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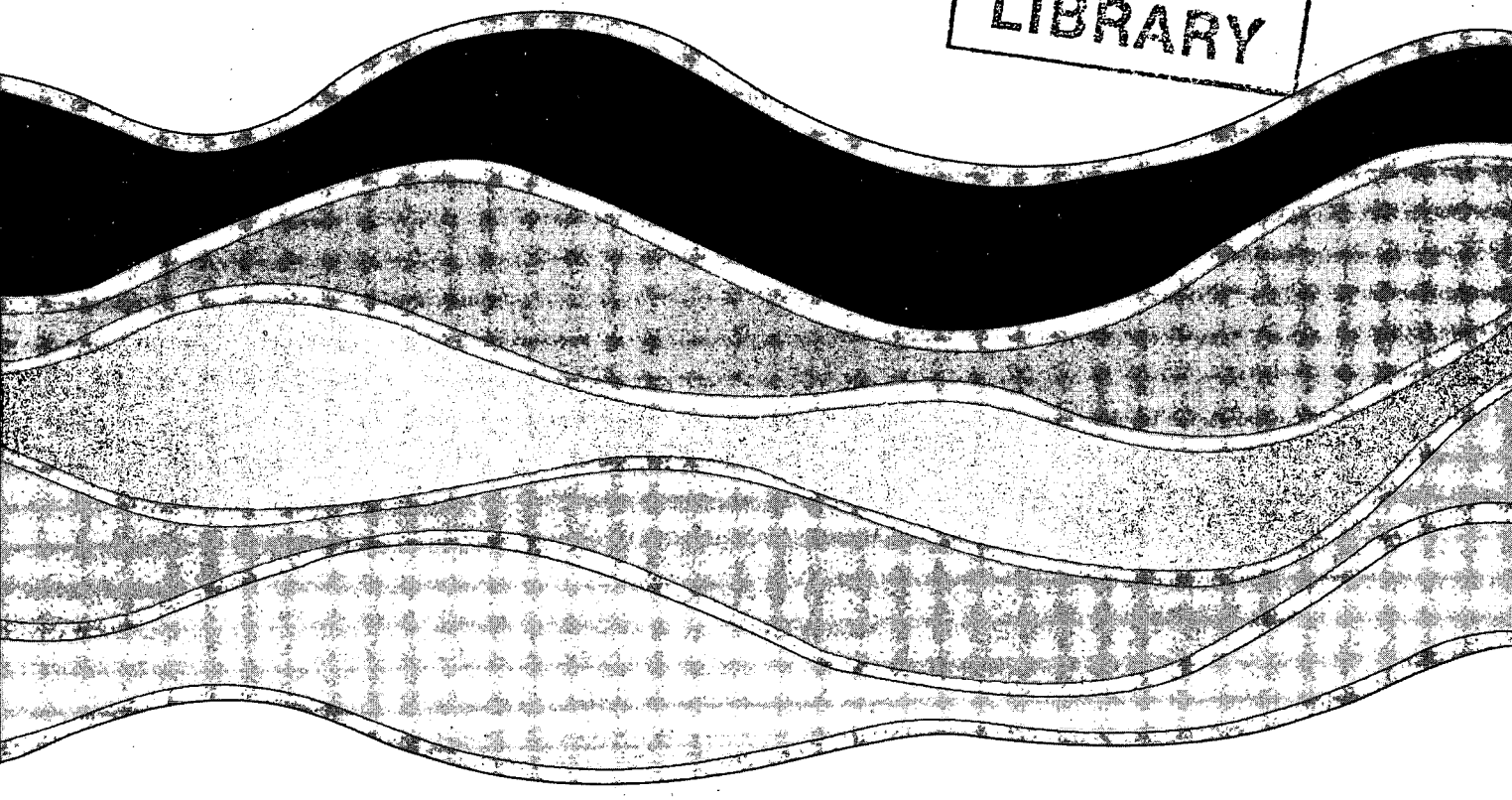

COLLOIDAL ORGANIC FIBRILS IN SURFACE WATERS: ELECTRON-OPTICAL CHARACTERISTICS, ACTIVITIES AND CHEMICAL ESTIMATES OF ABUNDANCE

G.G. Leppard

NWRI CONTRIBUTION NO. 96-151

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Gary G. Leppard
Aquatic Ecosystem Protection Branch
National Water Research Institute
Burlington, Ontario, Canada L7R 4A6

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MANAGEMENT PERSPECTIVE

This invited review paper presents suggestions for engineering applications of colloidal organic fibrils, discovered by NWRI scientists in 1977 to be an abundant component of surface waters. These are the same fibrils shown recently by NWRI scientists to be an important component of suspended flocs in engineered water treatment facilities.

The fibrils are defined chemically and morphologically in this review, accompanied by the current knowledge of their activities in natural waters. Means to isolate and characterize the fibrils are presented, along with techniques for quantifying them. Suggestions for engineered manipulations are offered for the purpose of producing flocs which might have optimal properties in water decontamination systems. Current scientific evidence indicates that some aquatic organisms secrete specific fibril types to answer specific biological needs (e.g., the need to immobilize toxicants, the need to increase the cell surface affinity for nutrients). Thus, living cells are actively engineering the nature of the flocs that they create; they appear to be responding to environmental signals in the bulk water (limiting nutrient stress, ratios of essential nutrients, stressful concentrations of certain cations), signals which can be manipulated by engineers at water treatment facilities.

SOMMAIRE À L'INTENTION DE LA DIRECTION

La présente communication sollicitée présente des suggestions relatives à des applications techniques des fibrilles organiques colloïdales, découvertes en abondance dans les eaux de surface par les chercheurs de l'INRE, en 1977. Il s'agit des mêmes fibrilles qui, les chercheurs de l'INRE l'ont constaté, sont des constituants importants des flocs en suspension dans les installations modifiées de traitement de l'eau.

Dans cet article, on définit les fibrilles sur les plans chimique et morphologique et on présente les données que l'on possède à l'heure actuelle sur leur activité dans les eaux naturelles. On indique en outre les moyens de les isoler et de les caractériser, ainsi que les techniques servant à les mesurer quantitativement. On présente des suggestions quant aux manipulations techniques à utiliser pour produire des flocs qui pourraient avoir des propriétés optimales dans les systèmes de décontamination des eaux. Les données scientifiques actuelles indiquent que certains organismes aquatiques sécrètent des types particuliers de fibrilles répondant à certains besoins biologiques (p. ex la nécessité d'immobiliser les toxiques, la nécessité d'augmenter l'affinité de la surface des cellules pour les nutriments). Ainsi, les cellules vivantes modifient activement la nature des flocs qu'elles créent; elles semblent réagir à des signaux environnementaux dans l'eau libre (stress résultant de la limitation des nutriments, ratio des nutriments essentiels, concentration nuisible de certains cations), des signaux que les ingénieurs des installations de traitement des eaux peuvent mettre à profit.

ABSTRACT

Many organisms in fresh waters secrete carbohydrate-rich substances which facilitate growth and survival. Some of these substances are acid polysaccharides of high molecular mass; they are multifunctional and their molecular architecture can be engineered by many organisms (e.g. algae, bacteria, plants) to combat specific environmental stresses (e.g. high toxicant levels, low nutrient levels). These polyanionic extracellular polymers represent families of macromolecules, rather than a specific class of carbohydrate polymer. They aggregate in water to form colloidal fibrils which can reside at the organism-water interface, participate in biofilm formation or enter the bulk water phase as colloids. Their manifold activities include the natural decontamination of water bodies; in the form of flocs, fibril aggregates are implicated in the functioning of engineered water treatment systems. The decontamination activity begins with the binding of toxicants in various ways (e.g. ion-exchange, sorption, chelation) by fibrils, followed by aggregation processes (e.g. flocculation, coagulation) which produce suspended particles (>1 μm) having a density different from that of the bulk water. Dense particles can aggregate further to form settling particles, thus removing the "bound" toxicants to the sediments. These processes have not been described well for real systems; the same can be said for detailed descriptions of fibril chemistry in terms of the various acid polysaccharides comprising them. While various fibril activities have been described by many laboratories encompassing many scientific disciplines, fibril impacts on surface water quality are only vaguely understood. The vagueness is a result of technical difficulties in classifying fibrils and in quantifying both abundance and native activities. The technical difficulties have remained insufficiently addressed despite some obvious examples of profound ecosystem and economic impacts thrust upon man by colloid systems in recent decades. The problem of quantifying the chemical features of polyanionic extracellular polymers can be overcome now by technology transfer, employing combinations of techniques already extant in the literature. A strategy to combine separation technology, biochemical concepts and the tools of analytical chemistry is outlined in this presentation. A specific feature essential to the use of the strategy is

the use of transmission electron microscopy to monitor and "tune" the isolation and purification methods for quantitative analyses of extracellular polymers. An improved quantitative understanding of fibril chemistry and activities should lead not only to a better understanding of colloid impacts, but also to an improved capacity to "engineer" flocs for the treatment of polluted waters.

Keywords: fibril, extracellular polymer, acid polysaccharide, transmission electron microscopy, engineered aquatic systems

RÉSUMÉ

De nombreux organismes d'eau douce sécrètent des substances riches en glucides qui facilitent leur croissance et leur survie. Certaines de ces substances sont des polysaccharides acides de haut poids moléculaire; elles ont plusieurs fonctions, et de nombreux organismes (p. ex. les algues, les bactéries, les plantes) peuvent les modifier pour lutter contre certains stress environnementaux (p. ex. teneurs élevées en toxiques, faibles teneurs en nutriments). Ces polymères extracellulaires polyanioniques constituent des familles de macromolécules plutôt que des classes spécifiques de polysaccharides. Ils s'agrègent dans l'eau et forment des fibrilles colloïdales qui peuvent se localiser à l'interface organisme-eau, participer à la formation d'un film biologique ou entrer dans la phase d'eau libre sous forme de colloïdes. Leurs activités multiples comprennent la décontamination naturelle des nappes d'eau; sous la forme de flocs, les agrégats de fibrilles participent au fonctionnement des systèmes modifiés de traitement de l'eau. L'activité de décontamination commence avec la fixation des toxiques de diverses façons (p. ex. par échange d'ions, sorption, chélation) par les fibrilles, puis se poursuit par des processus d'agrégation (p. ex. floculation, coagulation) qui sont à l'origine de particules en suspension (>1 μm) dont la densité diffère de celle de l'eau libre. Les particules denses peuvent s'agréger davantage pour former des particules qui sédimentent, les toxiques ainsi «liés» se retrouvant dans les sédiments. Ces processus n'ont pas fait l'objet d'une description précise dans les systèmes réels; on n'a pas non plus décrit en détails les propriétés chimiques des fibrilles, c'est-à-dire les divers polysaccharides qui les composent. De nombreux laboratoires, oeuvrant dans diverses disciplines scientifiques, ont décrit les diverses activités des fibrilles, mais les effets de ces dernières sur la qualité de l'eau restent assez mal compris. Cela provient des problèmes techniques qui entourent la classification des fibrilles et la mesure de leur abondance et de leurs activités intrinsèques. On ne s'est pas penché suffisamment sur ces problèmes malgré les exemples évidents, révélés au cours des dernières décennies, des effets écosystémiques et économiques profonds que peuvent avoir les systèmes colloïdes sur les activités humaines. On peut maintenant surmonter le problème de la mesure des propriétés chimiques des polymères extracellulaires polyanioniques par un transfert de

technologie, en faisant appel à une combinaison de techniques qui existent déjà dans la littérature. Dans cet exposé, on présente une stratégie combinant les techniques de séparation, les concepts biochimiques et les outils de la chimie analytique. Un des éléments essentiels de cette stratégie est l'utilisation de la microscopie électronique à transmission pour contrôler et «accorder» les méthodes d'isolement et de purification servant à l'analyse quantitative des polymères extracellulaires. Une meilleure connaissance quantitative des propriétés chimiques des fibrilles et de leurs activités devrait non seulement augmenter nos connaissances sur les effets des colloïdes, mais également améliorer notre capacité de modifier les flocs pour les faire participer aux traitement des eaux polluées.

Mots clés : fibrille, polymère extracellulaire, polysaccharide acide, microscopie électronique à transmission, systèmes aquatiques modifiés

1. Introduction

1.1 Extracellular polymers as biologically engineered particles

Many microorganisms in fresh waters secrete carbohydrate-rich substances which facilitate growth and survival. These organisms include eukaryote algae (Lewin, 1962), cyanobacteria (Drews & Weckesser, 1982) and non-photosynthetic bacteria (Beveridge & Graham, 1991). Some of these substances form extracellular materials serving functions which are principally skeletal, prominent examples being cellulose (Cook & Stoddart, 1973) and some complex heteropolymers of the bacterial cell wall (Beveridge, 1989). Others, such as acid polysaccharides of high molecular mass (Leppard et al., 1977; Geesey, 1982; Costerton et al., 1987; Leppard, 1992a), are multifunctional in many senses (Boney, 1981; Costerton, 1984; Costerton et al., 1987; Leppard, 1992a, 1993). Their molecular architecture appears to be genetically engineered by at least some secreting organisms (Whitfield, 1988; Sutherland, 1990; Leppard, 1995) for the purpose of responding to specific environmental stresses. The quantity of extracellular acid polysaccharide produced can also be related to stresses such as high toxicant levels (Brown & Lester, 1979; Leppard, 1993), desiccation (Roberson & Firestone, 1992) and low nutrient levels (Costerton, 1984; Strycek et al., 1992). Such secreted polyanionic substances represent families of macromolecules, rather than a specific class of carbohydrate polymer. Their sometimes great abundance and biologically engineered aspect make them the sole focus of this brief review. Because there are many diverse chemical species of acid polysaccharides (Aspinall, 1983; Kennedy & Sutherland, 1987), exhibiting a variety of different chemical and biological activities in both natural and engineered aquatic ecosystems, any single chemical measure of quantity which lumps them all together on the basis of a given general attribute (e.g. presence of uronic acid moieties) is likely to be disappointing in relating quantities to specific activities. In nature, the acid polysaccharides are often found in association with neutral polysaccharides and mucopolysaccharides; a consideration of such associations would take us beyond the scope of this brief review.

Many organisms which secrete acid polysaccharides into surface waters assemble them first into "packages" of colloidal dimensions (submicrometre particles), with the package usually taking the form of an extremely thin ribbon or "fibril" (Fig. 1). The polysaccharides within or comprising the fibril are often called exopolysaccharides, which is a class of exocellular polymer or extracellular polymer. The fibril (or fibrillar extracellular polymer) and its contents represent a non-living and variable component of natural organic matter; fibril detachment from cells can occur without internal cell damage (Leppard et al., 1977; Leppard, 1993). On an episodic basis, in a small aquatic ecosystem, the fibril can be more abundant, both in terms of number and the mass of organic carbon contained within them, than all of the conventionally-defined organic particles taken together (those larger than ca. 1 micrometre, including the biota). This situation can occur during the collapse of an algal bloom in a small lake (Burnison & Leppard, 1983). Fibril dominance also appears possible in water treatment systems which are rich in suspended flocs (Liss et al., 1995), and in shallow marine ecosystems (Heissenberger et al., 1995). On a planetary scale, fibrils are suspected of playing significant roles in industrial waters (Geesey, 1982) and in oceanic waters (Passow et al., 1994; Leppard, 1995; Heissenberger et al., 1995).

1.2 Detection of fibrils in aquatic ecosystems

The term fibril refers to distinctive ribbon-like colloids (Leppard et al., 1977) whose least dimensions are in the smallest portion of the colloidal size range and whose low native electron-opacity can be augmented by heavy metal stains for transmission electron microscopy. They are not to be confused with certain other elongate microscopic structures found in aquatic ecosystems. Such other structures include specialized hair-like projections on the surface of bacteria (van Iterson, 1969; Fletcher & Marshall, 1982), crystalline microfibrils of algal and plant cell walls (Preston, 1974), flagellar hairs on eukaryote algae (Dodge, 1973), fragments of algal extracellular scales and coccoliths (Dodge, 1973), elongate fragments of bacterial cell walls or zooplankton exoskeletons (Massalski & Leppard, 1979a), linear fractal aggregates of humic substances (Leppard et

al., 1990) and pre-humics (Leppard et al., work in progress) or the very much larger plant fibres (true particles) and elongate fragments of them (Preston, 1974). They are also not to be confused with filamentous microbes (bacteria, fungi, cyanobacteria) whose least dimensions can approach or even extend down into the upper portion of the colloidal size range.

The various types of fibrils, as defined morphologically (Leppard, 1986), have a unique combination of dimensions and shape when analyzed by high-resolution electron-optical means, the sole means to detect them as individual physical units in water (Leppard, 1992a, 1992b). Almost all known types have a diameter in the range of 0.002 to 0.020 μm; they can be hundreds of times longer than wide and can be branched. Those whose "appearance" is most ribbon-like can associate into flocs of high water-to-polymer content, flocs which spontaneously convert into dense settling particles under the influence of any dehydrating perturbation (Leppard et al., 1990; Leppard, 1993). Individual fibrils in flocs are visualized as individuals by transmission electron microscopy or TEM (ca. 0.001 μm resolution), even for the case of collapsed dense flocs (Leppard, 1992b). Within an individual fibril, the molecular arrangement and packing of the acid polysaccharides is unknown, although the fibril's length-to-width ratio suggests that some individual macromolecules might be aligned with the fibril's long axis as extended linear entities. Attempts to visualize their internal structure using energy filtered TEM techniques (Mavrocordatos et al., 1994) could lead to an improved classification scheme if used in conjunction with specific chemical markers. Specialized technology to determine the nature of the macromolecules making up the core of a fibril is in place (Behmlander & Dworkin, 1994); this involves observations, by field emission scanning electron microscopy, of fibrils digested selectively by enzymes.

Historically, the fibril has been defined by its image (usually a stained image to improve its weak contrast) in micrographs derived from TEM applied to ultrathin sections (ca. 0.05 or 0.07 μm thick) of embedded aquatic samples (Leppard, 1992a). Detecting them on the surfaces of microbes, especially for the case of large microbial aggregates, has not

presented serious technical difficulties (Leppard et al., 1977), nor has detecting them on the surface of suspended minerals (Massalski & Leppard, 1979a), suspended debris (Massalski & Leppard, 1979a) or submerged plant surfaces (Leppard, 1984b). Most of the detection work done to date with TEM has been qualitative. However, the detection of detached fibrils in water (the "apparently dissolved" fibrils) has been put on a quantitative basis using hollow-fiber filtration to concentrate them from a filtrate of lake water which had been passed through a cascade of filters designed to remove the various size classes of conventionally-defined particles (Burnison & Leppard, 1983). The mass of organic carbon in the fibril fraction was measured by conventional limnological chemistry while the TEM was used to monitor the fractionation scheme and subsequent purification steps (Burnison & Leppard, 1984) in a successful attempt at tuning all steps to achieve a high yield with minimal losses (Fig. 2). The use of specific chemical markers (e.g., informational molecules labelled with colloidal gold) for macromolecular components of fibrils is expected to aid in bridging the gap between TEM qualitative and semi-quantitative analyses of fibril abundance and a much less costly technology based on wet chemistry. The optical microscope technologies of monoclonal antibody reactions and carbohydrate hybridization probes (Vreeland et al., 1987) would be helpful if adapted to TEM characterizations. When a quantitative chemical technology will be in place, there will still be a need for TEM to monitor (Fig. 2) fractionation schemes for purposes of fine-tuning them (Leppard, 1985). TEM will also be necessary for making the distinction between extracellular and intracellular fibrils, both of which may contribute to a chemical measure applied to raw water. The TEM detection is currently so refined that, with sections taken through eukaryote algae, one can even visualize fibril genesis within the secretory apparatus of a single cell (Leppard, 1995).

For the detection of detached fibrils and small fibril flocs in water, a less costly TEM technology has come into use in the 1990's. This new TEM technology defines the fibril in terms of "whole mount" images (Leppard, 1992a) in electron-optical searches for fibrils ultracentrifuged onto (or into) electron-transparent films which had been mounted on grids for holding the specimens to be inserted into a TEM (Perret et al., 1991; Filella et al.,

1993). Chemical markers for use with these whole mount ultracentrifuge preparations are currently under development (Perret, personal communication). Some of the quantitative TEM technology described recently for the enumeration and shape analysis of carbohydrate polymers (Stokke & Elgsaeter, 1991) should be adapted to the analysis of fibrils.

1.3 Cost effective coupling of TEM detection to quantification by chemistry

TEM and its accessory and derivative instrumentation (Leppard, 1992a), used for the detection of fibrils, is best employed to monitor and "tune" fractionation schemes applied to source waters, an idea developed for environmental samples more than a decade ago (Leppard, 1985) but difficult to apply until recently. A monitoring and tuning operation, guided by TEM, can permit the water quality analyst to derive fibril-rich fractions of high yield and high relative purity for standard chemical analyses. Fibril-specific markers are being developed as colorimetric detectors of fibril suspensions in water (Figueroa & Silverstein, 1989; Strycek et al., 1992). For some markers, such as ammoniated ruthenium oxychloride (the stain, ruthenium red), the validity of use for a given type of source water can be assessed by TEM, with a consequent minimization of costly TEM use in subsequent monitoring for fibrils. Colorimetric measures, related to the acid polysaccharide component of fibrils, appear to be a good but crude means to quantifying them in source waters at minimal cost. The disadvantages and artifacts of this approach, used in combination with TEM verifications of fibril-marker associations, are discussed in the appropriate section below.

2. Fibrils: electron-optical images, activities and general chemistry

Fig. 1 shows some examples of fibrils as visualized in ultrathin sections by TEM. These are the preparations most relevant to the development of chemical quantitative assays. Micrographs illustrating a wide variety of morphological types can be found in Massalski & Leppard (1979a; 1979b); a classification scheme is outlined in Leppard (1986). Some

natural activities and biological functions of fibrils and fibril associations are set out in Fig. 3. A review of their activities in relation to contaminant dispersion is found in Leppard (1993). Roles as floc formers (Leppard et al., 1990; Leppard, 1995; Heissenberger et al., 1995) and as matrix components of biofilms (Geesey et al., 1977; Geesey, 1982; Leppard, 1986; Costerton et al., 1987) have been investigated, as have roles in microbial adhesion to surfaces (Fletcher & Floodgate, 1973; Geesey et al., 1977; Sutherland, 1983; Van Loosdrecht et al., 1990; Leppard, 1993) and colloid scavenging (Filella et al., 1993; Perret et al., 1994; Buffle & Leppard, 1995a). Also, fibrils can: bind extracellular enzymes in their active form (Ghiorse & Hirsch, 1979); scavenge the bulk water for metals (Decho, 1990; Leppard, 1993); immobilize toxic substances (Costerton et al., 1987); alter the surface characteristics of suspended particles (Leppard, 1984a); and change the solubility status of associated molecules (Leppard, 1993). Decontamination activities have been known for some time (Leppard, 1993) as have organic coating effects on the flocculation of suspended particles (Bernhardt et al., 1985). Undoubtedly, fibrils are important to the ecology of many aquatic microbes, both bacterial and algal, although attempts to quantify their importance are plagued by technical difficulties.

To understand better the behaviour and functions of fibrils in water, a greater understanding of their chemistry is required (Leppard, 1995). A chemical representation (Cook & Stoddart, 1973) of a major subcomponent of some eukaryote fibrils is shown in Fig. 4. This generalized formula, revealing considerable complexity in chemical fine structure, shows a segment of a long macromolecule with side chains. This macromolecule belongs to a common class of acid polysaccharides called pectins; chemical descriptions of pectins and structurally-related acid polysaccharides are found in Aspinall (1982, 1983). Such polyanionic polymers consist essentially of covalently linked sugars, with a varying proportion of the sugars bearing carboxyl groups; each individual chemical species of carboxylated sugar is called a uronic acid, with uronic acid nomenclature relating back to the formal name of the sugar. The formula of Fig. 4 relates to plant and some algal contributions to natural waters; for a guide to bacterial contributions, one can consult Whitfield (1988), Kenne & Lindberg (1983), Sutherland

(1983) and Drews & Weckesser (1982).

There is a growing literature pertaining to the chemistry of those cell surface polymers which are both rich in acid polysaccharides and related to polymer bridging/adhesion phenomena. This literature is scattered among many disciplines, with most of it consisting of specialized researches which fall within various fields of biochemistry, biomedical science, botany, engineering, environmental science, microbiology, phycology and ultrastructural science. The marine and freshwater researches have often been done in isolation from each other, despite similar concepts and techniques being employed, and one cannot always ascertain whether or not the emerging chemical details relate to fibrils per se. One general conclusion on natural variation is clear, however. Details of polysaccharide chemistry differ with fibril source; this includes differences attributed to biological speciation and to environmental factors. While a pectic substance was chosen as a representative macromolecule in Fig. 4, several other acid polysaccharides could also have been selected.

Other macromolecules can be associated with acid polysaccharides to yield fibrils which are mixed materials. For example, proteins and neutral polysaccharides readily associate with pectins; pectin/protein/neutral polysaccharide associations are typical and quantitatively important associations of many plant and algal cell walls (Cook & Stoddart, 1973). Mixed mucilaginous materials, taken directly from nature and examined chemically in the absence of fractionation, can reveal associations between acid polysaccharides, mucopolysaccharides, lipopolysaccharides, mucoproteins, glycoproteins and proteins (Leppard, 1995); for brief periods on an episodic basis, there is even a potential to find nucleic acids as an important component of such mixtures. Because of difficulties with fractionation procedures applied to natural aquatic mixtures of fibrils and other macromolecular aggregates, there are few detailed chemical analyses of fibril chemical components, for fibril isolates in which the fibril fraction has been characterized morphologically by TEM analyses. The fact that the complications can be overcome is shown by recent literature from the medical sciences where, unfortunately, the chemistry

refers to extracellular polymers without reference to fibril morphology. Also, using artificially cultured cells, intensive investigations were successful as far back as a quarter century ago (Leppard et al., 1971; Colvin & Leppard, 1973).

3. Naturally engineered fibrils and the human engineer

Diverse fibril types, considered as naturally engineered particles of colloidal dimensions, reveal activities in aquatic ecosystems which are a function of:

(1) the specific chemical entities within and/or associated with an individual fibril (e.g., carbohydrate polymers, proteins including enzymes, sorbed organics, inorganic counterions);

(2) the capacity to associate with other fibrils and other small colloids/particles to give rise to highly porous and adhesive microflocs, ones which can aggregate into settling particles or contribute to biofilms.

Specific environmental signals can be used experimentally to generate large quantities of extracellular polymers which have been assembled by biota into polysaccharide-rich fibrils (Strycek et al., 1992). Specific situations can be set up to generate sufficient quantities of a functionally specific type of extracellular (Corpe, 1970; Vandevivere & Kirchman, 1993) polymer for limited chemical analyses. For a given organism, the nature and quantity of extracellular polymer can be manipulated selectively in the laboratory (Christensen et al., 1985; Leppard, 1993). Relatively pure isolates of fibrils from fresh water bodies and mesocosms can now be perturbed experimentally to mimic likely fibril contributions to floc formation, and provide simplified scenarios for analyzing the mechanics of the aggregation process (Leppard et al., 1990). These recent advances are offered in support of long-term interests by public health engineers (Brown & Lester, 1979) in the activities of extracellular polymer systems in which acid polysaccharides are important.

The time is ripe for human engineers to use the information available on biologically-engineered extracellular polymers and the colloids derived from them, especially the

fibrils, to take better advantage of nature's approach to water purification. Biological flocs established for use in water decontamination have tended to be treated as "black boxes" too complex to understand in terms of internal architecture and mechanics. Current information suggests that engineers, working in close collaboration with microscopists, microbiologists and chemists, could redefine a bacteria-rich floc in terms of all the major components and their native associations (Liss et al., 1995). This could lead in turn to the description of a floc as a miniature decontamination machine, with specific functions attributed to specific morphological components of the machine. To some extent, progress has already been made in terms of microbe speciation and its relation to specific bacterial roles. Such progress has not been made for the fibrils which make up much of the floc matrix structure; the matrix governs what the microbes "see" and (given architectural similarities between flocs and biofilms) the rates at which microbes receive substances from the bulk water phase (de Beer et al., 1994). It is conceivable that, with a better understanding of a suspended floc as a decontamination machine, a water treatment engineer could manipulate the bulk water environment of a treatment system so as to get the most effective floc in terms of both microbe speciation and fibril speciation. Savings from the increased cost-effectiveness of applying the new information might offset by orders of magnitude the cost of the research needed to refine the new information.

Considering water decontamination solely in terms of fibril activities and focussing on the contribution by bacteria, one can postulate the following sequence of events; the role of microbes in this case would be that of providing the appropriate fibril types and quantities. Decontamination would begin with the binding of toxicants in various ways (e.g., sorption, ion-exchange, chelation) by fibrils. This would be followed by aggregation processes (e.g., flocculation, coagulation) for freely-suspended fibrils and toxicant accumulation on the surfaces of fibrils within well-developed porous flocs. As aggregation and accumulation processes proceeded, there would be an increase in number of suspended flocs accompanied by floc growth into particles large enough to be sedimented by gravity, provided that their density was greater than that of the bulk water.

Large dense flocs containing fibril/toxicant associations would tend to settle out of the water column, thus transporting bound toxicants to the bottom of a treatment container. Such a sequence of events, compatible with the current understanding of water column decontamination, has not been described in great enough detail for real systems. The sequence, in terms of fibril types and activities, is now amenable to detailed analysis (Leppard, 1992b); a comparative analysis of fibril systems, sampled from water treatment plants in different states of effectiveness, should provide morphological correlates of effectiveness. Relating such correlates to microbial physiology, manipulations of the bulk water parameters and the chemistry of extracellular polymers could provide information for more cost-effective engineering of water treatment systems (Liss et al., 1995).

4. Fibrils in source waters: influence on water treatment

Coagulation and flocculation processes are essential for drinking water treatment. The raw source water entering the treatment system has characteristics which are important to the cost-effectiveness of operation. The raw water chemistry, the concentration of natural organic matter and the chemical speciation of the natural organics are all important (Bernhardt et al., 1985; Rebhun & Lurie, 1993; Edzwald, 1993; Dolejs, 1993). The natural organics traditionally exert impact in three ways (Edzwald, 1993). Depending on speciation and on concentration, these organics can:

(1) determine the use of coagulants in treatments (both dosage and selection);

(2) influence the processes selected for water treatment plants (coagulation and flocculation processes as well as downstream solid-liquid separation processes);

(3) and have human health effects, because some natural organic species are precursors of undesirable byproducts of disinfection.

The role of fibrils in the impacts of other natural organics is not known. For massive fibril secretion episodes (Burnison & Leppard, 1983; Leppard, 1984b) in a source water, it is conceivable that a large change in the toxicant load could occur, thus changing briefly the requirements of a water treatment facility. Such an episode could change the dispersion mechanisms and the bioavailability (Leppard & Burnison, 1983) of toxicants.

Polymer bridging effects and coating effects on suspended debris and mineral particles could also contribute to abrupt changes in source water quality during an episode.

During one episode in a small lake, the colloidal organic carbon concentration was measured as 2.88 mg C per litre (Burnison & Leppard, 1983). This compares to a measure of 0.88 mg C per litre for the conventionally-defined fraction of particulate organic carbon. Thus, the organic carbon of the colloidal particles was three times greater in mass than the organic carbon of all the conventionally-defined particles (algae, zooplankton, fish, debris, etc.) taken together. This colloidal organic carbon fraction consisted mainly of fibrils as they are defined by TEM (Burnison & Leppard, 1983; 1984). Given that the surface area per unit volume of 0.005 μm fibrils must certainly be greater than the surface area per unit volume of particles larger than 0.45 μm, and given that fibril mass was greater than conventional particle mass, it is obvious that fibrils could dominate water quality changes which are dependent on surface activity. Assuming that organic carbon made up approximately 40% of the organic components of the fibrils, and ignoring the inorganic component, then the mass of fibrils present during the episode would have an upper limit near 7 mg per litre. Experiments with mesocosms sometimes generate much higher fibril concentrations than this (Leppard, personal communication with several laboratories).

5. Chemical estimates of fibril abundance

5.1 TEM images and fibril chemistry: what is the connection?

For more than two decades, the visualization of fibril/fibril, fibril/cell and fibril/surface relationships by TEM has relied upon the cytological stain called ruthenium red (Luft, 1971). This stain, known to chemists as ammoniated ruthenium oxychloride, permits the light microscopist to visualize the location of structures/regions rich in acid polysaccharide on and in the living cell and other relatively delicate structures found in natural waters. It also minimizes an artifact of acid polysaccharide extraction in the preparation of aquatic cells and particles for TEM analysis, while imparting electron-density (and thus contrast)

to the specimen; these two features allow one to compare the acid polysaccharide distribution among particles in a fresh sample with the distribution in a related sample which had been prepared for TEM, in the conventional manner for viewing the sample in an ultrathin section (Fig. 1). While one can see only fibril-rich regions (stained red) using a light microscope (resolution ca. 0.2 μm) to observe fresh samples, one can see individual fibrils down to 0.002 μm diameter with the TEM (Leppard, 1992a). The ruthenium red is not a necessity for the visualization of fibrils by TEM, but it is very helpful because of its role in stabilizing fibrils (Liss et al., 1995; Leppard et al., 1995); fibrils are not artifacts of ruthenium red usage (Cagle et al., 1972; Leppard et al., 1995), although ruthenium red might alter the finest details of their substructure. The staining mechanisms are discussed in Hanke and Northcote (1975).

For freshwater aquatic samples, at a pH near neutrality, ruthenium red will stain only polyanionic macromolecules. Given the relative proportions of the different kinds of polyanionic macromolecules in most freshwaters at most times (Buffle, 1988), it will almost always be staining acid polysaccharides, with all of these being types bearing uronic acid moieties. In unusual circumstances, such as immediately after the collapse of a bloom, it will stain the unstable nucleic acids released from lysing cells. If one suspects the presence of polyanions other than acid polysaccharides, including minerals, one can assay the water sample for them to assess the limits on the utility of using ruthenium red as a marker.

There are sources of confusion in relating a structure shown by a TEM image to a specific class of polysaccharide. Acid polysaccharides can be extremely rich in uronic acids or relatively poor in them, the uronic acid moieties can be present as acids per se or in a modified form (Fig. 4), and the uronic acid moieties can be consecutive in a chain, at branch points or away from them, or alternating in a chain with neutral sugars. It is not clear how these differences affect the staining reactions as they occur in the special circumstances of TEM preparation. Thus the use of staining intensity or fineness/coarseness to make hypotheses about chemical details is unwise unless

accompanied by other sources of data in an independent study. As an extension of this argument, a fibrillar gel which is a mixture of neutral and acid polysaccharides might possibly stain as if it were a gel of acid polysaccharides only, even if the ratio of acid sugars to neutral sugars were low. Exceptional circumstances with respect to the marking of extracellular structures are discussed in Sutherland (1983). The fact that intracellular structures can receive enhanced electron-opacity from ruthenium red staining has been shown (Cagle et al., 1972; Strycek et al., 1992) as has the fact that not all acid polysaccharides take on the form of discrete fibrils (Leppard & Colvin, 1971b). The extent to which exceptions can impact on the assessment of a raw water sample for marked fibrils and chemical estimates of abundance is a problem which can be addressed by the application of TEM to ultracentrifuged water (Burnison & Leppard, 1983).

5.2 Practical problems in the chemical classification of acid polysaccharides

The analysis of acid polysaccharides according to chemical species is extremely specialized, costly and time-consuming. The macromolecule chains can be very large (up to millions of daltons), the chains are not of fixed length, the repeating features of the primary structure can exhibit a lack of regularity, the number of monomeric species (including derivatives) can be considerable, the branching pattern can be inconsistent, and there may be several kinds of covalent linkages (Cook & Stoddart, 1973; Aspinall, 1982, 1983). A breakdown of molecular types according to "families" may be as far as an environmental chemist can afford to proceed; however, a classification in terms of families may be sufficient for the moment, and would be useful to certain engineering projects currently in progress, such as those attempting to relate extracellular polymers to sludge settleability (Andreadakis, 1993). A focus must be placed on imaginative classifications, those which relate a limited number of quantitatively important families to specific activities (e.g., aggregation behaviour leading to settling, cation exchange capacity, sorption of organic contaminants, mineral precipitation activity, creation of microbial habitat). For assessing which activities require attention, one must consider the activity of the entire fibril. The type of acid polysaccharide will determine in part the intrinsic properties of the fibril as a material and as a natural entity in a complex aquatic

medium. Estimating the abundance of fibrils by chemical means for acid polysaccharides is useful, but it is the fibril per se which is responsible for fibril-based activities and aggregation behaviour. Reiterated once again for those who model the activities of colloids in water, a "dissolved" polyuronate is merely one important component of a complex colloidal material known as a fibril (Leppard & Colvin, 1971a; Colvin & Leppard, 1973; Ghiorse & Hirsch, 1979).

5.3 Standards for fibril quantification

The sparse knowledge extant about fibrils and fibril constituents suggests that the establishment of fibril standards will be difficult in the early efforts. Much of the difficulty resides in the complex relations between microbial physiology, water chemistry and ecosystem stress. The level of complexity is sufficient to require several standards for a given aquatic organism and a set of standards for all major secretors in a given class of ecosystem, at least in principle. A given biological species can secrete more than one kind of acid polysaccharide (Leppard, 1995) and more than one morphological type of fibril (Massalski & Leppard, 1979b). Organisms can alter the structure of their extracellular polymers according to their physiological state at the time of secretion and, for mesocosms, according to the phase of the culture cycle (Uhlinger & White, 1983). The secretion of discrete fibrils can be related to nutrient stress, which is in turn a function of water chemistry (Leppard et al., 1977; Strycek et al., 1992). Fibril quantity varies with the phase of the culture cycle, with secretion maxima occurring in different parts of the culture cycle, depending on which organism species is examined (Strycek et al., 1992). Whether or not the organism being tested is attached to a surface can also make a big difference (Vandevivere & Kirchman, 1993).

Appropriate secretor organisms for testing can be sought from various living culture collections specializing in bacteria and algae. From such sources, it is best to select among those collections which are continually replenished with freshly isolated species. Microbes can adapt to culture conditions over the long term in ways which render them

metabolically dissimilar to the same species in the wild; as a general rule, cultured species can make inappropriate responses to stress (Costerton, 1984; Costerton et al., 1987) and long term culture can increase the extent of response artifact.

Selecting standards for fibril quantification from known cultures in experimental laboratory mesocosms will require some water fractionation to obtain concentrated fibril samples of high relative purity. Assuming that acid polysaccharides are likely to dominate fibril chemistry, one will have to adapt to certain realities in the form of complications. The ratio of uronic acid monomers to neutral sugars (and other monomers), within a macromolecule, is likely to show great variation from one fibril fraction to another. Consequently, in the use of uronic acid markers for quick estimates of fibril quantity, one will have to have dry weight measures for relating uronic acid measures back to a measure of fibril mass. Such conversion factors should not be difficult to calculate when there are sufficient fibrils present for accurate dry weight measures. A related problem comes from fibril fractions which are mixed fractions, mixed with neutral polysaccharides which are difficult for the non-specialist to separate from the fibrils. Such a mix could confound analyses if the ratios of polysaccharide types are variable in the starting raw water samples, be it from a culture or from a natural ecosystem. When proteins are present in appreciable amounts as a fibril constituent, they should be characterized in a general qualitative manner, especially when enzymatic activity is present. One might consider from the complications above that one cannot progress without a huge budget and an army of technicians. However, if one confines oneself to the analysis of aquatic ecosystems in which fibrils are quantitatively dominant (high ratio of fibril mass to the mass of interfering materials) or are the result of a collapsed bloom (only one or a few species of organisms involved in fibril production), then one can make progress (Burnison & Leppard, 1983, 1984; Leppard, 1984b). For fibril effects on water treatment operations, this is a reasonable scenario with which to start.

5.4 Available chemical measures for aquatic samples: strengths and weaknesses

According to Decho (1990), "There is ..... no single quantitative method which can accurately and precisely estimate exopolymer biomass under all situations"; this is also my experience. Many decades ago, the reaction of uronic acids with carbazole was, for the times, a flawed but satisfactory method of estimating uronic acids, a method improved for general use by Bitter and Muir (1962). In 1973, Blumenkrantz and Asboe-Hansen responded to the growing need for improved quantitative assays. They elaborated a new, rapid, sensitive and specific method based upon the appearance of a chromogen, when uronic acid heated to 100°C in concentrated sulfuric acid/tetraborate is treated with meta-hydroxydiphenyl. As increasing attention became focussed on the extracellular polysaccharides of microbes, Fazio et al. (1982) developed a means to estimate uronic acids in microbe-rich environmental samples. They addressed the facts that polymers containing uronic acids are resistant to quantitative hydrolysis, and that individual uronic acids, once released, form lactones irreproducibly. These problems were obviated by methylating the uronic acids and reducing them with sodium borodeuteride to the corresponding alcohol while they were still in the polymer. Quantitative recoveries were achieved and subsequent chemical and analytical manipulations accurately provided the proportion of each uronic acid. The overall procedure is, however, time-consuming and requires considerable technical skill. In response to the need for a non-destructive, rapid and simple means to measure acid polysaccharides in environmental samples of interest to engineers, a ruthenium red adsorption method was devised in 1989 by Figueroa and Silverstein. This method was found to be more effective than extraction procedures for the measurement of extracellular polysaccharides in activated sludge flocs; it does not provide the details yielded by the methodology of Fazio et al. (1982). For those who require only the level of detail provided by the method of Blumenkrantz and Asboe-Hansen, and who must minimize interference from neutral sugars, the technique of Filisetti-Cozzi and Carpita (1991) is suggested. The use of chemical methodologies for the analysis of exocellular polymers in environmental samples has been reviewed recently (Decho, 1990), with a focus on strengths and weaknesses. With regard to engineered aquatic ecosystems, one can consult a current review of biofilm-specific components for

those biofilms pertinent to water and wastewater treatment (Lazarova & Manem, 1995).

5.5 Fibril isolation procedures: fractionation artifacts and their impacts on chemical estimates of abundance

Early attempts to concentrate dispersed fibrils and fibril-rich microflocs from natural waters were plagued by colloid aggregation artifacts. Small colloids inconsistently and uncontrollably aggregated so as to end up partially in large colloid and true particle fractions; storage problems (bacterial action, dehydration phenomena) compounded the artifact problems, with great impact on chemical analysts. Typically, the analyst never knew, for a given water sample, what fraction of the total fibril population was received from the field, or even if the sampling/fractionation protocol would provide a detectable amount. An estimate of the yield and a systematic means to improve it became vital considerations.

The initial success at isolating colloidal fibrils of known yield (Burnison & Leppard, 1983, 1984) was dependent on two factors: (1) a fortunate choice of water body in which the mass ratio of fibrils to other colloids was high; and (2) the use of TEM as a monitor, in association with the best filtration procedure of the time, for adjusting important parameters of multiple runs to achieve maximum yield with minimum contamination (Fig. 2). Since that time, the rules-of-the-game have been elaborated for the detection of specific filtration artifacts and the minimization of them (Buffle et al., 1992). This improved understanding, including the need to couple sampling to sample fractionation without a storage phase, removes much of the costly trial-and-error aspect of such research. The concept of artifact-free "windows" in filtration and ultrafiltration procedures for deriving size fractions of aquatic colloids (Buffle et al., 1992; Buffle & Leppard, 1995b) is especially helpful. For a given type of natural water and a given apparatus, one can ascertain a flow rate which is slow enough to minimize surface-induced aggregation but fast enough to avoid bulk coagulation. The window concept in conjunction with TEM monitoring provides a soundly-based and systematic means to obtain colloid fractions which in fact contain what was intended. This progress has been

coupled to an increased selection and versatility of TEM instrumentation (Leppard, 1992a) and preparatory technology (Perret et al., 1991; Buffle & Leppard 1995b; Leppard et al., 1995).

Chemical estimates of fibril abundance now can be (potentially) related to specific colloidal entities which have a specific ecological significance. The activities shown for fibrils in Fig. 3 are now amenable to analysis on a quantitative basis. The methodological arsenal is advancing beyond the isolation of fibrils by filter fractionation. Perret et al. (1994) have established a basic ultracentrifugation protocol for fractionating aquatic colloids. They can relate their fractions to a parallel filter fractionation protocol which permits some correlative quantitative analyses by conventional wet chemistry. Preliminary work suggests that their ultracentrifugation protocol can be tuned to derive fibril-rich fractions (Perret, personal communication). Additionally, field-flow fractionation (FFF) techniques have a potential to separate fibrils from other colloids for physico-chemical analyses (Beckett & Hart, 1993) and the time is appropriate to assist FFF research with TEM observations. When it is important to remove fibrils from microbes and their colonies/biofilms/flocs for quantitative chemical analyses, some promising extraction techniques are available (Platt et al., 1985).

6. Conclusions

Colloidal organic fibrils which contain acid polysaccharide as an important component are ecologically-significant particles in natural waters, contributing to both biofilm and floc formation. Some researches indicate that the chemical fine structure of fibrils is varied in response to the biological needs of the fibril-producing organisms, being adapted in function to combat specific stresses. Different fibrils have different functions and switches in secretion from one fibril type to another can be engineered experimentally through the use of environmental perturbations.

A hypothesis is developed here from new information about the importance of fibrils to certain biological flocs which can alter water quality. This hypothesis suggests that fibril-

rich flocs in engineered water decontamination systems might themselves be engineered by man to yield flocs with optimal properties for decontamination. Currently, there is sufficient understanding and technology to test this hypothesis.

With the ideas above as a focus, this paper describes the general chemical characteristics and known biological activities of fibrils. It also describes their electron-optical characteristics in terms of (1) the TEM-based means to detect them and of (2) tuning fractionation schemes to isolate them quantitatively. The fibril isolates of the past were difficult to obtain for analyses of colloidal behaviour and chemical structure. We now know the bases for the difficulties and how to overcome many of the conceptual and technological problems of the past. We can begin again our quest to understand the roles of fibrils in nature.

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FIGURE LEGENDS

FIGURE 1. The morphology of fibrils found in several Canadian surface waters. The fibril types shown were abundant at the time of sampling and do not necessarily represent the most important types over the course of a year. The preparation for electron-optical imaging is that of Burnison & Leppard (1983) and the bar represents 0.45 μm. (A) water from the Severn River of Ontario; (B) water from a Halton Region Wastewater Treatment Plant (Burlington, Ontario); (C) water from Erickson-Elphinstone District Lake 318 in Manitoba.

FIGURE 2. A fractionation procedure for colloidal fibrils. When the mass ratio of fibrils to other colloids is high, this procedure has produced a fibril fraction of high yield and purity from lake water. The details of the procedure were established according to monitoring of fraction quality by TEM.

*Monitored, assessed and documented by transmission electron microscopy.

†Clarification step removed a pellet of coarse materials negligible in quantity.

Reprinted with permission from Burnison & Leppard, 1984.

FIGURE 3. Fibrils, fibril components and fibril aggregates; their potential impacts on geochemical, physicochemical and biological processes in surface waters. Quantification of these impacts is in its infancy.

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FIGURE 4. A detailed chemical structure of part of a pectin molecule, a carbohydrate polymer rich in uronic acid. The entire branched molecule is larger and essentially linear; most molecules in the pectin family would be less densely substituted than this one. The linear portion is a galacturonorhamnan chain showing some methyl esterification. Many variations on this molecular architecture occur in natural materials which enter surface waters.
Reprinted from Cook & Stoddart, 1973, with permission.

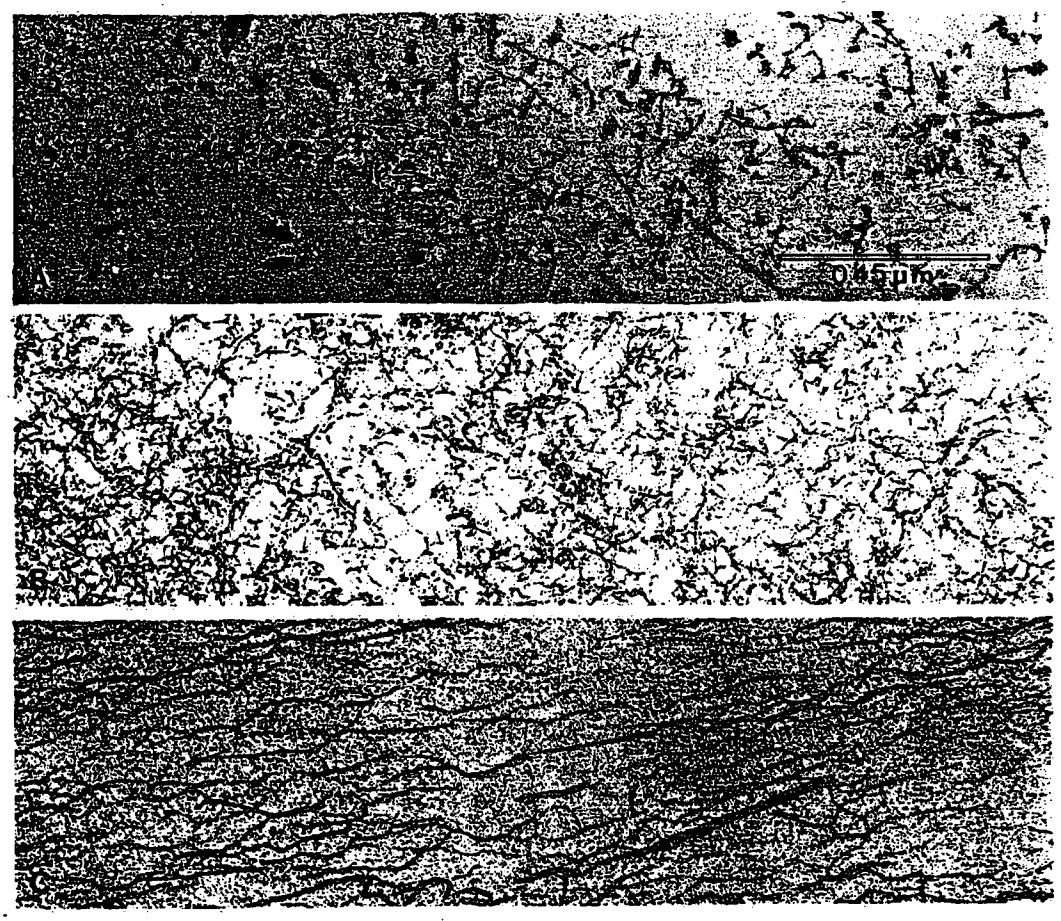


FIGURE 1.

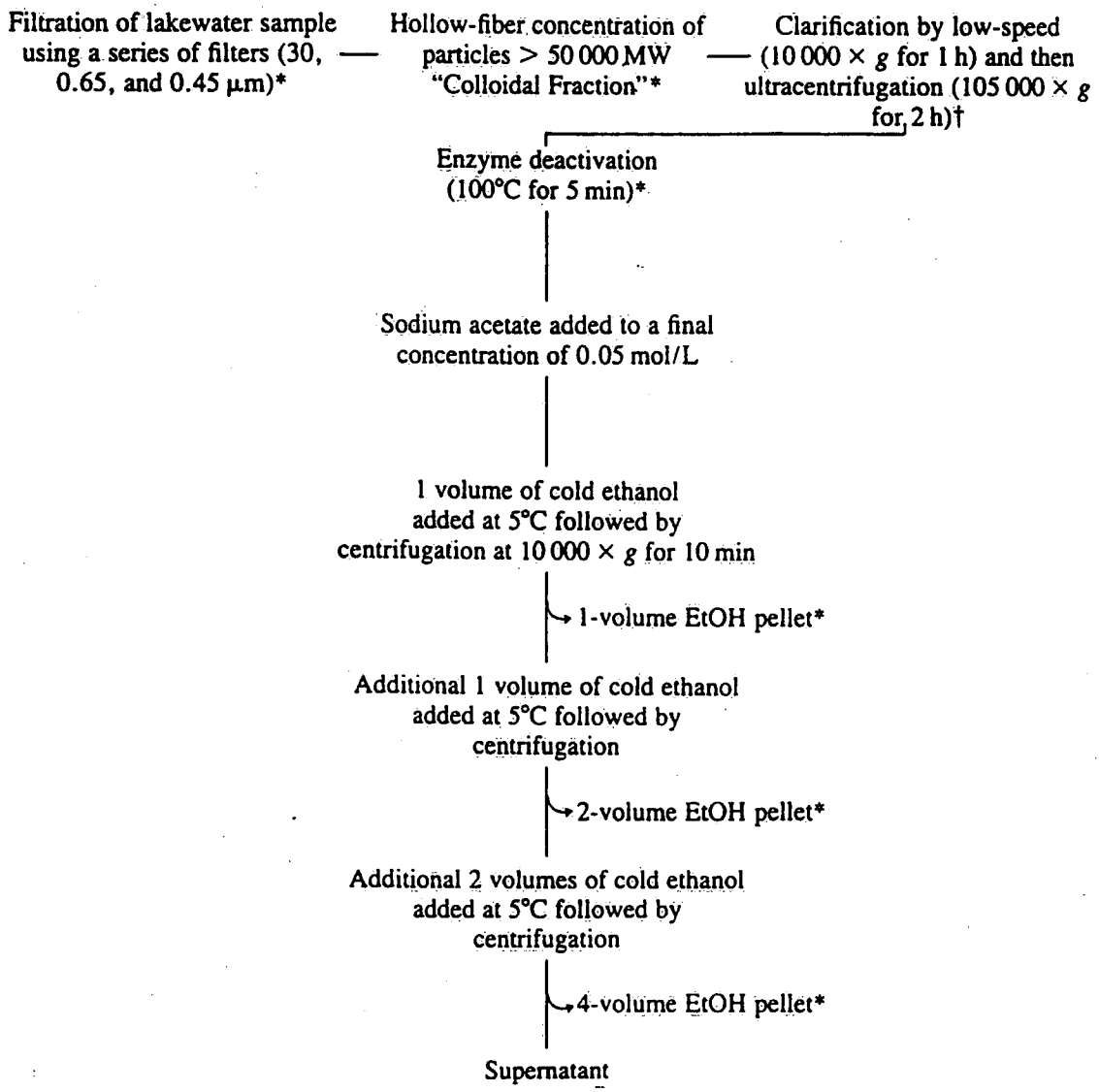

Filtration of lakewater sample using a series of filters (30, 0.65, and 0.45 μm)*
Hollow-fiber concentration of particles > 50 000 MW "Colloidal Fraction"*
Clarification by low-speed (10 000 × g for 1 h) and then ultracentrifugation (105 000 × g for 2 h)†
Enzyme deactivation (100°C for 5 min)*
Sodium acetate added to a final concentration of 0.05 mol/L
1 volume of cold ethanol added at 5°C followed by centrifugation at 10 000 × g for 10 min
1-volume EtOH pellet*
Additional 1 volume of cold ethanol added at 5°C followed by centrifugation
2-volume EtOH pellet*
Additional 2 volumes of cold ethanol added at 5°C followed by centrifugation
4-volume EtOH pellet*
Supernatant

FIGURE 2.

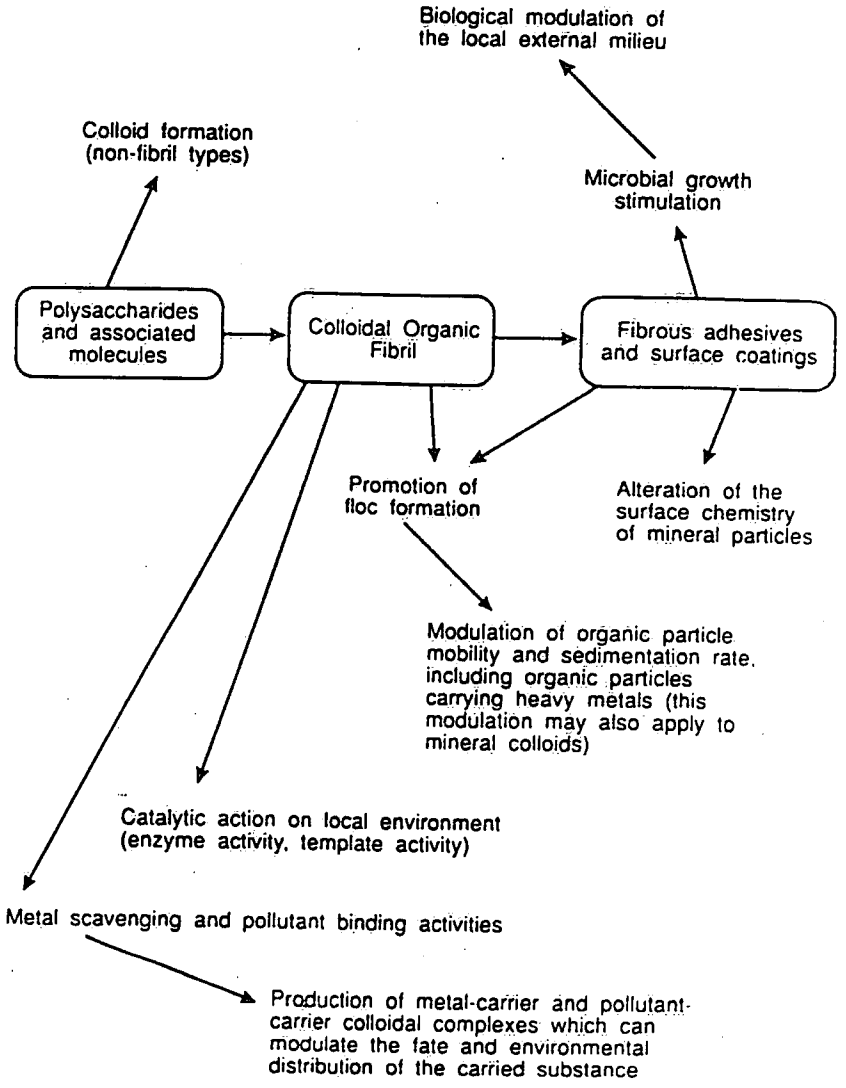

Biological modulation of the local external milieu
Colloid formation (non-fibril types)
Microbial growth stimulation
Polysaccharides and associated molecules
Colloidal Organic Fibril
Fibrous adhesives and surface coatings
Promotion of floc formation
Alteration of the surface chemistry of mineral particles
Modulation of organic particle mobility and sedimentation rate, including organic particles carrying heavy metals (this modulation may also apply to mineral colloids)
Catalytic action on local environment (enzyme activity, template activity)
Metal scavenging and pollutant binding activities
Production of metal-carrier and pollutant-carrier colloidal complexes which can modulate the fate and environmental distribution of the carried substance

FIGURE 3.

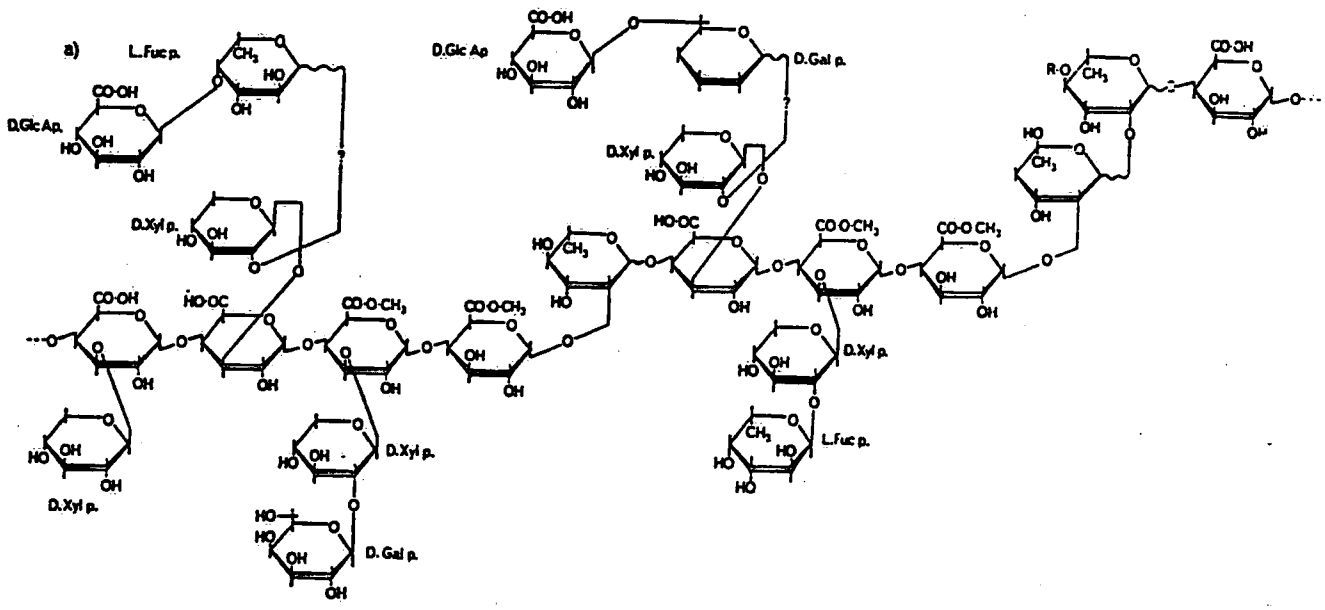
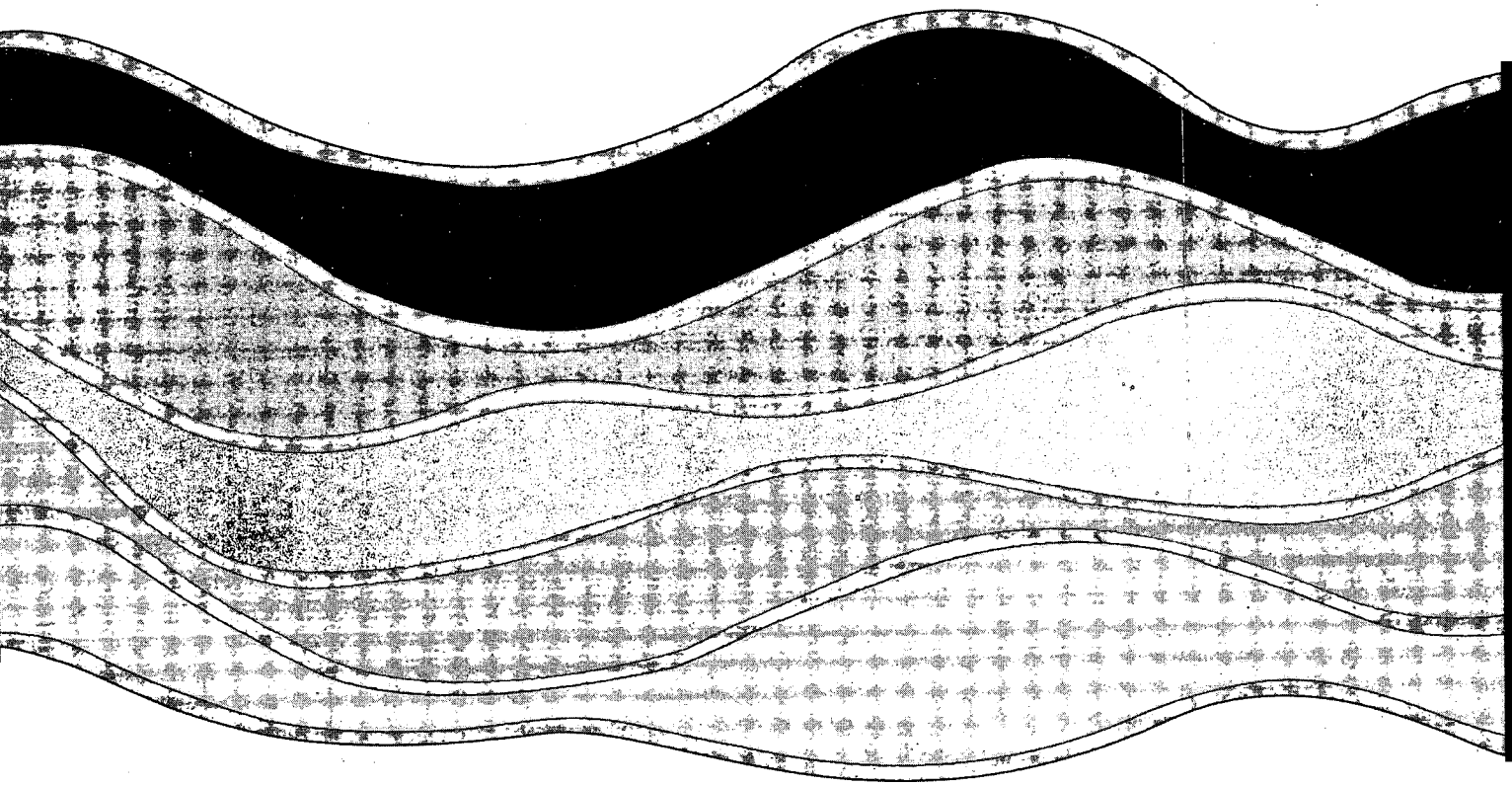

a)
L.Fuc p.
D.Glc Ap.
D.Xyl p.
D.Xyl p.
D.Xyl p.
D. Gal p.
D.Glc Ap
D.Gal p.
D.Xyl p.
D.Xyl p.
L.Fuc p.

FIGURE 4.



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