TRANSMISSION ELECTRON MICROSCOPY OF THE NATURAL ORGANIC MATTER OF SURFACE WATERS

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NWRI Contribution No. 89-48

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December 1989

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MANAGEMENT PERSPECTIVE

The technology outlined in this review allows one to analyze, by electron-optical visual means, the flocculation of dissolved organic carbon at a resolution of 0.001 μm. As a consequence, the factors which control flocculation in surface waters (pH, concentration factor, hardness, turbulence, surface activity, organo-mineral associations) can now be analyzed in greater depth than previously was possible. The scavenging by colloidal particles of contaminants and subsequent sedimentation of colloid flocs are important phenomena in the dispersion of contaminants in lake waters. This review presents new technology for assessing such phenomena, and analyzing the mechanisms.

PERSPECTIVE GESTION

La technologie décrite ici permet d'analyser, par des moyens visuels optiques-électroniques, la floculation du carbone organique dissous à une résolution de 0,001 um. Les facteurs qui contrôlent la floculation dans les eaux superficielles (pH, facteur de concentration, dureté, turbulence, activité à la surface, associations organo-minérales) peuvent ainsi être mieux analysés que précédemment. La fixation de contaminants aux particules colloïdales et la sédimentation subséquente des flocs colloïdaux sont des phénomènes importants dans la dispersion des contaminants dans les eaux lacustres. Le présent article passe en revue de nouvelles technologie permettant d'évaluer ces phénomènes et d'en analyser les mécanismes.

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SUMMARY

Some components of aquatic natural organic matter (NOM) can be analyzed effectively by particle analysis methods employing transmission electron microscopy in conjunction with multimethod analytical approaches in the field, minimal perturbation techniques for sample handling and technology transfer from the biomedical sciences. The NOM components include fulvic acids, colloidal fibrils and organic polymers >30,000 MW. The use of a water compatible embedding resin permits shape and size analyses of colloidal NOM (1-1000 nm) in ultrathin sections which minimize the misleading dehydration artifacts of the past. Experimentally induced perturbations allow one to follow aggregation/coagulation events at 1 nm resolution, while permitting the analyst to relate some components of coagulum structure to chemical entities. This review presents the current status of attempts to optimize a blend of analytical chemistry and transmission electron microscopy for describing NOM and its behaviour in surface waters.

RÉSUMÉ

Il est possible d'analyser efficacement la matière organique aquatique naturelle (MON) à l'aide de méthodes d'analyse particulaire par microscopie électronique par transmission combinée à des techniques d'analyse multiple sur le terrain, des techniques de perturbation minimale pour la manipulation des échantillons et le transfert des technologies des sciences biomédicales. Les composantes de la MON comprennent les acides fulviques, les fibrilles colloïdales et les polymères organiques de P.M. 30 000. L'utilisation d'une résine d'inclusion soluble dans l'eau permet de procéder à des analyses de taille et de forme de la MON colloïdale (1-1000 nm) en sections ultraminces ce qui minimise la possibilité d'obtenir les artéfacts dus à la déshydratation obtenus par le passé. Des perturbations provoquées au cours des expériences permettent de suivre les épisodes d'aggrégation/coagulation à une résolution de 1 nm, tout en permettant de faire le lien entre certaines composantes de la structure du coagulat et des entités chimiques. Le présent article traite de l'état actuel des tentatives visant à optimiser l'utilisation combinée de chimie analytique et de microscopie électronique par transmission en vue de décrire la MON et son comportement dans les eaux superficielles.

INTRODUCTION - GENERAL

The search for solutions to the problems of sampling and characterization of environmental particles is attracting the attention of many scientific disciplines. A greater knowledge of small particles is required to analyze more effectively the dynamics of many environmental compartments. Aquatic scientists now focus their efforts on submicron particles and particles whose nature is susceptible to excessive and/or uncontrolled alteration by accepted-but-inadequate techniques for sampling and characterization. Transmission electron microscopy (TEM) shows great promise in addressing the major concerns of the aquatic particle analyst; its resolution permits sizing analyses down to the nanometer range, and in combination with energy-dispersive spectroscopy (EDS), it can provide elemental analyses of particles well into the lower portion of the colloidal size range. In conjunction with improved technologies for minimizing sample alteration, TEM can now be exploited fully, even to the extent of using it to monitor the status of unstable particles in experiments on particle behavoiur.

The non-living natural organic matter or NOM [1,2] of surface waters is increasingly implicated in the availability of nutrients and toxic substances to biota [3], in the modulation of geochemical cycles [1,4] and in the dispersion of contaminants [5]. Among the constituents of NOM, there has been an environmental/analytical focus on fulvic acids and on some of the refractory colloidal organics [2,3,6]. Electron microscopy in various modes has been useful for more than two decades in supplementing chemical and limnological analyses of some NOM components with ultrastructural correlates [7,8,9,10].

Recently, TEM has begun to play a lead role in the analysis of NOM particles in the colloidal size range. This lead role has come about as a result of:

(A) improved resins for sample embedding which permit the TEM preparation of fresh NOM samples in the presence of water, thus yielding a physically stable NOM which can be sectioned for high resolution studies by TEM [13] and a NOM preparation which precludes the most severe of the dehydration artifacts of the past;

(B) continued development of minimal perturbation technology to minimize artifacts of sample handling at the sample site and in the laboratory [14,15], thus maximizing the likelihood of embedding a natural sample in its native state;

(C) an increased understanding of the variables which promote artifactual aggregate formation in NOM-rich samples and an accompanying capability to assess and reduce this artifact [16,17,18];

(D) new information from physics [19,20] and field applications of the multi-method analytical approach [21,22], which permit improved interpretation of the information content of TEM images.

This review will focus on TEM analyses of lacustrine NOM, with specific reference to fulvic acids (FA), high-molecular-weight fractions of "dissolved" natural organic matter (HMW-NOM) and colloidal polyanionic fibrils [10,18]. It will also outline the potential of TEM for analyzing the colloidal particulate component of water fractions in conjunction with the techniques of analytical chemistry, EDS and cytochemistry/selective staining [23,24]. In the past it has often appeared that TEM technology is either "too qualitative" or "too much like magic" for consideration by analytical chemists. To address these attitudes, we append below a summary review of the basic strengths, weaknesses and future prospects of TEM as a tool for the particle analysis of NOM. Because of the high content of biological terms and concepts, we append a brief glossary at the end of the paper.

II STATE OF THE ART IN TEM TECHNOLOGY

II.1 Discriminating artifacts from real structures

There are many different TEM technologies; we will confine this paper to those developed by biologists for analyzing unstable organic-rich materials (usually cells) and we will outline only that technology transfer which applies to NOM particle analysis. Specific biological techniques of sample preparation were developed originally in response to the need to: stabilize cells for examination of specific features under the conditions of high vacuum which are required by a TEM specimen chamber; reduce the thickness of specimens to permit an optimal transmission of the electron beam [1]. The development of chemical preservatives (fixatives) and gentle dehydration techniques became mandatory at the outset, and, the embedding of "fixed" materials in hard resins (which held individual components together in correct spatial orientation) became a valuable adjunct when microtomes were improved to permit ultrathin sectioning of such resins. The problems of stabilization and thickness reduction were solved early through the use of fixatives which preserved cell contents in a life-like state and through replacing the water of cells by molecules of resin monomer which could be polymerized gently to produce a block of resin-embedded cells for ultrathin sectioning (ca. 50 nm thick). One drawback to this success story was the finding that all the best resins were incompatible with water, thus requiring an extra step in the preparation of cells, the use of organic solvents to exchange for cell water and to be exchanged in turn for resin monomers.

As a result of the extensive sample manipulation, the problem of preparatory artifact was also recognized from the start. Artifact assessment by trial-and-error received the attention of thousands of biologists for many years. This enormous level of exploratory activity brought about a rich assemblage of techniques for minimizing

artifacts. A brief list of the major artifacts brought under control is as follows: extraction of components, redistribution of small components, shrinkage, rupture of membranes, creation of components via interactions between cell substances and preparatory fluids, rearrangement of membrane subunits, distortions in components sensitive to coagulation, autolytic reactions.

Cell biologists have a standard approach to preparing cells for TEM analyses of their colloidal components; it is useful for this review to describe this approach briefly and point out modifications which facilitate realistic TEM analyses of non-living NOM. The first step is fixation, the application of a chemical agent for the simultaneous killing and preservation of cells in a life-like manner. The ideal fixative (which exists in theory only) would preserve every detail of structure, right down to the molecular level, exactly as it was in life the instant before the cell was killed. The ideal fixative would also protect every detail of structure from alteration by the subsequent steps in preparation. The development of standards for assessing the quality of fixation of the various colloidal components has been the subject of many thousands of scientific projects involving many disciplines and a great diversity of technologies. At present, there are multi-method cytological approaches and alternative unrelated technologies which can be used routinely to check on the results shown by a specific variation of the standard approach (see below). The second step is dehydration, which consists of removing all the water from the cells by solvent exchange, using a chemically inert fluid miscible both with water and with the monomers of the plastic resin of choice (usually an epoxy resin). Once the water of the cell has been replaced completely by resin monomers, the polymerization reaction is initiated. The resultant block of plastic with its life-like cells inside is then sectioned (sliced) on a microtome and the sections are mounted on support grids for insertion into the TEM. Prior to insertion, the sections

can be placed in solutions of substances which can increase in a selective manner (counterstaining) the existing differential electron scattering power (contrast) of given colloids [23]. In fact, one can even carry out some conventional chemical analyses, including immunochemistry, in combination with the use of heavy metal counterstains (cytochemistry) so as to localize a given type of macromolecule in sections [23,24]. This capacity for refined localization can be further augmented, after insertion, by EDS (for organo-mineral colloids and organic colloids counterstained by heavy metal) or by electron diffraction (for crystalline organics).

In transferring the technology of cell biology into the particle analysis of NOM, one is faced with the problem of having to either (1) create a literature on NOM specimen perturbation resulting from the dehydration step in TEM preparation or (2) create a high quality resin which permits embedding in the presence of water. Fortunately, an experimental resin developed several years ago for biomedical research [13] now permits both creative pursuits to be carried out simultaneously. This resin, Nanoplast FB 101, can be used directly with non-living NOM fractions derived in the field, thus presenting us with the most non-perturbing preparatory technique available for the production of ultrathin sections. The drawbacks are that some differential stains become inapplicable and there are likely to be some colloid-resin interactions whose assessment will dilute one's energies for environmental pursuits.

While the systematic electron-optical analysis of cells and cell compartments today still has an artifactual component, the extent of the artifact for a given type of analysis can be measured and accounted for satisfactorily in the interpretation of TEM images. In contrast to the analysis of living cells and cell parts, however, the extent of artifact caused by earlier TEM preparations of non-living organic-rich materials of soils and surface waters is less readily

assessed by a consideration of the scientific literature. Until a more extensive literature on NOM materials and molecules has been created, one must proceed by selecting complementary techniques which minimize artifact, in the context of a multi-method approach, and then confirm the principle observations through the use of alternative unrelated technologies.

II.2 The present capabilities of TEM

What can one ascertain in TEM images of ultrathin sections which sets TEM apart from other optical techniques?

(A) One can resolve colloids throughout the entire size range of 1-1000 nm.

(B) One can select a section thickness, by adjusting a microtome, which will facilitate a detailed shape analysis of many colloid types in conjunction with an elemental analysis by EDS.

(C) One can prepare sections of a thickness appropriate to isolating, spatially, a specific colloid type from other colloid types in a heterogeneous aggregate, for the purpose of doing EDS on a "per colloid" basis within the aggregate. In this situation, individual colloids above and below the section are excluded , and one can use the morphological image to select individuals which are not adjacent to dissimilar colloids (different morphotypes). Analyses of the inorganic content of NOM and of the growth of natural organo-mineral associations involved in sedimentation phenomena could very well be assisted by such TEM-EDS examinations of embedded NOM [25].

(D) Morphological analyses of flocculation/coagulation and biofilm formation at 1 nm resolution, in relation to specific colloid morphotypes, is feasible now for lacustrine NOM.

(E) The relationships of some NOM components (e.g., secreted pectic/uronic acid-rich fibrils [10], secreted microfibrils of cellulose [26]) to cytological/physiological events and structural

changes at the cell surface/external milieu interface has received a profound analysis for decades, much beyond what was possible through the use of other optical technologies [24].

(F) For samples of NOM which are rich in relatively undegraded cell debris and/or rigid cell components, one can split the sample for two kinds of TEM preparations; one portion can be added to an embedding resin requiring no sample dehydration and the other portion can be treated according to the standard approach of the cell biologists. By this means, one can frequently relate morphological entities with minimal dehydration artifact to the standard image of the same entity which in turn can be related to the ultrastructure literature of cell biology based on ultrathin sections.

(G) By extending the blend of multi-method approach and minimal perturbation technique of point (F) above, one can borrow the technology of cytochemistry [24] to prepare molecule-specific "markers" which are identifiable by high resolution TEM, apply these markers in solution to a section of NOM containing the molecules of interest, and then use TEM to record the distribution of the marker relative to the various colloids in the TEM image. At its most sophisticated, the marker could be an antibody coupled to a heavy metal. Of wider application would be a marker consisting of a reagent which reacted with an extremely limited number of functional groups (e.g., the disulphide groups of proteins) and which either contained a heavy metal or could be coupled to one. The heavy metal, by virtue of its high contrast relative to organics would represent a highly selective stain verifiable by EDS.

II.3 Classification of particles by TEM-EDS

Clearly, a particle definition for TEM must be arbitrary, but particles are always defined arbitrarily no matter what methods

are used to define them [27], be they physical, chemical or limnological methods. We define a TEM particle as any visualized entity whose least diameter is greater than 1 nm [27]. When a relatively large distinctive entity is shown to be an assemblage of smaller units, the large entity is considered a particle and the subunits are considered both as subunits and as potential particles. Aggregates of particles are often readily recognized as such because one can recognize within them some distinctive entities known from the ultrastructure literature to be particles. Some aggregates are natural and some are artifactual; distinguishing between the two is elucidated in section III.

What kinds of structural entities does one see in high resolution TEM views and how can one construct a NOM classification scheme from them? Almost all views show one or more of the following types of entities at high magnification: (1) granules; (2) fibrils; (3) sheets (including membranes); and (4) particles of irregular shape (often fragments of crystalline polysaccharide or mineral fragments with adhering organics). At lower magnifications, one sees them displayed in random array or in specific patterns. Sometimes an aggregate is packed well enough that it appears as a unit of structure. Microbiota included in a NOM sample are readily recognized by their distinctive internal order and differentiated compartments [24]. A classification scheme begins by considering living cells separate from the NOM proper. The NOM proper can be divided into the four major categories above and then, depending on the nature of a given water sample, it can be subdivided into the various morphotypes present (considering size, details of shape and electron-opacity/increased contrast after counterstaining). Further subdivisions can be made (1) according to low magnification views of aggregates showing various distinctive patterns (2) according to selective staining/cytochemistry for specific

functional groups and (3) according to elemental composition as revealed by EDS spectra. If a certain particle type is revealed as numerically important, detailed analyses can be made of its size distribution, internal heterogeneity, porosity and frequency of association with other numerically important particles. Potentially, by EDS, one might also examine a particle for adsorbed species. Specific examples of particles having received detailed analyses are found in section IV.

III AQUATIC NOM CHARACTERIZATION: EXPERIMENTAL MEANS OF MINIMIZING ARTIFACTS

The isolation of a specific type or size fraction of NOM can be accomplished only by a major effort, in most cases. When a project requires a detailed TEM analysis in a quantitative chemical and limnological context, however, the effort is necessary and it can be highly rewarding. The case studies described below (sections III and IV) show the most important TEM, analytical and limnological approaches we have chosen to characterize aquatic NOM with a minimum of artifact. These approaches are based on the highest standards of TEM preparation and minimal perturbation sampling procedures, a maximum of control over sample manipulation, and a battery of multi-method techniques transferred from analytical chemistry, limnology and cytology/cell biology.

Artifactual production of NOM aggregates during sample preparation for TEM has been a major problem; a large part of the discussions of sections III and IV attest to this fact. Unambiguous demonstrations of aggregation artifacts are the more difficult because such aggregates may resemble those produced naturally in lakes and other surface waters. Despite this problem of discrimination, the problem of artifact minimization is not hopeless. Section III will describe and discuss experimental conditions which allow one to avoid (or at

least to control) the formation of technique-induced aggregates. In the context of artifact minimization, section IV will attempt to describe the presently known characteristics of the major types of NOM in their native state.

III.1 TEM preparation and documentation

To avoid denaturation of samples (resulting from the solvent exchange dehydration step of the classical TEM procedure), fresh hydrated samples were embedded in the water compatible resin, Nanoplast FB 101 [13]. To optimize the cutting properties of the resin block, limits were set on the sample:resin volume ratio (maximum of 10:1) and total volume of Nanoplast polymerized per embedding mold [15,28]. Sectioning was done extensively and systematically [10] to assure representative images for particle analysis (sizing, morphology, elemental composition, etc.) by TEM and associated technology (EDS, selective counterstaining, etc.). For samples presenting unusual features of interest, an independent confirmation of size and morphology was sought by the freeze-etching technique [14,18] and by a multi-method cytological approach involving different chemical fixatives and dehydrating agents and alternative embedding procedures [17]. The freeze-etching technique [29] consists of a physical fixation of the sample by freezing it rapidly enough to vitrify it (at a freezing rate greater than 10,000 K°/sec) followed by TEM examination of a metallic replica of a fracture surface generated by the experimenter through the vitrified NOM maintained below the recrystallization temperature. The replica corresponds approximately to an ultrathin section except that it contains no NOM per se and its morphological features are topographical. It is an ideal alternative confirmatory technique whose cost and complexity do not permit its use in the field. To relate new TEM information to the literature of ultrastructure, samples are also prepared according to the classical approach of the cell biologists.

Analyses of small aggregates of colloids and unusually large colloids (least diameter of 5 μm or less) are supplemented by observations from alternate thick (200 nm) and thin (50 nm) sections, the latter being documented at ca. 1 nm resolution. The EDS spot size can be changed from 50 nm to 330 nm by the microscopist according to the specific needs for sensitivity and coverage exacted by the specimen. Information on spatial relations can be supplemented further by a judicious use of scanning electron microscopy which, unfortunately, requires an additional effort put into artifact analysis and minimization. Optical microscope techniques and the great versatility of cytochemical techniques designed for optical microscopy also present a means to supplement observations by TEM.

III.2 Analysis of NOM aggregation

The aggregation of selected/enriched components of NOM can be studied by different means as follows.

(A) One can increase the solution concentration factor into the range of 10-1000 fold that of the lacustrine value just prior to embedding the sample in Nanoplast. Such a concentration increase can provoke aggregate formation in lacustrine NOM. A series of experiments using different concentration factors allows one to estimate the minimum concentration below which artifactual aggregation is negligeable. This approach is in its infancy but it is being used currently to study the behaviour of HMW-NOM (see section IV.2).

(B) One can do a series of filter captures of NOM with decreasing flow rates in the range 1000 $cm.h^{-1}$ down to less than 0.1 $cm.h^{-1}$ [15]. Immediately after the filter capture is completed, the still wet filters are embedded in Nanoplast, sectioned and observed by TEM. The TEM image shows clearly (Fig. 1) that aggregates (organo-mineral in this example) are formed on the upper surface of the filter. The average size of the

aggregates decreased with decreasing flow rate and artifactual aggregation was found to be minimal for flow rates below 1 $cm.h^{-1}$ [30]. The use of EDS and heavy metal counterstains in this example were of considerable use in discriminating the various components of the aggregates. It was shown that the colloids of Fig. 1 were mainly clays and iron and NOM. Of limnological interest, there were no calcium/magnesium carbonates despite the fact that the aggregates resulted from filtering water from a productive lake where calcium carbonate particles are important.

(C) One can use split samples to compare and contrast the results obtained from the minimal perturbation approach with the results obtained from a multi-method cytological approach based on the classical TEM sample preparation of the cell biologists. Often, the TEM images of a mixed aggregate prepared by Nanoplast embedding have many features in common with the same type of mixed aggregate prepared in the standard way using epoxy embedding (see sections II.1 and II.2) [25]. One can integrate the two images by using the following considerations; the Nanoplast image of the NOM and the disposition of its components within the aggregate will reflect reality (except for any intact microbiota present which will be degraded unless the Nanoplast was used in conjunction with a fixative), whereas the epoxy images of the cells of the biota and of all rigid extracellular materials and dehydration-resistant NOM will be interpretable in terms of both literature images and Nanoplast images. A little detective work will reveal what changes occurred in parts of the dehydration-sensitive NOM (the numerically important components of distinctive aspect in Nanoplast images) and what effects the dehydration artifact had on spatial rearrangement of components within an aggregate. Further detective work could enable one to derive three-dimensional models of the relationships between microbiota, NOM and other particles as they exist in a native aggregate. To this end, cytochemistry has already proven

useful in demonstrating organic sulfur compounds in extracellular plant products (at 3 nm structural resolution) and in demonstrating a manganese oxidizing factor associated with the extracellular fibrils (acid polysaccharide fibrils) of a bacterium. Through the use of a silver-hexamine reagent applied to sectioned plant cell walls [31], the spatial distribution of a disulphide-containing protein was mapped with respect to the various polysaccharidic structures within a 1 μm wall. Through the use of bacterial cultures capable of making particulate manganese oxides from manganese sulfate added to the culture medium [32], the following environmental observations could be made. The cells produced fibrils extending into their aquatic milieu and particulate manganese oxides formed in association with the fibrils, with manganese particle formation being blocked by treatments which inhibit enzymes such as manganese oxidase. In related experiments [32], it was shown that fibrils were important in generating colloidal particles of iron oxides.

III.3 Approaches for studying well-defined NOM components

Surface waters usually include a mixture of different types of NOM (FA, humic substances, organo-mineral particles, polysaccharides, proteins, cell debris) in various proportions. The means to separate them, one from another (filtration, centrifugation, chromatographic methods), all produce artifacts (e.g., aggregation, irreversible adsorption on a filter or stationary phase, etc.) and there is no single means to fractionate a natural water sample to get "pure" NOM fractions which relate to specific chemical entities. Therefore, combinations of approaches, as needs dictate, should be employed with an eye to minimizing sample/equipment interactions. Three approaches to obtaining meaningful NOM fractions with a minimum of NOM perturbation are being explored by the authors of this

paper, and are outlined briefly below.

(A) As a result of competing natural processes and seasonal cycles, the various types of aquatic NOM are not in constant proportions over time and space. As has been shown [4], relatively "pure" fulvics and humics of aquagenic type (formed in the water body) can be found at the surface of the open ocean, whereas "pure" fulvics and humics of pedogenic type (formed in soils and accumulated by water bodies) are found in non-productive ponds with large values for conventionally-defined, dissolved organic carbon [4]. Inside some lakes, pedogenic fulvics and humics are predominant in the hypolimnion and in winter, whereas,aquagenic ones exist mainly in productive lakes in the surface waters of summer [33]. Furthermore, the aliphatic content of pedogenic FA follows a seasonal cycle [34]. All these observations can be used to collect samples as enriched as possible in a given type of FA (and probably other NOM components). This approach, in which the lake provides a great assist with the fractionation and concentration of a specific NOM component, thus minimizing the need for technology, has been used for the results reported in section IV.1.

(B) A comparison of NOM fractions, sampled from a series of headwater lakes which vary by a small number of relevant parameters, can reveal some basic differences between predominant NOM species which relate to water quality. The investigation of multiple distant sites over a short time frame, however, imposes logistical constraints which, in turn, require that great care be used in the selection of water sampling and processing equipment. Ultrafiltration appears to be the process best suited for isolating HMW-NOM from its naturally dilute environment under the constraints of a comparative investigation. The best apparatus for this process in our experience is the tangential flow apparatus, which has a unique sweeping action of flow across the surface of the ultrafiltration membrane and thus

minimizes undesirable particle accumulations and aggregation at the filter surface. Microporous membranes (0.65, 0.45, 0.2 and 0.1 μm pore sizes) and various molecular weight cutoff membranes (with 300,000 and 30,000 and 10,000 MW being useful ones) can be combined to derive a variety of fractions [34]. Some HMW-NOM fractions are useful in analyzing flocculation/coagulation and results from such analyses are reported in section IV.2.

(C) The generation in the laboratory of specific NOM colloids using living lacustrine organisms has a great potential, provided that the colloids are not anomalous. Many environmental scientists interested in the particle analysis of NOM have abandoned the classical approaches to obtaining specific NOM components because of the technical difficulties listed earlier and an inability to cope with changing ratios of NOM components through time. As a consequence of these frustrations, there has been a focus on some fibrillar colloids secreted by phytoplankton, especially those which are secreted both in lakes and in laboratory cultures [10,12,14]. Since isolating fibrils from NOM-rich lake waters can yield an isolate in various stages of aggregation [35,36] and/or degradation [16], their isolation from surface waters has received second priority, by us, to the generation of fresh fibrils from specific algae under well-defined laboratory conditions. Manipulation of the nutrient regime of some algae can be a useful tool to induce fibril secretion into water [10]. The major barrier to success in this work comes from the fact that algae tend to be inconsistent in their response to the nutrient deprivation scenarios used to induce fibril secretion. Nevertheless, the production of quantitatively significant amounts of a given NOM component in fresh condition shows a potential to assist studies on particle reactions and on particle behaviour, as exemplified in section IV.3.

IV AQUATIC NOM CHARACTERIZATION: A DESCRIPTION OF SOME MAJOR TYPES

IV.1 Pedogenic fulvic acids (FA)

In a survey of surface waters for high ratios of FA to other substances [34], a pond was discovered to be so rich in pedogenic FA (MW=1750) relative to other organics, that the pond water could be treated almost as a dilute solution of one class of FA [37]. Very little pretreatment was needed to make the sample amenable to experiments, consequently minimizing handling artifacts [37]. Thus this pond (Mare aux Evees, near Fontainebleau in France) provided a novel situation for experimentation, a situation yielding samples of undegraded FA whose native aggregation behaviour could be subjected to both standard physico-chemical analyses and a TEM visualization of aggregation phenomena [17]. Although a water-compatible embedding resin of high quality was not available to us at the time, a multi-method cytological approach did permit some pertinent observations [17]. In particular, it was observed (Fig. 2) that FA in the concentration range of 20-200 mg/L formed a continuum of aggregated particles of widely varying size in rather fast equilibrium. At surfaces, dehydration occurred, thus making the aggregation process a less readily reversible one. The data collection showed many features in common with earlier size and shape analyses of soil FA which had been chemically degraded during purification [38,39]. The use of native FA in conjunction with Nanoplast embedding in the field is anticipated to expand the scope of that earlier experimental work.

Specific details of note regarding the FA particles from Mare aux Evees [17] are as follows. The multi-method approach of analytical chemistry (including ultrafiltration, fluorescence, diffusion in solution and surface tension measurements) was in general agreement

with TEM on the size of unit particles of FA (near 1 nm) despite the resolution impediment imposed by the grain structure of the best embedding resin available at the time. Many of the small aggregates were in the 13-35 nm size range as measured directly by TEM. Large aggregates tended to give rise to a continuum resembling a sponge. Within the "sponge" were included large well-packed aggregates (Fig. 2) which were spherical with diameters approaching the upper limit for colloids (ca. 0.6 μm). TEM analyses of such aggregate formation, currently in progress, could very well improve our understanding of some mechanisms of surface water instabilities.

IV.2 High molecular weight organic compounds (HMW-NOM)

Many of the cell-derived HMW compounds, such as polysaccharides [40], lignins [41], proteins [42], mucopeptides [40], tannins [43] and undegraded cellular debris [44],were characterized many years ago with regard to their chemistry and general aspects. Among these materials/compounds, the polysaccharides have considerable quantitative significance for some surface waters [8]; important ones are cellulose [26], chitin [42] and families of polymers containing uronic acids [10,12,45] which can form extended macromolecular aggregates in water [46]. An example of the latter is the fibril described in section IV.3. Fibrils can be distinguished from other components of HMW-NOM by their ribbon-like shape in combination with their very narrow range of diameters and staining properties [12,18]. They aggregate so readily in response to filtration that they are often excluded from HMW-NOM fractions whose cutoff filter was much below 0.45 μm, despite individual diameters typically near 0.005 μm.

The chemical and general characterizations of the cell-derived HMW materials above were followed up by demonstrations of ultrastructural

correlates for them (dehydrated versions). A large selection of electron micrographs, prepared by dozens of biological and biophysical laboratories on an international scale, which show these ultrastructural correlates, can be found in references [44,47,48]. Also, the texts of these works present a useful guide to specific TEM references in the ultrastructure literature which relate to the visualization of chemically-known HMW biologicals relevant to analyzing NOM. Most of these references still have considerable utility, even though HMW-NOM also includes humic substances(and degraded versions of the biologicals listed above) which have not as yet been described. Despite their utility, these references are used only rarely by aquatic particle analysts. Thus, until recently, attempts to survey lake water by TEM for the major colloidal components of HMW-NOM have been preliminary [49] and restricted to rigid materials and components whose ultrastructure can be interpreted despite dehydration artifacts. At the moment, however, there is no technological impediment to attempting realistic TEM analyses of HMW-NOM.

We have therefore begun such analyses, focussing on calm epilimnetic waters. By employing a tangential flow apparatus (TFA) with fresh samples, fractions of NOM can be prepared which are enriched (according to the efficiency of the specific apparatus chosen) in HMW-NOM from a pre-determined size range. A given fraction can be concentrated sufficiently to provoke aggregation. Fig. 3 illustrates aggregates of 0.2 μm > HMW-NOM > 30,000 MW which resulted from increasing the solution concentration factor of samples from different lakes by ca. 1-4 x 10^2 times the lacustrine concentration, using a stainless steel Millipore TFA in which the water sample does not come into contact with any lubricating substances. Embedding of these fractions, from a series of headwater lakes, was done in Nanoplast FB 101 [28]. On a "per lake" basis,

the morphology of the major types of aggregates (often many times the size of the upper cutoff filter), taken in conjunction with the relative abundance of each major type and the morphological aspect of other size fractions from the same lake, shows some promise of yielding a crude means of "fingerprinting" each lake type. Whenever a high contrast (electron opacity) indicates heavy elements within an aggregate (in this example Si, S, Fe), a secondary investigation can be done by EDS. Searches for internal heterogeneity can be conducted by counterstaining for polyanions and, when the cost is justified, by cytochemistry.

In the context of limnological investigations on the same lakes, one now has the potential to relate the following:

(A) ultrastructural correlates of the physical events which occur during the flocculation/coagulation of NOM;

(B) the growth of submicron aggregates in relation to settling/sedimentation;

(C) the behaviour of NOM in relation to biological correlates, such as changes in the major secreting organisms caused by toxic contaminants or the presence of an algal bloom;

(D) the nature of HMW-NOM in relation to water chemistry;

(E) the mode of NOM aggregation in relation to the mode of dispersion of selected contaminants.

IV.3 Fibrils rich in polysaccharide

Fibrils are elongate aggregates of organic macromolecules secreted by some aquatic biota and the roots of land plants [10,11,12], not to be confused with crystalline cellulose microfibrils [26]. They have diameters in the narrow range of 3-10 nm [10] and serve often as natural adhesives promoting microbial colony formation and biofilm formation in surface waters [11,12,18]. They are defined by a distinctive TEM image, typically as the ultrastructural units of some extracellular coatings (capsules, sheathes, slimes, mucilages and

the matrices of biofilms) [12,18]. The few chemical investigations of them have shown them to be rich in acid polysaccharide where the acidic monomer is an uronic acid [10,11,12]; more chemical analyses are needed. Because fibrils contain uronic acids and because they bind cationic counterstains in sections, they are often considered by aquatic scientists as colloidal polyanions. Research on their functions has proceeded slowly because of their chemical complexity, the difficulties of isolating and storing large quantities of a given morphotype of fibril for experiments, and the episodic appearance of them in the water column.

Currently there is an interest in studying the partitioning of contaminants between fibrils and lake water. In this context, their aggregation behaviour is of considerable importance. Dispersed fibrils of ca. 5 nm diameter and ca. 1000 nm length can generate particles (essentially sheets of tightly packed individual fibrils plus occluded small colloids) of ca. $10^5 \times 10^5 \times 10^3$ nm when they are concentrated by dehydration. Storage of fibril-rich water for extended periods of time (weeks) can also lead to aggregation phenomena, such as the formation of a fibrillar film on the walls of the storage container (accompanied by whatever the fibrils were able to sequester from the bulk water phase). Fig. 4 illustrates such transitions and suggests the relevance of considering the degree of fibril aggregation when fibrils are used in partitioning experiments.

Fig. 4 can also be considered a study in fibril behaviour. Fig. 4B shows a portion of a well packed film of "aged-in-water" fibrils which had "grown" on and become loosely associated with the glass container wall of an algal culture used for fibril secretion experiments [10]. Fig. 4C shows the extreme packing of fibrils (and their organization into sheets) which occurs under conditions of severe dehydration. Since well packed fibril aggregates are difficult

to disperse in the laboratory, the possibility of occlusion of environmentally significant substances within developing fibril aggregates presents interesting scenarios [50]. To the best of our knowledge,there is not yet an experimental literature on fibril aggregates which employs Nanoplast embedding to minimize sample handling artifacts.

V CONCLUSION

No component of NOM has been analyzed systematically by all the new techniques available up to now. However, the components under review here are approaching such a high level of scrutiny. Since enough is understood now about artifact identification, assessment and minimization to give us confidence in future undertakings in aquatic particle analysis, it is useful to make a brief and optimistic commentary on the current status and future directions of the research. The aforementioned TEM information on NOM sizing is compatible with sizing information found in the literatures [10,17, 38,51] of analytical chemistry, agricultural chemistry and ultra-structural biology. Size and shape analyses of NOM aggregates and the microheterogeneity within them
have produced some noteworthy observations (above). These analyses show definitively some advantages gained by treating NOM components as complex physical structures suspended in water, as opposed to large molecules dissolved in water [14,50].

A consideration of the usefulness of TEM in the analysis of sectioned NOM leads naturally to a consideration of future potential and to a caution about its weaknesses. The drawbacks are that TEM, both of itself and in conjunction with multi-method approaches developed by limnologists and analytical chemists, is very costly and time-consuming and dependent on combinations of technical

specialists which are rarely found in a single institute. Those who have difficulty in relating to semi-quantitative data and to a detective's mentality should not enter this field at this time. A scientific drawback resides in the fact that many interesting features of aquatic particles are likely to fall in the 1-5 nm size range, a range in which interpretation problems are maximal.

The following progress is likely to be evidenced in the near future.

(A) The development of an ultrastructural literature on the principal NOM components of lakes will provide a basis for more enlightened investigations into contaminant dispersion via colloids.

(B) Morphological analyses of aggregates and aggregation artifacts will be supplemented by TEM analyses of the processes of aggregation. When sections will have revealed the major stages of aggregation for a given NOM, one will be able to turn to cryotechnology, such as freeze-etching [29] and high pressure modifications of it [52], to attempt closely-spaced time sequence studies of aggregate formation/flocculation in relation to various experimental manipulations.

(C) High resolution analyses will challenge the classic means of defining what is and what is not amorphous. Ordered regions can be visualized in "amorphous materials" even when these regions are only tens of nanometers across and represent less than 5% of the material in the field of view.

(D) Analysis of the occlusion of mineral nutrients and contaminants which contain heavy elements, during the genesis of NOM flocs/coagula, in relation to bioavailability phenomena and natural lacustrine purification mechanisms, will receive an impetus from TEM-EDS.

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GLOSSARY

part one - definitions

NOM - natural organic matter
- all the organic molecules and materials in an aquatic ecosystem other than living organisms and man-made compounds

HMW-NOM - natural organic matter of high molecular weight
- an arbitrarily defined fraction of NOM confined to the colloidal size range

Pedogenic (OM) - organic matter formed in soils and leached out into water bodies

Aquagenic (OM) - organic matter formed within a water body

Morphotype - an arbitrarily defined species of colloidal material whose definition is based entirely on morphological differences between it and other colloidal materials in the same sample

Cytology/Cell Biology - the study of living cells, including the methods of cytochemistry and the use of chemical fixatives (see below)

Cytochemistry - the identification and localization of the chemical components of the cell, with a view to relating functional changes to chemical changes

part two - technical procedures

Chemical Fixation - the use of chemicals (such as glutaraldehyde and/or osmium tetroxide) to preserve as faithfully as possible every detail of cellular ultrastructure down to the molecular level exactly as every detail was in life at the instant before the cell was killed by the "fixative"

Cryotechnology - an alternative and complementary technology to chemical fixation, one which produces "fixed" cells by physical means (such as rapid freezing and vitrification)

Freeze-etching - a technique of cryotechnology which consists of freezing a sample rapidly enough to vitrify it, mechanically generating a fracture plane through it, and making a metallic replica of the fracture surface, all the while maintaining the vitrified NOM below the recrystallization temperature
- the product of the technique is a replica which presents a topographical image of NOM untouched by chemical agents and amenable to high resolution analyses by TEM

Embedding - the replacement of the water molecules of a sample by molecules of a material which can be hardened to provide a matrix appropriate to making sections thin enough for optimal use of TEM
- the most versatile embedding material of the cell biologists is the epoxy resin which requires the use of an intermediary solvent (dehydrating agent) since epoxy monomers cannot be used directly with fixed cells
- for analyses in which avoidance of the dehydrating agent is desireable, one can use a water compatible resin provided that its cutting properties can be optimized and providing that it does not cause artifacts more difficult to cope with than the artifacts produced by dehydrating agents
- the experimental melamine resin, Nanoplast FB 101, is our choice of water compatible resin for high resolution TEM analyses of sectioned aquatic NOM in minimally-perturbed state

Counterstaining - the application of a stain, usually containing a heavy metal, to a section so as to increase the density of a cell part or colloid relative to the embedding matrix, thus increasing its contrast so as to make viewing easier

Cytochemical staining - a technology employing the use of various chemical and physical methods to detect or measure specific chemical components within a cell or cell parts
- it includes the use of selective counterstains

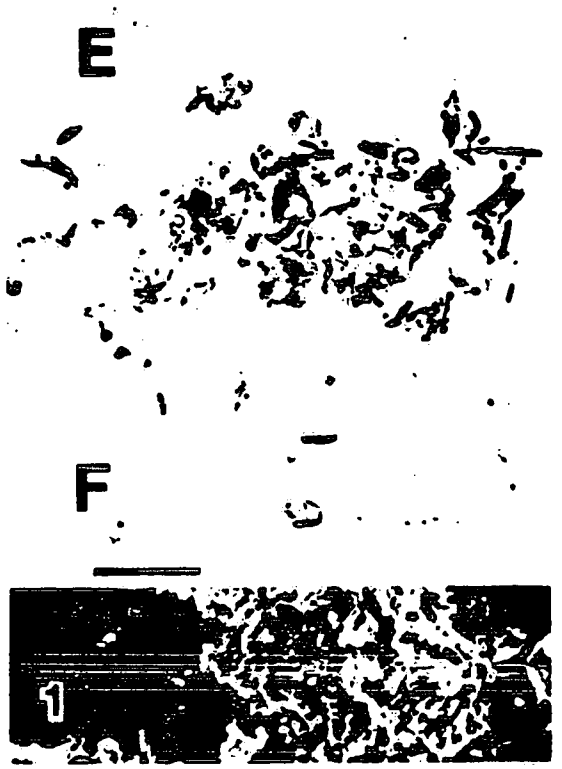
FIGURE LEGENDS

Fig. 1. A TEM view of a section (upper figure) taken through a heterogeneous collection of colloids induced to aggregate into a porous "conventional size particle" by a high filtration flow rate applied to a 0.45 μm cutoff filter. "F" represents the filter body and "E" represents the space between the cutoff filter and a larger size screening filter. The artifactual aggregate is at the interface between filter and oncoming lake water, and most of its individual components are actually smaller than 0.45 μm .Below this section, we present a scanning EM view which looks down upon the upper surface of the filter, a view at right angles to the section. This low resolution view by scanning EM gives the impression that the loose aggregate of colloids, as shown by high resolution TEM, has become a single rough-surfaced particle nearly 10 μm wide. The implications of this double artifact scenario are discussed in ref. 15 from which Fig. 1 was adapted. The bar represents 3 μm.

Fig. 2. A TEM view of a section taken through aggregates of pedogenic FA. Note that the dense aggregates have diameters near the pore size of the cutoff flter used to define conventional particles. The bar is 0.45 μm.

Fig. 3. Thin sections of several types of aggregates of HMW-NOM taken from epilimnetic waters of lakes in Ontario, Canada. These aggregates were induced to form in the laboratory by increasing the concentration factor of 0.2 μm > HMW-NOM > 30,000 MW by more than 100-fold. (A) a fibrous continuum from Wolf Lake. (B) aggregates showing nucleating centres, from Bay Lake. (C) aggregates showing uniform density, from Williams Bay of Jack Lake. The bar represents 3 μm.

Fig. 4. Fibrils prepared for electron microscopy according to Leppard et al. (1977). These micrographs show counterstained ultrathin sections of fibril-rich NOM captured from suspension by gentle centrifugation. (A) fresh fibrils. (B) fibrils stored for three weeks in water at room temperature. (C) fibrils stored as a freeze-dried concentrate and then rehydrated.


E
F
1


2


3B
3A
3C


4 A 0.1μm
4 B 0.1μm
4 C 1.0μm