey), amylopectin (red), albumin (blue), and cuticle (green) overlaid on each other; (B) all biopolymers illustrated in black; (C) IHSS HA extract from peat; (D) biopolymers (black) overlaid on IHSS peat (green) (the highlighted areas in 2D are referred to in the text); (E) an HSQC NMR simulation for a mixture of ~15 randomly linked sugar units (the simulation is used to demonstrate that the carbohydrate region quickly becomes crowded even when a relatively simple mixture of sugars are present); (F) comparison of an HSQC NMR simulation for a humic model *(36)*, with the IHSS PEAT HS. Note that this figure is not intended to validate or negate the model in any way. It simply demonstrates that when structural units are linked in a way not natural in the original biopolymers, numerous extraneous peaks appear (especially in the aromatic region where additional linkers are introduced in the model), that are not present in the HSQC of the PEAT HA.

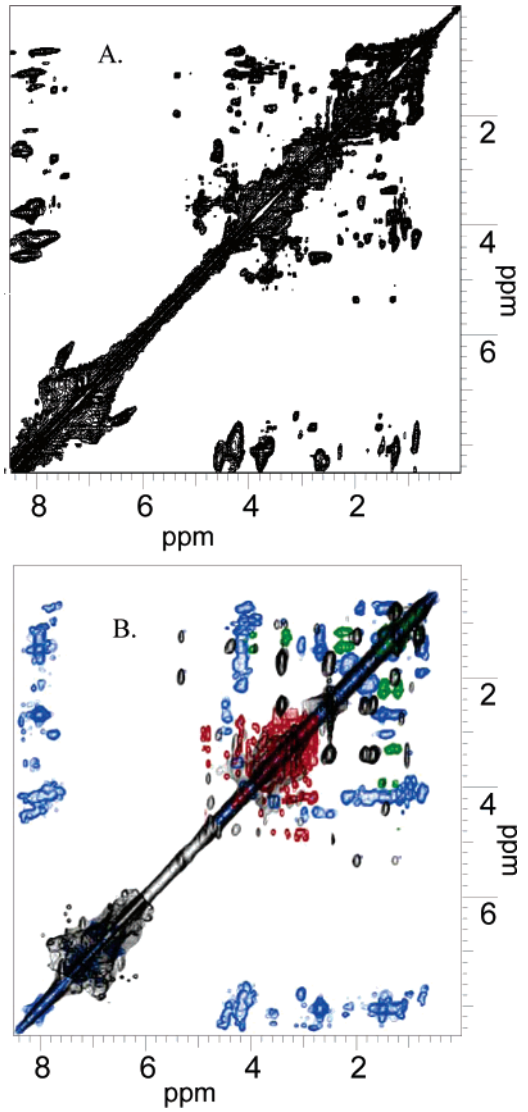

FIGURE 3. TOCSY spectra of the IHSS peat HA (A) and biopolymers (B). The four biopolymers, lignin (grey), albumin (blue), amylopectin (red), and cuticle (green).

for each of the 256 increments in the F1 dimension. There were 2K data points collected in F2, and a 1J $^{1}H-^{13}C$ (145 Hz) and a relaxation delay of 2 s were employed. Both TOCSY and HSQC were processed using sine-squared functions with phase shifts of 90° and a zero-filling factor of 2. Spectral predictions (and colored overlay plots) were carried out using Advanced Chemistry Development's (ACD/Labs') Spec Manager (version 9.06). Parameters used for prediction including line shape, spectral resolution, sweep width, and base frequency were chosen to match those of the real data sets as closely as possible.

Results and Discussion

High-resolution solution state 2D NMR approaches were applied to identify the signatures of known biomolecules in humic materials. If a distinct chemical category of humic materials exists in soils, and in abundances reported in the literature (*1*−*3*), once the signals from biopolymers (and their related degradation products) have been eliminated, the signals from the humic category should be easily observed.

Representative 2D HSQC NMR spectra of lignin, polysaccharide, cutin, and protein are shown in Figure 1. Assignments given in the caption are based on NMR predictions and simulations for the major groups present in each biopolymer

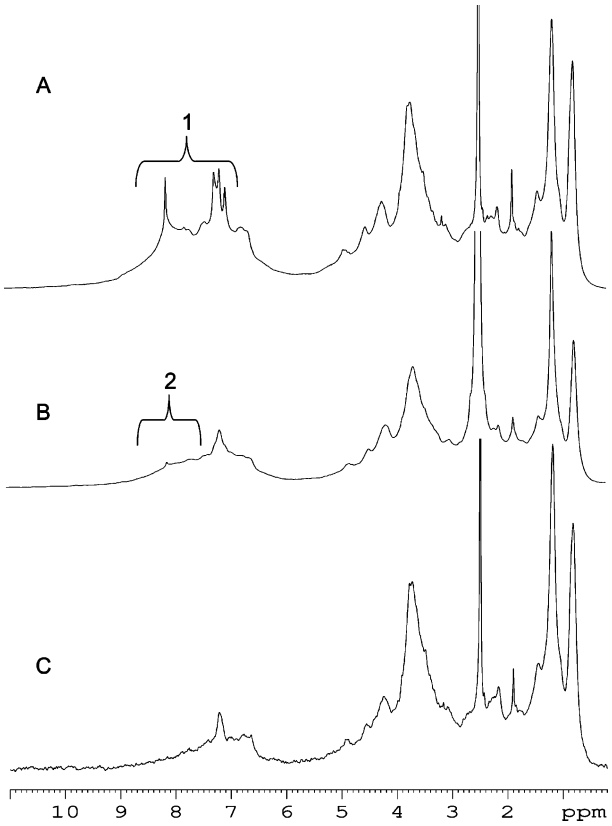

FIGURE 4. Comparison of 1D NMR spectra of the HA with the projections from the 2D experiment: (A) ^{1}H spectrum of the IHSS peat HA in DMSO-d_6; (B) ^{1}H spectrum of the IHSS peat HA in DMSO-d_6 after the addition of ~5 μL of D_2SO_4 (deuterated sulfuric acid) used to shift signals from N−H and O−H, thus reducing signals from exchangeable groups; (C) sum projection from the HSQC representing protons that can be detected in the experiment (note that only H bound to carbon is detected, see main text for additional discussion).

as well as additional 1D and 2D NMR experiments (data not shown). The HSQC experiment simply detects the H−C bonds in an organic structure. A cross-peak in an HSQC spectrum represents the chemical shift of both carbon and proton atoms in a C−H unit (*25*, *28*, *29*). When considered together, the cross-peaks form a specific pattern that can be thought of as the "molecular fingerprint" of a specific structure or class of structures. Basic assignments for the major structural units within each chemical category are highlighted in Figure 1. The representative biopolymers are overlaid in Figure 2a, retaining their respective colors from Figure 1 and are illustrated together in black in Figure 2b. The HSQC spectrum of an International Humic Substance Society (IHSS) standard humic acid (HA) is presented in Figure 2c. Humic acid is a major component of HS (*14*) and is considered to be one of the most recalcitrant fractions in soil (*30*). All experiments shown in this communication were run for extensive periods of time, and even signals that are much less than 1% of the total NMR integral were easily observed in the 2D NMR experiments shown here (Figure 4). Thus, while it is not possible to guarantee all structures present at trace levels were detected, the major constituents which in turn represent the vast majority of the humic material are well represented.

Generally, there is good agreement between the pattern produced by the biopolymers and the signature observed for the HA standard. This is clearly shown in Figure 2d which overlays the four biopolymers (black) on the HA spectrum (green). It is important to point out that an exact match between the biopolymers and the HA is not expected. The chosen biopolymers cannot represent the hundreds or even

thousands of plant and soil biopolymers that may be present in the HA. The purpose of the overlay is simply to show the general regions where the major biopolymers resonate and in turn demonstrate the similar patterns, indicating closely related structures (including partially degraded biopolymers), that dominate the spectrum of the HA. The box highlighted in Figure 2d is especially under-represented in the HA spectrum by the mixture of biopolymers. This region results mainly from carbohydrate signals, and the amylopectin used to represent carbohydrates in the HSQC is a relatively simple polymer of primarily linked glucose units. Thus, amylopectin can in no way account for the range of carbohydrates that will be abundant in the soil environment from both plant and microbial sources. In nature, there is an abundance of sugar residues and potential linkages that can occur, and many different complex and simple sugars will contribute to this region in the HA spectrum. Figure 2E shows a simulation for a very small number of simple sugars. Considering the numerous microbial and plant carbohydrates (simple and polymeric) that will be extracted by NaOH (the extractant commonly used to obtain the operationally defined humic fraction), it is logical that this carbohydrate region will be highly crowded as clearly shown in Figure 2C. A comprehensive correlation between this region in the HA spectrum and amylopectin was not expected. The same is true of the encircled region in Figure 2D where the representative lignin biopolymer does not appear to account for some cross-peaks in the HA. This is because the commercial lignin used in this experiment has a low *p*-hydroxybenzoate content, which are components abundant in many lignin types but absent from others (*31*, *32*). In addition to the biopolymers shown, numerous others were tested, in particular a range of tannins and polyphenolic biopolymers that have been suggested as the building blocks of HS in the "polyphenol theory" of HS formation (*33*). In all cases, we found very poor correlations between polyphenolic biopolymer signals and those signals in the HA spectra, indicating that these polymers, if present, are very low in abundance. Such structures, while present in plants, are probably leached from soil (due to their high solubility) or undergo rapid chemical and biological degradation. If one considers that HA is simply an operationally defined extract, it is logical that the majority of HA will be a complex mixture, consisting of the most abundant structural components found in plants and soil microbes. Figure 2F compares the spectrum of the IHSS peat HA to the simulated spectrum of a popular model structure for humic acid. The significance of this will be discussed in detail later in this paper (see point 2 under "Justification of the NMR Approaches" below).

To further support the theory that HA is simply a mixture of plant and microbially derived structures, total correlation spectroscopy (TOCSY) was applied. Like HSQC, TOCSY produces a molecular fingerprint of a molecule or mixture, but in TOCSY peaks arise from the interactions of protons over numerous bonds. In simple terms, HSQC describes the H−C units in a mixture, and TOCSY describes how these units are linked together. Considered together, the two experiments can be used to describe the complete H−C framework of any organic structure. Figure 3 shows the TOCSY spectrum of the HA (Figure 3a) and that of the biopolymers (Figure 3b). Clearly, the vast majority of peaks in the HA TOCSY spectrum can be described if the HA is considered to be a mixture of biopolymer derived structures.

We can find no specific evidence that the major categories of structures which constitute the standard HA undergo any novel cross-linking to form a new structural category. If novel linkages are formed in the soil environment, additional patterns from these bonds will be apparent in the NMR spectrum of HA. Such signals are not clearly apparent in either the HSQC or TOCSY spectra of the HA.

Justification of the NMR Approaches. Before proceeding further in the discussion, it is important to address the following two questions: (1) In the 2D NMR approaches shown, is the vast majority of material being detected? (2) If a distinct chemical category of humic material was present, would it be discernible by the methods employed?

Point 1. In 2D HSQC NMR, both the carbon and proton atoms are dispersed into two dimensions. This is achieved by a series of radio frequency pulses that form the basis of the 2D experiment. First, as the HSQC experiment observes only ^{1}H attached to ^{13}C (note that only the ^{13}C nucleus is NMR active, which is one in ∼100 carbons), the NMR experiment is therefore insensitive in comparison to its 1D dimensional analogues. Second, it is at least theoretically possible that some species could relax during the train of radio frequency pulses used in the 2D experiment and be underestimated or not detected at all in the final spectrum. Fortunately, it is relatively simple to estimate the amount of material that is actually being detected and then relate this back to the total material in the sample (i.e., the quantitative 1D ^{1}H NMR experiment). This is achieved by adding all the slices from the 2D experiment and comparing this sum projection to the 1D ^{1}H experiment. However, it is important to remember that only protons attached to carbons are detected in the HSQC experiment; thus, exchangeable protons (OH, NH, etc.) that are detected in the ^{1}H spectrum will not be detected in the 2D version. One way to reduce the intensity of exchangeable protons is to simply add a small amount of strong acid to the DMSO solvent, so that these exchangeable groups are shifted from the spectral region (*34*). Figure 4 compares the 1D NMR spectra of the HA with the projection from the 2D experiment. It is important to note that due to many factors, including reduced digital resolution, window function manipulations, and the $^{1}H-^{13}C$ coupling constant used (note that 145 Hz is commonly used as a good estimate for all 1 bond $^{1}H-^{13}C$ correlations, but in practice this varies for different structures), a perfect correlation is not expected between the 1D and the 2D NMR projection. However, if the vast majority of material is being detected in the 2D NMR, the projection will show a general similarity to the exchanged proton spectrum. The region labeled 1 is dominated by signals mainly from H−N groups in amides (possibly some phenolic OH as well). The addition of acid shifts the majority of exchangeable protons to ∼12 ppm. However, the process of unsealing the tube and adding the acid leads also to the introduction of a small amount of water which in turn produces a large peak at 12 ppm. These protons are in constant exchange with the amide region; therefore, while the addition of acid clearly reduces signals from exchangeable groups, they cannot be eliminated completely. Thus, in region 2 in particular, there is still a significant contribution from N−H groups after the addition of the acid. It is important to understand these signals are mainly from residual exchange and not from aromatic protons. It is clear from a comparison of Figures 4B and 4C that signals detected in the 2D NMR are very similar to those observed in the 1D NMR after chemical exchange. This confirms that the vast majority of material in the humic mixture is being detected in these experiments.

Point 2. Over the years, numerous models have been suggested for HA. The model suggested by Schulten and Schnitzer is probably the most accepted and is featured in many modern soils textbooks (*35*). The authors would like to make it very clear that the goal of this section is not to verify nor invalidate the model of Schulten and Schnitzer. The aim is simply to ask the question that if cross-linked macromolecular humic molecules do exist where would they resonate on an HSQC spectrum and could they be discerned by this approach. To test this, we carried out state-of-the-art

NMR predictions for the entire 2D model reported by Schulten and Schnitzer in 1997 [see Figure 1 in the cited reference *35*]. We must point out that the predictions are based on HOSE code matches and incremental algorithms derived from a large database of assigned chemical shifts (nearly 4,000,000 (including both ^{1}H and ^{13}C)) that represent the majority of published NMR data. Predictions using this approach have been shown to be accurate within 0.3 ppm (^{1}H) and 3 ppm carbon (^{13}C) for ~ 90% of positions in any structure (*36*) and have been used to produce very accurate predictions for soil components (*37*). Figure 2F shows that there is a generally poor correlation between signals in the HA and those simulated for the proposed structure of Schulten and Schnitzer (*35*). While many of the signals fall in the region of the HA (this is expected as the Schulten/Schnitzer model contains many subunits that have plant/microbial origins) the majority of novel linkers introduced to form the "humic" macromolecule (for example many of links from the aromatic bonds) do not correlate at all to those found in the IHSS HA sample. However, it is very important to point out that such cross-linked humic material may exist as trace components (<1%, i.e., below 2D NMR detection limit) in the samples studied but, more importantly, could be major components in older soil or sediment samples that have undergone more extensive transformations. Thus, while the evidence supports the theory that organic matter in "fresh" soils/peats at a very early stage of diagenesis is predominantly derived from biopolymers at varying stages of humification, it does not negate the model proposed by Schnitzer and Schulten, and it is feasible that such cross-linked recalcitrant structures may form abundantly in certain aged environments.

With the evidence described in the paper [mainly that the vast majority of humic signals can be described in terms of parent biopolymers (and their degradation products) and that novel cross-linkages cannot be found], the standard HA is best described as a complex mixture of plant and microbial residues present in soil at the time of extraction. Logically, there will be a range of residues present that will closely represent those found in living plants or microbes, and some of this will have undergone extensive degradation. However, in this sample we cannot find evidence that degradation products are in any way linked to form a specific novel humic entity that is present in any abundance for the samples in this study. In addition to the standard HA considered in detail here, we also studied the total alkaline extracts (which represent both the Fulvic and Humic fractions combined) from soils under differing vegetation (grass and aspen). In both cases, the HSQC spectra in Figure 5 are generally similar to each other, and signals from biopolymers are again by far the most dominant spectral features. It is apparent when the contour levels are studied in detail that the relative abundance of the major biochemical classes differs between the different soils but that the bulk of these HS extracts can still be described in terms of structures derived from their parent biopolymers. The exact ratios of the various compound classes, and individual structures within these classes, will to a large extent reflect the differing chemical composition within the specific soil microenvironment.

In considering the contribution to soil HS structure of four representatives of the principle compound classes in plants and microbes, we have not explicitly shown less abundant biochemical groups such as nucleic acids and pigments or black carbon (*22*, *37*). However, while these and many other biochemical classes were considered, we found that four chemical categories (protein, lignin, carbohydrate, and aliphatic biopolymers, including fatty acids, waxes, etc.) dominated the NMR spectra of the HS and thus represent the principle contributors to HS composition in the samples

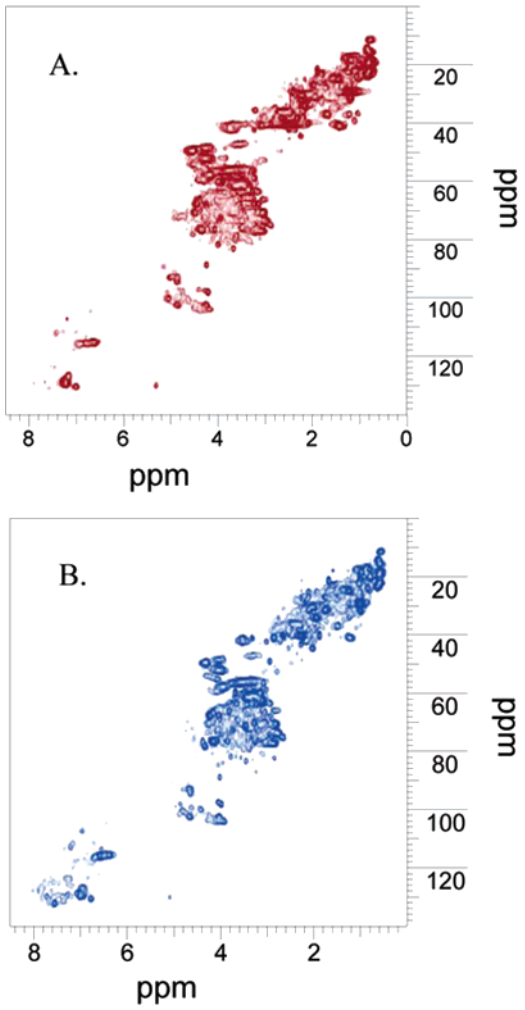

FIGURE 5. HSQC spectra of HS extracts from soils of under different vegetation: (A) chernezem grassland soil; (B) Aspen forest soil.

studied. This by no means rules out the presence of novel compounds in low concentration and certainly does not define them as simple or well understood systems. Finally, it is important to note that the NMR data presented here only contradict the abundance of a humic material that is chemically distinct and drastically different from the native parent microbial or plant materials in fresh soils. This does not rule out the formation of humic materials that are closely related to the parent biopolymers (for example, oxidized lignin, etc.).

We have shown that HS are predominately mixtures of plant and microbial derived components. It is now anticipated that this information can be used for more accurate prediction of their chemical transformations and also to determine the mechanisms by which this fraction exhibits increased environmental recalcitrance. Resistance to biodegradation may partially be described in terms of the particular properties of SOC components such as specific categories of compounds or polymers. For example, the rate-limiting factor in the slow response of SOC to temperature variation may be due to the inherent resistance of lignin to biological or chemical decomposition. On the other hand, physical protection of species, for example sorption to a clay surface, may be central. Increasing the understanding of humic components will enable more focused research to predict, explain, and understand the responses of soil organic carbon to climatic change, and ultimately its feedback to global warming.

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