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Review

# The molecularly-uncharacterized component of nonliving organic matter in natural environments<sup>☆</sup>

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## Abstract

Molecularly-uncharacterized organic matter comprises most reduced carbon in soils, sediments and natural waters. The origins, reactions and fates of these ubiquitous materials are relatively obscure, in large part because the rich vein of geochemical information that typically derives from detailed structural and stereochemical analysis is yet to be tapped. This discussion highlights current knowledge about the origins and characteristics of molecularly uncharacterized organic matter in the environment and outlines possible means by which this structurally uncharted frontier might best be explored. © 2000 Elsevier Science Ltd. All rights reserved.

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<sup>☆</sup> On 21 June 1999, 12 scientists met at the Hanse Wissenschaftskolleg in Delmenhorst, Germany, to discuss how the molecularly uncharacterized component of the Earth's organic matter reservoirs might be better studied. In addition to analytical and organic chemists, the participants of this first "Hanse Round Table" included scientists with expertise in petrology, microbiology, archaeology and petroleum geochemistry. Their deliberations helped identify the major conceptual gaps and potentially rewarding research strategies highlighted in the following discussion.

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## 1. Introduction

Organic matter is a thermodynamic anomaly atop a free energy precipice that drops off on all sides to dispersed, stable ingredients such as carbon dioxide, water, nitrate and phosphate. Given the high free energy yield when electrons are passed from organic matter to molecular oxygen, nitrate, transition metal ions and sulfate, it is little surprise that only one or two carbons out of one thousand in organic molecules ultimately escape oxidation to be preserved in marine sediments (Berner, 1989). Nevertheless, nonliving organic molecules persist in essentially all natural environments, where on average they greatly outweigh biochemicals in the living organisms from which they derive. On a global basis (Hedges and Keil, 1995), more organic matter occurs in soil humus ( $1600 \times 10^{15}$  gC), recently deposited marine sediments ( $1000 \times 10^{15}$  gC) and dissolved in seawater ( $700 \times 10^{15}$  gC), than in all land plants ( $600 \times 10^{15}$  gC) and marine organisms ( $3 \times 10^{15}$  gC) combined. These molecular survivors play immensely important roles in the natural world, including involvement in such key processes as modulating temperatures at the globe's surface, weathering rocks to soils, complexing toxic metals, and composing precursors for eventual formation of coal and petroleum. Organic compounds embedded in marine sediments and paleosols also provide exquisitely detailed records of natural history, even where macroscopic physical fossils are rare (Engel and Macko, 1993) or absent (as in petroleum).

Growing recognition of the critical environmental roles and information potential of organic molecules has given birth to a branch of biogeochemical study which focuses on the short- and long-term fates of organic substances following death of the source organisms. The foundations of this line of research were established in the early part of the 20th century when Waksman (1936) and other pioneers recognized that organic matter in soils is a complex mixture of substances formed largely by microbial degradation of plant tissues. Since the demonstration by Treibs (1934) that petroleum and ancient shales contain organic molecules retaining the unmistakable tetrapyrrole structure derived from chlorophyll pigments, molecular-level analysis has been a mainstay of biogeochemical studies. The basis of such research has been chromatographic separation of the small (typically  $<1000$  amu) molecules directly extracted, or chemically released, from environmental mixtures. This step is often followed by characterization of the distinctive fragmentation patterns these extracts produce in mass spectrometers. This general strategy rests on the fact that microorganisms, plants and animals synthesize an extremely small subset of the billions of molecules that can be assembled from all possible covalent combinations of C, H, O, N, S and P. In addition, many structurally unique biochemicals are pro-

duced only within specific organisms and/or environmental settings, and hence can be used to trace the remains of these different sources through space, time and considerable degradative alteration. Such molecular "biomarkers" also carry embedded information in the unique stereochemical "handedness" of their atoms in space and in the ratios of the stable and radioactive isotopes they contain. Importantly, detailed knowledge of the structure of an organic molecule allows strong inferences to be drawn about the types of reactions it can undergo, which are often dauntingly complex and difficult to observe directly in natural environments.

In spite of over half a century of effort and rapidly increasing analytical sophistication, more than half of all the organic matter in soils, sediments, and seawater still remains uncharacterized at the molecular level. For example, a recent survey of over 100 amino acids, sugars and lipids in the water column of the central Pacific Ocean (Wakeham et al., 1997) left  $\sim 15\%$  of the molecules composing plankton unidentified and missed greater than 75% of the organic molecules in particulate debris raining in a matter of days to the ocean floor (Fig. 1). Similarly large fractions of the organic constituents of soil humus (Stevenson, 1994), organic matter dissolved in seawater (Williams and Druffel, 1988) and wastewater treatment effluents (Dignac et al., 2000) remain to be identified. Although broad structural features of the complex mixtures composing these huge carbon reservoirs can be inferred from "bulk" elemental (CHNOS) and spectral (IR and NMR) analyses, these average characterizations carry a miniscule fraction of the geochemical information that might be gleaned from knowledge of the detailed structures of the component molecules. Biogeochemists of today are playing with an extremely incomplete deck of surviving molecules, among which most of the trump cards that molecular knowledge would supply remain masked.

## 2. Sources and formation pathways

Identifying the origin of the molecularly uncharacterized component (MUC) of organic matter in natural environments is fundamental to understanding its subsequent distribution and reactions. MUC was once assumed to be formed primarily by spontaneous "heteropolycondensation" reactions among small reactive intermediates released during enzymatic breakdown of biomacromolecules (Tissot and Welte, 1978; Hedges, 1988). Such "humification" theories are based on the observation that many simple biochemicals (e.g. amino acids, phenols and sugars) abiotically condense (especially at high concentrations and temperatures) to produce extremely complex assemblages of molecules that exhibit the brown color and many of the physicochemical

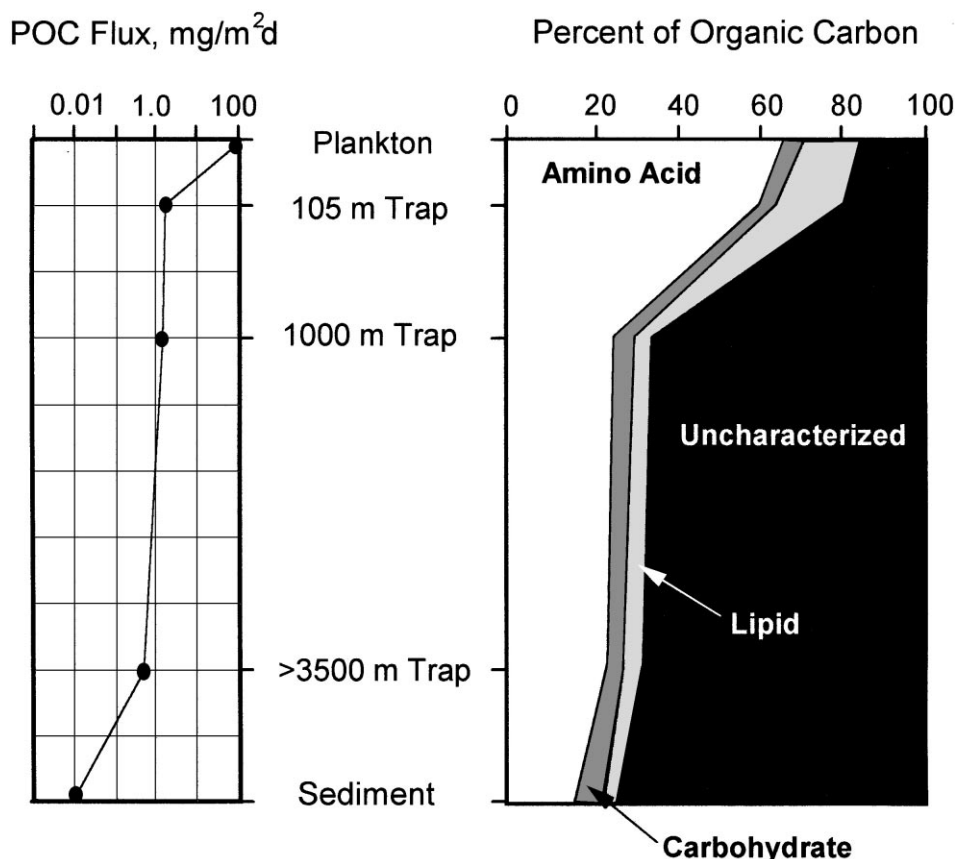


Fig. 1. Particulate organic carbon (POC) fluxes and corresponding fractions of amino acid, carbohydrate, lipid and molecularly uncharacterized organic carbon in plankton, sediment trap and sediment samples from the Equatorial Pacific Ocean (after Wakeham et al., 1997). The fraction of molecularly uncharacterized organic carbon increases with more extensive degradation to become the major constituent in deeper POC samples.

properties of sedimentary and soil organic matter. These condensations include Maillard (or “browning”) reactions between carbohydrates and amino acids (or proteins) that form dark, often aromatic, “melanoidins,” as occurs during baking, maple syrup production and within foodstuffs from archaeological sites (Hoering, 1973; Evershed et al., 1997). Artificial humic substances, even those produced from well-defined precursors, also resemble natural counterparts in defying essentially all attempts at chromatographic separation and structural definition. However, many analytical tools necessary to specifically test different proposed humification pathways and products were simply not available more than 20 years ago when the theories for heteropolycondensation reactions were introduced.

A recent shift in paradigm away from humic substances has been driven by a variety of techniques and observations. One impetus for change has been that conventional solubility-based fractions of organic matter, such as fulvic acid, humic acid and residual humin, cannot be related to microbial turnover rates as directly

as fractions that are physically isolated based on size or density characteristics (Christensen, 1996; Hedges and Oades, 1997). Thus physical form appears to be at least as important as chemical state (as reflected by solubility behavior) in determining the reactivity of natural organic matter. In addition, “solids” nuclear magnetic resonance (NMR) analyses of the chemical characteristics of <sup>1</sup>H, <sup>13</sup>C and <sup>15</sup>N in complex organic mixtures are now possible for milligram amounts of solid samples (Fig. 2). Because such procedures are often applicable to unprocessed bulk samples, they can sidestep many of the complications arising when samples must be dissolved for conventional liquid-phase NMR spectroscopy.

An important observation coming from <sup>15</sup>N NMR analyses is that organic nitrogen in soils (Knicker and Lüdemann, 1995; Knicker et al., 2000), modern sediments (Knicker et al., 1996) and seawater (McCarthy et al., 1997) is primarily in amide form. In contrast, aromatic heterocycles, are typically found in ancient sediments (Patience et al., 1992; Derenne et al., 1998) and advanced abiotic condensation products (melanoidins)

of the reaction of amino acids with carbohydrates (Benzing-Purdie et al., 1983). Because amide linkages are difficult to form abiotically, their predominance in nonliving organic matter from contemporary environments points strongly toward a remnant biochemical component of MUC. A second line of evidence against abiotic heteropolycondensation in the process of biodegradation is that extensive *in situ* formation of new chemical compounds is seldom evident from either NMR (Fig. 2) or molecular-level analyses (Hatcher et al., 1983). Organic matter degradation appears to be predominantly a process of attrition, during which relatively resistant biochemicals are selectively concentrated into MUC.

Another key development has been identification over the last decade of a variety of biomacromolecules that are resistant to biodegradation and laboratory methods by which biopolymers (e.g. proteins, polysaccharides and cutins) are hydrolyzed to small structural units for chromatographic analysis (Tegelaar et al., 1989; de Leeuw and Largeau, 1993). Analytical pyrolysis, which yields structural units of biomacromolecules as specifically identified molecules, has been particularly useful in revealing structural features among the wide distribution of hydrolysis-resistant biomacromolecules in vascular plants and algae (Largeau et al., 1986). Fossil counterparts have been recognized in kerogens isolated from ancient shales, some of which still retain the characteristic

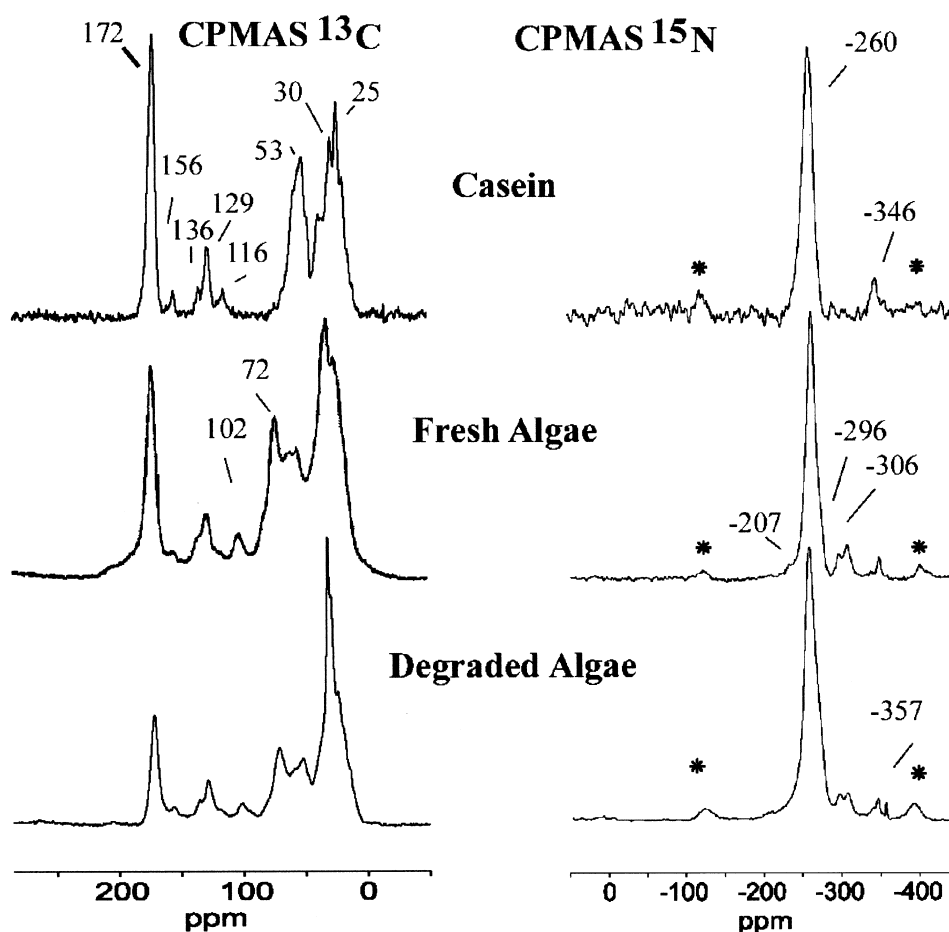


Fig. 2. Solids CP-MAS  $^{13}\text{C}$  and  $^{15}\text{N}$  NMR spectra of casein (a milk protein), fresh algae and biodegraded algae (from Knicker, 2000). The given numbers correspond to the chemical shifts (in ppm) of the indicated resonances from a spectral standard and asterisks indicate spinning side bands of the larger central resonance. Within the  $^{13}\text{C}$  spectra, the nominal chemical shift regions and corresponding major carbon types are: 0–45 ppm (alkyl), 45–60 ppm (*N*-alkyl and methoxyl), 60–95 (*O*-alkyl), 95–115 (di-*O*-alkyl), 115–145 (aromatic), 145–160 (*O*-aromatic) and 160–210 (carboxyl/carbonyl). In the  $^{13}\text{C}$  spectra, the predominance of protein in fresh algae is evident, as is the loss of non-alkyl carbon during algal degradation. For the  $^{15}\text{N}$  spectra in the right column, amide nitrogen (–260 ppm) constitutes the main resonance of all samples. Such spectra can be obtained for mg-size samples of organic matter with little preparation other than drying and grinding and yield broadly representative information about the major forms of carbon and nitrogen in the component organic material.

morphology of their biological sources, including phytoplankton and vascular plants (e.g. Largeau et al., 1986; Goth et al., 1988; Derenne et al., 1991). Because less than one percent of local biosynthate is preserved in soils and sediments, even trace amounts of resistant biomacromolecules in living organisms can be concentrated by degradation to become major components of MUC residues in these matrices. Evidence for the photochemical formation of MUC-like materials from oxidative crosslinking of polyunsaturated fatty acids in natural waters (Harvey et al., 1983; Gatellier et al., 1993), and for diagenetic incorporation of protein-derived moieties into hydrolysis resistant organic matter in marine sediments (Zegouagh et al., 1999), suggests that some spontaneous linkages between molecules do contribute to MUC formation. Such crosslinking and repackaging, however, do not necessarily produce large changes in the bulk chemical composition of the precursor molecules.

A fast-developing line of research into MUC origins is growing evidence for the presence in many natural environments of substantial amounts of “black carbon” derived from biomass burning. Carbon combustion products have many natural forms (Fig. 3) ranging from charcoal residues that often retain the physical form of the parent fuels, to highly graphitized soot spheroids derived from extensive recombination of small free radicals (e.g. of acetylene) within flames (Goldberg, 1985). Soots and charcoals are extremely difficult to identify chemically within natural organic mixtures because they are insoluble and resist hydrolysis and essentially all other chemical degradations. Black carbon has few established molecular tracers, excepting aromatic hydrocarbons (and possibly fullerenes) in soots and benzenepolycarboxylic acids formed by nitric acid oxidation of charcoal (Glaser et al., 1998). Moreover, black carbon often is not representatively detected by conventional “solids”  $^{13}\text{C}$  NMR spectroscopy, which relies on the vicinity of hydrogen atoms through which carbon is detected. The graphitic component of black

carbon can be quantified as the residue of treatments with strong oxidizing agents (Gustafsson and Gschwend, 1998), but represents less than half of the total carbon in soots and may be completely missing from charcoal. Although recent evidence has been presented for a major component of black carbon in various soils (Golchin et al., 1997; Schmidt et al., 1999), marine sediments (Masiello and Druffel, 1998) and solids suspended in rivers (Masiello and Druffel, 2000), greatly improved analytical methods will be necessary to chemically quantify and characterize this elusive MUC form.

Despite its chemical opacity and many forms, black carbon is readily observed and morphologically characterized by direct microscopic observation. In fact, palynologists and sedimentologists have long used optical and electron microscopes to identify pollen, chars and soots, thereby extracting related paleoenvironmental information on vegetation, climate and proximity of continental masses (Littke and Sachsenhofer, 1994; Meyers, 1997). In particular, high-resolution transmission electron microscopy (HRTEM) is a powerful, but sparingly applied, tool for direct observation of black carbon in cokes (Rouzaud and Oberlin, 1990) and soils (Poirier et al., 2000). Also evident microscopically in marine sediments are “reweathered” pollen grains, coals and kerogen fragments that have survived at least one cycle through sedimentation, uplift and erosion on the continents (Lueckge et al., 1996). Such “precooked” geopolymeric materials should be particularly resistant to biodegradation and chemical detection but can be structurally recognized by characteristically rearranged carbon skeletons and stereochemistries. These recycled forms of reduced carbon may be important in global balances of bioactive elements over geologic time. Since reburial of ancient organic matter releases no new  $\text{O}_2$ , the delicately balanced reservoir of atmospheric oxygen requires less stringent negative feedback controls over geologic time than would be necessary if only newly-formed reduced carbon were preserved (Bernier, 1989).

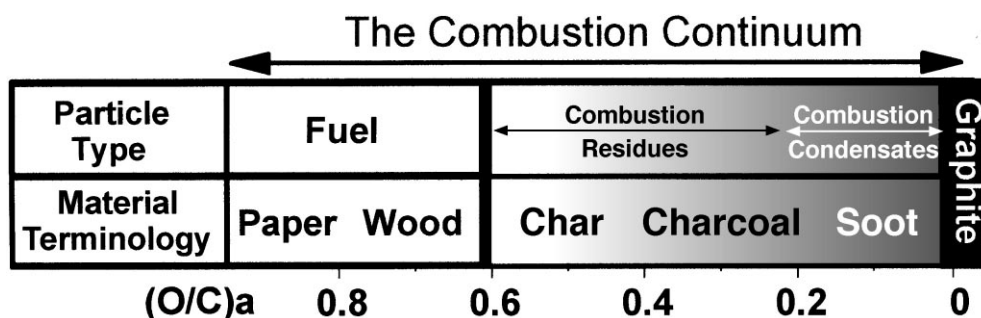


Fig. 3. The combustion continuum of black carbon (from Jones and Chaloner, 1991; Goldberg, 1985). Increased heating and chemical reformation yields a spectrum of progressively carbon-rich and refractory organic materials. (O/C)<sub>a</sub> indicates typical atomic ratios of oxygen to carbon in the various black carbon types.

The compositional features of dissolved organic matter (DOM) in seawater, three-quarters of which typically is uncharacterized at the molecular level (Benner, 1998), offer clues with regard to MUC sources and formation pathways. The constituents of this dilute ( $\sim 1$  mg/l) but huge carbon reservoir (as big as atmospheric  $\text{CO}_2$ ) may be particularly informative because the  $^{14}\text{C}$  content of DOM isolated from the abyssal Pacific ocean corresponds to an average “age” of roughly 6000 years (Druffel et al., 1992). Some fraction of the molecules making up deep seawater DOM thus have persisted during multiple mixings through the world-ocean circulation system without benefit of protective associations with mineral surfaces or (apparently) complexing ions. These aged molecules should strongly embody the chemical characteristics that contribute toward extreme resistance to environmental degradation, including such severe processes as photolytic attack and cycling through submarine hydrothermal vent systems. A major challenge in studying seawater DOM is that most methods of its isolation (e.g. adsorption onto synthetic resins and ultrafiltration) from 1,000,000 times more water and 35,000 times as much salt are incomplete and potentially fractionating. The most complete and gentle isolation method, retention by ultrafilters of molecules larger than roughly 1000 amu, is only 25–35% efficient for seawater (Benner et al., 1992). Thus, oceanic DOM components lower in molecular weight than a six-sugar oligosaccharide remain poorly characterized.

Bulk analysis of DOM components that can be isolated from seawater by ultrafiltration indicates chemical characteristics consistent with a marine, rather than riverine, origin (Hedges et al., 1997). The recovered molecular mixtures contain roughly 1 nitrogen per 15 carbons, are relatively enriched in  $^{13}\text{C}$ , and exhibit a more highly aliphatic structure than DOM in river water. Although  $^{13}\text{C}$  NMR analysis indicates a high relative abundance of carbohydrate-like substances (Fig. 4), less than half of this oxygen-rich material is measurable by conventional colorimetric and chromatographic methods (Benner, 1998). A key observation is that the amide-rich nitrogen constituents of ultrafiltered DOM from throughout the ocean yield high ratios (versus the common L-counterparts) of D-alanine, D-serine, D-glutamic acid and D-aspartic acid (McCarthy et al., 1998). This pattern does not correspond to what would be expected for thermally induced stereochemical inversion, but does match with the characteristic high abundances of these same amino acids in the peptidoglycan-rich cell walls of eubacteria. Peptidoglycan, a highly crosslinked “fabric” of nitrogen-containing sugar strings bridged by compositionally unusual peptides (Fig. 5), only occurs in eubacterial cell walls and hence is characteristic of this source. The *N*-acetylglucosamine and *N*-acetylmuramic acid links of this chainmail-like polymer are relatively resistant to strong acidic hydrolysis. If released by hydrolysis at elevated temperatures, however, amino

sugars can readily react with each other (and with peptide-derived amino acids) to form heteropolycondensates. Thus, these ubiquitous components of bacteria could be easily missed by conventional analyses involving chemical degradation and subsequent chromatographic separation.

Our extremely limited current understanding of the distributions, compositions and ecological functions of microorganisms, cascades into parallel uncertainties about the origins, physical forms and reactivity of MUC. Early comparisons of plate and direct counts, and more recent methods in molecular biology, indicate that over 99% of heterotrophic microorganism species in natural environments are not amenable to current culturing techniques (Amann et al., 1995; Pace, 1997; Ward et al., 1998) by which their substrate specificities, biochemical compositions and life strategies typically are studied. We do know, however, from studies on the minority of species that can be grown in the laboratory, that prokaryotes (eubacteria and archaea) are marvelous synthetic chemists, as well as awesome disposal units. In addition to recycling immense quantities of MUC, heterotrophic microorganisms (and their remains) may be important direct sources of these molecularly uncharacterized “leftovers” (McCarthy et al., 1998). Archaea, the least studied microorganisms on earth, are characterized by diverse cell wall structures with a variety of

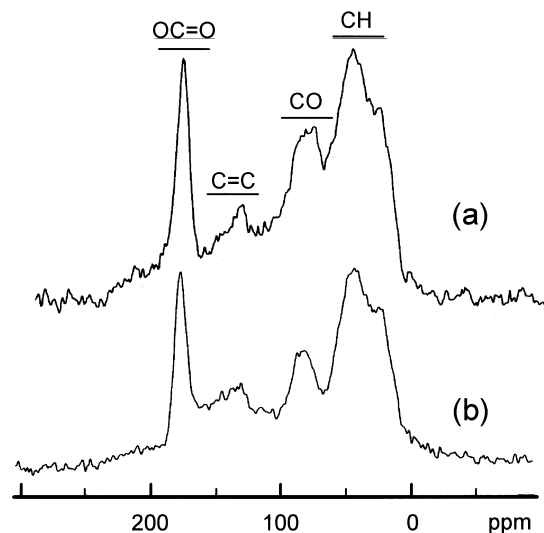


Fig. 4. Solids CP-MAS  $^{13}\text{C}$  spectra of dissolved organic matter (DOM) isolated by tangential-flow ultrafiltration from surface (a) and deep (b) seawater (from Benner et al., 1992). As applied here, this method isolates molecules in the “colloidal” size range of approximately 0.001–0.500  $\mu\text{m}$ , based on the cutoff ranges of the ultrafilter and preparatory filter, respectively. In general, seawater DOM isolated by ultrafiltration is rich in aliphatic (0–50 ppm) and “carboxyl/amide/ester” ( $\sim 175$  ppm) carbon, along with aliphatic carbons bonded to oxygen, as occur in carbohydrates ( $\sim 70$ –75 ppm).

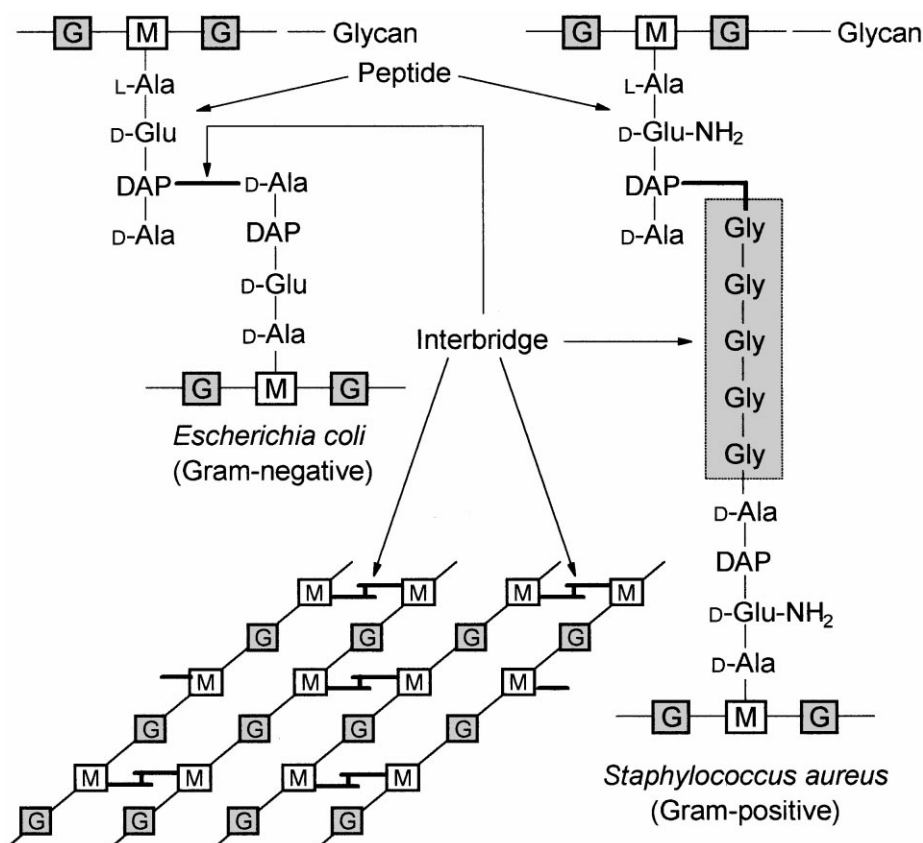


Fig. 5. Schematic representation of the structure of bacterial peptidoglycan (after Brock et al., 1994). The upper two images illustrate the linkages of structural units within peptidoglycans of species of Gram-negative and Gram-positive bacteria. The network to the lower left shows how these units are assembled into a peptidoglycan sheet (peptide crosslinks in bold) that is relatively resistant to biodegradation. Abbreviations: G = *N*-acetylglucosamine, M = *N*-acetylmuramic acid, DAP = meso-diaminopimelic acid, Ala = alanine, Gly = glycine, Glu = glutamic acid.

peptidoglycan replacements and by membranes incorporating ether-linked lipids (De Rosa et al., 1986; DeLong et al., 1998). These typically robust biochemicals apparently allow hyperthermophiles and halophiles to adapt to the extremes of their chosen habitats and may have been important sources of MUC over Earth history.

### 3. Chemical recalcitrance

In addition to the issue of source, is the question of why the various forms of MUC are difficult to break down into simple structural units that microorganisms can degrade and scientists can analyze in the laboratory. A number of factors can be identified, in addition to the previously discussed aspects of inherent chemical stability that may contribute toward such resistance. One fundamental constraint is accessibility of different regions of MUC molecules to enzymes and inorganic chemical reagents. A critical factor in enzymatic degradation is

that most microorganisms are unable to routinely transport molecules greater than about 600 amu through their cell walls. Larger substrates must first be dismantled outside the cell by exoenzymes. Any external substrate that cannot be broken down by exoenzymes into molecules smaller than a tetrasaccharide will require abiotic scission prior to assimilation. For this reason, the collection of dissolved molecules smaller in size than 600 amu might be compositionally distinct from larger entities that are not subject to direct import across cell walls for dismantling by the formidable phalanx of intracellular enzymes. It is thus unfortunate that current methods for isolating DOM from seawater are largely restricted to molecules bigger than 1000 amu. Because of low salt content, DOM from rivers and lakes can be isolated with smaller pore-size ultrafilters, as well as by evaporation, and might more readily provide fresh insights on how substrate size and composition affect microbial metabolism.

The limitations incurred by our inability to isolate a representative fraction of bacteria in pure culture are

paralleled by a lack of information about the substrate specificities and activities of the extracellular enzymes produced by these bacteria. Although the production, function, and structure of enzymes that are useful in biotechnological applications have been studied extensively, these enzymes are not likely representative of those used by non-hyperthermophilic bacteria found in a diverse array of natural environments. Measurements of extracellular enzyme activity in marine systems routinely rely on small substrate analogs, whose relationship to macromolecules is questionable (Helmke and Weyland, 1991; Pantoja and Lee, 1999). Development of new methods for measuring activities of polysaccharide and peptide-hydrolyzing extracellular enzymes (Arnosti, 1995, 1996; Pantoja et al., 1997) may provide new perspectives on the nature and rates of hydrolytic processes in seawater and sediments.

Since most enzymes and ions must be compatible with water, processes that remove water from the immediate surroundings of macromolecules will often slow their degradation. This is one of the reasons why biodegradation is characteristically delayed in desiccated materials (Evershed et al., 1997) and within such anhydrous matrices as amber (Bada et al., 1994). In water, folding of large molecules, and aggregation of small molecules, can both lead to internal hydrophobic microenvironments whose isolation is strongly favored by the polarity and highly ordered “structure” of the surrounding water network. The energetic imperative for minimal interruption of water networks by hydrophobic materials is the driving force behind the formation of oil droplets and the assembly of micelles and membranes. Hydrophobicity, along with hydrogen bonding and ion pairing, are strong contributing factors toward the exquisitely complex three-dimensional folding patterns of proteins. Proteins in fact have become valuable models for studying the interplay of molecular conformation and reactivity, owing in part to the efforts devoted to solving the “protein folding problem” of predicting secondary and tertiary protein structures from primary amino acid sequences. One telling observation from such evaluations is that high-energy reactants, such as the hydroxyl radical ( $\text{OH}\cdot$ ), selectively attack DNA and RNA at exposed (external) hydrogens, in preference to more chemically reactive counterparts buried within the macromolecule (Pogozelski and Tullius, 1998). Physical availability to attack, rather than intrinsic bonding energies, often determines organic matter reaction rates during chemical (and enzymatic) degradation. This conclusion is also consistent with the observation that intrinsically reactive biochemicals are detectable within ancient sediments of many types and hence appear to be “encapsulated” (Fig. 6) in some type of protective matrix (Knicker et al., 1996; Eglinton, 1998).

Another useful guideline for assessing reactivity/conformation relationships pertinent to MUC cycling is

comparison of structural characteristics among proteins of the same generic type occurring in hyperthermophiles and low-temperature counterparts. Although the enzymes of hyperthermophilic bacteria are characterized by a variety of structural features such as increased hydrogen bonding, larger ion bridging arrays and more extensive hydrophobic interactions among segments. The overall differences versus low-temperature counterparts are surprisingly minor (Danson and Hough, 1998). Relatively subtle chemical changes leading to minor modifications in low-energy bonding can cause profound changes in enzyme function and stability. The corollary for MUC reactivity is that similarly minor changes in molecular content or architecture may lead to sharp contrasts in biochemical reactivity and preservation (Eglinton and Poinar, 2000). Evidence along these lines comes from the observation that a portion of protein dissolved in filter-sterilized seawater loses reactivity toward microbial degradation in a matter of days (Fig. 7), although subsequent hydrolysis indicates little if any alteration in amino acid composition (Keil and Kirchman, 1994). As another example, acidic hydrolysis typically proceeds via hydrogen-bonded intermediates in which the added proton must be aligned with the axis of the bond holding the target oxygen or nitrogen. Many chemical subunits therefore must also be in a very specific alignment, as well as being generally accessible, to react. For this reason, portions of a protein spread over a particle surface may

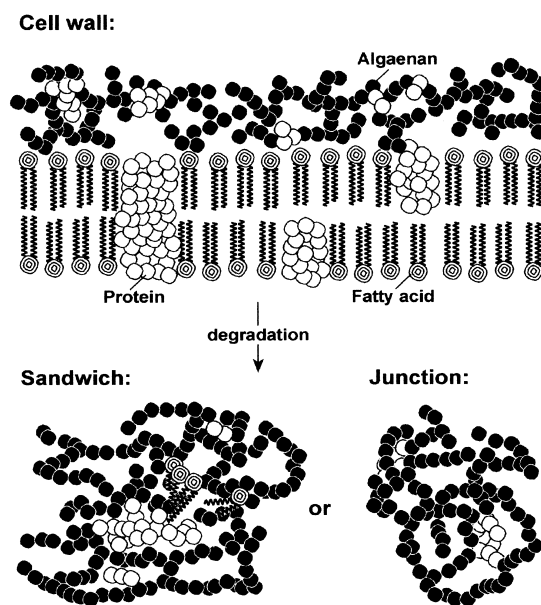


Fig. 6. Schematic illustrations of (above) the structure of an algaenan-containing algal cell wall, and (below) proteinaceous organic matter “encapsulated” within degraded algal cell wall material (from Knicker, 2000b). Encapsulation has been suggested as a mechanism by which intrinsically labile proteins and peptides might be physically protected from biodegradation.



be resistant to reaction, even though they are physically accessible to enzymes and reagents (Nagata and Kirchman, 1996). One implication of such observations is that structural or conformational changes which greatly affect chemical reactivity may not be detectable by many bulk and molecular-level analyses (see next section).

#### 4. Chemical characterization

New analytical methods and experimental strategies for MUC characterizations are clearly needed. In this regard, it seems that recent developments from outside the bounds of classic biogeochemistry might be brought into play. An outstanding example of huge potential for such technology transfer is from molecular biology and the health sciences, where burgeoning research on genetics, protein structure and immunology provides a wealth of new tools. In particular, ribosomal RNA mapping (Giovannoni et al., 1990) offers a powerful means for assessing the types and distributions of microorganisms living in different natural environments where MUC may be formed or altered. Fluorescent in

situ hybridization (FISH) techniques have advanced sufficiently to now locate and identify specific microbial cells in sediments and other matrices (Amann et al., 1995; Llobet-Brossa et al., 1998), providing information about the numbers and locations of specific microorganisms, most of which cannot be cultured at present (Button et al., 1993). In part, these types of fast-developing capabilities should help compensate for our inability to culture most microorganisms.

An additional front where technical imports and new strategies may prove helpful is in fundamental analytical chemistry. For example, pyrolysis has proved a fast, sensitive and reproducible tool for releasing diagnostic structural units from large organic molecules, especially those which are not readily hydrolyzed (Larter and Horsfield, 1993). The breadth and specificity of such methods can be increased by temperature programming pyrolysis filaments within the source of a mass spectrometer, complemented by mild chemical or electron ionization procedures (e.g. Boon et al., 1998). Pyrolysis methods, however, are often limited by extensive structural alterations, low and uncertain yields, and potential artifacts from secondary reactions and sample matrix effects. Some of these shortcomings can be sidestepped by use of chemolytic variations, in which reactants that favor cleavage and/or formation of volatile products can be added directly to the sample before it is heated. One such reagent is tetramethylammonium hydroxide (TMAH) which allows simultaneous chemolytic cleavage and methyl derivatisation of such recalcitrant materials as ether-linked lignins and carbon-linked coals (Hatcher and Clifford, 1997). TMAH also can be tagged with  $^{13}\text{CH}_3$ , thereby allowing distinction between pre-existing and added methyl functionalities (Filley et al., 2000). Because vicinal diphenol products from the fungal decay of lignins still fall within the “analytical window” of TMAH, it is possible to trace microbial degradation products of this likely MUC progenitor considerably further than has been possible with oxidative reagents (e.g.  $\text{KMnO}_4$ ,  $\text{RuO}_4$  and  $\text{CuO}$ ) that alter this precursor. Other potentially rewarding lines of development for pyrolytic methods include the use of recovery standards and offline methods by which the yields of both volatile and residual reaction products can be directly assessed.

Structural elucidation techniques that can be applied to large molecules (up to 100,000 amu in weight) are particularly well suited for the analysis of MUC because they do not require extensive scission to yield fragments small enough for analysis. Chemical alteration is proportionately less and patterns of chemical “connectedness” are retained. Techniques for the mass spectrometry of biomolecules have rapidly advanced over the last decade (McLafferty et al., 1999). Multi-stage formation and fragmentation of organic ions “trapped” in sequential mass spectrometers (e.g. MS–MS techniques) have proven a powerful tool for characterization of complex organic

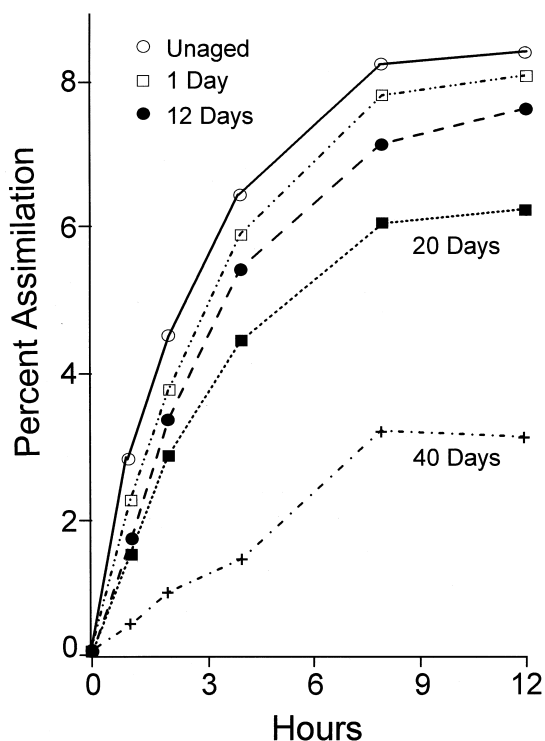


Fig. 7. The deactivation of bovine serum albumin aged for up to 40 days in seawater, as reflected by subsequently decreased percent assimilation (over 0–12 h) of the dissolved protein by bacteria (after Keil and Kirchman, 1994). No such aging effect was obtained with organic-free (UV-treated) seawater, indicating that organic–organic interactions likely produced the refractory protein.

molecules. Very large molecules now can be routinely ionized and analyzed within mass spectrometers with the aid of sampling techniques such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). In MALDI, the sample is “exploded” from its matrix into the gas phase by laser energy. For ESI, a solution of the analyte is sprayed into a low-pressure chamber, where droplets of the liquid evaporate to yield charged ions. Using time-of-flight and Fourier transform ion cyclotron resonance spectrometers, these two methods have been applied to proteins at attomolar concentrations ( $10^{-14}$  g of a 10,000 amu molecule) with masses in excess of 100,000 amu (McLafferty et al., 1999). Due to their high speed, resolution and accuracy, these mass spectral techniques can resolve the components of complex molecule mixtures. In many cases, however, the absence of well-defined reference materials and the challenge of resolving a multitude of chemical details within such large molecules have thwarted specific structural assignments for natural materials.

NMR spectroscopy has been a particularly revealing analytical approach for defining the major structural characteristics of natural organic mixtures, and hence their predominant MUC components. Cross-polarization/magic-angle spinning (CP/MAS) methods for directly analyzing dried bulk solids have in particular advanced the study of insoluble macromolecular materials such as lignins, coals and kerogens (Maciel and Dennis, 1981; Hatcher et al., 1983; Golchin et al., 1997). This versatile method not only reveals the chemical environment of H, C and N, but also can be expanded to two-dimensional resolution to reveal the linkages and proximities of atoms in their natural sample matrices. In addition, dipolar-dephasing (DD) and proton spin relaxation editing (PSRE) routines greatly aid NMR discriminations of organic carbon forms and microenvironments (Kögel-Knabner, 1997). Because  $^{13}\text{C}$  and  $^{15}\text{N}$  represent a percent or less of their total elements, molecules artificially enriched in these isotopes can be detected with greatly enhanced sensitivity. As they undergo environmental processes (e.g. biosynthesis, degradation, and sorption) these magnetic centers sometimes provide telltale clues about their immediate position in complex soil and sedimentary matrices. However, NMR only gives an averaged record of distribution patterns among major functional groups in organic compound mixtures and is often insufficiently sensitive to detect more subtle chemical changes. In addition, time-consuming “Bloch” decay techniques (which do not necessitate H–C “cross-talk”) are often needed to detect highly aromatic carbons that predominate in most chars and soots. Because NMR measurements comprehensively outline the major chemical structures in most natural mixtures, they can track whether specific compound types have been efficiently accounted for in a given molecular-level analysis. Spectroscopic analyses, such as XANES, of reaction

mixtures can indicate where the missing structures went and what specific chemical treatments might next be tried to convert them to chromatographically resolvable forms (G. Sarrett, pers. comm.). Combinations of NMR methods with those for high-resolution mass spectrometry of large molecules (McLafferty et al., 1999) should be particularly rewarding.

Another strategy for better defining MUC forms and functions is the use of synthetic chemicals as probes that can be sensitively traced in natural environments where MUC predominates. This class of xenobiotic molecules includes a chemically diverse array of pesticides, herbicides, PCB's, and aliphatic halocarbons such as chloroform, carbon tetrachloride and freons. Because most of these industrial compounds have no natural counterparts, they can be sensitively detected against a conveniently blank natural background. Such tailor-made compounds also often contain halogens that render them detectable at minute concentrations by electron capture and mass spectrometric methods. Because their structures and production history are known, the distributions and reactions of man-made chemicals reflect the mechanisms and rates of the natural processes that disperse and degrade natural counterparts. Thus, the uptake of anthropogenic compounds by soil and sedimentary organic matter provides comparative information as to how biochemicals might be protectively incorporated into MUC (Richnow et al., 1997). Oxidative cross-linking of unsaturated lipids by free radicals (Harvey et al., 1983) or sulfur (Sinninghe-Damsté et al., 1989), decomposition of lignite wastes in soils (Rumpel et al., 1998) and degradation of plastics, paper and polyesters in landfills (Pichler and Kögel-Knabner, 2000), also establish possible models for how MUC might form and degrade in nature. Such big-scale controlled experiments, under well-defined environmental conditions, are revealing complements to much smaller laboratory simulations that sometimes fail to encompass key natural components or conditions. Synthetic organic compounds, however, may have different geographic sources, reaction histories and physical forms than MUC analogs.

An additional rewarding research strategy for MUC would be to develop more techniques to characterize complex insoluble organic materials at the micron scale in their natural settings. In particular, such methods would help meld microscopic and chemical studies of discrete small bodies such as pollen grains, dinoflagellate cysts, soot framboids, and reweathered coal and kerogen fragments. An advantage of analyses at this scale is that many of the targeted bodies have known biological origins, growth environments and initial compositions, all of which aid in mechanistic evaluations and environmental reconstructions. Although reflectance (Mastalerz and Bustin, 1996) and transmission (Landais et al., 1993) infra-red and Raman (Roberts et al., 1995) spectro-

scopy can now be applied to micron-size objects, and a method for laser-induced volatilization for stable isotope analysis has just been described (Wieser and Brand, 2000), other promising techniques such as laser ablation mass spectrometry need to be resolved to the critical scale of 10–50  $\mu\text{m}$  targets. Such chemical resolution might make it possible to directly define the distribution of organic matter on mineral surfaces, as well as the chemical reactions that cause slow erosive degradation of recalcitrant materials such as charcoal and kerogen. Stable isotope analysis on the scale of a single pollen grain or dinoflagellate cyst could reveal the concentration and isotopic composition of inorganic carbon in ancient atmospheres or oceans, or the altitudes at which specific land plant species lived on past continental surfaces.

Laboratory simulation experiments provide a strategy for dealing with the complexity of MUC formation and reaction under controlled conditions that are logistically advantageous. Such approaches are especially practical for testing hypotheses about how MUC is formed on short time scales. In addition, standardized means of assessing the physical accessibility and substrate quality of large organic molecules are necessary. In the former case, reaction rates with high-energy species such as hydroxylradical,  $\text{H}_2\text{O}_2$  and alkyl peroxides might prove useful indicators of the physical accessibility of macromolecules (or their components) on surfaces (Nagata and Kirchman, 1996) and in capsules, cavities and molecular folds (Pogozelski and Tullius, 1998). Standard “cocktails” of hydrolytic exoenzymes, or bacterial assemblages, might prove handy for comparing the substrate potential of different organic materials that could represent MUC, or its progenitors. Likewise, particles with well defined surface areas, chemistries, and roughness characteristics would be useful (in batch and flow systems) for comparing the extents and rates with which different organic molecules assume protective sites on various solids. Finally, “combinatorial” synthetic techniques involving a limited variety of reactants (Wilson and Czarnik, 1997) could be used to produce suites of molecules that vary systematically from each other in specific structural features. These complex mixtures of engineered homologs could then be subjected to various degradations and physical transformations (such as described above) to identify those shared structural characteristics which lead to biological inertness or transform given molecule types outside their conventional analytical windows. Similar approaches have been used to great advantage in pharmaceutical testing.

Clearly, greater teamwork and international organization are necessary to make significant advances in characterizing MUC and defining its role in global biogeochemistry. In particular, reference materials representing a variety of soils, sediments and organisms need to be agreed upon by the geochemical community and

then routinely included in study sets. Because the biochemicals that comprise living organisms are diverse and require different analytical procedures, comparative analyses among a large number of cooperating laboratories are required. In particular, *quantitative* methods need to be developed and applied to measure such challenging biochemicals as lignin, tannins, acidic and basic sugars, algaenans, cutans and other hydrolysis-resistant biomacromolecules (de Leeuw and Largeau, 1993). Only by sequentially targeting these potentially important molecule types for precise quantification in a small number of widely studied samples can the community nibble away at the molecularly uncharted territory of MUC in living organisms and environmental samples. Such comparisons also are badly needed to evaluate analytical results among laboratories and methods, so that data can be shared more confidently across the biogeochemical community.

The saying that “What you don’t know can’t hurt you” is becoming ever less true in the Earth Sciences. Humans are increasingly altering the chemistry of the planet’s surface and many of its complexly-related processes such as climate, soil fertility and the reproductive success of organisms. The stakes of not knowing the detailed chemical composition, and hence the origins, reaction pathways and fates, of over half of all organic matter on Earth are becoming increasingly great. On the basis of a molecular understanding of nature, Earth scientists will increasingly need to address such questions as when soils should remain fallow, what industrial chemicals should never be made and which natural processes are teetering on fragile geochemical underpinnings.

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