



# PROGRESSION FROM MODEL STRUCTURES TO MOLECULAR STRUCTURES OF NATURAL ORGANIC MATTER COMPONENTS

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## ABSTRACT

Natural organic matter (NOM) found in soil, sediment and water has diverse mixture and molecular structural complexity. This complexity has been simplified by number-average molecular models, but the documental validity of these models decreases as the heterogeneity of the mixture increases, coupled with chemical and physical alterations that occur during NOM extraction and analysis. NOM models based on assumptions of extensive condensation and oxidative coupling reactions in humification processes portrayed NOM as macromolecular random-coil shapes, but more recent NOM models based on analytical evidence indicate that NOM is an aggregate of small molecules. Various organic matter fractionation approaches have been developed that separate NOM molecules into relatively homogeneous compound classes. Compound-class model structures developed from data synthesis of elemental, titrimetric, and nuclear magnetic resonance, infrared, and mass spectral analyses are better approximations of chemical structures than obtained previously on unfractionated NOM. The progression from model structures to molecular structures of NOM requires improved purification and fractionation methods, application of complementary and quantitative spectral characterizations, and ultra-high resolution mass separations combined with tandem spectral analyses. Syntheses of standards are required for final confirmation of NOM molecular components.

**Keywords:** Natural organic matter, humic acid, fulvic acid, model structures, molecular structures

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## 1. INTRODUCTION

Natural organic matter (NOM) found in soil, sediment and water has extreme mixture and molecular structural complexity. Attempts to determine molecular structural components of NOM by chromatographic and mass spectrometric resolution of NOM into pure compounds required for structural analyses have not yet been successful. Because various molecular structural figures are the language of chemistry, the chemist is severely handicapped if the chemical properties of NOM cannot be communicated in terms of structural figures. Therefore, many chemists who investigate NOM have resorted to chemical model structures that approximate NOM mixtures as if NOM were a pure compound or a limited mixture of compounds that can be represented by various molecular structures. NOM structural models have generated considerable controversy because of the subjective nature of the exercise of model construction with sparse objective data and the representation of complex molecular mixtures by a few discrete chemical structural models. Defenders of structural NOM models state that they are necessary to communicate chemistry, to put analytical constraints on NOM structures, to serve as dynamic successive approximations that can generate new analytical approaches to separate NOM mixtures, and to lead to improved hypotheses of NOM structures. The purpose of this paper is to present an historical overview of successive approximations of model structures that hopefully will lead to molecular structures of NOM components with improved analytical and interpretive methods.

## 2. HUMIC MODELS

Early NOM models focused upon the humification process that produces humus, its fractions (fulvic acid, humic acid and humin) and subfractions (apocrenic acid, glucic acid, humatomelanin acid, ulmic acid, etc). Extensive classification schemes that fractionated humus on the basis of acidity, color, and solubility were developed [1-3]. A number of humic subfractions were given molecular formulas based upon the belief they were pure compounds. Humic acid models proposed by Fuchs and Flaig were highly aromatic phenol-rich structures [4]. The Fuchs model was similar to coal models, which featured condensed planar polyaromatic core structures with carboxyl and phenol groups at the periphery. The Flaig model featured polyphenol ethers formed from oxidative coupling of phenols which were known to exist in lignin and tannin precursors. Cheshire et al. [5] pro-

posed that phenolic acid precursors of humus also could be derived by aldol condensation reactions of furfural derived from dehydration of carbohydrates. Maillard [6] believed humic substances resulted from the abiotic chemical reaction between reducing sugars and amino groups of proteins to produce melanins that decomposed to humus with significant nitrogen content. The various theories (lignin theory, polyphenol theory, sugar-amine condensation theory, and a process combination theory) with structural models and reaction pathways for each theory are presented by Stevenson [2].

The extensive condensation reactions leading to these humus models and the polymeric nature of certain humus precursors such as lignin led to the perception that humus consists of macromolecular heterogeneous substances in which it was pointless to express the integrated chemical information in terms of chemical structure [7]. Therefore, humus models based on a macromolecular perception were represented as flexible polyelectrolytes that randomly coiled into various shapes depending on charge, pH, and composition of the humus macromolecule [8]. Organic contaminant sorption studies indicated humus in soils exists as an amorphous glassy solid with black carbon that explains nonlinear and irreversible sorption behavior [9]. The complexity and macromolecular weight characteristics of the humic models led to the perception that characterization of NOM, especially humic NOM, at the molecular level was practically impossible. Only various chemical and biological degradation products of humus could be characterized as chemical structures.

### 3. ANALYTICAL MODELS

Advances in analytical chemistry have played a major role in influencing NOM models. Oxidative degradation of soil humic substances coupled with gas chromatography resulted in an aggregate model in which aromatic phenolic acids detected by gas chromatography are held together by hydrogen bonds [10]. Aquatic humic substances responsible for color in water were oxidized with alkaline cupric oxide, methylated to enhance volatility, and analyzed by gas chromatography [11]. The products were phenolic acids shown to be derived from lignin and tannin precursors. These low-molecular weight acids were components of a heterogeneous aggregate model proposed by Gjessing [12] in which a humus core was associated with low-molecular weight aromatic molecules (carboxylic acids, phenols and quinones), metals, and metalloid elements such as silicon. The cupric oxide oxidation method [13] has seen extensive

application for analyses of lignin components in aquatic NOM, but a review of dissolved organic matter (DOM) literature by Perdue [14] found that lignin-derived phenols only accounted for an average of 0.6% of dissolved organic carbon (DOC) in fresh waters. Degradative techniques based upon thermochemolysis with tetra-alkyl ammonium hydroxide [15] and pyrolysis [16, 17] coupled with gas chromatography and mass spectrometry also have detected lignin-derived phenols along with numerous aliphatic acids, amino acids, amino sugars and neutral carbohydrates as significant components of NOM in soil and water.

Nuclear magnetic resonance (NMR) investigations of NOM composition [18] have resulted in radical revisions of NOM structures and models. NOM in soil, sediment and water was shown by NMR spectra to have a much greater percentage of aliphatic structures and lower phenol content than were seen in degradative studies. The aliphatic structures seen by NMR in marine humic substances were modeled by Harvey et al. [19] as cross-linked aliphatic chains terminated by carboxylic acids. This model results from hydrolysis and oxidative coupling of polyunsaturated triglyceride lipids of marine planktonic organisms. Aluwihare et al. [20] concluded from NMR measurements that a significant fraction of DOC in sea surface water consists of structurally related and biosynthetically derived acyl oligosaccharides that persist after more labile organic matter has been degraded.

Terpenoid precursors were found to be a major contributor to aliphatic structures of DOM in various waters [21]. Considerable effort was expended acquiring <sup>13</sup>C-NMR spectra that quantitatively represented the distribution of carbon structures in NOM [22]. Using NMR information, Wershaw [23] proposed a humic substance model of molecular amphiphiles that self-assemble on a mineral surface with a polar exterior and a non-polar interior. This model is popular for explaining partitioning of non-polar contaminants into humic substances. Two-dimensional solid-state NMR has been useful in assigning specific molecular linkages between carbon, hydrogen, oxygen, and nitrogen in NOM structures [24]. Using data derived from multidimensional NMR spectrometry and diffusion ordered NMR spectrometry that gives molecular-weight information, Simpson et al [25] concluded that terrestrial humic substances are molecular aggregates of relatively low molecular weight (<2000 Da) held together by complexation with metal cations. Thus, NMR investigations resulted in radical revisions of NOM composition and a radical downward revision of molecular weight estimates of primary molecular

structures as compared to earlier humic models that emphasized condensation and coupling reactions. These findings rekindled hope that the relatively low molecular weight of NOM components would enable molecular identification of primary NOM structures.

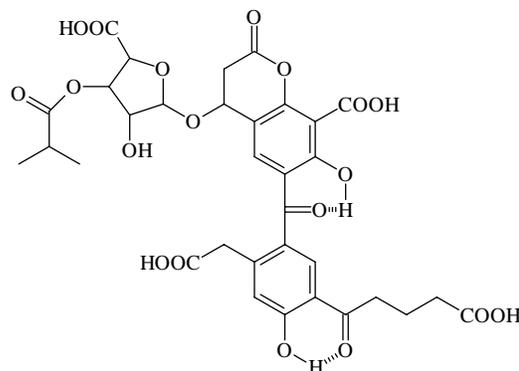
Electrospray ionization/mass spectrometry (ESI/MS) is a recently developed analytical technique that is revising molecular models of NOM structures. Mass spectral information on non-volatile components of NOM can be obtained with minimal degradation, and multiple mass spectral fragmentation can yield primary structural model approximations [26]. However, ESI/MS is a qualitative method in which the distribution of ions produced depends on relative ionization efficiency, multiply-charged ions, molecular-aggregate ions, positive versus negative ionization sites in analyte molecules, and various instrumental parameters used to charge, store, and separate the ions [27]. Ultra-high resolution ESI/MS using a ion-cyclotron resonance mass spectrometer (ICRMS) was used to assign molecular formulas to 4626 molecules in Suwannee River fulvic acid [28]. Numerous ESI/MS studies of NOM extracts from soil, sediment and water have generally confirmed the <2000 Da upper molecular weight limit for primary NOM components with median mass/charge ions being most abundant in the 300-500 Da range. Additionally, NOM derived from thermogenic "black carbon" was found by ESI/ICRMS and explained a portion of the aromatic carbon component in soils and sediments that could not be ascribed to lignin components [29]. Despite the ability of ESI/ICRMS and multiple MS methods to provide molecular-level information of NOM composition, the lack of analytical standards to confirm this information is a major limitation.

#### 4. SUCCESSIVE MODEL APPROXIMATIONS OF SUWANNEE RIVER FULVIC ACID AND ITS FRACTIONS

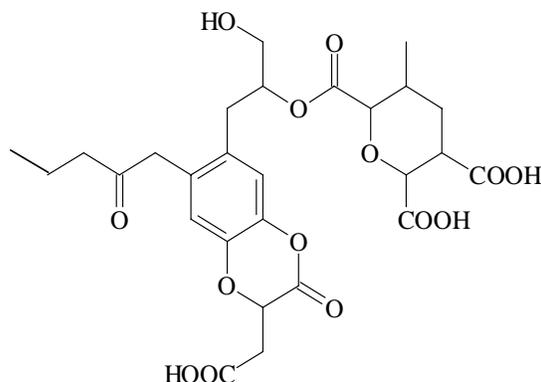
Suwannee River fulvic acid (SRFA), isolated for the International Humic Substances Society standard and reference samples, was extensively characterized by the U.S. Geological Survey [30]. Part of this characterization was assembling the characterization data [31] into an average structural model presented below.

This and other structural models were derived without considering possible organic precursors and diagenetic processes that produce fulvic acid. However, this was the first model that combined quantitative oxygen functional-group and carbon structural data with average molecular weight data.

Nitrogen, sulfur, and phosphorus were not incorporated into this and subsequent models because they are minor elements that do not occur (on the average) in every molecule [30]. The average molecular weight (800 Da) measured by multiple methods was sufficiently low to present the model as specific chemical structures rather than representing molecules as ill-defined macromolecular shapes. This model revealed that fulvic acid molecules are enriched in ring structures, both aliphatic and aromatic, and that they have an average of four carboxylic acid groups per molecule.



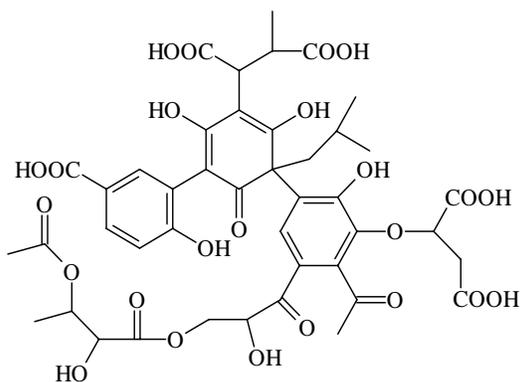
The second model was derived from quantitative characterization of fractions resulting from two-stage normal-phase chromatography of SRFA on silica gel [32]. This model, presented below, revealed that the strong-acid acidity with  $pK_a$  values as low as 2 resulted from carboxylic acid groups being clustered together next to aliphatic C-O linkages in ethers and esters:



The third model was derived from quantitative characterization of a SRFA fraction that was assayed to have the greatest binding constants for  $Ca^{2+}$ ,  $Cd^{2+}$ ,

Cu<sup>2+</sup>, Ni<sup>2+</sup> and Zn<sup>2+</sup> [33]. This fraction was derived from a pH-gradient fractionation of SRFA on XAD-8 resin followed by a normal-phase fractionation of a high-affinity metal-binding fraction on silica gel.

This third model had greater molecular weight, phenol content and carboxylic acid group content than the average model structure for unfractionated SRFA. The carboxylic acid, ether and phenol groups were clustered to favor metal-chelate structures with large binding constants. The model is presented below.

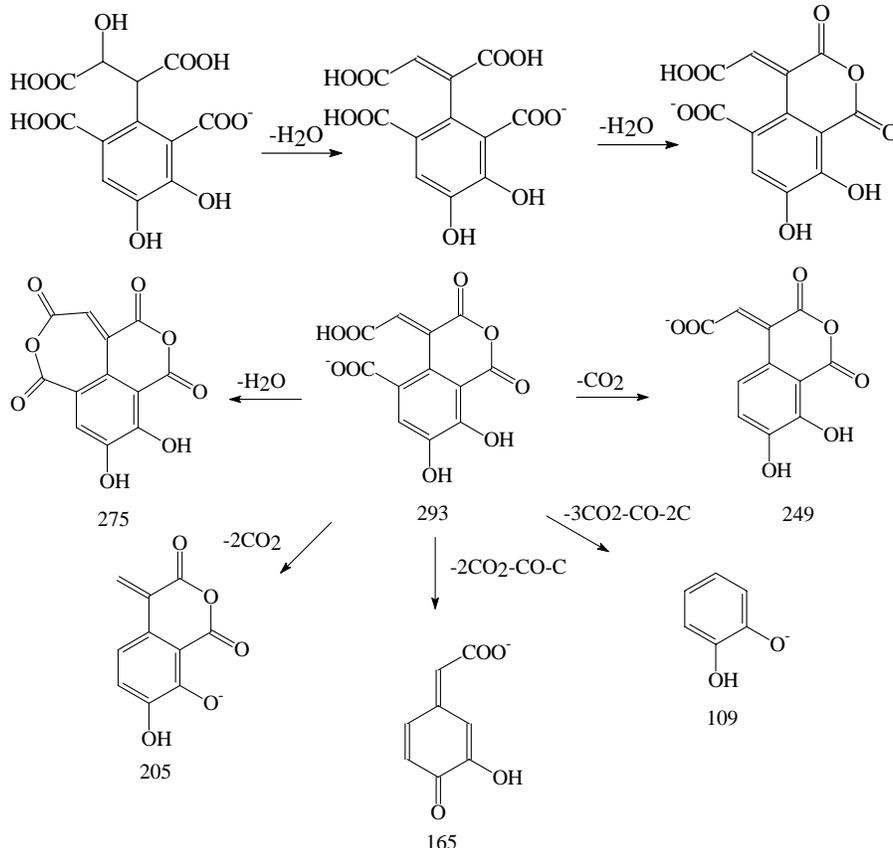


This was the first model designed with a possible precursor structure since the m-hydroxybenzene aromatic ring is present as condensed tannins.

The fourth model was derived from electrospray ionization/multistate tandem mass spectrometry separation of SRFA negative ions [26]. The negative ion at 329 m/z was selected from the ion trap and was fragmented through four successive stages selecting ions resulting from water-loss at each stage. A hypothetical fragmentation pathway with model structures ions is presented below. This model points to a lignin precursor because the final product ion at 109 m/z was *o*-dihydroxy benzene that is indicative of lignin structure.

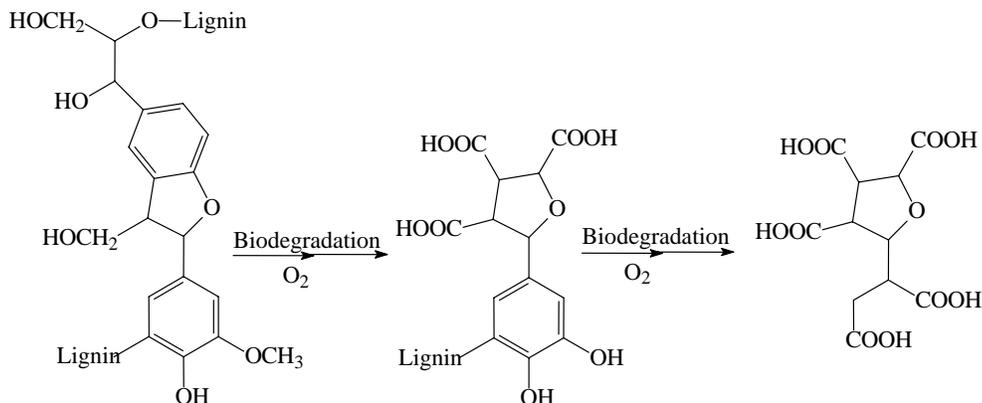
The fifth model represented another fraction from the pH-gradient fraction followed by the silica-gel fractionation of SRFA [33]. The selected fraction was exceptionally acidic (pKa1 = 0.5), and was composed of aliphatic structures with an average of five carboxyl groups per molecule [34]. The strong acidity was attributed to electrostatic field effects of clustered carboxylic acid groups on a tetrahydrofuran ring structure.

For the first time, a diagenetic reaction sequence was considered along with a lignin precursor in the derivation of this model presented below.

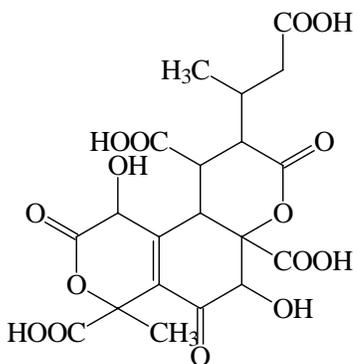


The sixth set of models was based on a study of precursors from which SRFA was derived [35]. The fractionation sequence was normal-phase fractionation on silica-gel followed by reverse-phase fractionation on XAD-8 resin. Characterization of the fractions by

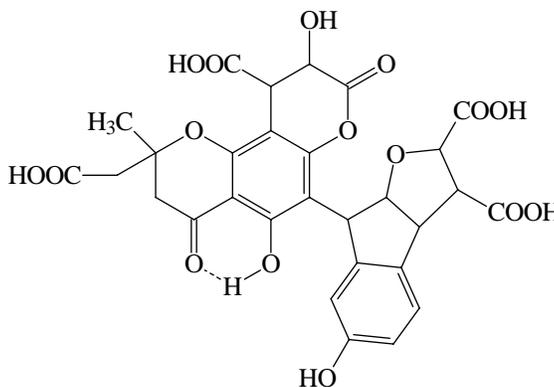
infrared, <sup>13</sup>C-NMR, and ESI/MS indicated that SRFA was primarily derived from terpenoid and tannin precursors with lignin precursors present in lesser amounts. Models of the tannin- and terpenoid-derived fulvic acids are presented below.



**The fifth model**



Terpenoid Fulvic Acid Model



Tannin Fulvic Acid Model

**The sixth set of models**

These models have recognizable structures that can be related back to parent terpenoids and tannins based upon plausible diagenetic reaction sequences.

The six models of SRFA presented in this section show progressive refinements in structural model approximations. The first model was based entirely upon quantitative chemistry data. Carboxylic acid group acidity and metal-binding properties were added

as analytical constraints in models 2 and 3. ESI/MS data was added in model 4, and organic precursor and diagenetic reaction sequences were added as model constraints in models 5 and 6. Several different fractionation sequences were developed based upon the objective of the study, and the decreased mixture heterogeneity of the fractions greatly improved the validity of the models.

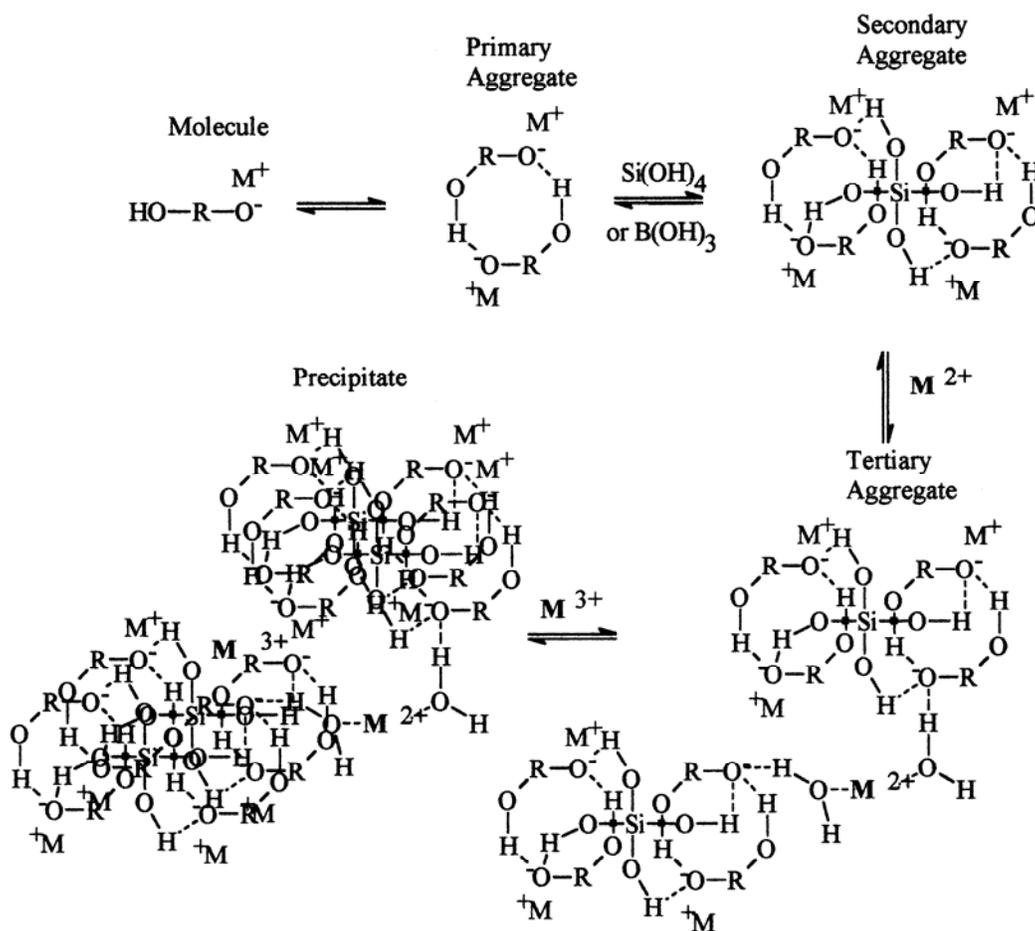
### 5. AGGREGATE, POLYMER, AND COMBINATORIAL MODELS

Different viewpoints exist on the molecular size, weight, shape and interaction of NOM molecules with themselves, with mineral constituents and with various contaminants in the environment. The older “polymer model” represents humic constituents of NOM as randomly coiled macromolecules that result from various chemical condensation and coupling reactions of smaller biomolecular constituents [36]. These coiled NOM macromolecules change their shape as a function of pH, ionic strength, and interactions with cations and minerals. A newer “aggregate model” view of humic constituents of NOM is that they are “relatively low molecular mass components forming dynamic associations stabilized by hydrophobic interactions and hydrogen bonds” [37]. The “combinatorial model” represents humic molecules as complex supramolecular assemblies of small molecules, oligomers, and polymers aggregated

into supramolecular assemblies by non-covalent forces [38]. This combinatorial model likely has the greatest applicability to NOM from various environments based on polydispersity measurements of NOM molecular sizes and weights by various methods.

In natural waters, soils and sediments, the NOM molecules (represented as HO-R-O<sup>-</sup> in the figure below) exist in equilibrium with aggregates and precipitates that form by hydrogen bonding, covalent bonding with silica and boron, and metal complexation.

The degree (number of molecules in the aggregate) of NOM aggregation depends on the inorganic chemistry of water and soil minerals. These NOM aggregates act as an amorphous phase in certain of its properties, such as organic contaminant sorption. The existence of these NOM aggregates in certain molecular weight measurements, such as size exclusion chromatography [39], explains differences in NOM molecular and size measurements by various methods.

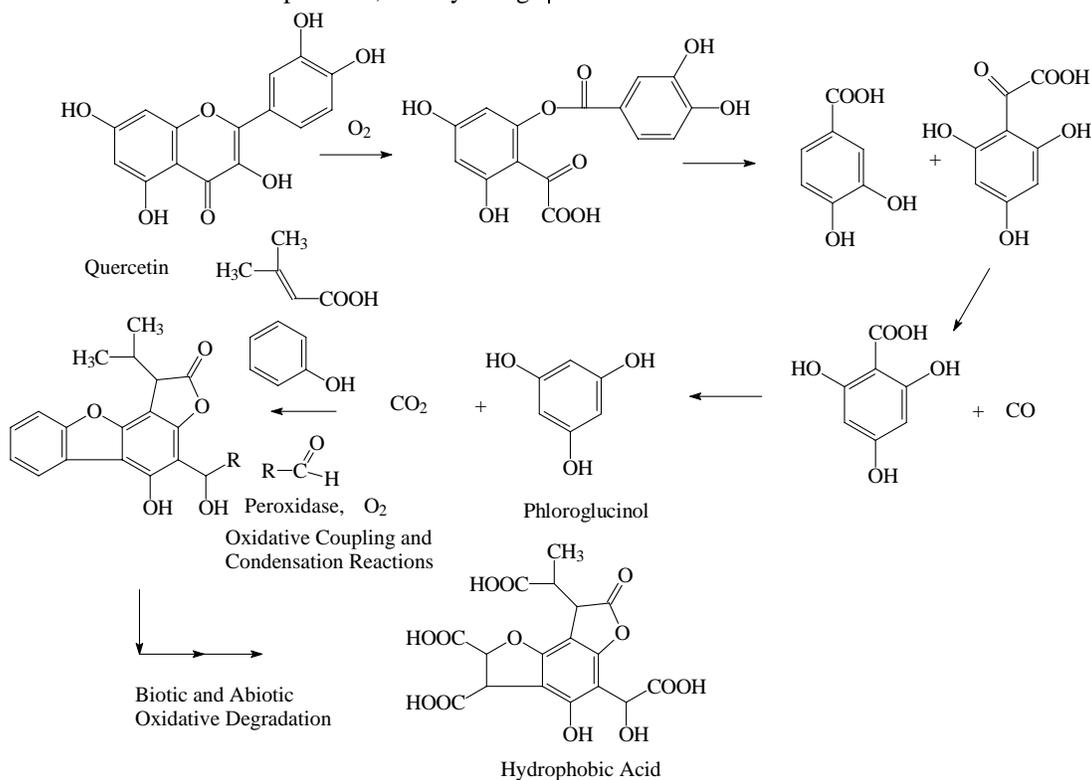


## 6. DISSOLVED ORGANIC MATTER DIAGENESIS MODELS

Dissolved organic matter (DOM) from a variety of surface-water and ground-water environments was comprehensively fractionated, isolated, and characterized [40]. A comprehensive assessment of precursors, diagenesis, and reactivity to water treatment of dissolved and colloidal organic matter was recently published [41]. Using average structural models of various DOM fractions as end products, and by using

natural products derived from terrestrial plants [42] and microbiota [43], diagenetic reaction pathways were constructed based upon biotic oxidation [44] and abiotic oxidation [45] pathways. Possible diagenetic pathways for fulvic acid derived from lignin were presented in the previous section.

Diagenesis of a hydrophobic acid DOM fraction (aquatic fulvic acid isolated on XAD-8 resin) from condensed tannins is shown by the models presented below.

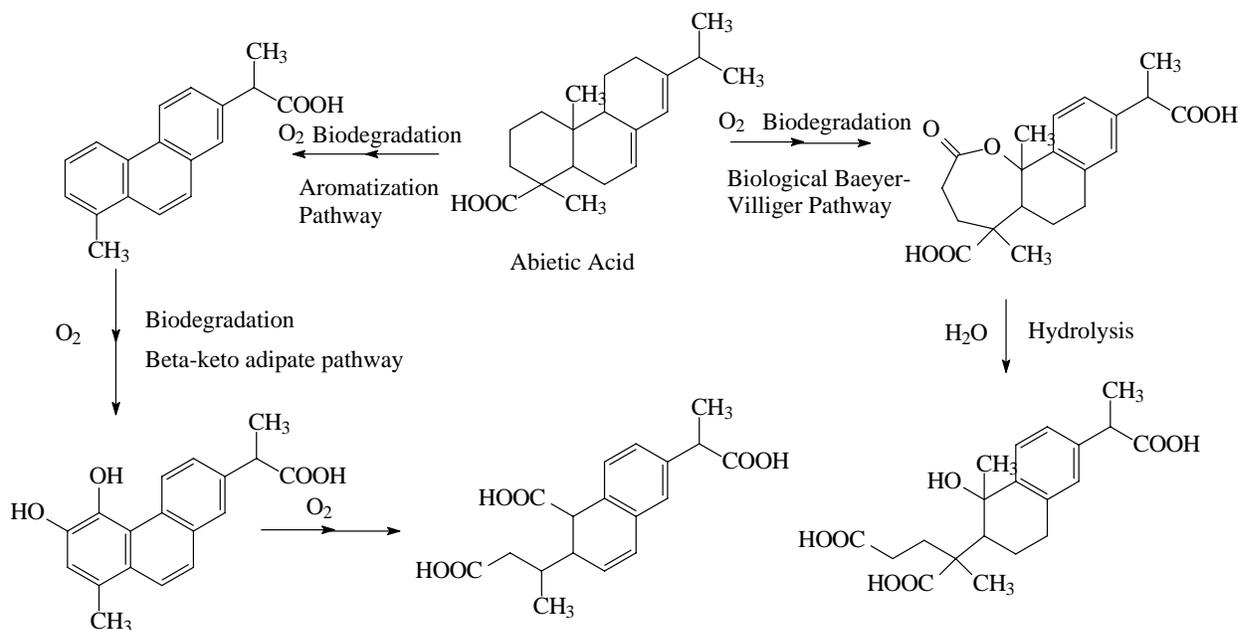


This model has a hydrophobic core that interacts with XAD-8 resin and a hydrophilic exterior of carboxylic acids and hydroxyl groups that render these acids water soluble. Condensed tannins and flavonoids are antioxidants that rapidly degrade to phloroglucinol and phenolic acids when exposed to molecular oxygen [45]. Phloroglucinol is a very reactive phenol that condenses with aldehydes and oxidatively couples with phenols and alkenes to form carbon- and oxygen-substituted structures to completely substitute the aromatic ring [42]. Lastly, biotic oxidation [44] of labile organic structures results in carboxyl groups accumulating on refractory-ring and branched-chain structures. This process leads to a metastable and water soluble hydrophobic acid component of DOM.

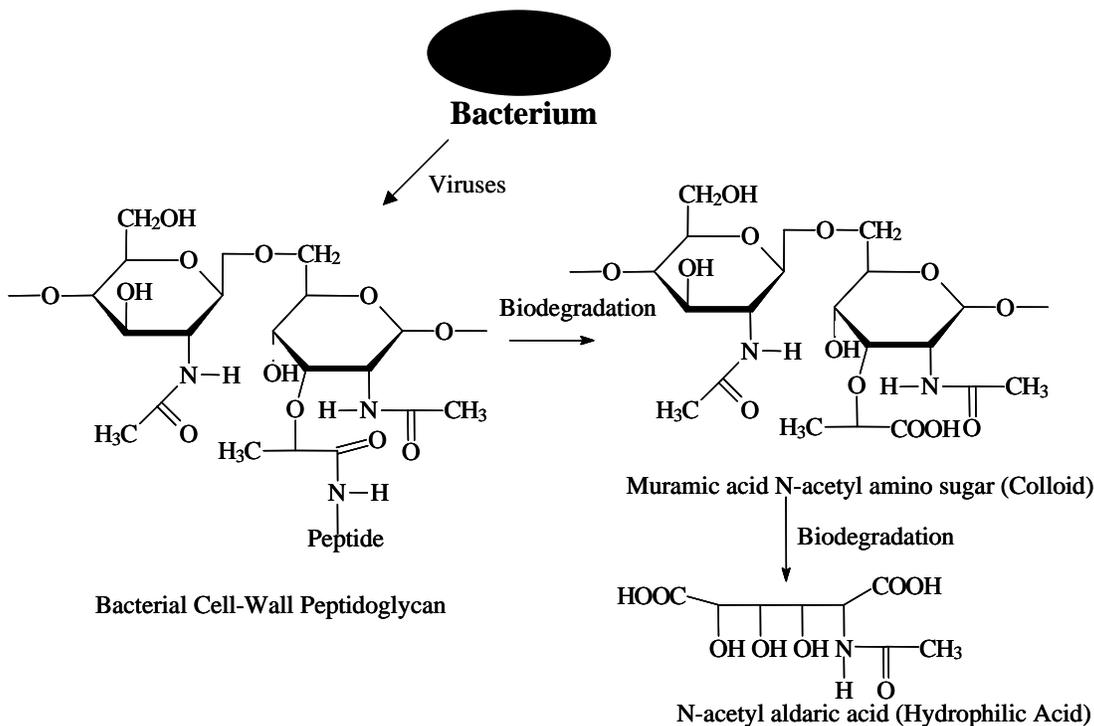
Diagenesis of a hydrophobic acid DOM fraction

from a terpenoid precursor, abietic acid, is shown by the models presented on the next page.

These models are based upon known metabolites from oxidative microbial degradation of aliphatic, alicyclic rings [46]. The aromatization pathway may explain a portion of the aromatic carbon structures observed by  $^{13}C$ -NMR spectra that cannot be readily explained by phenolic acids derived from lignins and tannins. Both pathways produce hydrophobic fulvic acid structures in which carboxylic acid groups are scattered around the perimeter of the rings. This scattered carboxylic acid group arrangement does not facilitate metal binding or adsorption on mineral sesquioxide coatings. Therefore, terpenoid derived DOM acids are not removed by coagulation/flocculation by iron(III) chloride or alum [21].



In certain surface-waters, bacterial cell wall precursors are quantitatively most important for DOM components [41]. Molecular models for DOM diagenesis from bacteria are shown below.



Bacterial cell-wall peptidoglycans contain peptides that strongly interact with mineral surfaces and become part of the soil and sediment humin and humic acid fractions [2, 47]. Peptidoglycans were found as major components of particulate (>1 micrometer size) and colloidal (3,500 Da to 1 micrometer size) organic matter in wastewater from a swine waste-retention basin [48]. When the peptide side chain is degraded from bacterial peptidoglycans, N-acetyl amino sugars are produced that are the major DOM component of the colloid fraction [40]. Further degradation of these amino sugars produces N-acetyl aldaric acids that are major components of the operationally defined hydrophilic acid DOM fraction, as was found in the Great Salt Lake [49].

## **7. APPROACHES FOR NOM MOLECULAR STRUCTURE DETERMINATION**

Determination of NOM components as specific molecular structures is beginning to be perceived as a tractable scientific endeavor. The molecular weight of primary molecules of NOM in soil, sediment and water is generally less than 2000 Da, and much of this NOM can be ionized and separated into molecules, and molecular formulas can be computed by ESI/ICRMS. The Chelsea soil humic acid has been modeled in 3-dimensions using quantitative experimental characterizations, computer assisted structure elucidation and atomistic simulations [50]. However, computer models of humic acids are limited by complex mixture characteristics, molecular formulas do not distinguish between isomers, and some NOM precursors such as condensed tannins and lignins are different isomers of various hydroxyphenylpropane reactants. Therefore, additional chromatographic and spectral information is needed for molecular characterization. The following are recommended steps to approach NOM molecular structure determination:

1. Select "end member" environments for NOM characterization. These environments have well-defined autochthonous or allochthonous inputs to better define precursors and have limited processes that degrade NOM. These environments should be selected to both bracket and constrain environmental variables for the sake of a comprehensive understanding and for simplification of complexity.

2. Develop improved NOM extraction, fractionation, isolation and purification methods. Much has already been done, but the finding that NOM molecules exist as aggregates held together by a combination of hydrogen bonds, metal complexes, and non-polar interactions means these interactions must

be eliminated to separate the molecules. Derivatization such as acetylation or methylation is effective in disrupting hydrogen bonds, and use of reagents (hydrofluoric acid, citric acid, ethylenediamine tetraacetic acid) that form stronger complexes with metals than NOM can remove complexes. A new fractionation method was successful in concentrating organic nitrogen constituents in certain fractions of NOM in a natural lake water, a wastewater effluent, and in algal and bacterial cultures [51]. Certain organic solvents and solvent mixtures are also effective in disrupting both polar and non-polar interactions, and they aid in extraction and molecular separations [52].

3. Couple preparative column chromatography with preparative high performance liquid chromatography (HPLC) with a universal detector such as refractive index to generate compound-class NOM fractions. This recommendation is very similar to the standard analytical approach used to separate petroleum hydrocarbons into compound classes before chromatographic and spectrometric analyses. Normal-phase chromatography of SRFA on silica-gel has already proved successful in separating terpenoid-derived fulvic acid from lignin and tannin-derived FA [35].

4. Obtain infrared and NMR spectral analyses on compound-class fractions. Both of these spectral analyses are non-destructive and can be performed on small samples, especially with high-field NMR spectrometers. Compound-class composition can be established by these spectral analyses, especially by multidimensional NMR methods. Complementary structural-group information is provided by comparing infrared and NMR spectra. Compound class molecular models can be derived to guide subsequent mass spectral studies.

5. Obtain both high resolution ESI/ICRMS and ESI/multistage tandem MS data on the compound-class fractions. High-resolution ESI/ICRMS data give elemental composition of specific NOM molecules. Coupling HPLC columns with a mass spectrometer is recommended to improve molecular separations before mass spectrometry. The MS methods need to be calibrated with internal standards to better correlate ionic fragments with molecular structure, and to calibrate variable response factors. The mass spectral data on compound-class fractions with known infrared and NMR spectral data will enable postulation of specific molecular structures.

6. Synthesize standards based upon selective postulated NOM molecules from step 4. Confirm these molecules by high resolution chromatographic and spectral methods. This final step is likely to be expensive and may require several attempts for

success; however, natural product chemists have experience in the approach outlined in this section, and they have separated and identified many complex molecules that exist in complex matrixes.

## 8. CONCLUSIONS

Molecular model approximations of NOM components are improving as new analytical techniques, and a better understanding of NOM precursors, diagenetic processes and NOM properties is applied to constrain these models. A paradigm shift has occurred in the perception of NOM, especially the humic component, from being a heterogeneous macromolecular substance that is impossible to characterize at the molecular level, to being an aggregate of small- to medium-sized molecules that is potentially tractable to characterization by advanced chromatographic and spectral methods. In general, the analytical evidence as synthesized by NOM models indicates NOM degrades from high-molecular weight biopolymers to low-molecular weight molecules. A better understanding of NOM molecular structures will lead to better understanding of various biogeochemical processes that produce NOM. This understanding can be applied to engineered systems such as water treatment and constructed wetlands.

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