

## Molecular Structure in Soil Humic Substances: The New View

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A critical examination of published data obtained primarily from recent nuclear magnetic resonance spectroscopy, X-ray absorption near-edge structure spectroscopy, electrospray ionization–mass spectrometry, and pyrolysis studies reveals an evolving new view of the molecular structure of soil humic substances. According to the new view, humic substances are collections of diverse, relatively low molecular mass components forming dynamic associations stabilized by hydrophobic interactions and hydrogen bonds. These associations are capable of organizing into micellar structures in suitable aqueous environments. Humic components display contrasting molecular motional behavior and may be spatially segregated on a scale of nanometers. Within this new structural context, these components comprise any molecules intimately associated with a humic substance, such that they cannot be separated effectively by chemical or physical methods. Thus biomolecules strongly bound within humic fractions are by definition humic components, a conclusion that necessarily calls into question key biogeochemical pathways traditionally thought to be required for the formation of humic substances. Further research is needed to elucidate the intermolecular interactions that link humic components into supramolecular associations and to establish the pathways by which these associations emerge from the degradation of organic litter.

### Introduction

Humic substances, perhaps best described as refractory, dark-colored, heterogeneous organic compounds produced as byproducts of microbial metabolism, are among the most widely distributed organic materials on the planet (1). This assembly of organic “leftovers” contributes vital properties to soils, including sequestration, mobilization, and oxidative or reductive transformation of organic xenobiotic molecules, trace gases, and trace metal contaminants. Early efforts to characterize this material resulted in the following fractionation scheme based on solubility under acidic or alkaline conditions (1): humin, the insoluble fraction of humic substances; humic acid (HA), the fraction soluble under alkaline conditions but not acidic conditions (generally pH < 2); and fulvic acid (FA), the fraction soluble under all pH conditions. Although chemical and physical differences do underlie these variations in solubility, the separation of humic substances into three fractions is operational, and does not

indicate, for example, the existence of three distinct types of organic molecule (2).

Early concepts, informed by the developing field of polymer science [see, e.g., the review by Piccolo (3)], posited that humic substances comprised randomly coiled macromolecules that had elongated shapes in basic or low-ionic-strength solutions, but became coils in acidic or high-ionic-strength solutions (1). Supporters of this “polymer model” of humic substances [e.g., Swift (4)] point to the careful ultracentrifugation study of a Sapric Histosol HA by Cameron et al. (5), in which the sedimentation velocity technique was used to monitor the solute–sedimentation boundary of subfractions with reduced polydispersity (i.e., similar average molecular mass). The data suggested that the molecules in humic substances had mass-weighted average molecular masses of 20 000–50 000 Da, radii of gyration of 4–10 nm, and random coil conformations.

However, recent information gathered using spectroscopic, microscopic, pyrolysis, and soft ionization techniques is not consistent with the “polymer model” of humic substances. Fresh scrutiny of soil processes active in the formation and preservation of humic substances also casts doubt on this model (3, 6). A new concept of humic substances has thus emerged, that of the supramolecular association, in which many relatively small and chemically diverse organic molecules form clusters linked by hydrogen bonds (H-bonds) and hydrophobic interactions (3, 7). A corollary to this model is the concept of micellar structure, i.e., an arrangement of organic molecules in aqueous solution to form hydrophilic exterior regions shielding hydrophobic interiors from contact with vicinal water molecules (8).

That these new models emphasize molecular interactions over molecular components suggests to us, as it has to others (6, 9–11), that traditional—and, it must be said, largely unsuccessful—attempts to purify humic substances through the removal of strongly associated biomolecules may be misguided, and that an empirical alternative to traditional conceptualizations (1, 12–13) may be more relevant. This alternative conceptualization encompasses all molecules found to be intimately associated with a humic fraction—including recognizable biomolecules that cannot be removed without significant alteration of the chemical properties of the fraction (1, 9–10, 14–16). Furthermore, the prevalence of amide forms of nitrogen within humic fractions (1, 6) implies that key synthesis reactions postulated by the “polymer model” (1), which suppress amide N functional groups as part of the pathway for the creation of humic substances, are not dominant processes in natural systems. A synthesis of research contributing to this new view of the molecular structure and origins of soil humic substances is the objective of the present paper.

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## Humic Substances Are Supramolecular Associations

Piccolo (3, and the references therein) and his colleagues provide evidence, using both gel permeation chromatography and high-pressure size-exclusion chromatography (HPSEC), that the apparent size of humic fragments changes drastically with addition of simple organic acids, as compared to addition of HCl as a reference. Ultraviolet-visible spectra of organic acid-treated humic substances show hypochromism (decreased absorbance due to increased distance between the absorbing chromophores). Evidently, the apparent sizes of humic materials do not change due to tight coiling (or uncoiling), as suggested by the "polymer model," but instead change due to disaggregation (or aggregation) of clusters of smaller molecules. The effects of additions of carboxylic and mineral acids on humic fractions with differing hydrophobicities indicate that aggregate disruption is greatest when more hydrophobic humic materials are combined with organic molecules containing both hydrophobic and hydrophilic segments (17). This result, in turn, suggests that humic materials are held together by hydrophobic interactions, which are easily disrupted when simple organic molecules penetrate large hydrophobically bonded associations and separate them into smaller, higher-energy H-bonded associations. The low hydrophobicity and high negative charge of FA, relative to HA, would not support large hydrophobically- or H-bonded associations, thus accounting for the observed minimal change in the apparent average molecular size of FA under a variety of solution conditions (17).

Recent criticism of the work of Piccolo et al. (3, 17) provides alternative interpretations of their results based on ionic exclusion and specific adsorption effects (4, 18). Varga et al. (19) revealed the potentially confounding effect of extreme changes in pH within the HPSEC column that accompanied the addition of organic acids similar to those described by Piccolo et al. (17). In response, Piccolo et al. (20) performed similar analyses on both humic substances and well-characterized macromolecular polymers (e.g., polysaccharides and polystyrenesulfonates) demonstrating that changes in column performance upon addition of acids were not responsible for the behavior of humic substances that they had documented.

Simpson (21) examined solutions of humic substances with a multidimensional nuclear magnetic resonance (NMR) spectroscopic technique and observed both aggregation and disaggregation behavior consistent with that described by Piccolo (3). Two-dimensional diffusion-ordered spectroscopy (DOSY) involves use of a pulsed-field gradient that modifies the NMR signals of nuclei such that the intensity of a resonance can be related to a diffusion coefficient and, therefore, to a hydrodynamic radius and approximate molecular mass. A concentrated solution of peat HA (100 mg/mL) contained aggregates with diffusivities corresponding to an average molecular mass >66 000 Da, whereas a less concentrated solution (5 mg/mL) contained aggregates with diffusivities corresponding to an average molecular mass between 2500 and 6100 Da (21). Addition of acetic acid to the latter solution caused disaggregation like that observed by Piccolo (3), and made possible the identification of DOSY signals associated with chemically distinct molecular fragments with differing degrees of mobility. The diffusivities of these humic components corresponded to average molecular masses ranging from 200 to 2500 Da (21). Because acetic acid is known to denature proteins, Simpson (21) suggested that this organic acid may instigate dispersion similarly by triggering conformational changes in humic substance fragments. Examination of spectra of FA solutions suggested typical average molecular masses of ~1000 Da and provided little evidence of aggregation (21), as also suggested by Piccolo (3).

In another set of DOSY experiments, Simpson et al. (7, 22) extensively purified a series of humic substances through repeated cation exchange treatment to remove associated metals, then dissolved these samples in either D<sub>2</sub>O or dimethyl sulfoxide (DMSO). By contrasting the spectra of the purified humic substances within the two solvents, Simpson et al. (7, 22) again identified different diffusion coefficients for chemically distinct fragments in several samples, though this time the largest of them featured molecular masses of only about 1500 Da. Liquid chromatography of these samples combined with <sup>1</sup>H NMR spectroscopy showed substantial separation of different structural groups, in part a result of aggregate disruption after the removal of metals during purification (7). Further discussion of experimental results supporting the existence of discrete domains with distinct molecular motional behaviors within humic materials is provided in our Supporting Information.

Soft desorption ionization techniques, such as electrospray ionization (ESI) and laser desorption ionization (LDI), volatilize large ions for identification via mass spectrometry (MS), providing positive or negative ion mass/charge distributions that may represent the true mass distribution of molecules within humic fractions, if the volatilized organic ions are singly charged, all constituents undergo ionization, and the ionized constituents represent their parent molecules (23). Humic cations volatilized by ESI-MS have molecular mass distributions with number- or mass-weighted averages near 1000 (24–25) or 2000 Da (26–27), respectively. Laser desorption ionization-MS provides mass/charge distributions with number-weighted averages closer to 500 Da, perhaps due to fragmentation, decreased ionization efficiency, or poor resolution of signals representing ions with masses greater than 1000 Da (23, 28). These ESI- and LDI-MS average masses are far lower than those that have been measured by traditional methods, such as ultracentrifugation (1). Methylation of an aquatic FA did not change the ESI-MS number-weighted average molecular mass of the sample, but did reduce the mass-weighted average molecular mass, suggesting that methylation of carboxyl groups had reduced the aggregation of humic materials through elimination of H-bonds (29). Thus an array of independent analytical techniques has consistently revealed relatively small, independent moieties in humic substances, providing significant evidence to support the concept of these organic materials as collections of diverse, low-molecular-mass molecules.

## Humic Substances Can Form Micelles

Examination of the same interactions that promote supramolecular associations led to development of the micellar model of humic substances, in which intra- or intermolecular organization produces interior hydrophobic regions separated from aqueous surroundings by exterior hydrophilic layers, analogous to the micelles formed by surfactants in aqueous solution (30). For example, the fluorescence of pyrene in solutions of humic substances is increased within a few hours by the addition of Mg<sup>2+</sup>, then decays over a period of several days (31–33). The initial increase is thought to result from cation-enhanced formation of micelles featuring hydrophobic domains created by charge neutralization and cation bridge formation, inducing protection of the pyrene probe from quenching anions such as Br<sup>-</sup> (8). The drop in fluorescence then occurs as Mg<sup>2+</sup> slowly migrates to higher-energy, directly coordinated positions within the humic material, and the previously formed hydrophobic regions disaggregate. Nanny and Kontas (34) used the fluorescence of 6-propionyl-2-dimethylaminonaphthalene (Prodan), another probe sensitive to polarity, to investigate the formation of micelles in humic substances over periods of days to weeks. Within humic solutions, Prodan experienced a polarity between that of ethanol and cyclohexane, indicating

that it had been shielded from water molecules (34). More micellar structures were formed under neutral to acidic pH conditions than were formed under basic conditions, and FA formed the structures more quickly than did HA.

Martin-Neto et al. (35) also detected increasing hydrophobic microsities with decreasing pH using electron spin resonance (ESR) spectroscopy. The width of the ESR absorption line of organic free radical species provides an indication of the relaxation time of these species. The line width of ESR signals produced by stable semiquinone-type free radicals within humic substances decreased with decreasing pH, indicating the presence, under acidic conditions, of protective hydrophobic regions that increased relaxation time. The hydrophobic ESR probe 5-SASL (stearic acid with a nitroxide free radical in position 5 of the hydrocarbon chain) was used by Ferreira et al. (36) to monitor humic substances as a function of pH. Under acidic conditions (pH < 5), ESR spectra indicated that, in a matter of hours to days, the probe was immobilized due to hydrophobic interactions with humic moieties, while at higher pH the probe molecule remained mobile. That this response was reversible also suggests that hydrophobic regions form under acidic conditions, but disperse under basic conditions.

Chien et al. (37) studied HA solutions containing the herbicide atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine) labeled with  $^{19}\text{F}$  in a trifluoromethyl group on the ethylamino side chain. They used  $^{19}\text{F}$  NMR spectroscopy to assess the effect of paramagnetic probes with differing hydrophobicities on the intensity of the  $^{19}\text{F}$  signal. When located within a few nanometers of NMR-active nuclei, paramagnetic species cause efficient relaxation of these nuclei, resulting in line-broadening and, therefore, loss of NMR signal intensity. The hydrophilic Gd-EDTA anion remained in aqueous solution and caused no paramagnetic relaxation of the  $^{19}\text{F}$  signal, whereas the 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) probe, which sorbed within hydrophobic humic structures, produced rapid relaxation. These data indicate that atrazine occupied nanometer-scale domains accessible only to hydrophobic molecules, which is consistent with a proposed micellar structure. Simpson et al. (38) obtained high-resolution magic angle spinning (HR-MAS) NMR solution-state spectra of a soil sample containing humic materials that were not fully water-soluble. Humic substances suspended in  $\text{D}_2\text{O}$  showed a reduced aromatic signal, as compared both to those suspended in the more highly penetrating DMSO and to soluble humic extracts—further evidence for hydrophobic regions. Tombácz (39) noted that HA fractions having large hydrocarbon contents, as assessed by NMR, also have high critical micelle concentration values, suggesting that the colloidal properties of humic associations are influenced by the presence of associated alkyl biomolecules such as fatty acids. Trends in both surface tension and enhanced solubility of *p*-dichlorobenzene (40) or  $^{14}\text{C}$ -labeled DDT (2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane) (41) within peat HA solutions were consistent with formation of micelle-like aggregates, especially under acidic conditions.

Transmission electron microscopy of dissolved organic matter, an aquatic organic fraction that displays functional properties that overlap with those of soil humic substances (1), revealed formation of 400–800-nm micelle-like colloids over a 10-day period (42). Addition of EDTA (ethylenediaminetetraacetic acid) reduced aggregation by only 20%, suggesting that multivalent cation binding is not essential to the formation of these colloids (42). Images from this study, and those from atomic force microscopic examinations of HA and FA adsorbed to mineral surfaces from aqueous solutions of varying properties (43–45), provide no evidence for coiled structures under either acidic or high-ionic-strength conditions, or for elongated structures under either basic or

low-ionic-strength conditions, as would be posited by the “polymer model” of humic substances.

Wershaw (9) further suggests that soil minerals provide surfaces upon which amphiphilic humic moieties form bilayer, membrane-like coatings, a conclusion based in part on infrared attenuated total reflectance linear dichroism measurements indicating that the carboxylate groups of compost leachate organic acids exposed to model soil surfaces such as alumina have very limited rotational mobility, and are likely bound to mineral Al. Adsorption behavior of the hydrophobic fraction of this leachate on alumina was similar to that displayed by simple surfactants forming layered structures on alumina surfaces, suggesting similar self-organization may occur as humic materials adsorb to soil minerals (9). This broad set of data provides considerable evidence supporting a view of humic substances as large, dynamic associations capable of exhibiting micellar properties in aqueous solutions.

### **Biomolecular Fragments Belong in Humic Substances**

Humic fractions extracted from soils contain recognizable biomolecular fragments that have been specifically excluded from traditional definitions of humic substances (1, 13, 16). Many of these biomolecular moieties are intimately associated—even covalently bonded—with the humic fraction and cannot be separated effectively (9–12, 14–15, 46). If we recognize humic material as a supramolecular association of component molecules, why must we exclude an entire category of component molecules simply because they can be identified as biomolecules? We suggest that the drive to purge humic fractions of strongly associated, recognizable biomolecular components (10, 46) may be inappropriate and misleading for these complex, heterogeneous mixtures. A definition of humic components that is relevant to experimental studies should include all molecules that are strongly associated within a humic fraction, i.e., those that cannot be removed without significant alteration of the chemical properties of the fraction. This definition would necessarily include recognizable biomolecules that play important structural roles.

Does this broader definition of humic molecules conflict with the acknowledged refractory nature of these materials? Soil humic substances typically possess average  $^{14}\text{C}$  ages ranging from hundreds to thousands of years, while many biomolecules are readily decomposed by microbes seeking energy and nutrients (1). However, binding to humic fractions can result in the protection of biomolecules from microbial degradation (11, 47–49), producing recognizable, but refractory, biologically derived moieties. Alternatively, MacCarthy (46) posits a “two-compartment” view of humic substance stability. While most humic material is persistent, some humic components may be transient. The persistent material provides the dominant contribution to the  $^{14}\text{C}$  age, whereas the transient components may provide the dominant contribution to the C turnover rate. It is possible that strongly associated—but more easily degraded—biomolecules make up a large portion of the transitory, frequently replenished humic components. The concept of humic components as members of associations that may protect individual molecules from microbial degradation, and may include a minority population of relatively short-lived constituents along with more resistant moieties, is thus not inconsistent with the observed refractory nature of humic substances.

Another argument for the classification of associated biomolecular fragments as humic components relies on the fact that biomolecular moieties contribute noticeably to the functional behavior of humic associations (6, 10–11, 50). Obviously, biomolecular components of humic fractions affect analytical properties, such as elemental composition and functional group distribution (6, 10–11). A recent

characterization of the optical spectroscopic properties of humic substances indicates that fluorescence absorption and emission spectra arise from a continuum of coupled states formed through charge-transfer interactions of a few distinct chromophores, rather than from a superposition of many independent chromophores (51). It is reasonable to suppose that biomolecules associated with humic materials contribute to the charge-transfer interactions that lead to the complex optical properties of humic substances. Thus, the potential role of intermolecular interactions in determining humic behavior indicates that removal of specific components, such as biomolecules, may substantially alter the emergent properties of humic associations. Reviews of current research regarding the composition of the individual fragments of humic material produced through the degradation of biomolecules and, therefore, which are relevant to the supramolecular association model of humic substances, are included in the Supporting Information. These biomolecules are derived primarily from lipids, lignin, nonlignin aromatic species, carbohydrates, and proteins (e.g. 23, 52–57).

### Functional Group Distributions Are Inconsistent with the “Polymer Model”

Discussions of current research on C, O, N, and S functional group distributions that are relevant to the supramolecular association model of humic substances are included in the Supporting Information. Here we shall focus on recent data concerning the most important of these distributions for the objective of our review, the N-containing functional groups. Examination of these data provides further evidence to support the concept of humic substances as supramolecular associations that include biomolecules.

Early chemical methods for differentiating organic N functional groups, which relied on acid hydrolysis and subsequent isolation of amino acids, amino sugars, and ammonium, suggested that amino acids made up less than half of the soil N content (1). Some pyrolysis-based discussions of humic N (10, 58) attribute uncharacterizable fractions, termed “non-hydrolyzable” or “acid-insoluble N” (20–35%) and “hydrolyzable unknown N” (>20%) (1), to heterocyclic N. This latter N, whose existence suggests conversion of proteinaceous precursors to nonamide moieties during humification, is consistent with synthesis pathways requisite to the “polymer model” of humic substances (1, 6). However, recent spectroscopic and thermochemical studies indicate that, while heterocyclic N compounds are present, protein-derived forms of N in fact dominate soil humic fractions. Supramolecular associations of humic components derived from biological precursors could possess N functional group distributions dominated by amide N (6), consistent with this recent experimental evidence.

Solid-state natural-abundance  $^{15}\text{N}$  cross polarization (CP) MAS NMR spectra of humic materials purified through extraction or removal of mineral components (59) indicate that humic N exists predominantly as amide functional groups, with a small contribution from free amino groups (48, 59–66). Studies of non-hydrolyzable N fractions indicate further that acid hydrolysis leaves behind a substantial portion of peptide structures (61–62) that have perhaps been protected by organo-mineral association (62, 67–68) or hydrophobic entrapment (48, 62, 68–69). Most spectra of humic substances do not feature signals for typical heterocyclic N functional groups, such as indoles, pyrroles, and imidazoles (chemical shifts from –180 to –239 ppm, relative to the  $^{15}\text{N}$  NMR reference, nitromethane), and pyridines (chemical shifts from –25 to –90 ppm) (63), implying that less than 10% of the N resides in heterocyclic forms (62).

The CP-MAS NMR technique, however, may be less sensitive to heterocyclic N than to amide N, as it depends

on transfer of magnetic energy from  $^1\text{H}$  nuclei to  $^{15}\text{N}$  nuclei, and heterocyclic N atoms are not often directly protonated. Despite this limitation, detection of signals from similarly unprotonated nitrates (61) suggests that the technique may be sufficiently sensitive, and neither longer CP-MAS contact times (63) nor direct polarization (DP)  $^{15}\text{N}$  NMR analysis [in which the  $^{15}\text{N}$  nuclei are excited directly] (62) has resulted in improved detection of heterocyclic N in humic materials. On the other hand, a study of model compounds indicated that detection of unprotonated N atoms via CP-MAS NMR spectroscopy is dependent on both applied magnetic field strength and contact time (70). In fact, an examination of soil organic matter associated with clay fractions using the  $^{15}\text{N}$  CP-MAS spin-counting NMR technique, which expresses the amount of NMR signal detected per unit mass of the nucleus in a reference material with a quantitative signal, indicated that 25–50% of soil N was not detected by  $^{15}\text{N}$  CP-MAS NMR spectroscopy and may consist of unprotonated, heterocyclic N functional groups (71). These spectra also demonstrated that most NMR-visible  $^{15}\text{N}$  nuclei are in amide functional groups (71).

Data obtained through a variety of other NMR techniques similarly suggest that most humic N is present as amide N derived from peptidic structures. Dipolar dephasing (DD) solid-state  $^{13}\text{C}$  NMR spectra, which reveal C atoms affected by strong proton dipolar coupling due to the presence of bonded amino groups, have been used to estimate the relative amounts of N-substituted alkyl C atoms in humic materials from peat soils (62). Subsequent calculation of the contribution of peptides to the total C and N content of these materials corroborates the dominant role of peptides (62). Two-dimensional double cross polarization (DCP) MAS  $^{13}\text{C}$ – $^{15}\text{N}$  NMR spectroscopy has been used on  $^{15}\text{N}$ -enriched humic materials to detect  $^{13}\text{C}$  nuclei close to  $^{15}\text{N}$  nuclei (65). These spectra show significant coupling between amide-C and N-substituted-alkyl-C atoms, demonstrating again the prevalence of amide functional groups. Fan et al. (52) performed several solution-state 1D and 2D NMR analyses on a HA treated with Tiron (4,5-dihydroxy-1,3-benzene disulfonate) to reduce exchangeable metal ions and increase solubility. Signals from  $^1\text{H}$  NMR spectra indicated abundant amino acids, while 2D NMR spectra obtained using both  $^1\text{H}$  total correlation spectroscopy (TOCSY), which features off-diagonal scalar couplings marking protons connected by three or fewer covalent bonds, and  $^{13}\text{C}$ – $^1\text{H}$  heteronuclear single-quantum correlation (HSQC) NMR spectroscopy, which features off-diagonal scalar couplings marking protons directly bonded to C atoms, identified the atoms as components of peptides. Additional NMR spectra obtained using DOSY and 2D  $^1\text{H}$  nuclear Overhauser effect spectroscopy (NOESY), which allows identification of protons located near each other, support the presence of peptidic N in humic matter (7, 22, 55).

A few  $^{15}\text{N}$  NMR spectra do feature peaks produced by heterocyclic N components. Mahieu et al. (63, 66) detected a shoulder representing indole, pyrrole, and imidazole groups in “labile HA” (extracted via alkalization followed by acidification) and recalcitrant Ca-humates (extracted after a lengthy acid-washing treatment to remove polyvalent cations, followed by alkalization and acidification) that accounted for as much as 22% of the total N content. These functional groups, more common in recalcitrant Ca-humate fractions and in aerated soils, may have formed as part of long-term humification processes. After detecting a pyrrole/indole  $^{15}\text{N}$  NMR shoulder only in a sample from the deepest, oldest layer of a peat soil, Knicker et al. (72) suggested that abiotic conditions during advanced humification promote formation of heterocyclic N compounds. Mahieu et al. (63) noted that this shoulder does not include signals from all forms of heterocyclic N, as other forms may be present in quantities

less than the detection limit of 10%. A small signal attributed to pyridinic N atoms was found in a HA spectrum by Zang et al. (48). A combination of selective NMR techniques applied to labile HA fractions detected the presence of pyridinic and pyrrolic N atoms, as well as amide N atoms bound to aromatic rings (73). A  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectra of a forest soil FA indicated that, while most N took the form of ammonium, amide and indole functional groups were also present (50).

Pyrolysis of humic substances produces fragments including pyrroles, free and substituted imidazoles, pyrazoles, pyridines, substituted pyrimidines, pyrazines, indoles, quinolines, N-derivatives of benzene, alkylamines, and alkyl and aromatic nitriles (58). However, the relation of these fragments to parent humic structures is unknown. Some interpretations of humic structure based on pyrolysis data conclude that there is nearly as much heterocyclic N (35%) as proteinaceous N (40%) (58). Pyrolytic arguments for the presence of heterocyclic N within humic substances include the following: (a) detection of these groups in aquatic humic substances under low-temperature pyrolysis conditions [200–300 °C] (58), (b) measurement of consistent intensities of these groups after additions of amino acids or use of pyrolysis temperatures as high as 700 °C (74), and (c) identification of heterocyclic N in soil and humic substance pyrolysates, but not in plant or microbial pyrolysates, implying formation within soil (75). Others interpret the results to be indicative of proteinaceous materials within humic substances (48, 76), as amino acids yield similar pyrolysates (77–78). While pyrolysates of acid hydrolyzates and hydrolysis residues of humic substances contain many heterocyclic N compounds that cannot be characterized by chemical methods (75), examination of hydrolysis residues using a dithionite–citrate–bicarbonate treatment to remove pedogenic Fe and Mn oxides and mineral-bound N functional groups, followed by hydrolysis and amino acid analysis, revealed substantial amounts of amino acids initially protected from hydrolysis by association with soil minerals (67).

The addition of catalytic chemicals to facilitate molecular fragmentation at lower temperatures reduces confounding secondary reactions. Amino acids and proteinaceous materials characterized with tetramethylammonium hydroxide (TMAH) thermochemolysis at 250 °C provide products indicating effective cleavage of peptide bonds, yielding individual amino acid methyl esters, and few secondary reaction products (79). Knicker et al. (80) used TMAH thermochemolysis to analyze the acid-hydrolyzed humin fraction of an algal sapropel, generating products, including methyl esters of amino acids, indicating the dominance of proteinaceous N even after hydrolysis. The HA sample examined by Zang et al. (48) also generated methyl esters of amino acids. Given the greater yield as compared to conventional pyrolysis, Zang et al. (48) posited that TMAH disrupts the three-dimensional structure of the HA, releasing entrapped proteinaceous moieties. Further experiments indicate proteinaceous materials can become encapsulated within hydrophobic domains in the HA and thereby be protected from complete hydrolysis (48). Thermochemolytic results suggest that most of the heterocyclic N groups detected via pyrolysis are indeed the products of secondary transformations.

Using Gaussian fits to N 1s X-ray photoelectron spectra of 11 soil HA samples, Abe and Watanabe (81) conclude that most N is peptidic, a classification that includes amide, secondary and tertiary amine, and pyrrole functional groups, while 3–19% of the N could be categorized as aromatic, i.e., derivatives of pyridine, imine, and aniline functional groups. A study of several humic substances using N K-edge X-ray absorption near-edge structure (XANES) spectroscopy verified the dominance of amide functional groups, based on comparisons with model compound peak positions (82).

Pyridinic N accounted for 20–30% of total N, a portion of which was identified as oxidized derivatives of pyridine, perhaps substituted with carboxyl groups. Myneni (83) argues that more work with model molecules is required before these functional groups can be assigned unambiguously to specific XANES peaks.

Nevertheless, it appears that amide N in peptides is the dominant chemical form of N in humic substances, and that free amino acids are present as well. Heterocyclic N is a less significant contributor to humic composition. Positively charged amide groups within humic substances, which have a net negative charge under typical soil pH conditions (1), may have important impacts on humic conformation, interaction with mineral surfaces, and retention of nutrients or contaminants (84). Supramolecular associations of relatively low-molecular-mass humic components derived from biological precursors could possess a considerable amount of amide N functional groups (6). In contrast, the “polymer model,” which posits the destruction of amide groups during the formation of humic macromolecules (1, 6), is inconsistent with the N functional group distributions dominated by amide N that are suggested by extensive experimental evidence.

### Future Research Needs

Given that humic substances are best described as supramolecular associations of low-molecular-mass organic molecules, including recognizable biomolecules, continued characterization of these materials should emphasize identification of key properties arising mainly through intermolecular interactions, not molecular composition. In addition, because the supramolecular association model does not explain changes in functional group composition that occur with the separation of humic substances into different apparent mass or size fractions (see Supporting Information), we need better information about the hydrophobic and H-bonding interactions that hold together humic aggregates of various sizes. As an adjunct to the experimental techniques applied to this problem, molecular modeling (85) may prove useful in exploring the collective strength and endurance of these intermolecular interactions, as well as in understanding their effects on aggregate structure and composition. Finally, the new view of the structure of humic substances suggests a need to probe and reevaluate the biogeochemical pathways of their formation, and ultimately to redefine the concept of humification. Further investigation of soil humic substances may provide insights regarding other complex natural organic fractions defined by alternative operational means (86).

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### Supporting Information Available

Discussion of evidence supporting the existence of domains with different degrees of mobility within humic substances. Descriptions of biomolecular fragments detected within humic materials via spectroscopic, pyrolytic, and soft ionization techniques. Information regarding humic N functional groups not discussed in the main text, followed by a thorough review of humic nonpolar, O, and S functional groups. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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