6 Mapping Biopolymer Distributions in Microbial Communities

John R. Lawrence, Adam P. Hitchcock, Gary G. Leppard, and Thomas R. Neu

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6.1 INTRODUCTION

Microbial communities or aggregates also known as biofilm systems may be divided into stationary ones and mobile ones. Stationary ones are the classical microbial films usually on solid surfaces. Mobile ones have been named with a variety of terms such as assemblages, aggregates, flocs, snow, or mobile biofilms.[1] The techniques described in the following chapter apply to both biofilms and flocs. Aquatic aggregates (river, lake, marine, technical) may be very different in terms of size, composition, density, and stability.[2] Lotic aggregates are structurally very stable as they are exposed to a constant shear force resulting in relatively small aggregates ($\approx$ 5 to 300 μm), whereas lake or marine snow may be very fragile and much larger (millimeters to meters). Both environmental aggregates are colonized to a certain degree by prokaryotic and eukaryotic microorganisms (bacteria, algae, fungi, protozoa). The bacterial composition of environmental aggregates was studied *in situ*, for example, by Weiss et al.[3] In comparison to natural aggregates, technical aggregates are heavily colonized mainly by bacteria, for example, in activated sludge.[4] The microbial population structure of activated sludge was first analyzed *in situ* by Wagner et al.[5] Another example for man-made aggregates are mobile biofilms growing on carrier material, for example, in fluidized bed reactors. Due to high shear force, these immobilized aggregates are extremely dense and stable.[6] A major understudied component of all these microbial systems is their exopolymeric matrix.

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Exopolymeric substances (EPS) have correctly been referred to as the mystical substance of biofilms and aggregates[7] and a challenge to properly characterize.[8] The extracellular polymeric substances (EPS) are defined as organic polymers of biological origin which in biofilm systems are responsible for the interaction with interfaces.[7] Although EPS are understood as extracellular polymers mainly composed of microbial polysaccharides, by definition other extracellular polymeric substances may also be present, for example, proteins, nucleic acids and polymeric lipophilic compounds.[8–11] In biofilm systems we can expect two types of structural polymeric carbohydrate structures. First, those associated with cell surfaces and second, those located extracellularly throughout the extracellular biofilm matrix. The importance of EPS in flocs and biofilm systems is fundamentally twofold, (i) they represent a major structural component of flocs and (ii) they are responsible for sorption processes.[12,13]

Particularly in complex environmental systems, the EPS are difficult if not impossible to chemically characterize on the traditional basis of isolating single polymer species. Chemical approaches are limited to pure culture, chemically defined systems. Despite this problem, chemical quantification of EPS constituents in biofilm systems have been reported.[14] These confirm the complex nature of the material and the extensive range of polymers present. Increasingly attempts have been made to examine natural biofilm and floc polysaccharides *in situ*.[1,8,15–18]

The critical need for *in situ* analyses and visualization of EPS is due to its complex chemical nature and the importance of its molecular structure in its behavior. Indeed, the challenge remains to characterize its chemical composition in the context of its biological form. To do this we have proposed a variety of *in situ* methods based on the application of chemical probes and 1P (1-photon) and 2P (2-photon) laser microscopy. In addition, synchrotron radiation using the interaction of x-rays with

the molecular structure of intact hydrated biofilms has proven an effective approach. In this overview we assess *in situ* analyses of EPS using light of various wavelengths ultraviolet, visible, infrared, and x-ray in combination with targeted probes to assess the structure of biofilms and flocs.

6.2 METHODOLOGY

6.2.1 Handling Flocs for Microscopic Examination

Due to their size, location and relative fragility, river, lake, or marine flocs are difficult to examine under *in situ* conditions. Lotic aggregates are often sampled in bottles with, for example, or volume. Similarly, lake or marine flocs maybe sampled directly into special containers by scuba divers.[19] However, within 30 min, these sampling procedures will result in settling and co-aggregation of smaller flocs into larger loosely associated aggregates of several 100 μm diameter thus analyses of these specimens are extremely time sensitive. Leppard[20] reported the occurrence of artifactual aggregation where small aggregates combine to yield a few large aggregates. In addition, it was noted that rough handling (high flow, centrifugation) storage longer than 24 h, and most concentration steps will all result in coagulation of the flocs.

In order to maintain structural integrity of the sample some care must also be exercised in the preparation for microscopic examination. In general, biofilm and floc samples are exposed to physical stress in the real-world environment, therefore in most instances they are resilient enough to be manipulated and mounted for staining and observation. However, laboratory treatments such as drying, freezing, washing, dehydration etc. will all perturb the native structure of the floc. Leppard,[20,21] Leppard et al.,[22] and Droppo et al.[23] provide useful instruction on the handling of flocs for microscopic examination and preservation of their native state and properties. Staining may be carried out by careful addition of the stain and its withdrawal using tissues or small sponges, with subsequent replacement and washing with sterile medium (variously 3× to 5×) or environmental water (river, lake, pond, etc.). In some instances removal of excess stain must be carried out by centrifugation of the floc and resuspension in stain/probe free water. Only careful evaluation can determine at what point these treatments will alter the floc under investigation and this should be assessed for each type of floc examined. Conventional wet mounts and other slide preparations may also be usefully performed to examine flocs.[24] Floc or aggregate samples may be fixed to the bottom using flowable silicon adhesives or allowed to settle to the bottom of a small petri dish (diameter 5 cm). In these cases an upright microscope may be used to examine the preparation. In the case of flocs an inverted microscope in combination with a settling chamber having a cover slip bottom such as those provided by NalgeNunc International, Denmark, may be a preferred method of preparation for 1-photon laser scanning microscopy (1P-LSM), 2-photon laser scanning microscopy (2P-LSM), or fluorescence microscopy.[1] Although if an inverted microscope is used, access to the sample is limited and the working distance of the objective lens may further limit examination of the biofilm. It is also possible that lotic aggregates be collected directly in the LabTek coverslip chambers (NalgeNunc International). By this sampling procedure the settling and co-aggregation of small flocs is kept to

a minimum. Subsequently, the aggregates can be microscopically examined using LSM for reflection signals and autofluorescence (general, algal, cyanobacterial). In addition, flocs may be stained inside the chamber using nucleic acid specific stains to record bacterial distribution and fluorescently labeled lectins to record glycoconjugate distribution.

In the case of synchrotron based imaging such as scanning transmission x-ray microscopy (STXM) the sample must be prepared on an x-ray transparent holder. STXM measurements must be performed on a wet cell constructed with a silicon nitride window (Silson Inc, Northampton, U.K.) by placing the sample onto one half of the silicon nitride cell and sealing it with the other half. Figure 6.1 shows a typical completed wet cell with enclosed biofilm material. The wet cell is then placed directly in the beamline for imaging.[25,26]

6.2.2 Epifluorescence Microscopy

Conventional widefield epifluorescence microscopy provides simple effective means to examine the results of most staining of the exopolymers of microbial cells, flocs, and biofilms provided a suitable range of optical filters are available. Optical sectioning may be achieved using epifluorescence, a stepper motor, and a digital video imaging device. The major limitation of the image series collected is poor axial resolution, however, this may be improved by computing intensive restoration procedures or deconvolution (see Section 6.2.3).

6.2.3 CLSM and 2P-LSM

Confocal laser scanning microscopy (CLSM or 1P-LSM) has become an indispensable technique for the study of interfacial microbial communities.[27] This is particularly due to the increasing number of fluorescent stains and reporter

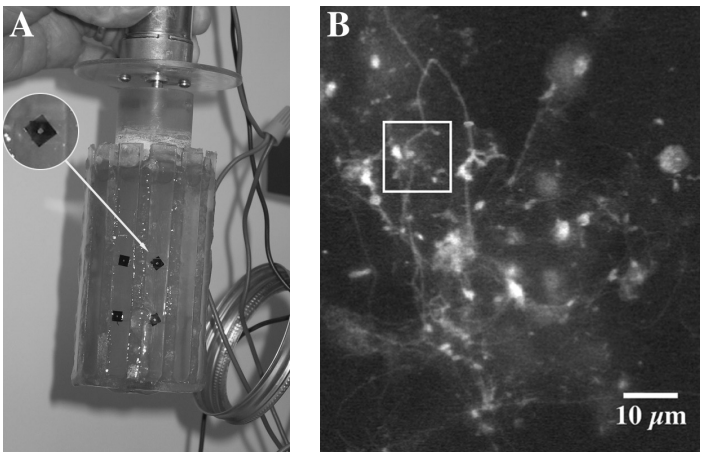

FIGURE 6.1 (A) Image showing a silicone nitride window attached to a rotating annular biofilm reactor, detail in inset shows window and central x-ray transparent region for STXM imaging, (B) CLSM image of x-ray transparent region showing biofilm development on the window

systems suitable for application in the study of flocs and biofilms. Specific techniques include those for detection and quantification of cellular and polymeric compounds in biofilms.[9,16,27] In addition, Neu et al.[28] demonstrated that 2P-LSM could be effectively applied to the study of highly hydrated microbial systems such as flocs and that a range of fluorescent reporters for both cell and exopolymer identity could be applied in combination with this imaging approach. Figure 6.2 provides a comparison of the excitation for 1P versus 2P for the common fluor fluorescein illustrating the different response of the fluor in the two forms of LSM. Extensive details of these microscopy techniques and their use in combination with biofilms and flocs are provided in Lawrence et al.[27] Neu,[1] Lawrence and Neu,[29] and Lawrence et al.[30]

6.2.3.1 CLSM Limitations

A limitation of 1-photon excitation is laser penetration of samples (excitation) and detection of emission signal in thick samples. This problem is very much dependent upon the density and light scattering properties of the sample. Consequently thick samples have to be embedded and physically cut into slices using embedding resins or cryosectioning.

6.2.3.2 2P-LSM Limitations

The major problems are the overall stability of the laser system, maintenance of signal intensity, and excessive noise in the image. In addition, images may be degraded by reaction of the light source with the substratum or mounting materials causing, for example, streaks in the image due to adsorption of infrared light (e.g., plastics). Although laser penetration is better (twofold) in 2P-LSM over CLSM, light scattering in thick biological samples remains a problem.

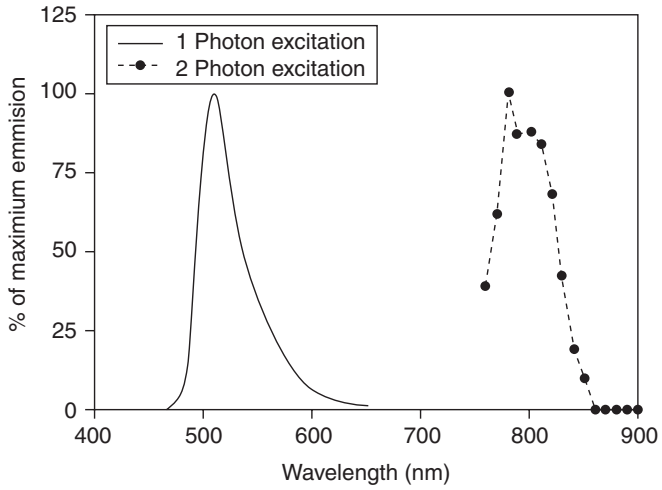

FIGURE 6.2 Comparison of the 1P and 2P emission for fluorescein when excited at wavelengths between 400 and 900 nm

6.2.4 Synchrotron Radiation (Soft x-ray Imaging)

Scanning transmission x-ray microscopy (STXM) is a powerful tool that may be applied to fully hydrated biological materials. This is due to the capacity of soft x-rays to penetrate water and have minimal radiation damage relative to electron techniques. In addition, soft x-rays interact with nearly all elements and also allow mapping of chemical species based on bonding structure.[31] Soft x-ray microscopy also provides suitable spatial resolution and chemical information at a microscale relevant to bacteria. Most importantly, the method uses the intrinsic x-ray absorption properties of the sample eliminating the need for the addition of reflective, absorptive, or fluorescent probes and markers which may introduce artifacts or complicate interpretation. Figure 6.3 shows the representative absorption spectra for protein, nucleic acid, saccharide, lipid, and calcium carbonate. The potential of soft x-rays for imaging early stage *Pseudomonas putida* biofilms using a full field transmission x-ray microscope with synchrotron radiation was demonstrated by Gilbert et al.[32] They measured at single photon energy and did not explore the analytical capability of x-ray microscopy. Lawrence et al.[25] demonstrated the application of analytical soft x-ray microscopy to map protein, nucleic acids, lipids, and polysaccharides in biofilm systems. Hard

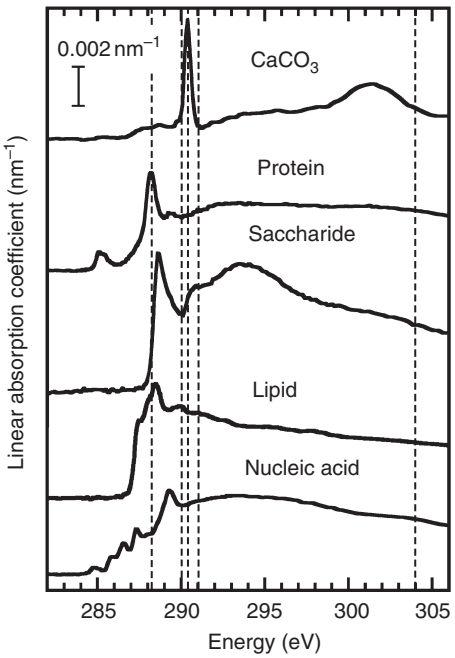

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FIGURE 6.3 C 1s NEXAFS spectra of protein (albumin), polysaccharides (sodium alginate), lipid(1-Palmitoyl-2-Hydroxy-sn-Glycero-3-Phosphocholine), and nucleic acid (calf thymus DNA). All spectra except that of DNA were recorded with the ALS 7.0.1 STXM. The spectrum of DNA was recorded on ALS 5.3.2 STXM. (copyright American Society for Microbiology, Lawrence, J.R. et al. *Appli Environ Microbiol*: 69: in press, 2003)

x-ray analyses also have potential for application to biofilm–floc materials having been used for bacterial cell–metal interaction studies.[33]

6.2.4.1 STXM Limitations

Limitations to STXM include: suitability of the model compounds relative to biofilm/floc material, data acquisition without undue radiation damage, requirement for very thin samples (<200 nm equivalent thickness of dry organic components, less than 5 micron of water when wet), use of fragile silicon nitride windows, sample preparation, that is, encapsulation in a wet cell, and absorption saturation distortion of analysis in thick regions of a specimen.

6.3 TARGETS AND PROBES

The *in situ* analyses of hydrated biofilms may be carried out using a variety of probes targeted generally at polysaccharides, proteins, lipids, or nucleic acids. In addition, other probes such as dextrans, ficols, and polystyrene beads may be used to assess general properties such as charge, hydrophobicity, permeability, or the determination of diffusion coefficients. Probes are most frequently conjugated to fluors although colloidal reflective conjugates (gold, silver) may be used.[27] Recently, quantum dots (QDs) have shown great promise as multiwavelength fluorescent labels. Colloidal QDs are semiconductor nanocrystals whose photoluminescence emission wavelength is proportional to the size of the crystal. Kloepfer et al.[34] reported that cell surface molecules, such as glycoproteins, made excellent targets for QDs conjugated to wheat germ agglutinin. This new class of fluorescent labels may open opportunities for *in situ* detection of matrix chemistry. As indicated above, the option exists for probe independent examination of major biopolymers and other constituents in hydrated biofilm and floc material providing a basis for detailed examination of these structures and ground truthing of the fluorescent and reflection based probe dependent approaches.

6.3.1 POLYSACCHARIDES

6.3.1.1 General Probes

A range of stains with specificity for beta-D-glucan polysaccharides are used as general stains, these include calcofluor white and congo red. Ruthenium red has also been used as a light microscopy stain for detection of EPS. Probes for glycoaminoglycan such as Alcian blue may also be used as a general stain for "polysaccharides." Wetzel et al.[35] demonstrated its use for determination of total EPS in microbial biofilms, in this case it was used indirectly and not for microscopy. Due to the complexity of the EPS the likelihood of finding a true total polysaccharide probe appears to be very limited.

6.3.1.2 Lectins

Lectin-like proteins have a long history of application in the biological sciences.[36] Currently, lectins are regarded as proteins with a lectin–carbohydrate and a lectin–protein binding site and are characterized on the basis of their interactions with specific monosaccharides. Lectins are produced by many organisms including plants, vertebrates, protists, slime molds, and bacteria where they function as cell/surface-recognition molecules.[37] Recognition of the specific site is controlled by stereochemistry, however, the carbohydrates also interact with lectins via hydrogen bonds, metal coordination, van der Waals, and hydrophobic interactions.[38] (See also review articles and comprehensive books on lectins.[39–42])

The difficulty of isolating a single polymer type from a complex biofilm matrix may be comparable to the situation at the cellular level.[43] Neu et al.[16] noted that if one considers the potential of carbohydrates to encode information in terms of saccharides it is even larger than that of amino acids and nucleotides. The latter two compounds can only build 1 dimer whereas one type of monosaccharide can form 11 different disaccharides. Further, 4 monosaccharides, a common number in the repeating unit of polysaccharides, may form 35,560 different disaccharides.[37] If each of the estimated number of bacterial species (4,800,000) secretes one protein and one polysaccharide this would be 9,600,000 EPS compounds; a very conservative estimate.[44] As a consequence, there is a need to establish an *in situ* technique for the assessment of glycoconjugate distribution in floc systems. At present the most promising approach to achieve this is the application of fluorescent-lectin-binding-analysis (FLBA) in combination with CLSM. Labeled lectins have been successfully used in many microbial pure culture studies to probe for cell surface structures.[45–48] Fluor conjugated lectins have also been used fairly extensively in complex environments including, marine habitats[49] and freshwater systems.[1,15,16,18,30,50,51]

As noted by Neu and Lawrence[9] lectins may represent a useful probe for *in situ* techniques to three-dimensionally examine the distribution of glycoconjugates in fully hydrated microbial systems. The many lectins available, offer a huge and diverse group of carbohydrate specific binding molecules waiting to be employed for an *in situ* approach.[52] The above listed studies all suggest that lectins may be applied successfully to extract information regarding the nature of the EPS. Fluor-conjugated lectins effectively reveal the form, distribution, and arrangement of EPS in three dimensions. Figure 6.4 illustrates this phenomenon showing the distribution of EPS using *Solanum tubersosum, Cicer arietinum*, and *Tetragonolobus purpureas* lectins and confocal laser microscopy to examine a microcolony in a river biofilm, note the multiple layers of EPS identified by each lectin and their spatial distribution. Figure 6.5 illustrates the distribution of binding sites for lectins within a river floc from the Elbe River. As also shown in Figure 6.5, FLBA has been combined with fluorescent *in situ* hybridization (FISH; see review by Amann et al.)[53] to allow localization and identification of bacteria associated with the binding of specific lectins.[17] This visualization is extremely useful as a starting point for additional questions regarding the EPS. However, the major goals of quantification and chemical identification remain more elusive. Neu et al.[16] evaluated lectin binding in complex habitats in detail. They showed that it was possible, through digital

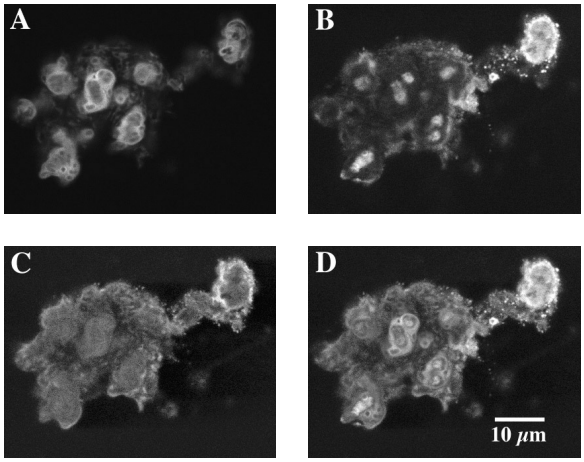

FIGURE 6.4 CLSM micrographs of a bacterial microcolony stained with lectins (A) *Cicer arietinum*-Alexa-568 (red); (B) *Solanum tuberosum*-FITC (green); (C) *Tetragonolobus purpureas*-CY5; and (D) the overlay image of all three channels showing the layers and differential lectin binding

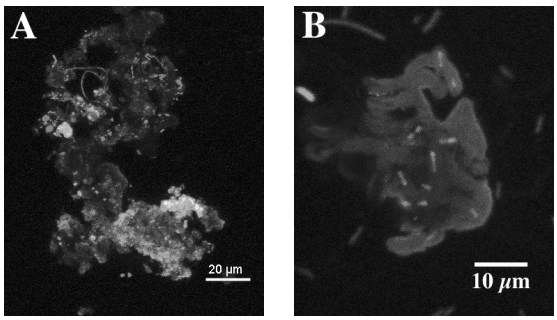

FIGURE 6.5 (**Color Figure 6.5 appears following page xx**) Images of the combined FLBA–FISH approach showing (A) staining of an Elbe River floc where the gene probe EUB-CY3 (red) and lectin *Canavalia ensiformis*–FITC (green) were applied; and (B) the binding of the lectin *Cicer arietinum*–Alexa-568 (red) and the lectin *Arachis hypogaea*–CY5 (blue) with localization of beta-proteobacterial cells (green) using the probe Bet42a

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image analyses of confocal image stacks, to quantitatively evaluate the binding of different lectins spatially and with time. Neu et al.[18] were able to detect clear statistically significant effects of nutrient treatments and time on the EPS composition of river biofilms using CLSM and FLBA. Figure 6.6 shows a typical data set with variation in lectin binding in response to the addition of nutrients during biofilm development. There were however, effects of the fluor, the matrix, and the lectin on the apparent specificity of lectin binding and limitations on the interpretation of the nature of binding site of the lectin. Recent comparative STXM–CLSM studies of biofilms demonstrated significant agreement between the probe target dependent

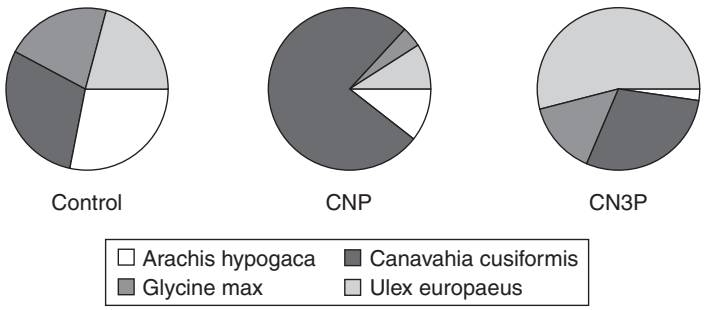

FIGURE 6.6 Sample data set illustrating the effect of nutrient additions on the EPS composition as determined by a panel of fluor-conjugated lectins. Note the increase in *Canavalia ensiformis* lectin binding in the carbon, nitrogen, phosphorus treatment versus the increase in *Ulex europeaus* lectin binding when 3x phosphorus is added to the river water during biofilm development

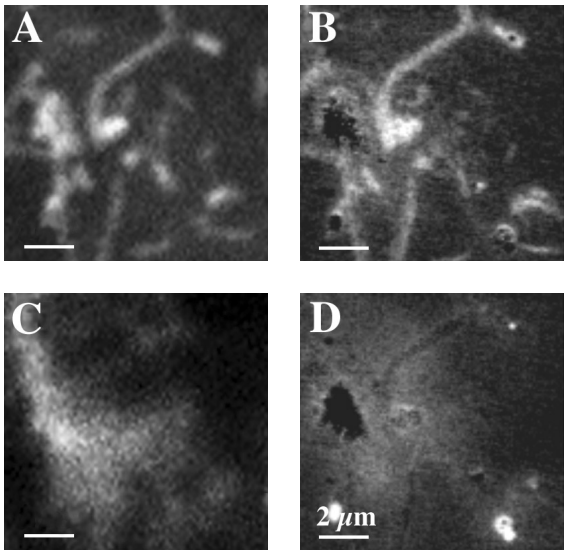

FIGURE 6.7 (A) CLSM image of mixed species river biofilm stained with nucleic acid sensitive stain Syto9; (B) STXM image of the same location showing the location of nucleic acids as detected by fitting models based on spectra in Figure 6.3; (C) localization of fucose containing polysaccharide using the fucose sensitive lectin *Tetragonolobus purpureas*; and (D) the same area imaged using STXM and fitting of general polysaccharide

identification of polysaccharide by CLSM and the probe independent detection based on soft x-ray spectroscopy. Lectin binding could be shown to identify subsets of the total polysaccharide regions detected using STXM (Figure 6.7). Significant questions remain however regarding the precise chemical interpretation of the binding of a specific lectin.

6.3.1.3 Antibodies

Antibodies have been suggested as potential probes for sugars and carbohydrates, however there are limitations to their application in complex microbial communities. For example, (i) the production of antibodies against carbohydrates is in general difficult relative to proteins, (ii) it requires the isolation of pure polysaccharide material from the complex polysaccharide matrix of a complex microbial biofilm community, and (iii) if the antibody could be produced its specificity would allow only the detection of a very limited fraction of the carbohydrates present in a complex biofilm community. Thus the application of antibodies presents significant technical and interpretative barriers for *in situ* characterization procedures.

6.3.2 Proteins–Lipids

Proteins are major constituents of the exopolymeric matrix of floc and biofilm systems. Particularly in activated sludge flocs, protein can be the most important contributor representing 50% or more of the extractable EPS.[14,54] Both extractive analysis and *in situ* detection of protein is complicated by the presence of lipoproteins and glycoproteins, molecules that have a chemistry representative of more than one class of biomolecule.

Neu and Marshall[55] applied a "protein specific" probe Hoechst 2495 to detect bacterial footprints on surfaces. In this case these remnant structures were readily detected with this dye, consistent with the presence of a high level of protein in the EPS. The SYPRO series of protein stains, although developed for protein in gels and solutions have been proposed for application *in situ*. Lawrence et al.[25] applied SYPRO orange alone and in combination with other macromolecular stains. These SYPRO stains bound extensively in the biofilm system, both in a cell associated and matrix distributed pattern. They found strong colocalization of protein, lipid, and polysaccharide. Parallel studies using STXM verified that colocalization was a valid interpretation and representative of conditions in the biofilm matrix. Again this may reflect the lipoprotein, glycoprotein distribution in the matrix polymer.

The hydrophobic lipid stain Nile Red has also been used extensively to detect lipids in algal and bacterial cells and associated materials. Wolfaardt et al.[56] reported using Nile Red to detect hydrophobic cell surfaces within a degradative biofilm community, while Lamont et al.[57] indicated that lipid deposits associated with *Frankia* could be localized.

6.3.3 Nucleic Acids

Nucleic acids are also abundant in biofilms both as cell associated DNA–RNA and within the extracellular matrix of the biofilm or floc. Although noncellular binding of nucleic acid stains has often been classified as nonspecific staining, it has become apparent that DNA may be a structural component of biofilms. Indeed extractive studies have often indicated that a considerable fraction of the biofilm EPS is DNA. Some reports reviewed in Nielsen and Jahn[14] indicate that nucleic acids may comprise 5 to 15% of the extracellular materials pure culture biofilms and activated sludge. Recently,

Whitchurch et al.[58] indicated using pure cultures that this extracellular DNA may be structural in nature and required for biofilm development. Similarly, Lawrence et al.[25] detected extracellular nucleic acids in biofilm materials using STXM. Correlation of fluorescent nucleic acid staining with the results of soft x-ray analyses indicated that both detected regions of non-cellular nucleic acids within a biofilm.

6.3.4 Charge/Hydrophobicity

The essential approach to *in situ* determination of surface charge involves the application of probes with known characteristics with assessment of their binding patterns in the floc matrix. The use of fluorescent beads with sulfated or carboxylated surface chemistry has been used for determination of hydrophobicity and hydrophilicity of bacterial cells and may be used for flocs. Zita and Hermannson[59] describe the essential method using beads obtained from Molecular Probes Inc. (Molecular Probes, Eugene OR). Fluorescent beads have also been used to analyze under *in situ* conditions the surface properties of filamentous bacteria in activated sludge flocs.[60] As noted above the binding of the hydrophobic dye Nile Red a lipophilic compound may also be interpreted as recognition of hydrophobic regions. Similarly, dextrans may be obtained with anionic, polyanionic, neutral, or positive charges, these may also be reacted with microbial EPS to assess charge and charge distribution. This approach has been applied by Wolfaardt et al.[56] Figure 6.8 are three-dimensional (3D) stereo pairs of the binding of 100 nm carboxylate and 20 nm sulfate modified beads at the same location in a river biofilm. The image shows differential binding based on hydrophobicity and penetration of the biofilm material based on hydrated radius of the probe.

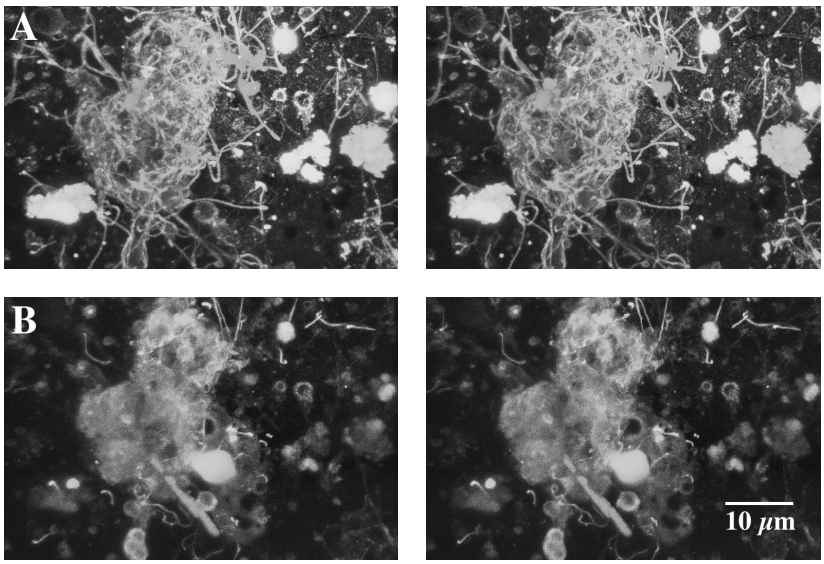

FIGURE 6.8 CLSM stereo pairs A and B showing the differential sorption of the 100 nm carboxylate modified beads and 20 nm sulfate modified beads in river biofilm

6.3.5 Permeability

There are a number of fluor conjugated probes that may be used to assess permeability and diffusion coefficients of bacterial cells and polymer, these include ficols, size fractionated dextrans, and a range of fluorescent beads (10 nm to 15 μm diameter) (see, e.g., Molecular Probes, Eugene, OR). Lawrence et al.[61] used 1P-LSM to monitor the migration of fluor conjugated dextrans to determine effective diffusion coefficients for biofilm systems. Microinjection and 1P-LSM has been developed by De Beer et al.[62] to determine diffusion coefficients in biofilm materials. The standard FRAP (fluorescence recovery after photobleaching) approach may also be applied to bacteria, aggregates, and biofilms. Figure 6.9 (see also Section 6.3.4) illustrates the penetration of biofilm microcolonies by 100 and 20 nm diameter fluorescent beads.

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6.4 EXAMINATION OF EPS BOUND AND ASSOCIATED MOLECULES

Due to its chemical heterogeneity the EPS of biofilms is an important site for the sorption of carbon, metals, contaminants, and enzymes.[12] A number of studies indicate that the EPS of biofilms functions to maintain long-term reserves of metabolizable carbon.[63,64] Wolfaardt et al.[65] demonstrated that autofluorescence could be used to localize the herbicide diclofop methyl in biofilm polymers and to follow its sorption and subsequent metabolism. Antibodies may be used to map the locations of specific enzymes, specific compounds such as pesticides, and a variety of other biologically relevant molecules. Specific enzyme activity associated with EPS may be localized using antibodies or fluorescent reporters of enzyme activity.[66] Figure 6.10 shows the results of incubation of biofilm material with ELF 97 (Molecular Probes, Eugene, OR), illustrating the binding of the lectin *Phaseolus vulgaris*–TRITC and the combination of the exopolymer image with ELF positive locations in the biofilm. This shows the presence of both cellular and EPS localized phosphatase activity. Lawrence et al.[67] localized the herbicide atrazine within river biofilms using antibody staining. As indicated in the publication of Wuertz et al.[68] ion or metal sensitive probes such as

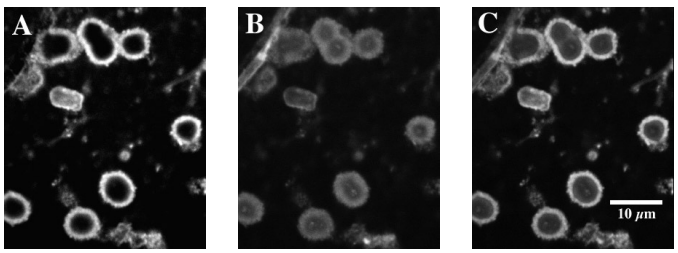

FIGURE 6.9 CLSM images showing the differential penetration of microcolonies with (A) binding and retention of 100 nm sulfate modified beads on the outside; (B) penetration of the 20 nm aldehyde–sulfate modified beads to the inside of the colony; and (C) the overlay of the two channels showing localization of the two probes

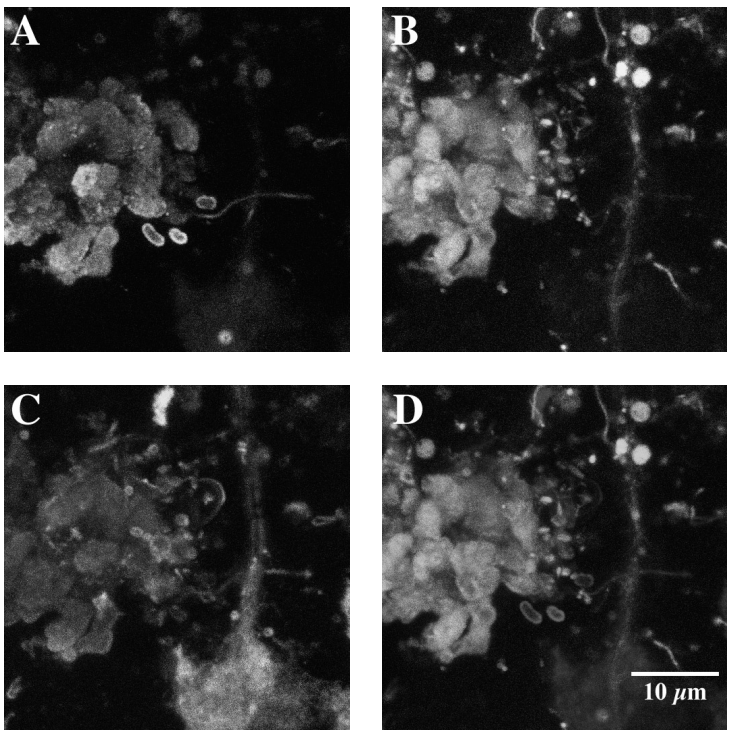

FIGURE 6.10 CLSM micrographs illustrating (A) the binding of *Phaseolus vulgaris*–TRITC lectin the development of ELF97 (Molecular Probes, Eugene, OR) phosphatase activity reporting fluorescence; (B) and (C) *Arachis hypogaea*-CY5 lectin binding pattern; and (D) the combination of the three signals shows the presence of both cellular and EPS localized phosphatase activity

Newport Green may be used to detect the presence of metals in biofilms and within the EPS matrix.

6.5 DIGITAL IMAGE ANALYSES

Digital images may be collected by a wide range of options, digital camera or digital video on wide field epifluorescence microscope, 1P-LSM, and 2P-LSM. Collection and analyses of synchrotron images is a specialized area not covered in detail in this chapter.[25,26] Once an image series or image stack is generated there are many options to analyse these images and depending on the specific requirements many commercial or freeware systems can be chosen. Key points to consider include: gray level resolution, programming language, capacity for modification, design of macros or plug-ins, and memory requirements, capacity to perform operations on serial image stacks produced in confocal microscopy, or to perform object-based image analysis. Software may be from the microscope company directly or special software companies. All the major companies constantly extend the features on

their microscope software. However, in general no software is suitable for every data set and can perform every type of analysis. Consequently, the data has to be treated with different software. The most sophisticated general packages are Imaris (Bitplane), Amira (TGS), and Volocity (Improvision). All of these packages have the usual options plus rendering capacity. Others available include, Quantimet System, Leica (Heidelburg, Germany), MicroVoxel (Indec Systems, Sunnyvale, California), VoxelView (Vital Images, Fairfield, Iowa), or VoxBlast (VayTek, Inc., Fairfield, Iowa), a complete listing of commercial image analyses software may be found most easily by an internet search. Alternately freeware may meet the requirements the following are widely used systems, Comstat, a program for UNIX workstations (see http://www.im.dtu.dk/comstat/), NIH Image for Apple platforms (http://rsb.info.nih.gov/nih-image/), or the Windows-based version (ScionImagePC at www.scioncorp.com), and the new Java version ImageJ from NIH or ImageTool (http://ddsdx.uthscsa.edu/dig/itdesc.htlm). There is also Linux based software produced by the French INRA, Nantes, called QUANT3D. An example of how macros developed in NIH Image were used to achieve quantitative measurements in FLBA is provided in Section 6.5.1.

6.5.1 Quantitative *In Situ* Lectin Analyses

Neu et al.[16] provided a detailed analysis of lectin binding in complex systems and proposed a standardized method for digital imaging, image analyses, and calculation of lectin binding. Image analysis was used to define the area of the biofilm binding a specific lectin. In addition, the average gray value of the defined area was determined. These two parameters were used to quantify the area binding a specific lectin according to Equation (6.1).

$$\%\text{ICBA} = \frac{\text{TA} \times \text{AGV} \times 100}{255 \times 393216} \tag{6.1}$$

% ICBA is the Intensity Corrected Binding Area, TA is the Thresholded Area of lectin binding, AGV is the Average Gray Value within thresholded area, 255 the gray value of saturated pixels, and 393216 is the number of pixels in a full image (768×512).

They noted the importance of incubation time, lectin concentration, the nature of the fluor labeling, presence of carbohydrate inhibition, order of addition, and lectin interactions. An incubation time of 20 min was found to be sufficient; tests indicated that fluorescein isothiocyanate (FITC) conjugated lectins had more specific binding characteristics than tetramethyl rhodamine isothiocyanate (TRITC) or cyanine dye (CY5) labeled lectins. They concluded that the selection of a panel of lectins for investigating the EPS matrix required a full evaluation of their behavior in the microbial system to be studied. Neu et al.[16] used macros developed in NIH Image and the above equation to analyze 1PLSM image stacks and determine the quantitative abundance of specific lectin binding sites in river biofilms. Neu et al.[18] has applied this approach to examine the impact of nutrients on the glycoconjugate make up of the EPS of river biofilms an approach that may be easily applied to flocs.

6.6 DECONVOLUTION

Deconvolution may be required to remove out-of-focus information from the images in the stack. Essentially, deconvolution is an algorithm for calculation that places extended signals in the *z* direction to their correct *xy–xz* location in the image stack. A variety of programs are available that allow the user to carryout this mathematical process including, AutoDeblur (AutoQuant), HazeBuster/Microtome (Vaytek), Huygens (SVI), Amira DECONV (TGS), TILLvisION deconvolution (TILL Photonics), 3-d deconvolution (Zeiss). The 3D rendering may be carried out using ray tracing or surface contour based programs such as Huygens (SVI). An example of application of deconvolution to images of bacterial and biofilm associated with sponges is presented with color illustration in Manz et al.[69]

6.7 3D RENDERING

The use of visualization techniques such as confocal, 2PLSM, or synchrotron based imaging necessitates the presentation of the data in the most meaningful form. In

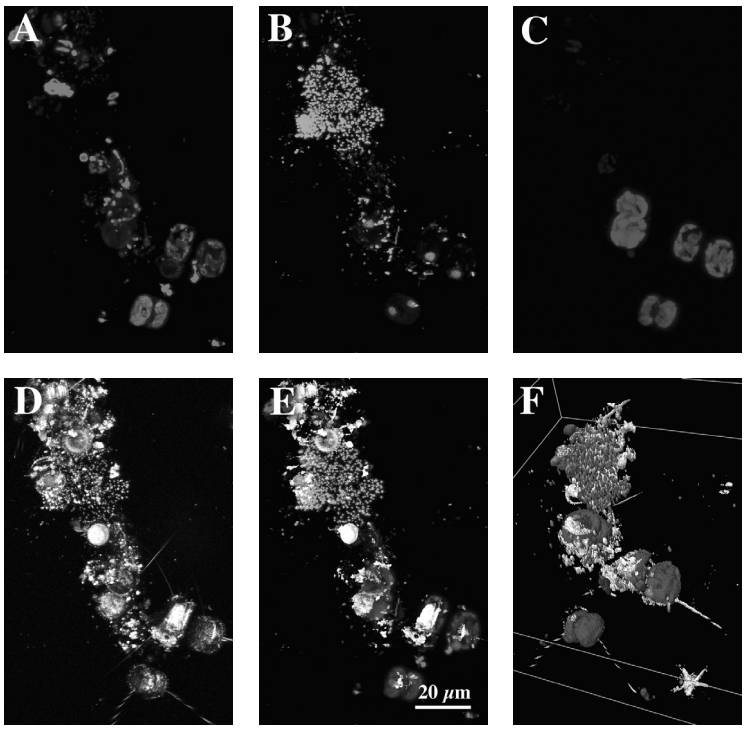

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FIGURE 6.11 (**Color Figure 6.11 appears following page xx**) 3D rendering of Elbe river floc showing the individual channels (A) staining with the lectin *Triticum vulgaris*–TRITC; (B) nucleic acid staining with SYTO9; (C) an autofluorescence signal for algae; (D) reflectance of particulate colloidal materials; (E) merged 3D image; and (F) oblique view of the 3D rendering of the four image stacks (red — lectin *Triticum vulgaris*-TRITC, green — syto9, blue — autofluorescence, white — reflection, all colors — overlay, 3D — isosurface/rendering)

general, this has meant the use of a range of color and three-dimensional presentation formats including: simulated fluorescence, stereo pairs, red–green anaglyph projections, and two or three color stereo pairs. These approaches allowing the presentation of multichannel information sets providing a synthesis of large data sets for the readers examination. Additional approaches involve the application of 3D rendering of these data sets. Figure 6.11 provides an example of a series of images of an Elbe river floc showing: four images, the reflection image of particulate matter in the floc, autofluorescence showing the presence of autotrophic algae and cyanobacteria, staining with the nucleic acid stain Syto 9 showing all bacteria, and staining with a lectin *Triticum vulgaris*–TRITC revealing the exopolymer matrix of the floc as single channel maximum intensity projection (MIP) and then combined as MIP and a rendering image all of the same floc. Finally, images are often adjusted in terms of color balance for publication using programs such as Photoshop (Adobe Systems Inc., San Jose, California)

6.8 CONCLUSIONS

What is needed to further fine tune the *in situ* analysis of exopolymeric substances of microbial communities? Primarily, we require a more detailed characterization of the probes and a greater understanding of the nature of their affinities for targets such as protein, lipid, nucleic acids, and carbohydrates. Further studies of their behavior in complex microbial communities must also be carried out in order to establish the "ground truth" for imaging based studies. This has been carried out in part through the correlative application of STXM and 1P-LSM imaging[25] which indicated considerable agreement between the probe free and the probe dependent mapping of major biopolymers in biofilm systems. However, new probes specific for major biopolymers will have to continue to be developed and evaluated in complex systems. These highly specific probes will be critical for the assessment of floc properties as well as for structural examination of these microbial communities.

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