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Methods for Analyzing Floc Properties

Steven N. Liss, Timothy G. Milligan, Ian G. Droppo and Gary G. Leppard

ABSTRACT

Flocs are major transport agents for many environmentally significant substances in freshwater and marine ecosystems, as well as being essential components of many engineered water treatment systems. There is a well recognized need for a compilation and synthesis of the existing methods for analyzing floc properties and the structure/function relationships within flocs. A detailed compilation and synthesis of the extant methods is presented here, in a format which should stimulate the three major users of the methods. These users will be engineers, freshwater scientists and marine scientists from many diverse disciplines within the government, university and private sectors of many countries. Of particular interest will be novel methods for dissecting flocs microscopically and for analyzing microbial systems and processes at the molecular level.

Méthodes d'analyse des propriétés des flocs

Steven N. Liss, Timothy G. Milligan, Ian G. Droppo et Gary G. Leppard

RÉSUMÉ

Les flocs sont au nombre des principaux transporteurs de nombreuses substances importantes pour l'environnement dans les écosystèmes dulcicoles et marins; ce sont aussi des composantes essentielles dans de nombreux systèmes d'épuration artificiels. Il est bien connu qu'une compilation et une synthèse des méthodes existantes d'analyse des propriétés des flocs et de leurs relations structure/fonction sont nécessaires. On présente ici une compilation et une synthèse détaillées des méthodes existantes, dans une forme qui devrait intéresser les trois principaux utilisateurs de ces méthodes : les ingénieurs et les scientifiques de diverses disciplines s'intéressant aux milieux dulcicoles et aux milieux marins dans les ministères, les universités et le secteur privé d'un grand nombre de pays. Les nouvelles méthodes présenteront un intérêt particulier pour la dissection des flocs au microscope et pour l'analyse des systèmes et procédés microbiens à l'échelle moléculaire.

NWRI RESEARCH SUMMARY

Plain language title

Methods for analyzing floc properties

What is the problem and what do scientists already know about it?

Flocs are major transport agents for contaminants and nutrients in freshwater ecosystems and in the oceans. They also play essential roles in engineered water purification systems. A better understanding of their properties and internal structure/function relationships is certain to lead to an improved understanding of contaminant transport and biogeochemistry on a planetary scale, and to improved cost-effectiveness in the operation of water treatment plants. The key to this better understanding resides in improved methods of analysis.

Why did NWRI do this study?

There has been a well recognized need for a compilation and synthesis of the existing methods for analyzing floc properties, especially because the three main groups of floc-oriented scientists (engineers, freshwater scientists and marine scientists) have tended historically to ignore methodological improvements not designed for the current needs of their group. The subsequent problems of institutionalized poor communication and reduced technology transfer became exacerbated in recent years, as biology became an increasingly larger factor. NWRI is a world leader in floc methods development and consequently had no difficulty getting support or an enthusiastic publisher for a book which would focus on the analysis of flocculation and floc properties. This chapter on methods, with two NWRI co-authors, is a central chapter of the book-to-be.

What were the results?

A detailed compilation and synthesis of the extant methods is presented for the first time, in a format which should stimulate the three major users of the methods.

How will these results be used?

The information will be used by engineers, freshwater scientists and marine scientists from many diverse disciplines within the government, university and private sectors of many countries. Of particular interest will be novel methods for dissecting flocs microscopically and for analyzing microbial systems and processes at the molecular level.

Who were our main partners in the study?

Lewis Publishers/CRC Press, NWRI, DFO, WTC/ETAD, Ryerson University, Brockhouse Institute for Materials Research, International Association for Sediment Water Science

Sommaire des recherches de l'INRE

Titre en langage clair

Méthodes d'analyse des propriétés des flocs

Quel est le problème et que savent les chercheurs à ce sujet?

Les flocs sont au nombre des principaux transporteurs des contaminants et des éléments nutritifs dans les écosystèmes dulcicoles et dans les océans. Ils jouent également un rôle essentiel dans les systèmes d'épuration artificiels. Si on comprend mieux leurs propriétés et les relations internes entre structure et fonction, il est certain qu'on comprendra mieux le transport et la biogéochimie des contaminants à l'échelle planétaire et qu'on améliorera le rapport coût/efficacité des stations de traitement des eaux. La clé réside dans l'amélioration des méthodes d'analyse.

Pourquoi l'INRE a-t-il effectué cette étude?

Il était bien connu qu'une compilation et qu'une synthèse des méthodes existantes d'analyse des propriétés des flocs sont nécessaires, en particulier parce que les trois principaux groupes de scientifiques spécialisés dans les flocs (ingénieurs, scientifiques des milieux dulcicoles et scientifiques des milieux marins) ont eu tendance dans le passé à ne pas tenir compte des améliorations méthodologiques qui n'étaient pas conçues pour les besoins immédiats de leur groupe. Les problèmes subséquents de communication institutionnalisés et de réduction du transfert de la technologie se sont exacerbés depuis quelques années, au fur et à mesure que la biologie prenait de l'importance. L'INRE est un chef de file mondial dans la mise au point de méthodes d'analyse des flocs et, par conséquent, n'a aucune difficulté à obtenir du financement ou à trouver un éditeur enthousiaste pour publier un ouvrage sur l'analyse de la floculation et des propriétés des flocs. Le présent chapitre sur les méthodes, rédigé par deux personnes de l'INRE, est un chapitre fondamental du livre à paraître.

Quels sont les résultats?

On présente pour la première fois une compilation et une synthèse détaillées des méthodes existantes, dans une forme qui devrait intéresser les trois principaux utilisateurs de ces méthodes.

Comment ces résultats seront-ils utilisés?

L'information sera utilisée par les ingénieurs et les scientifiques de diverses disciplines s'intéressant aux milieux dulcicoles et aux milieux marins dans les ministères, les universités et le secteur privé d'un grand nombre de pays. Les nouvelles méthodes présenteront un intérêt particulier pour la dissection des flocs au microscope et pour l'analyse des systèmes et procédés microbiens à l'échelle moléculaire.

Quels étaient nos principaux partenaires dans cette étude?

Lewis Publishers/CRC Press, INRE, MPO, WTC/ETAD, Ryerson University, Brockhouse Institute for Materials Research, International Association for Sediment Water Science

Methods for Analyzing Floc Properties

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Introduction

The function-structure relationships of flocs are important to environmental scientists, microbiologists and engineers. Ultimately, their goals include being able to solve practical problems more effectively, and to provide better information for modeling ecological processes and contaminant transport in aquatic environments and in the operation of wastewater systems. Methods and analytical tools play a critical role in floc research and in achieving these goals. These are intended to do one of two things: i) to provide descriptive and quantitative information that may lead to a fuller understanding of flocculation; and ii) to have tools that may be applied to the management of floc processes in engineered and environmental systems.

At present, few standard methods with good reproducibility are available, although several physical, chemical, and microbiological measurement and analytical techniques have been developed. Earlier reviews give a comprehensive review of the methods and techniques for the measurement of physical characteristics for activated sludge [1] and an overview of the principles, methods and applications of particle size analysis in primarily marine systems [2]. Eisma et al. [3] and Dyer et al. [4] conducted a comparative study in the Elbe estuary to evaluate several different in situ methods for determining floc size and settling velocity. More recently, several entries in the Encyclopedia of Environmental Microbiology [5] provide overviews of methods, particularly advanced optical microscopy and molecular tools applied to the study of microbial structures including flocs [6]. Common to all these reviews is the wide range of methods employed to determine some of the most basic of parameters that describe flocs in the environment.

In engineered systems, advances have been achieved primarily in studying floc properties (ecology, structure and physicochemical characteristics) of individual flocs from full-scale systems and from laboratory-scale reactors run under well-controlled conditions. In contrast, studies in the marine and freshwater environments have concentrated on bulk properties such as gross morphology, size and settling velocity in samples collected with an emphasis on *in situ* measurements. One reason for the difference between measurements in the natural environment and engineered systems is the availability of flocs and the ease with which they can be sampled intact. Those involved with studying natural systems have tended to focus on the gross properties and behaviour of floc. Engineered systems are suited to detailed examination of surface properties and molecular determinants in floc behaviour.

In this chapter we present an overview of the principal methods presently being used in engineered, freshwater and marine systems. Some aspects of the methods presented can be applied to both natural and engineered systems. Our goal is to provide an insight into the work

being carried out in the different aquatic environments so that researchers can consider adapting the techniques presented to their respective fields.

Floc Size

Floc size is a widely measured floc characteristic. Floc size influences properties such as mass transfer (transport and settling) [7], biomass separation, and sludge dewatering [8,9,10]. Flocs are generally observed as two-dimensional projections, and there is no simple means of specifying size or shape [11]. However, Flocs are highly irregular in shape, porous, and 3 dimensional. Equivalent spherical diameter (ESD), frequently calculated from the two-dimensional area, is often used to characterize floc size due to its simplicity, and its application in Stokes' law [11,12,13]. Bache *et al.* [11] defined the effective diameter as the geometric mean $\sqrt{(d_{min}*d_{max})}$ based on the maximum (d_{max}) and minimum (d_{min}) dimensions across the 2-D floc image. Barbusinski and Koscielniak [14] and Li and Ganczarczyk [15] described floc size based on the average floc diameter defined as one half of the sum of the longest and shortest dimensions of the flocs measured.

Flocs in suspension are found over a range of sizes that describe a continuous distribution. Several standard parameters are available to describe floc size distributions. Median (d50), upper quartile (d25) and mode have all be used to describe the size distribution of flocs in suspension [13,16,17]. Due to the open architecture and poorly defined association of particles within a floc, researchers use fractal geometry to describe floc structure [18,19,20,21,22,23]. Depending on the nature and the sizing technique employed, there is no evidence to show which definition is the best representation of floc size. However, researchers should be clear in their definition of floc size when reporting results.

In general flocs range in size from a few microns to a few millimeters when measured by ESD. One exception is large assemblages of diatoms or other biologically derived material. Sometimes referred to as marine snow to differentiate it from more inorganic rich flocs, these patches of aggregated organic material can reach ESDs many orders of magnitude larger than what could be considered normal flocs. When marine snow becomes buoyant during decomposition, as was observed in the Adriatic Sea during the mucilage phenomenon, "floc size" can exceed 1m [24,25,26,27].

Many methods and instruments have been developed in the past to measure floc size distributions in natural and engineered systems. One of the earliest methods was the Coulter Counter, which determined the size distribution of particles in suspension. This method was popular in the marine environment as the electrolyte concentration in seawater permitted samples to be analyzed without alteration. However, stresses applied during the counting process can disrupt flocs which raises the issue that this method of may be of little value for estimating floc size [28,29].

The determination of floc size has relied primarily on imaging of flocs followed by image analysis to ascertain the parameters describing the size distribution [12,30,31]. Both microscopic observations and photographic techniques [13,32,33,34,35] have been used. In situ photography of flocs, although relatively easy to employ, does not allow measurement of very small flocs due

to resolution limits. Often these systems can only image down to 50 to 100 μm, although a 10:1 camera system with a resolution of 10 μ m has been developed [36,37]. Recent advances in digital photography should improve the resolution of *in situ* camera systems. The main advantage of these instruments is their ability to measure floc size with minimal disturbance to the natural stress environment of the flocs. However, they were developed for the natural environment, and may be difficult to apply in an engineered system as they are limited by the concentration of particles in suspension.

Microscopic methods usually incorporate a camera and computerized digitizer to provide images for analysis. The increased resolution of microscopic systems allows for accurate, reproducible and relatively fast estimates of floc morphological parameters. Specialized techniques such as confocal microscopy and electron microscopy (discussed in detail below) allow the internal structure flocs to be examined. The obvious drawback for microscopic analysis is the requirement to remove flocs from their natural environment and the associated instrument costs.

Common to both photographic and microscopic methods is the requirement to conduct image processing and analysis on the captured image to determine floc size and other descriptive parameters. Image processing and analysis comprises several steps [38]. Different algorithms are applied to the digital image to improve the quality of the image and to separate a floc from its background. Each area of coherent pixels with values within a selected range of threshold values is then used to calculate the different parameters used to describe floc size. There are differing views on the number of pixels required to define a particle with values ranging from 3 to 35 pixels [39,40]. Several different image analysis systems are available on the market but all are based on the same principals for manipulating a matrix of pixel values. Clear explanations of the methods employed in the analysis are critical for understanding how descriptive parameters such as ESD are generated.

Laser based sizing instruments are now being widely used to determine floc size in situ [41,42]. Two different laser techniques have been used, focused beam reflectance measurement (FBRM) and laser diffraction. FBRM instruments (modified ParTec) employ a rotating laser beam to determine the size of particles in the sensing zone [41]. When the laser encounters a particle the beam is reflected for the period of time it takes to traverse the particle. Using the angular velocity of the beam and the duration of the reflected laser pulse, the length of the intersecting particle chord is determined. A chord correction algorithm is then used to determine the size distribution of the particles in suspension. FBRM instruments were designed for process control and are not easily adapted to studies in natural systems. However they do have the advantage of working at higher concentrations than instruments that rely on light transmission [41].

Laser diffraction instruments were first used by Bale and Morris [43] who modified a Malvern particle size analyzer (Malvern Instruments, UK) for underwater use. Since then purpose-designed laser diffraction systems have become available, notably the LISST (Sequoia Scientific Inc., WA, USA) and the CILAS (CILAS, France). Laser diffraction instruments are based on the scattering of laser light by particles as the beam transits a known sample zone. The scattering angle is determined by the size of the particle with the scattering angle being small for large particles and large for small particles [42]. A series of concentric ring detectors sense the

amount of light they are receiving. Using the Mie or Fraunhoffer theory of scattering for spheres, these values can then be inverted to yield the particle size distribution [42].

Floc size has also been inferred from the settling behaviour of flocculated suspensions [4,44]. Settling column methods in general measure the equivalent hydraulic diameter of particles in suspension rather than the actual physical size of the suspended particles. Floc size is expressed in terms of the diameter of a sphere with the density of quartz, settling at the same speed as the particle in question [45].

Sample Handling and Stabilization

In situ measurement of floc size is clearly preferable due to the fragile nature of flocs. Sample handling may break up existing flocs or promote formation of larger flocs during storage [46]. Gibbs and Konwar [47] showed that common sampling methods disrupt flocs. Critical to any work with flocs outside of their natural environment is sample handling and preparation. The need for microscopic examination of flocs and for laboratory experiments with natural flocs has led to the development of new techniques for removing flocs from their ambient conditions with minimal change in floc size or structure. Considerable efforts have been given to overcome perturbation that may be associated with sampling and specimen preparation.

For floc size measurements not performed *in situ*, samples are collected in bulk suspension and transported to the laboratory for sizing. Essential to this first step is minimizing the stress applied to the flocs during sampling. Droppo and Ongley [12] employed traditionally laboratory used plankton chambers within fluvial systems. By using the plankton chamber as both the sampling and analytical chamber for image analysis, potential perturbations are minimized. Depending on the sizing methods, further floc sampling might be required. Some size measurements using image analysis systems or microscopic observation require sub-sampling of flocs onto microscope slides. This is normally done using a pipette for which the opening has to be wide enough (2 to 3 mm) to prevent floc breakage and disaggregation [48].

Floc stabilization prior to further sample handling has been shown to be effective in preserving floc structural characteristics. Droppo *et al.* [49,50] described a method of utilizing low melting point agarose to physically stabilize microbial flocs before analysis. This technique was found to have no significant effects on floc size distributions. Ganczarczyk *et al.* [51] used a similar approach in physically stabilizing microbial flocs. Optically clear polyacrylamide gels have been used in marine sediment traps to capture flocs intact for later processing [52].

Floc Settling Velocity

Settling velocity measurements of flocs are important for studying the fate of sediments within natural systems and for the evaluation of solids removal from treated effluents and in the estimation of floc wet density. Floc settling velocity has been found to increase with increasing floc size [53,54,55,56,57] but not necessarily in accordance with Stokes Law. Floc settling under gravity has been reported to be affected by a wide range of factors including the shape and

settling orientation of flocs being measured. The effect of fluid drag force on the settling velocity of a non-spherical particle is larger than that on a spherical particle [58,59]. The fastest settling rate is for particles of spherical shape, followed by cylindrical, needle-like, and disc-like [58]. Floc settling velocity may be affected by the settling orientations of the flocs because the drag force depends on the floc area facing the settling direction [53]. Fluid flow through the internal structure of flocs may also be important, as this would reduce hydrodynamic resistance and increase settling velocity [15]. Zahid and Ganczarczyk [54] stated that the computation of settling velocity by Stokes' law from the size and density measurements has to consider the effect of floc permeability. This, however, is in contradiction to the usual way of calculating wet density of flocs from the size-settling velocity measurements. The effect of floc permeability on settling velocity is considered negligible [55,60]. To complicate this picture from the floc structure viewpoint, Liss *et al* [61] showed that channels that appeared to be open by COM and CLSM were in many instances filled with EPS fibrils that could be seen only by TEM.

Floc settling velocity is most commonly determined by measuring the distance traveled by a floc over a known time using multiple exposure photographic and video imaging [34,35,53,57,62,63]. These techniques are effective in measuring floc size and settling velocity within the resolution limits of the imaging method used. Klimpel *et al.* [60] used a cinematographic technique to measure larger flocs (>100 μm), and the multiple exposure technique to measure smaller flocs (<100 μm). Droppo et al. [57] developed a videographic technique to measure floc settling velocity. This technique involves using a stereoscopic microscope or 1X tellecentric lens and a video camera to capture images of settling floc in a column filled with a media similar to the native environment of the samples. A small quantity (~ 1 ml) of floc samples is introduced at the top of the column. A sufficient travel distance is allowed for flocs to reach terminal velocity. Settling images of flocs are then recorded on a VCR as they pass though the focal plane of the microscope. These images are then analyzed using a computer imaging software for size and settling velocity.

Similar video imaging techniques have been developed to examine floc size – settling velocity relationships *in situ* in marine and freshwater environments [36,37]. All are based on video imaging of settling flocs within a stilled water column. Missing, however, from most studies has been an accurate estimation of floc density. A new instrument called INSSECT (IN situ Size and SEttling Column Tripod) has been designed to measure all the variables that at present are thought to influence the flux of fine-grained sediment to the bottom [52]. Comprising a rotating sediment trap and settling column, the rotating tripod is equipped with video and still camera systems, current meters, and polyacrylamide gels to capture settling flocs.

There is no simple equation relating the settling velocity of flocs to their size. Stokes' law or modified Stokes' law best describe the settling velocity of particles that approach a perfect sphere. Despite the limitations, estimations of other floc properties (e.g. density) derived from Stokes' law have proven useful in floc research. Stokes' law is defined as follows:

$$v = \frac{1}{18}\frac{g \cdot d^2 (\rho_f - \rho_w)}{\mu} \qquad (1)$$

where v = terminal settling velocity
ρ_f = wet density of particle
ρ_w = density of water (assume settling in water)

g = gravitational constant
μ = viscosity of water (assume settling in water)
d = diameter of particle

Li and Ganczarczyk [53] used a power function of the form, $v = A\,L^n$, and a linear function, $v = A + BL$, to correlate floc settling velocity (v) with its longest dimension as a characteristic size (L), where A, B and n are the equation coefficients determined experimentally. The power function is considered to be a better way to described the relationship because the power function predicts that the velocity will be zero when floc size approaches zero while the linear function does not. However, the measured settling velocities can yield coefficients lower than that predicted by Stokes' law ($n = 2$) [54,55,64]. The power law coefficients (n) calculated from the power function generally have ranged from 0.55 to 0.88. The number of flocs measured in these studies were as low as 21 and as high as 343. Lee *et al.* [56] managed to measure a total of 1385 flocs for settling velocity and size determinations and reported a power coefficient of 0.7-0.8. A modified linear model incorporating the floc settling shape factor was found to improve the correlation coefficient (R^2) of the linear relationship [64].

In the marine environment, settling velocities of flocs have been inferred from clearance rates in enclosed sedimentation tubes [4,44]. Commonly, open-ended tubes are submerged horizontally to permit free flow of particles in suspension and then closed, rotated to 90°, and retrieved. Subsamples are removed from the tube at set intervals during settling and the settling velocity is determined from the change in concentration with time [44]. Settling velocities calculated from clearance rates were found to be an order of magnitude less than those determined in situ using camera techniques during the Elbe Estuary intercallibration program [4]. However, the ease of use and the direct application of the results for determining vertical sediment flux have made settling columns a common instrument for nearshore studies.

In short, floc settling in any environment is highly related to size, but also related to floc shape and density. While Stokes law gives a reasonable approximation, the relationship between floc size and settling velocity is best described by a power law equation with the value of the exponent close to 1. Recent advances in digital imaging and image analysis and the ability to collect ancillary data have led to better understanding of the size-settling velocity relationship for flocculated suspensions [52].

Floc Density and Porosity

Floc density and porosity are two important floc characteristics in evaluating floc behaviour. Along with floc size and shape, floc density plays a strong role in influencing settling velocity with concomitant transport and industrial efficiency implications. As the porosity of a floc has consistently been shown to be negatively correlated to density [7], it to is an important parameter for floc behavioural assessment. Density is usually derived from the settling velocity-size measurements using Stokes' law or modified Stokes' law [34,35,53,54,55,56,57,60,65]. The validity of this approach has been questioned because it usually assumes spherical flocs and the settling velocity and size relationship do not follow Stokes' law. Zahid and Ganczarczyk [54] stated that there were a number of uncertainties involved in the density calculation from Stokes'

law, therefore the approach was regarded only as an approximation. Lee et al. [56] also supported this approach since it provides at least qualitatively valid density estimation.

The following equation is often used to calculate floc porosity from density [53]:

$$\varepsilon = \frac{\rho_s - \rho_f}{\rho_s - \rho_w} \qquad (2)$$

where ρ_s and ρ_f are the dried floc density (1.34 - 1.69 g/cm^3) and wet floc density, respectively, and ρ_w is the liquid density.

Andreadakis [66] made use of interference microscopy for floc density determination and used the above equation to calculate floc porosity. Density determinations for aggregates are usually based upon observations of terminal velocity, although a method based upon a series of sucrose solutions of incremented densities has been presented by Lagvankar and Gemmell [67]. Ozturgut and Lavelle [59] employed a linear-density stratified column which allows flocs to settle to their isopycnic levels to measure low density but settleable wastewater effluent flocs. Dammel and Schroeder [68] used a similar density gradient centrifugation technique, which allows the flocs to settle in a fluid of continuous increasing density until the flocs become stationary, to measure the density of activated sludge flocs. This technique, however, does not measure floc size concurrently with its density, thus, a size and density relationship might not be established easily. In addition, the ionic strength of the suspension medium and the nature of the medium itself have to be compatible and non-toxic with the biological flocs.

A variety of floc density models have been proposed. Magara *et al.* [34] proposed the following floc effective density (ρ_e) model based on Stokes' law,

$$\rho_e = \rho_f - \rho_w = 0.003698 \bullet \mu_w \, v \, d^{-2} \qquad (3)$$

where ρ_f and ρ_w are the floc density and liquid density respectively (g/cm^3), μ_w is the liquid viscosity (g/cm^3-s), v is the floc settling velocity (cm/s) and d is the floc ESD (cm). Tambo and Watanabe [35] suggest a model based on Stokes' law for effective floc density and size :

$$\rho_e = \rho_f - \rho_w = \frac{34\mu_w v}{g d_f^2} \qquad (4)$$

assuming a drag coefficient of 45/Re and a floc sphericity of 0.8. Andreadakis [66] suggested that the floc density (ρ_f) is a function of its size (d),

$$\rho_f = 1 + 0.30 \, d^{-0.82} \qquad (5)$$

assuming that the dried sludge density of 1.34 g/cm^3. Glasgow and Hsu [65] developed an empirical equation for kaolin-polymer aggregate to relate its density (ρ) to diameter (d) and pH,

$$\rho = 1.05 \bullet d^{(-0.0038pH + 0.00716)} \qquad (6)$$

assuming a sphericity of 1.0.

Zahid and Ganczarczyk [54] plotted effective density as a function of average diameter on a logarithmic scale and developed the following equation for the floc effective density and the average diameter (D),

$$\rho_e = \frac{0.005}{D^{1.21}} \tag{7}$$

where the two constants, 1.21 and 0.005, represent the slope of the straight line and the effective density of a 1.0 mm diameter particle, respectively. Accordingly, the size-porosity function was expressed as:

$$\varepsilon = 1.0 - \frac{0.007}{D^{1.21}} \tag{8}$$

Mikkelsen and Perjrup [69] presented a method for determining effective floc density and calculated settling velocity in coastal marine environments using data collected by a LISST. Assuming that floc fraction, the amount of material in suspension that is found in flocs, is high then effective floc density is equal to the total suspended mass divided by the total volume concentration of the flocs.

Although there are many empirical models available for the estimation of floc density and porosity, none of them can be considered as a universal model. This is simply because all these models were developed from their specific conditions such as the type of natural or engineered system, the type of microorganisms, the hydrodynamic conditions, and the experimental techniques used. Therefore, floc density and porosity must be experimentally determined in all situations. New instrumentation is now available that can determine the size – settling velocity relationship of flocs in suspension, determine the mass of the flocs, and capture them for microscopic analysis [52]. With these advances, it should be possible to determine densities in natural environments in situ.

Floc Structure: Correlative Microscopy

Leppard [70] defined correlative microscopy (CM) as a strategy of using multiple microscopic techniques which can include conventional optical microscopy (COM), confocal laser scanning microscopy (CLSM), and transmission electron microscopy (TEM), and allow one to detect, assess and minimize artifacts that might arise from using one technique only. CM has been successfully used by Liss *et al.* [61] with a minimal perturbation approach in studying natural and engineered flocs. A recent minimal perturbation approach [49,50] involves the use of sample stabilization in low melting point agarose and a four fold multi-preparatory technique. The use of a four-fold multiple preparatory technique and CM was found to maintain the structural integrity of the samples through the stabilization, staining and washing procedures. The use of only one microscopic technique can bias or limit the information acquired because of the artifacts that arise in specific sample preparations and the resolution constraint associated with a particular technique.

The use of COM is the most common microscopic approach in the analysis of external gross-scale floc structure [12,14,31,57,71,72]. High resolution transmission electron microscopy (TEM) is often used to investigate the fine structure of natural and engineered flocs (nm), especially in the study of EPS distribution within floc structure [26,27,72,73,74,75]. This is generally done by stabilizing samples in a fixing agent such as glutaraldehyde, then embedding in Spurr resin or alternatively a fixation and embedding in Nanoplast; an ultrathin section is then obtained from the embedded sample by slicing with an ultramicrotome and a diamond knife. This ultrathin section (50 -100 nm) is then placed on a copper grid for further staining (e.g. uranyl acetate) to give better contrast, although at TEM resolution, fibrils, bacterial cells and other components of floc are visible. TEM can be used in conjunction with energy dispersive spectroscopy (EDS) to detect metal accumulation and to give element abundance in EPS [61,76]. The thickness constraint of ultrathin sections (50 -100 nm or less) in the preparation of TEM images has restricted the floc sample volume, which has a diameter as large as 1 mm, that can be examined at high resolutiondue to the consideration of cost and time. COM and confocal laser scanning microscopy (CLSM) images are useful in indicating the number of TEM sections required to be collected for determining the representative images of flocs [61].

Nanoplast resin is particularly effective as a stabilization medium since it is a hydrophilic melamine embedding resin that holds the fibrillar EPS in native three-dimensional disposition. Nanoplast omits the solvent dehydration stage, and it forms cross-linkages between matrix colloids prior to structural water loss at the end of the embedding process. Measurements of the dimensions of colloidal matrix material and their three- dimensional disposition are realistic. Nanoplast has been recently shown to be useful for stabilizing sediment biofilms and the EPS matrix of these structures for observation by confocal laser scanning microscopy (CLSM) [77]. The environmental scanning electron microscope (ESEM) permits observations of fully hydrated samples but there have been no published reports to date that describe microbial flocs using ESEM, excepting a few reports using ESEM information as an aid for interpreting other observations [e.g. 76].

CLSM is one of the most recent microscopic techniques used to study flocs and has been shown to be a useful technique in bridging the resolution gap between COM and TEM [23,61]. Images are scanned with a laser beam and collected in a point-by-point fashion by a photodetector system [78]. These collected images are stored in the computer memory for further image processing and analysis. The advantages of CLSM over conventional light microscopy include the reduction of image blurring caused by light scattering, with a concomitant increase in effective resolution. CLSM also allows the examination of a thick specimen such as animal tissue and biological flocs by scanning a series of planar images (X-Y plane) along a vertical axis (Z) one at a time. These series of planar images can be reconstructed in a computer imaging analysis system into a 3D image of the sample [21,22,23,61]. Floc in excess of 500 μm can be visualized by CLSM. Two photon, or multiphoton, scanning laser microscopy (2P-LSM) permits examination of floc and films approaching 1 mm in thickness while minimizing photobleaching and phototoxicity. A particularly useful feature of CLSM and 2P-LSM is that these can be used in combination with a variety of fluorescent molecular probes to study the spatial distribution of extracellular polysaccharides, cell viability, pH gradient, proteins, RNA, lipids, and other components of floc nondestructively [77,79]. These techniques are increasingly

being adapted by researchers to further our understanding of flocs and the relationship between structure, ecology and the physicochemical properties of flocs and films [23].

Other advanced microscopic methods including Raman confocal microspectroscopy can potentially provide insights into the chemical composition of microbial cells and the heterogeneity associated with spatial distribution associated with structure and the conditions of growth [80,81]. Atomic force microscopy (AFM) has emerged as tool that can provide detailed information on topography of microbial surfaces as well as probing surface properties [82,83. Through the interaction of the AFM tip and the microbial surface attractive and repulsive forces can be explored (See Logan Chapter XX). Functionalizing the tip with molecular specific probes is a further advance that permits more detailed examination of molecular interactions. Synchrotron-based scanning transmission X-ray microscopy (STXM) has recently been used correlatively with TEM and CLSM on a rivering biofilm to gain high resolution information on the three-dimensional distribution of specific classes of extracellular polymers [84].

Extracellular Polymeric Substances

In aqueous environments bacteria may invest a substantial amount (>70%) of their carbon and energy in the production of extracellular polymeric substances [77,85], indicating an important role of EPS in the functioning of microbial communities and the structures they form. Over the past 20 years studies have emphasized the composition of the EPS based on analyses of whole sludge or the extracted EPS. More recently, investigators are beginning to focus on detailed investigation of the surface properties and EPS through direct examination, by microscopy, of the whole biopolymeric material rather than the extracted EPS as has been done previously [86] (see Chapter by John Lawrence).

EPS can be classified as capsular EPS (bound) and slime EPS (soluble). The bound EPS is attached tightly to the exterior cell wall, while the soluble EPS is the loosely attached or unattached 'slime' material that can be washed away by centrifugation [87,88]. In order to analyze the composition of bound EPS without inducing cell lysis, a variety of extraction methods have been developed. Brown and Lester [89] have compared bacterial EPS extraction methods from other sources including chemical methods such as ammonium hydroxide, sodium hydroxide, EDTA, sulfuric acid, and boiling benzene. Mechanical extraction methods such as high-speed centrifugation, ultrasonication, and boiling or autoclaving were also investigated. The influence of various separation and extraction processes, on the constituents and quantities of EPS being extracted, has been reviewed recently, along with a comparison of the analytical measures in common use [90].

Cation exchange resin (CER), utilizing both a mechanical and chemical means of extraction, has been found to be effective in terms of minimal cell lysis and non-disruptive effects on the EPS [91,92]. This extraction method removes divalent cations such as Ca^{2+} and Mg^{2+} from the EPS matrix and replaces them with monovalent cations. By removing the divalent cations the EPS becomes less stable thereby allowing the EPS to separate from the cellular material. Subsequent to capsular EPS extraction, proteins [93], carbohydrates [94], acidic polysaccharides [95], and DNA can be measured.

Although quantitative estimations of EPS in microbial structures have been traditionally accomplished through extraction and chemical methods, fluorescent probes are suitable for estimating EPS in situ. In the past, Calcofluor White M2R was used to measure exopolysaccharide production in single bacterial strains such as *Azospirillum*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [96,97]. Similarly, congo red was employed for general light microscopy staining of polysaccharides [98]. More recently researchers have begun to use fluorescently labeled lectins as a method to probe the spatial relationships of EPS within thick heterogeneous microbial structures [99,100,101,102]. Lectins are a large group of glycoproteins that bind to specific carbohydrates. They are prevalent in nature and are present in plants, bacteria, animals, and humans [103]. Plant lectins have been used as both specific and general stains to estimate EPS as well as to characterize the EPS left on surfaces after the removal of microbes [99,100,101,104].

Surface Charge and Hydrophobicity

As reviewed by Liss [6], methods used in the past to determine microbial surface charge include attachment to charge-modified polystyrene, fluorescent probe ion exchange resin, and electrophoretic mobility. At present, the most common and reproducible method to determine surface charge density of microbial aggregates is by a colloid titration [105,106]. Mikkelsen [107] recently compared surface charge determinations of sewage sludge by various methods in order to identify applications and limitations of the colloid titration method for analysis that tends to yield widely ranging values amongst different studies. The colloid titration method was found to be limited to conditions of low reactant doses and valid for charge determination of extracellular polymeric substances primarily. For determining whole floc or sludge surface charge estimates from zeta potential titrations may be more reliable than that determined from colloid titration.

Numerous methods have been reported in the literature for determining hydrophobic interactions of cells and have been summarized by Liss [6]. These include methods that measure actual binding to a hydrophobic ligand such as microbial adhesion to hydrocarbons (MATH) and those giving an estimate of an overall surface property, such as salt aggregation test (SAT) and contact angle measurement (CAM) of dry cell layers.

The MATH method is a simple method to rapidly quantify cell surface hydrophobicity [108,109]. This method is based on the partitioning of cells possessing hydrophobic surface characteristics at the interface of a biphasic hydrocarbon-aqueous system after brief mixing. The relative hydrophobicity is calculated from:

$$H = 100\,(1 - a/A), \qquad (9)$$

where a is the absorbance of the aqueous layer after phase separation; A is the initial absorbance of the aqueous phase at 400 nm before mixing with hydrocarbons. Attention must be given to the possibility that cell clumping may occur during the assay, which can result in a huge reduction in absorbance. Changes in the initial cell density can also affect the measurement.

CAM is one of the most common techniques for the measurement of hydrophobicity of bacterial cell surfaces and flocs because the surface free energy of these cells can be estimated from the measurement (see Liao et al., Chapter xx). Although different apparatus may be used, all the measurements involve the preparation of a thin bacterial lawn through the vacuum filtration of a bacterial suspension and the determination of sessile drop contact angles on the bacterial lawn, either by using a telegoniometer or by projecting a magnified image system. The application of axisymmetric drop shape analysis (ADSA) may overcome some of the problems inherent in contact angle measurements on biological cells [110,111].

SAT is based on subjecting cells to increasing concentrations of salting-out agents (e.g. ammonia sulphate) [112]. The order in which cells are aggregated and settled is a measure of their surface hydrophobicity. The most hydrophobic cells are aggregated first at a low salt concentration. All tests are compared to the reaction at the highest molarity of salt (positive control). Bacterial suspensions mixed with a 0.002M sodium phosphate (pH 6.8) are used as a negative control. A drop of methylene blue can be added to enhance the visualization of the aggregates [113]. Urbain *et al.* [114] used this method to study the internal hydrophobicity of sludge flocs. Limitations of SAT include the fact that many hydrophobic bacterial cells will clump in the absence of any added ammonium sulphate, and that it provides only a qualitative estimation of the relative rank of hydrophobicity. Electrostatic interactions may affect the results of SAT more than other hydrophobic measurement techniques [109].

Microbial Ecology

The identification and *in situ* detection of microrganisms within activated sludge flocs using DNA probes have been reported for the subclasses of proteobacteria [115], Gram-negative filamentous bacteria [116], *Acinetobacter* spp. [117], and nitrifying bacteria, ammonia- and nitrite-oxidizing bacteria [118,119,120,121]. Probes have been found to be effective in monitoring shifts in nutrient amended activated sludge samples and avoid biases associated with culture dependent methods.

Nucleotide probes are also being used in multimethod approaches to the analysis of marine snow. Grossart and Simon [122] studied the bacterial colonization and microbial decomposition of lake snow with rRNA-targed fluorescent oligonucleotide probes, used in conjunction with standard limnological parameters and measures of hydrolytic enzyme activities, bacterial production and growth rates, and changes in floc composition during aging and sinking. The flocs were shown to be hotspots of enhanced microbial activity, with the microbial community dominated by β-Proteobacteria. The overall results suggested that lake snow and activated sludge flocs have similar functions in their aquatic ecosystems. A review and comparative analysis of the microbial ecology of flocs from many ecosystems has been published recently by Simon et al. [123], who consider marine, lacustrine, riverine and estuarine environments. They describe methodology and reference it in considerable detail, and they conclude that the significance of bacteria in aggregation processes is much greater than previously estimated.

A variety of approaches permitting correlation of structure-function relationships with respect to specific metabolic and biogeochemical processes associated with flocs are available. Carbon-substrate oxidation patterns or phenotypic fingerprinting based on commercially available

BIOLOG microplates allow for simultaneous testing of 95 separate carbon substrates producing patterns of metabolic response based on the reduction of tetrazolium dye as an indicator of sole carbon utilization [124]. Brock and Brock [125] introduced microautoradiography (MAR) as a tool for looking at the activities of individual microorganisms in their natural habitats. This technique has been used since to address many important ecological questions regarding bacterial activities in natural aquatic systems [126,127.128,129]. MAR is a method that can be used to investigate the ability to assimilate organic or inorganic compounds by natural bacterial populations as well as assessing their abilities to be active under different operating conditions e.g. like the aerobic, anoxic or anaerobic conditions encountered in some activated sludge systems [130,131,132]. Radiolabeled (^{3}H, ^{14}C, ^{35}S or ^{33}P) substrates are used and can become associated with cells or biological structures of interest either as a result of adsorption or active uptake. After coating cells with a photographic emulsion and following an appropriate time of exposure, uptake is indicated by deposition of silver grains on the surface of radioactive cells.

Recently, direct combination of fluorescent *in situ* hybridization (FISH) and MAR for simultaneous *in situ* identification and determination of substrate uptake patterns of individual microbial cells within complex microbial community has been applied to activated sludge [131,132,133,134,135]. In the protocols used, an additional step of FISH prior to the 4',6-diamino-2-phenylindole (DAPI) stain, which is used to obtain total cell counts [115,130,136,137], and the MAR developing steps, is incorporated. This combination permits simultaneous *in situ* detection of DAPI signals, probe-conferred fluorescence, and silver grain formation (which indicates the presence of radioactive compounds within the fixed cells) at a single cell level. The technique has been used to examine the identity of unknown bacteria which carry out specific processes, including their roles in organic substrate uptake and biological phosphate removal [130,131]. Some filamentous bacteria from activated sludge have been studied with these techniques, and their roles in the uptake of several organic substrates determined [133,1354].

Conclusion

Recent advances in both microscopic and photographic imaging techniques have led to a much greater understanding of floc behaviour and floc structure. While the emphasis in the three different environments (marine, freshwater and engineered systems) differs, new techniques in each discipline present an opportunity to advance our understanding of flocs. Very significant advances have been made in the study of floc properties in engineered systems whereas the emphasis in natural systems has been on determining bulk properties that control the transport of sediment. Methods for dissecting flocs microscopically and understanding microbial systems and processes at a molecular level are relatively new to the marine and freshwater studies and offer an exciting new area of study provided floc sampling methods can advance at the same time.

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