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NOTE

Assessment by Microscopy of the Coverage of Sludge Flocs by a Nano-scale Surface Layer

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Abstract: Flocs are irregularly shaped suspended particles of complex structure and composition that occur naturally in aquatic systems. In water treatment facilities, the settling of flocs is extremely important. Transmission electron microscopy was used to quantify the formation of a nano-scale surface layer on flocs from two laboratory bioreactors modeling a biological wastewater treatment facility. By comparing different floc populations, we demonstrated that flocs which settled quickly out of wastewater had a significant amount of this layer, whereas those with little nano-scale surface layer had poor settling properties. This morphological feature of floc ultrastructure may permit engineering manipulations that promote floc settling.

Keywords: nano-scale surface layer, sludge, settleability, transmission electron microscopy, wastewater treatment.

Introduction

The flocs of natural surface waters and the engineered flocs used for water purification in biological water treatment facilities are irregularly-shaped sedimenting units composed of microorganisms, extracellular polymeric substances (EPS), trapped inorganic and organic materials, and water (Leppard and Droppo, 2005). Microorganisms within the floc are enmeshed within a three-dimensional network of EPS, much of which occurs in the form of nano-scale adhesive fibrils (ca. 5 nm diametre for most). These fibrils are predominantly composed of polysaccharide, sometimes being rich in acid polysaccharide; their nano-scale structural characteristics, activities and abundance have been reviewed in Leppard (1997). In general, with regard to the population of non-living EPS colloids within the matrix material of activated sludge flocs, proteins tend to dominate (Bura et al., 1998; Wingender et al., 1999; Leppard et al., 2003) while fibrils act as bridges. As is the case for complex biofilms (de Beer et al., 1994), the fibrils can promote the formation of (a) microbial consortia in specialized microenvironments, (b) intra-floc channels for transport of nutrients inwards and wastes outwards, and (c) fine-scale pore systems for the establishment of both diffusional gradients and barriers to exclude viral predators (Leppard and Droppo, 2005). The roles of fibrils at floc surfaces are currently a subject of investigation by water treatment engineers (Liao et al., 2002; Liss et al., 2002).

A microbe-rich floc can be partially covered by an expanding layer of nano-scale thickness (Liao et al., 2002; Liss et al., 2002), which involves the orientation of fibrils and other colloids (including individual bacteria) at the floc/bulk water interface and which modulates settling in treatment systems. Extensive coverage by this nano-scale layer has been implicated in increased floc/floc interactions and a consequent improvement in settleability. It has recently been postulated that dysfunctional settling in treatment tanks might be minimized by engineering the floc surface, via environmental adjustments to the water, so as to facilitate an optimal coverage of flocs by a surface layer which decreases roughness and increases stability (Liao et al., 2002).

We address here the problem of how to quantify, as a function of an engineered manipulation, the extent of a nano-scale layer which appears to provide such a coverage, as ascertained by previous research on the same experimental system (that of Liao et al., 2001). We perceive the layer as being dynamic and incomplete, because the flocs undergo a continuous aggregation-breakage-reaggregation-restructuring process. By correlating observations from optical and electron microscopy, the (incomplete) layer appears as a partial coverage by a collection of patches of aligned EPS fibrils, which can impose sometimes a consecutive alignment of many bacteria along the floc/bulk water interface. This note concerns itself with an assessment, by transmission electron microscopy (TEM), of the partial coverage of sludge flocs by a thin layer of particles in the submicrometre size range, as a function of solids retention time (SRT). Fibrils of ca. 5 nm diameter (Leppard, 1997; Wingender et al., 1999; Leppard et al., 2003) are a major component of this aligned layer of nano-particles/colloids.

Experimental

Microbe-rich flocs were obtained for microscopical analysis from biomass (as a gift from B. Q. Liao) which had been generated from parallel laboratory-scale sequencing batch reactors (SBRs) employed experimentally to model a biological wastewater treatment system (Liao et al., 2002). The SBRs had been seeded initially with activated sludge from a municipal wastewater treatment facility (Main Treatment Plant, City of Toronto, ON), fed a synthetic wastewater containing glucose and inorganic salts, and operated and monitored as described in Liao et al. (2001). The model treatment system was operated at different SRTs, to yield samples with different degrees of coverage of floc surface by relatively smooth patches of a nano-scale surface layer (Liao et al., 2002; Liss et al., 2002). All samples were chemically fixed on site to stabilize them for subsequent TEM analysis; there was no storage of fresh samples. The SRTs chosen for

comparison were 4 and 20 days; these SRT's were extremes in the earlier study of Liss et al. (2002). Each of the SRTs chosen had been shown earlier to yield populations of flocs which were fairly homogeneous in general characteristics for a given SRT. Comparison of the two floc populations (4 days vs 20 days SRT) revealed them to differ greatly in morphology, stability, degree of hydration and aggregation properties (Liss et al., 2002); they also appeared to differ greatly in surface roughness, a feature requiring TEM for detailed analysis.

With populations sampled from the two SRTs, nano-scale observations of the floc/bulk water interface of sludge flocs were made on ultrathin sections of whole flocs which had been prepared for correlative TEM by the multi-method preparatory technique of Liss et al. (1996). The searches of TEM views of flocs, to select representative images of floc surface ultrastructural features, were done systematically according to the protocol of Leppard et al. (2003). Lower resolution, multi-scale observations on relatively large sample volumes, made with two complementary microscopies (conventional optical microscopy and environmental scanning electron microscopy; Liss et al., 2002), were used to guide the much higher resolution TEM documentation so that it could be carried out in a cost-effective manner.

Using observations from the lower resolution complementary microscopies as a guide, a systematic search of TEM images, in many fields of view provided by ultrathin sections, yielded trend information based on tens of thousands of individual views. To initiate the selection of representative views, we began with a collection of many ultrathin sections (taken from multiple blocks of embedded flocs, representing multiple embeddings of each mode of sample preparation, sectioned at several depths from the tips of each block). Representative sections were searched initially using both low (4,000x) and medium (15,000x) primary magnifications to get an overview of the relative abundance and associations of the most common colloids, as defined by morphology and size (Leppard and Droppo, 2005) at the floc edge. Observations were then made in greater detail at a primary magnification of 50,000x. When comparing floc populations from the two SRTs, a trend became evident, as did a need to quantify it. A subsequent protocol was developed, as a result, for selecting (at random) a limited number of views of floc edges for costly and time-consuming detailed analyses. This derived protocol is outlined in the section "Results and Discussion".

To stabilize and embed samples for the subsequent ultramicrotomy (above), a proxy was selected from the four-fold multi-method preparation of Liss et al. (1996), a proxy whose preparatory protocol is outlined in detail in their methods section. This proxy preparation consisted of sample immersion in a cacodylate-buffered (pH 7.1) primary fixative containing both glutaraldehyde and ruthenium red, followed after several subsequent steps (including a secondary fixative, osmium tetroxide-ruthenium red) by embedding in epoxy resin (via solvent exchange using an ethanol series). The ruthenium red fixes fibrils in place; it minimizes extraction of fibrils during the many fluid exchanges. Embedded flocs in hardened resin were sectioned (50-80 nm thick) with an RMC MT-7 ultramicrotome.

The sections were collected on formvar-coated copper grids for viewing and documentation by TEM, with a JEOL 1200 EX II TEMSCAN scanning transmission electron microscope operated in transmission mode at 80 kV. Prior to viewing, counter-staining of sections on grids was done as follows. They were covered in a drop of concentrated uranyl acetate in 50% ethanol for 10 minutes in the dark and then rinsed with distilled water. The grids were then stained again for 5 minutes in Reynolds' lead citrate solution (Lewis and Knight, 1977), in a carbon dioxide-free environment, rinsed again and allowed to dry. These two counter-stains produce a high differential contrast for the documentation of nano-scale structures by TEM at high resolution (Lewis and Knight, 1977).

Results and Discussion

Protocol for the Measurement of Nano-scale Surface Layer Coverage

It is not possible to view an entire floc in a TEM image because, at a relatively high magnification, only a tiny portion of even a small floc can be seen in an ultrathin "physical" section. Therefore, a systematic measure of nano-scale surface layer coverage by TEM has a constraint; it must be achieved through analysis of only a small portion of the total floc surface. The correlative multi-method search protocol (above), and the specific protocol for high-resolution detailed observations presented here, guarantee that many different flocs are examined.

We found that all electron micrographs had to be taken at a primary magnification of 15,000x. This is the lowest magnification at which the layer was readily distinguishable from other nano-scale structures. Using a standard magnification of 15,000x allowed for a maximum portion of floc/bulk water interface to be examined while providing sufficient magnification to obtain appropriate resolution.

Micrograph negatives were placed in an enlarger that projected an image onto a digitizing pad, a pad equipped with a specialized mouse that allowed one to measure lengths by using the mouse to trace whatever was being measured. Calibration was done using the scale bars on the micrographs. Layer coverage was measured by tracing a line along the edge of the floc where the layer was located. Layer coverage was expressed as "the length of layer measured on a portion of the floc, divided by the total length of edge that could be ascertained on that micrograph". An edge at the floc/bulk water interface was defined as layer plus protruding recognizable colloids (bacteria, minerals, organic debris) plus arrangements of surface EPS which disturbed surface smoothness. Figure 1a, based on an SRT of 4 days, presents a micrograph exhibiting low layer coverage. Figure 1b is the same micrograph with the floc/water interface highlighted differentially; the solid line is layer, while the dotted line is edge not covered by layer. Figure 1c, based on an SRT of 20 days, presents a micrograph exhibiting high layer coverage, highlighted by the solid line in Figure 1d.

For comparing data from the two SRTs, a two-sample independent *t* test was done. This test is designed for two independent random samples of equal size, where the variances are unequal and there are more than 60 samples in both groups combined (Bourke et al., 1985).

Nano-scale Surface Layer Coverage as a Function of SRT

The amount of smooth surface per floc increased dramatically with a large increase in SRT. This is the result of the formation of a dynamic nano-scale surface layer, defined in terms of localized aligned nano-particles (yielding larger scale patches which partially enclose a floc over time).

Based on 120 total measures (99.99% confidence), the mean of percent coverage of flocs by layer was greater for an SRT of 20 days than for an SRT of 4 days. At 20 days the mean coverage was 47% (based on 464,800 nm of measured floc edge), while at 4 days the mean coverage was 11% (based on 451,700 nm of measured floc edge). More than half (53%) of floc images at an SRT of 4 days had no layer coverage, whereas, for an SRT of 20 days, only 7% had no layer coverage. These facts correlate well with observations on surface roughness made by the multi-scale complementary microscopies at lower resolution and magnification. For an SRT of 4 days, many TEM images (32%) showed between ten and thirty one percent layer coverage, while few images (7%) showed between forty and fifty one percent layer coverage. With the SRT set at 4 days, none of the flocs had more than 51% layer coverage, and the majority showed 0% coverage. In sharp contrast to these data, using an SRT of 20 days showed that more than half of the flocs (57%) had a layer coverage in the 40-70% range; only 28 % of individual flocs had less than 40% coverage while 13% of individuals had more than 70% coverage.

These data suggest that floc populations in the model systems consist of individual flocs at different stages of formation and maturity, and that floc ageing yields a population after 20 days which has a much higher proportion of mature flocs with a nano-scale surface layer (and also of desirable flocs, from an engineer's perspective). The bar graphs of Figure 2 are strongly indicative of this point.

Observations by the complementary microscopies, of entire flocs from the two sets of populations, were consistent with the surface measurements from TEM. At an SRT of 4 days, the average floc was irregular in shape (shape factor <0.2), whereas the average floc at an SRT of 20 days was nearly spherical (for specific data, see Liss et al., 2002). Both the increase in coverage by a nano-scale surface layer and the tendency to attain a more compact shape, with increasing SRT, correlated well with the increase in floc strength measured earlier (Liao et al., 2002) using the same model treatment systems.

Preliminary measures of layer coverage were repeated on floc populations with SRTs of 9 and 16 days; the initial results showed each of the two SRT populations to be much more heterogeneous (compared to populations of 4 and 20 days SRT), with intermediate coverages.

Conclusions and Recommendations

We describe a user-friendly technique to quantify the extent of floc coverage by a nano-scale surface layer, a layer of significance for the achievement of engineered improvements to water treatment facilities. While the images used for the nano-scale measurements typically represented less than 1/2000 of an individual floc, they yielded an overview of layer coverage which was representative of the entire floc. For a floc population, thousands of images were observed of different individual flocs and different regions of given flocs, sufficient to reveal obvious trends which could be documented independently and systematically, by fastidious TEM analyses of many individual floc surfaces. The degree of difficulty in obtaining statistically significant data would of course increase if a need arose to reveal smaller differences between floc populations (or to ascertain differences in more heterogeneous floc populations, a difficulty which appeared in preliminary work using SRTs of 9 and 16 days). The development of layer-specific probes (fluorescent electron-opaque probes for principal chemical entities comprising the layer's nano-particles) would address a practical difficulty which arose occasionally in making a decision on whether or not there was real layer coverage of a specific region of floc surface when the presumed layer was indistinct. Also, layer-specific fluorescent probes would permit more of the data collection to be done with the more convenient light microscopy, with TEM being used for confirmation. More research on the ultrastructure and exact chemical nature of the nano-scale surface layer will be needed to (a) improve decision making for engineering floc properties and (b) to reveal the exact mechanism whereby the layer facilitates settleability.

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Glossary/Nomenclature

Colloid – any particle with a least dimension in the range 1-1000 nm

EPS – extracellular polymeric substances, mainly polysaccharides and proteins, secreted by microbes, for use in optimizing their microhabitat

ESEM – environmental scanning electron microscope, which provides a topographical image of a hydrated environmental sample at a resolution intermediate between that of a light microscope and a TEM

Fibril – elongated string-like structure composed of aggregated EPS, with a least dimension in the nano-scale range

Fixative – a chemical solution designed to preserve as faithfully as possible the ultrastructure of a biological sample, exactly as that ultrastructure was in life at the instant before the cells were killed by the fixative

Floc – a suspended particulate, in the multi-micrometre to multi-millimetre size range, which is derived by aquatic aggregation processes, and which is typically rich in colloidal sub-components

SBR – sequencing batch reactor

SRT – solids retention time

TEM – transmission electron microscope, which uses transmitted electrons to form a high resolution image

Ultramicrotomy – the mechanical slicing of an embedded sample to produce ultrathin sections of uniform thickness (usually < 100 nm), free from distortions such as wrinkles, breaks or folds

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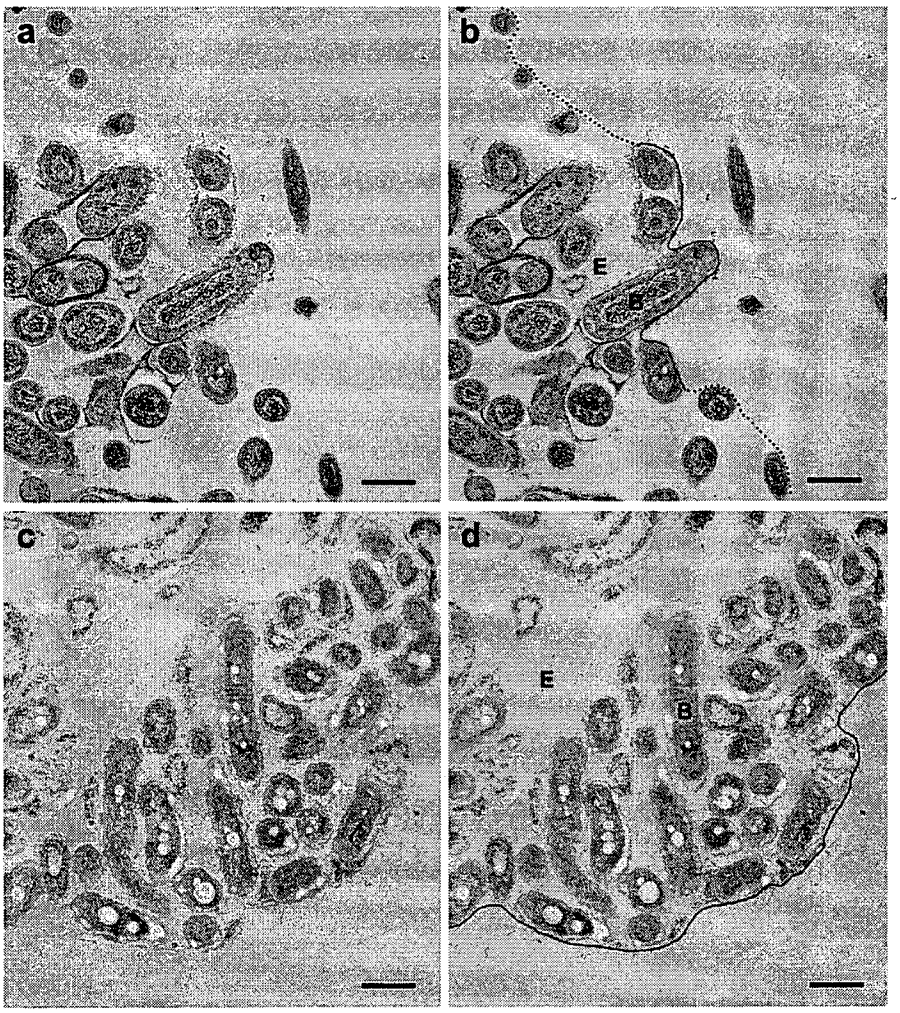
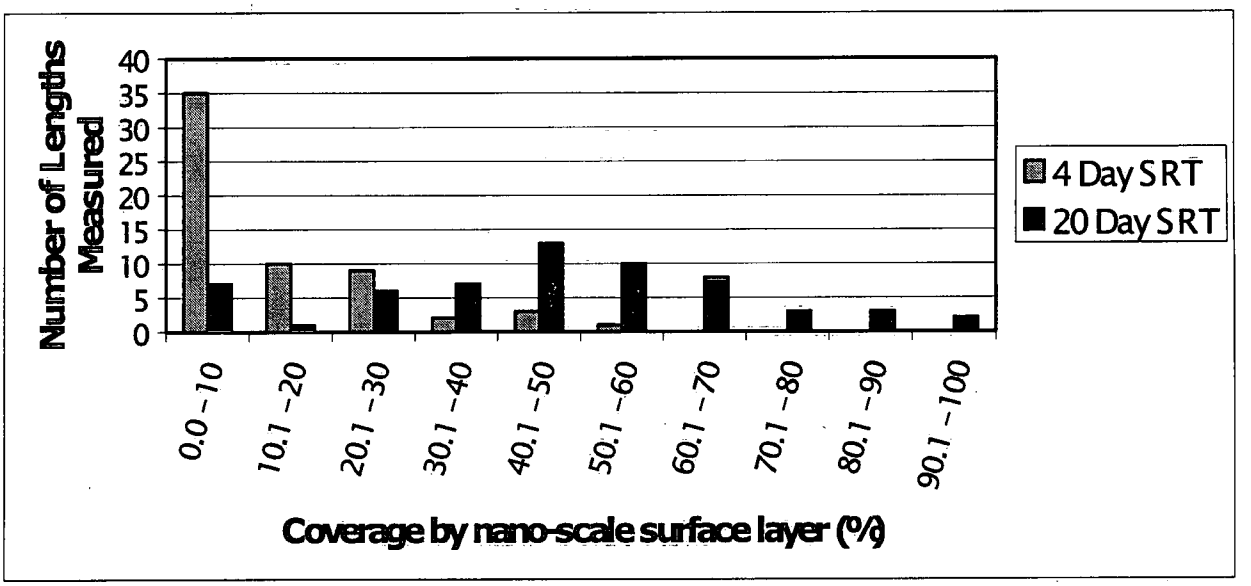
Figure Captions

Figure 1. (a) Portion of the edge of a floc from an SBR whose SRT was 4 days. (b) The same micrograph altered so that a drawn solid line is laid over the nano-scale surface layer (plus associated aligned bacteria), while a drawn dotted line is laid over a porous portion of the floc/bulk water interface. (c) Portion of the edge of a floc from an SBR whose SRT was 20 days. (d) Same micrograph (20-day SRT) with the nano-scale layer represented by a drawn solid line, which reveals that for this portion of floc/bulk water interface, the coverage by the nano-scale layer is maximal. The scale bars represent 0.5 μm. For (b) and (d), "E" represents a region rich in fibrils of EPS, and "B" represents a bacterium, of which there are many.

Figure 2. Bar graph illustrating the percent coverage of flocs by the nano-scale surface layer, comparing SRTs of 4 (grey) and 20 (black) days, based on edge and nano-scale surface layer lengths measured in micrographs for images of floc/bulk water interfaces.


a
b
E
B
c
d
E
B


Number of Lengths Measured
40
35
30
25
20
15
10
5
0
0.0 – 10
10.1 – 20
20.1 – 30
30.1 – 40
40.1 – 50
50.1 – 60
60.1 – 70
70.1 – 80
80.1 – 90
90.1 – 100
Coverage by nano-scale surface layer (%)
4 Day SRT
20 Day SRT

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