

# Characterizing *Dissolved*



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# *Aquatic* **ORGANIC MATTER**



**Understanding the  
unknown structures is  
key to better treatment  
of drinking water.**

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**W**hether it causes aesthetic concerns such as color, taste, and odor; leads to the binding and transport of organic and inorganic contaminants; produces undesirable disinfection byproducts; provides sources and sinks for carbon; or mediates photochemical processes, the nature and properties of dissolved organic matter (DOM) in water are topics of significant environmental interest. DOM is also a major reactant in and product of biogeochemical processes in which the material serves as a carbon and energy source for biota and controls levels of dissolved oxygen, nitrogen, phosphorus, sulfur, numerous trace metals, and acidity.

DOM and soil humus have similar chemistries (1). Indeed, operationally defined humic substances typically compose about 50% of the DOM of an average river (2). However, DOM concentration, composition, and chemistry are highly variable and depend on the sources of organic matter (allochthonous versus autochthonous); on the temperature, ionic strength, pH, major cation composition of the water; on the surface chemistry of sediment sorbents that act as solubility controls; and on the presence of photolytic and microbiological degradation processes.

This article will discuss specific advances in determining the generally unknown molecular structures of DOM. It provides an overview of aquatic DOM characterization studies, primarily in freshwater,

where the greatest percentage of the organic material has been characterized at the compound class level.

### Defining organic matter

DOM can range in molecular weight from a few hundred to 100,000 daltons (Da), which is in the colloidal size range. Humic substances have been regarded as macromolecular, but recent studies of aqueous humic extracts from soil (3), lignite (4), and water (5, 6) found relatively small primary molecular structures (100–2000 Da) with macromolecular characteristics resulting from aggregates formed by hydrogen bonding, non-polar interactions, and polyvalent cation interactions. DOM is a complex mixture of aromatic and aliphatic hydrocarbon structures that have attached amide, carbonyl, hydroxyl, ketone, and various minor functional groups. Heterogeneous molecular aggregates in natural waters increase DOM complexity.

Certain investigators have concluded that humic substances cannot be characterized at the molecular level (7). However, recent findings that show small primary structures compose the majority of DOM raise molecular characterization to a tractable problem. DOM research can be divided into two categories: whole water studies, in which DOM is characterized in water and its inorganic constituents, and studies of DOM fractions isolated from water and inorganic constituents.

### Whole water characterizations

**TOC, DOC, and BOM analyses.** Total organic carbon (TOC) is the most comprehensive measurement used to quantify the presence of organic matter (OM) in aquatic systems. TOC is often synonymous with natural organic matter (NOM) because organic contaminants in natural systems generally represent an insignificant fraction of the TOC.

The first characterization of NOM can be based on the subdivision of TOC into operationally defined fractions such as dissolved organic carbon (DOC) and particulate organic carbon (POC). POC is the fraction of the TOC that is retained on a 0.45-micrometer ( $\mu\text{m}$ ) porosity membrane. DOC is the organic carbon smaller than 0.45  $\mu\text{m}$  in diameter. POC generally represents a minor fraction (below 10%) of the TOC, according to Thurman (2). The proportion of POC increases with a river's size and flow rate. DOC con-



**DOM is a complex mixture of aromatic and aliphatic hydrocarbon structures that have attached functional groups.**

centrations range from 0.1 milligrams per liter (mg/L) in groundwater to 50 mg/L in bogs (2). DOC concentrations not only depend on the nature of the watershed, but are also influenced by seasonal variations and POC inputs such as runoff or algae bloom. Most of the NOM is considered to be refractory to rapid biodegradation. Biodegradable organic matter (BOM) can be measured based on operationally defined protocols, such as biodegradation over a given time. BOM is expressed as biodegradable dissolved organic carbon (BDOC) or assimilable organic carbon (AOC) that results in biomass production, depending on the analytical protocol used. Surveys conducted on rivers in the United States and France showed that the BDOC

content ranged from a few to about 40% (8). The BDOC content of rivers varies with the origin of the NOM. Autochthonous NOM, which is produced from macrophytes, algae, and bacteria, is more biodegradable than allochthonous NOM, which has a pedogenic origin. Chemical and biological treatments applied to drinking water production significantly increase or decrease the biodegradable fraction of NOM, depending on the treatment (9, 10).

**DOM profiling by resin sorbents.** DOM is commonly characterized by fractionating it into distinct categories with resin sorbents. A protocol with XAD-8 resin has been widely used to isolate humic substances (humic and fulvic acids [11]) and is the basis of a simple DOC analysis that determines the so-called humic/nonhumic distribution (DOC profiling) of raw and treated waters. Another fractionation approach requires a serial two-column array of resins (12), in which the nonhumic DOC fraction is operationally defined as "transphilic DOC". Two small, serial resin columns requiring only a few hundred milliliters of water have been used to determine DOC distribution between operational categories based on polarity (13, 14). The terms "hydrophobic", "hydrophilic", "acidic", "basic", and "neutral" refer to the predominant property of a DOM fraction while recognizing that many DOM molecules are both amphiphilic and amphoteric. A third anionic exchange resin column can further fractionate the hydrophilic DOC into charged and neutral DOC (15). In addition, spectrophotometric measurements, such as ultraviolet (UV) absorbance



and fluorescence measurements, can complement DOC measurement in DOM profiling studies. Examples of DOM profiling of selected waters are in the Supporting Information.

**Spectrophotometric analysis.** Surface water absorption of both visible and UV light is widely attributed to the aromatic chromophores present in NOM molecules—primarily humics—dissolved in the water. Humic-type molecules are also thought to be largely responsible for the fluorescence observed in natural waters.

As shown by numerous studies, UV-visible spectra of NOM are typically broad and nearly featureless because the number of possible types of chromophores is large and none possess an easily distinguishable spectrum. Several UV-visible absorbance ratios have been proposed to characterize the spectrometric profile of NOM in soil. However, most aquatic research has limited data collection to the absorbance at 254 nm, which serves as a rough indicator of overall NOM concentration.

Specific UV absorbance (SUVA or  $SUVA_{254}$ ) is defined as the sample's UV absorbance at 254 nm divided by the DOC concentration of the solution. Carbon-13 nuclear magnetic resonance ( $^{13}C$ -NMR) was used to determine a strong correlation between SUVA and the aromatic carbon contents of a large number of NOM fractions (16).

High SUVA waters are generally enriched in hydrophobic NOM, such as humic substances. Therefore, SUVA indicates aromatic compounds in the DOC and can be used to estimate the chemical nature of the DOC at a given location. The water industry also uses SUVA as a surrogate parameter to monitor sites for disinfection byproducts precursors (17). It should be noted, however, that high nitrate content in low DOC waters may interfere with this measurement. Additional examples of UV relationships with various parameters are given in the Supporting Information.

In addition to chromophores, fluorophores are associated with the humic portion of NOM. Coumarin photo-oxidation products from catechol degradation products of lignin were proposed as long-wavelength fluorophores of freshwater DOM (18). More recently, quinone moieties resulting from phenol oxidation were found to contribute significantly to the fluorescence of humic substances extracted from marine sediments (19). Two distinct classes of fluorophores are generally discussed, the humiclike fluorophores and the proteinlike fluorophores. For example, Coble referred to two humiclike fluorophores in addition to the specific marine humiclike fluorophore and one or two proteinlike fluorophores (tyrosine and tryptophan-like), depending on the origin of the water samples (Table 1) (20).

Although less than a 1% of the aromatic moieties in NOM actually emit light as fluorophores (21), three-dimensional fluorescence spectroscopy is an attractive analytical tool because it is at least an order of magnitude more sensitive to NOM than UV absorbance. Examples of various two-dimensional fluorescence spectra and their applications to various waters are given in the Supporting Information.

**Size characterization of NOM.** Methods to characterize NOM by size are highly dependent on their aggregation state and interactions with media used for size separations. Sequential ultrafiltration is used for low-resolution separations and size exclusion chromatography (SEC) is used for higher-resolution size separations.

A cascade of ultrafiltration membranes was used to characterize DOC by size with the understanding that the apparent molecular weight distributions are strongly influenced by a number of parameters, such as pH, ionic strength, membrane type, pressure, and calibration standards (22, 23). A recent study that compared sequential ultrafiltration with SEC found that neither technique gave absolute measures of molecular weight of NOM (24).

SEC is a fractionation method based on the dimensions of molecular or particle size, which means the largest molecules have the shortest retention times. UV detectors are favored for high-performance liquid chromatography (HPLC)/SEC characterization because NOM components strongly absorb in the UV and the appropriate detectors are commercially available.

Chin et al. made a major contribution to estimating NOM molecular size by this approach in presenting data for organic matter isolates and whole water samples that were size-fractionated by HPLC/SEC with UV detection and vapor pressure osmometry (a colligative property measurement) (25). There was agreement within a factor of 2 between the results obtained by the two methods for a given sample, although the SEC results had higher values. They optimized the SEC technique by suppressing charge effects with a 0.1-molar solution of sodium chloride in the water mobile phase and by using polystyrene

**TABLE 1**

### Major fluorescent components in excitation–emission matrix

According to fragments from pyrolysis studies, humic-type molecules are found in dissolved organic material and may partially explain the fluorescence of natural waters.

Range of excitation (nm)	Range of emission (nm)	Component type
330–350	420–480	Humiclike
250–260	380–480	Humiclike
310–320	380–420	Marine humiclike
270–280	300–320	Tyrosine-like, proteinlike
270–280	320–350	Tryptophan-like, proteinlike or phenol-like

sulfonates as calibration standards. The values obtained for dissolved NOM ranged from several hundred to a few thousand Daltons. In general, the molar absorptivity at 280 nm (25) or SUVA (26) were found to correlate well with both apparent molecular weight and aromaticity of the aquatic NOM isolates.

Recently, Piccolo et al. found that additional decreases in apparent molecular weight estimates (of a

lignite humic acid) by HPLC/SEC could be obtained by adding acetic acid to the water mobile phase at pH 3.5 (4). They attributed this decrease to disruption of molecular humic aggregates by acetic acid.

A problem associated with the use of UV detection with SEC is the low response it provides for NOM structures with low-UV molecular absorptivities, such

composition products. On-line pyrolysis performed in a pyroprobe is preferred to off-line pyrolysis because it requires only a few milligrams of lyophilized or freeze-dried NOM. Pyrolysis gas chromatography/mass spectrometry (GC/MS) has better separation than pyrolysis-MS techniques.

The presence of inorganic salts in samples isolated from natural waters has little effect on the fragmentation of the organic matrix. Consequently, simple concentration techniques, such as reverse osmosis and rotary evaporation, can be used to collect bulk NOM samples for pyrolysis GC/MS characterization.

The advantage of the pyrolysis approach is that natural biopolymers, which represent the bulk of NOM precursors, can be clearly identified because they yield very specific fragments with few interferences among the biopolymers. Bruchet assigned which NOM biopolymers are represented by the most predominant pyrolysis byproducts (31). Table 2 lists major pyrolysis fragments most commonly encountered in pyrochromatograms from aquatic NOM.

Independent of the method of isolation, NOM from different origins led to similar subunits under pyrolysis; however, the percentage of these structural subunits varies significantly among samples, which provides a specific fingerprint for a select organic material. The peak areas of select pyrochromatogram byproducts can also be used to estimate the relative proportion of the major biopolymers in the original organic matrix (32). The variability of the biopolymer distribution of NOM fractions determined by the pyrolysis approach agreed with the structural differences inferred from other analytical techniques, such as <sup>13</sup>C NMR, elemental analysis, and specific component analyses. However, because of the difference in the response factor of the biopolymers during the thermal stress (biopolymers and structural subunits behave energetically different during thermal stress), the fingerprint deduced from the pyrochromatogram probably does not reflect the exact relative proportions of the main types of biopolymers in the original sample. Degradation of NOM biopolymers by environmental processes also obscures the assignments of NOMs by pyrochromatogram fingerprints.

It has been demonstrated that benzenecarboxylic moieties undergo decarboxylation during conventional pyrolysis. During the past 10 years, new pyrolytic approaches using alkylating agents, such as tetraalkyl ammonium hydroxide, have been used to overcome this significant limitation and improve the detection of polar compounds (33). Because it appears that both hydrolysis and alkylation mechanisms occur at high temperatures in the presence of alkylated agents, such as tetramethyl ammonium hydroxide (TMAH), the procedure has been termed thermally assisted hydrolysis and methylation or thermochemolysis. Most of these studies have focused on humic substances.

Flash heating in the presence of tetraalkyl ammonium hydroxide avoids decarboxylation and releases carboxylic groups in aliphatic and aromatic structures. Following previous findings (34, 35), González-Vila et al. pointed out that pyrolysis TMAH of aquatic

**TABLE 2**

## Specific pyrolysis fragments of biopolymers

The following are the predominant pyrolysis byproducts from aquatic NOM.

Type	Common pyrolysis fragments
Polysaccharides	Methylfuran, furfural, acetylfuran, methylfurfural, levoglucosenone, hydroxypropanone, cyclopentenone, methylcyclopentenone, acetic acid
Aminosugars	Acetamide, <i>N</i> -methylacetamide, propionamide, acetic acid
Proteins	Acetonitrile, benzonitrile, phenylacetonitrile, pyridine, methylpyridine, pyrrole, methylpyrrole, indole, methylindole (from tryptophan), toluene, styrene (from phenylalanine), phenol, <i>p</i> -cresol (from tyrosine)
Polyphenolic compounds	Phenol; <i>o</i> -, <i>m</i> -, <i>p</i> -cresol; methylphenols; dimethylphenols
Lignins	Methoxyphenols
Tannins	Catechol
DNA	Furfuryl alcohol
Polyhydroxybutyrates	Butenoic acid

Source: Data from Reference (31).

as proteins, sugars, aminosugars, and aliphatic acids, which are ubiquitous components of aquatic NOM. However, HPLC/SEC with UV and on-line DOC detectors has been adapted and optimized during the past decade (27–29). Because both aromatic and nonaromatic NOM moieties in the chromatographic fractions are detected, this analytical tool provides a better understanding of the qualitative and quantitative NOM properties in natural and treated water samples without a preconcentration step (27–29).

Hesse and Frimmel recently created a similar system (HPLC/SEC) coupled with a dissolved organic nitrogen analyzer for the analyses of wastewaters (30). The results may determine the total organic nitrogen content of natural waters because organic nitrogen can be separated from inorganic nitrogen by SEC. Examples of HPLC/SEC of various waters are shown in the Supporting Information.

**Pyrolysis GC/MS.** Following rapid heating to a high temperature, natural and synthetic biopolymers are degraded into low-molecular-weight thermal de-

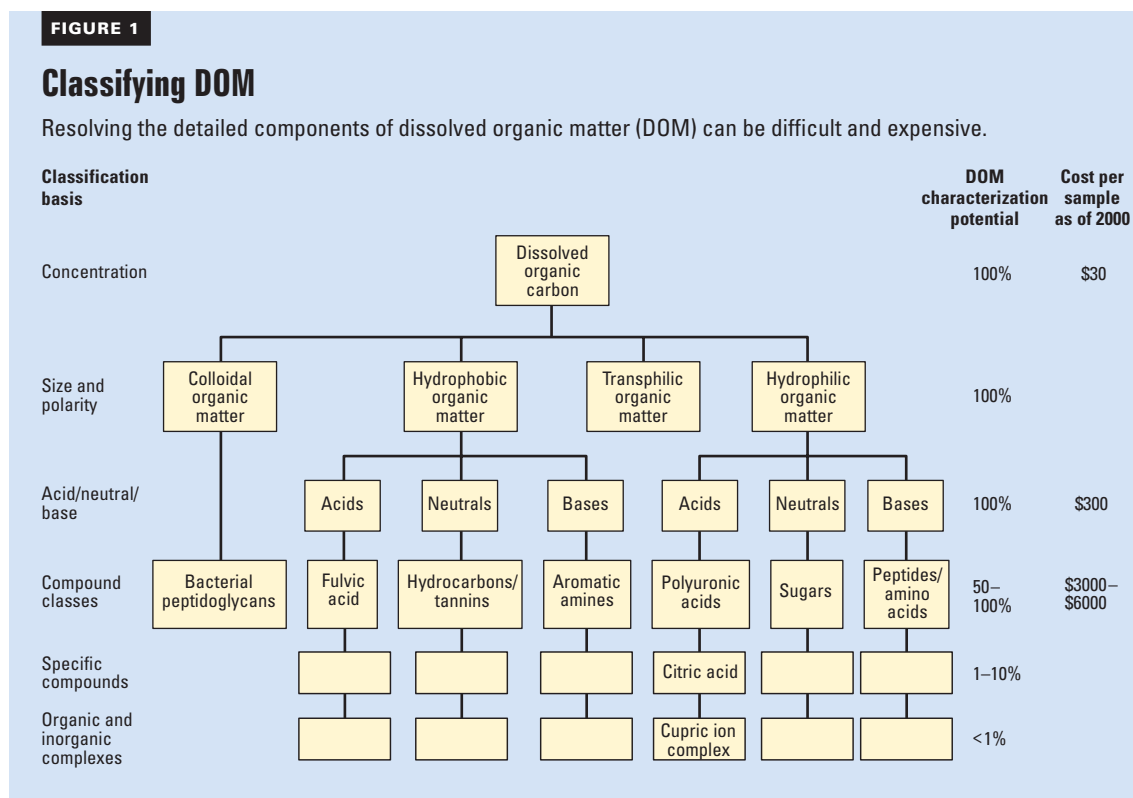
humic substances provides information on the presence of lignin markers and allows the identification of dicarboxylic acids (36). One drawback of the TMAH technique is that it does not differentiate between naturally occurring methyl ether or methyl esters and those formed during thermochemolysis. The use of tetrabutyl ammonium hydroxide made that differentiation possible. Using different ammonium salts (e.g., hydroxide and acetate forms), it is now possible to estimate the free, esterified, and total acid content (36).

### Fractionation, isolation, and characterization

A comprehensive, tiered approach to DOM characterization was initially developed as a DOC fractionation method that classified dissolved organics

brane. The dialysis permeate is passed through columns in series containing cation exchange resin, Amberlite XAD-8, and Amberlite XAD-4 to isolate hydrophilic base, hydrophobic acid, and transphilic acid/neutral DOM fractions. Those hydrophilic DOM fractions that do not adsorb on these sorbents are desalted from the sample by selective precipitation and evaporation methods, allowing the hydrophilic acid fraction to be separated from the hydrophilic neutral fraction by selective adsorption/desorption on a Duolite A-7 anion exchange resin. DOM fractions are frequently coisolated depending on the objectives of the study.

The costs for these types of analyses are listed in Figure 1 and are based on \$30 for a replicate set of DOC determinations, \$300 for an analytical-scale



based on their polarity (hydrophobic/hydrophilic), acid/neutral/base properties, compound-class characteristics, specific compound characteristics, and compound complex characteristics (37). This fractionation approach was improved into a preparative fractionation method in which DOM fractions can be quantitatively isolated as desalted, freeze-dried preparations and subsequently characterized (38). This preparative fractionation method led to the isolation of bacterial cell-wall peptidoglycan colloids that constitute significant percentages of DOC in most surface water samples. The DOM classification scheme is presented in Figure 1.

These DOM fractions are operationally defined. For a previously filtered water sample, the hydrophobic base and neutral fractions are first isolated by selective sorption/desorption on Amberlite XAD-8 resin. Colloids are next isolated by dialysis of the evaporated concentrate through a 3500-Da mem-

brane. The dialysis permeate is passed through columns in series containing cation exchange resin, Amberlite XAD-8, and Amberlite XAD-4 to isolate hydrophilic base, hydrophobic acid, and transphilic acid/neutral DOM fractions. Those hydrophilic DOM fractions that do not adsorb on these sorbents are desalted from the sample by selective precipitation and evaporation methods, allowing the hydrophilic acid fraction to be separated from the hydrophilic neutral fraction by selective adsorption/desorption on a Duolite A-7 anion exchange resin. DOM fractions are frequently coisolated depending on the objectives of the study.

As indicated in Figure 1, specific compound characterization of natural DOM typically can identify only 1–10% of the DOM. Mixture complexity and molecular complexity have defeated previous attempts to resolve natural DOM into discrete components with high-resolution chromatography. However, multistage mass spectrometry coupled with electrospray ionization is showing promise in giving molecular-level structural information about DOM components (6).

FIGURE 2

## Analyzing Suwannee River fulvic acid

(a) This full negative ion electrospray/ion trap mass spectrum is complex. The following multiple mass spectra were acquired: (b) MS/MS at mass-to-charge ( $m/z$ ) 329 ion, (c) MS/MS/MS at  $m/z$  311 ion [ $M-H_2O-H$ ], and (d) MS/MS/MS/MS at  $m/z$  293 ion [ $M-2H_2O-H$ ].

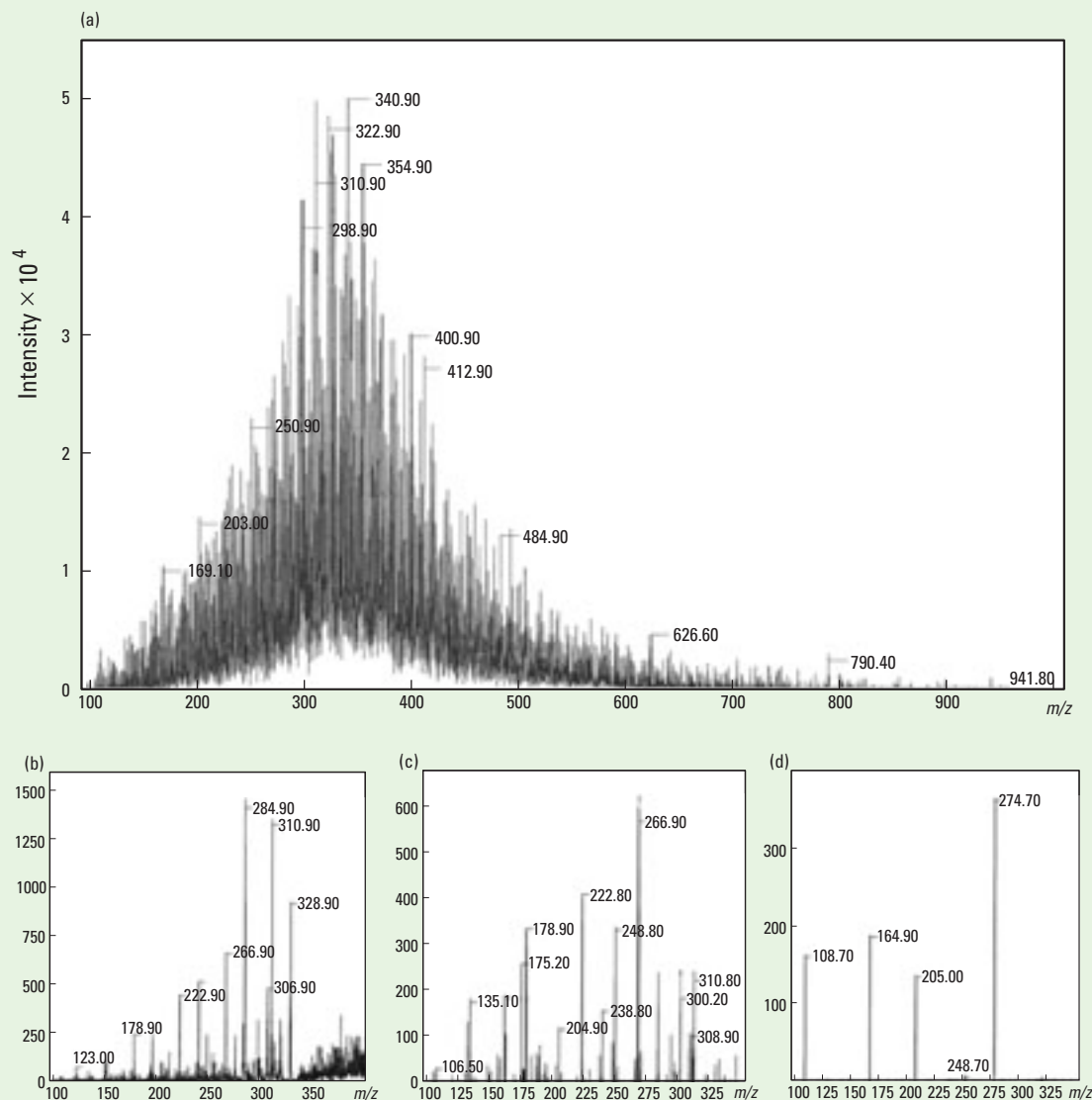


Figure 2 presents multistage mass spectra of Suwannee River fulvic acid. Figure 2a is a mixture of singly and multiply charged ions, as well as molecular aggregates and fragments of the original molecular species. Therefore, it cannot be regarded as a molecular-weight distribution. This mixture complexity in Figure 2a can be resolved into multiple mass spectra shown in Figures 2b–d. The spectrum in Figure 2b is still a complex mixture, but further simplification in Figure 2c suggests a single precursor compound can be postulated from the hypothetical fragmentation pathway shown in Figure 3.

The fragmentation pathway is based on multiple mass spectrometric studies of model compounds with similar structures. Confirming parent DOM structures and fragmentation pathways depends on synthesizing the parent structure for confirmatory mass spec-

tra. Synthesis is a costly process, so the major value of multiple mass spectral studies of DOM is in postulating structures that yield additional insights into the nature and properties of DOM. According to Figure 1, the most specific level of DOM characterization is the structural determination of its inorganic and organic complexes. The nature and measurements of these complexes are given in the Supporting Information.

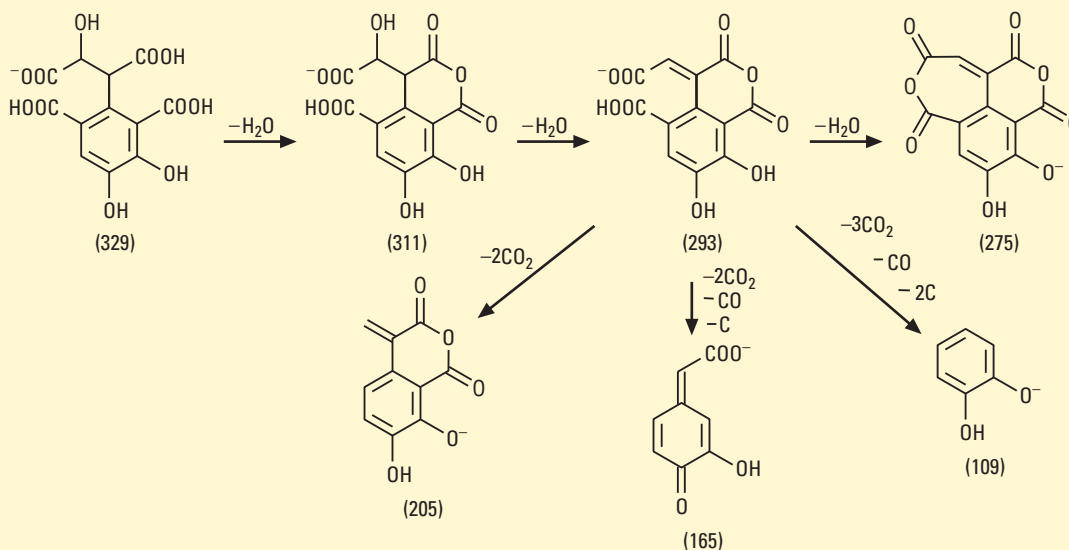
### Research challenges

Extending DOM characterization from the compound-class level to the specific compound level and synthesizing standards are the present research challenges. Molecular characterization of DOM will give specific information about precursors (lignins, tannins, terpenoids, proteins, amino sugars), the processes that produce DOM (biodegradation, con-

FIGURE 3

## Fragmentation pathway

This scheme describes a hypothetical parent compound for dissolved organic matter based on mass spectrometric results from Figure 2. Using mass spectral results is much less expensive than relying on synthesis. Molecular masses are in parentheses.



densation reactions, photolysis), and reactive structures in DOM (disinfection byproduct [DBP] precursors, metal-binding sites, surfactant structures). Anthropogenic components of DOM, such as polycarboxylic acid metabolites of various surfactants in wastewater, can also be distinguished from NOM at the molecular level.

The costs associated with the compound-class characterizations presented in Figure 2 are too expensive for DOM monitoring, but continued advances in pyrolysis/GC/MS and thermochemolysis/GC/MS have the potential for inexpensive characterization. To be relevant and comprehensive, these techniques need to be calibrated with model compounds that are closely related to DOM structures. Thus, the information from expensive DOM fractionations with spectral characterizations transfers to calibration and interpretations of inexpensive methods.

Understanding the structural chemistry of these hydrophilic DOM components, and especially the dissolved organic nitrogen (DON), can be useful in designing new water treatment processes to remove these components from drinking water. It has been generally accepted that hydrophobic DOM represents the major source of DBP precursor sites, but certain hydrophilic DOM components not removed by conventional water treatment have been found to react with disinfecting agents to produce undesirable DBPs (40). There is some evidence that the nitrogen-rich constituents (e.g., proteinaceous-type structures) represent an important class of the problematic hydrophilic NOM fraction. Macromolecular polysaccharide components of DOM also clog membranes used in water treatment. HPLC/SEC technology with multiple detectors (UV, fluorescence, DOC, and DON) may be used in combination with advanced characterization tools (electrospray/MS, pyrolysis GC/MS) to obtain detailed

structural information that will result in a better understanding of NOM structure and reactivity.

These are a few of the research challenges; there are undoubtedly more. DOM research is rapidly advancing with many exciting and useful applications. As the need for water resources and recycling continues to increase, so will the need for understanding the nature and properties of DOM.

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*Note:* Supporting information is available on the Web at <http://pubs.acs.org/est>.