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Research review paper

# Stereological assessment of extracellular polymeric substances, exo-enzymes, and specific bacterial strains in bioaggregates using fluorescence experiments

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ABSTRACT

This review addresses the introduction of fluorescent molecular tags into exo-enzymes and extra polymeric substances of bioaggregates and the use of confocal laser scanning microscopy (CLSM) to map their role, purpose and quantitative description of the biological processes they undertake. Multiple color staining coupled with CLSM and fluorescent *in situ* hybridisation (FISH) and flow cytometry have identified the individual polymeric substances, whether they are proteins, lipids, polysaccharides, nucleic acids or antibodies, as well as the microorganisms in the bioaggregate. Procedures are presented for simultaneous multicolor staining with seven different fluorochromes — SYTOX Blue for nucleic acids; Nile red for lipids; Calcofluor white [CW] for β-polysaccharides; concanavalin A [Con A] for α-poly-saccharides; fluorescein-isothiocyanate [FITC] for proteins; SYTO 63 for live microbial cells and Calcium Green for monitoring calcium levels in the microbial cells. For the distribution of certain microbial strains, metabolic enzymes and extrapolymeric substances to be quantitatively described the generated colored images are converted into digital forms under specific predefined criteria. Procedures and computer software programs (Amira; MATLAB) are presented in order to quantitatively establish grid patterns from the CLSM images. The image is digitized using a threshholding algorithm followed by a reconstruction of the image as a volumetric grid for finite element simulation. The original color image is first converted to a grey followed by resizing, detection and modification of bilevel images and finally a total reversal of the image colors. The grid file is then used by specific computer software (Gambit, Fluent) for further numerical studies incorporating chemical reactions, transport processes and computational fluid dynamics including intra-bioaggregate fluid flow, and heat and mass transfer within the bioaggregate matrix.



**Contents**



*Abbreviations:* BODIPY, bovine serum albumin conjugates of 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diazas-indacen-3; CARD, catalyzed reporter deposition; CFD, computational fluid dynamics; CLSM, confocal laser scanning microscope; COD, chemical oxygen demand; Con A, concanavalin A; CW, calcofluor white; DAPI, 4′,6-diamidino-2-phenylindole; DNA, deoxyribonucleic acid; EPS, extracellular polymeric substance; FISH, fluorescence *in situ* hybridation; FITC, fluorescein-isothiocyanate; IgG, immunoglobulin G; MAR, micro-autoradiography; NTSC, National Television System Committee; PHOs, protein-hydrolysing organisms; SCOD, soluble chemical oxygen demand; SEM, scanning electron microscope; STAR, substrate tracking autoradiography; TAMRA, tetramethyl-6-carboxyrhodamine; TEM, transmission electron microscope.

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## 1. Introduction

Bioaggregates such as activated sludge flocs, aerobic and anaerobic biofilms and marine snow are made up of numerous microorganisms, immobilized in EPS and/or matrices constituting polymers of proteins, polysaccharides, humic acids, and lipids (Nielsen et al., 1992). Over 99% of the bacteria are present in biofilms (Dalton and March, 1998) or granules that protects the incorporated bacteria from antibiotics (Goldberg, 2002), disinfectants (Peng et al., 2002), or threat by environmental shock (Chen et al., 1998). The intercellular communication within biofilms and granules regulates gene expression that enables temporal adaptation to phenotypic variation (Kjelleberg and Molin, 2002) and enhances survival rate in a nutrient deficient condition (Koch et al., 2001). The enzymes secreted by constituent cells accelerate biological reactions for functions of maintenance and reproduction. To quantitatively describe the biological processes that are involved within the bioaggregates acquires spatial distribution of functional bacterial strains, anticipated enzymes, and substrates and metabolic products inside the bioaggregates. For instance, Kloeke and Geesey (1999) determined the locations of phosphatase as well as phosphatase-excreting microorganisms in activated sludge using precipitated fluorescent crystals from the reaction between the enzyme and the ELF® 97 palmitate, using the FISH technique. With this combination of analytical tools, these authors used phosphatase activity to quantitatively describe the biological processes that occurred in the system.

Images of very high resolution could be obtained using SEM or TEM technique (Gerhardt et al., 1994; Erlandsen et al., 2004). However, these technologies were not able to differentiate the functional constituents of the bioaggregates such as protein, lipids, specific bacterial strains and enzymes. The basic concept of confocal microscopy was originally developed by Marvin Minsky in the mid-1950s (patented in 1961) (Amos and White, 2003). The use of CLSM has gained popularity since it can (1) control depth of field of scanning; (2) eliminate background information away from the focal plane; (3) collect serial optical sections from thick specimens; (4) eliminate out-of-focus light; (5) provide quality scanned images; and (6) in particular, provide fluorescent scanning of substances bound with different fluorochromes that would be excited using light of different wavelengths. The multiple color staining technique and CLSM together visualize the distribution of components of EPS in biological aggregates (Bockelmann et al., 2002; Strathmann et al., 2002; Lawrence et al., 2004; Boessmann et al., 2004; Staudt et al., 2004; Neu et al., 2004; Lawrence et al., 2005; McSwain et al., 2005; Wang et al., 2005, Chen et al., 2007a,b, Adav et al., 2007a,c,d; Adav and Lee, 2008). FISH coupled with CLSM were able to locate specific bacterial strain(s) in bioaggregates that were under investigation (Lemaire et al., 2008). Furthermore the immunohistochemical staining technique and CLSM were able to locate the specific enzymes in a sludge floc or a biological granule (Whiteley and Lee, 2006; Lee et al., 2009a).

To have a comprehensive three-dimensional (3D) view of the "functional map" of the bioaggregate under investigation was of great academic and practical interest. To achieve this goal, however, an integrated protocol with the above-mentioned techniques should be available to simultaneously allocate EPS, functional cells and enzymes on the same target. FITC labeled lectins have been used to stain the glycoconjugate fraction of biofilm (Lawrence et al., 1998; Michael and Smith, 1995; Neu 2000; Cerca et al., 2005) or of flocs and granules (De Beer et al., 1996; Wang et al., 2005). Schmid et al. (2003) applied FITC to stain the protein fraction in their sludge floc. Two fluorochromes were used to probe the EPS in single biofilms specimen (Strathmann et al., 2002; Boessmann et al., 2003, 2004; Staudt et al., 2004). Furthermore, three fluorochromes were applied to locate the EPS components in biofilms (Neu and Lawrence, 1997; Neu et al., 2001, 2004; Lawrence et al., 2003, 2004, 2005); aerobic granules (McSwain et al., 2005). Bockelmann et al. (2002) combined the FISH technique with a lectin fluorochrome in their double labeled experiments on river snow to provide fantastic, 3D images for visual observations. To quantitatively describe the spatial correlations among those probed substances, such as the distribution of distance of certain strain and of specific enzyme, the colored images have to be converted to digital forms with criteria set by the acquired analysis. The 3D grid model for the studied aggregate could thereby be established with locations of functional components realized for analyzing its steady-state behavior and transient response under changes in environmental conditions (Chu and Lee, 2004; Chu et al., 2005).

Chen et al. (2007b) proposed a six-fold labeling scheme for staining total cells, dead cells, proteins, lipids and α- and β-polysaccharides in bioaggregates. For instance, a merged image for a phenol-fed aerobic granule, scanned at 680 μm from the granule surface, is shown in Fig. 1.