An abstract of the dissertation of Gabriel C. Iltis for the degree of Doctor of Philosophy in Environmental Engineering presented on September 12, 2013.

Title: Visualization and Characterization of Biofilm Spatial Distribution in Porous Media using X-ray Computed Microtomography.

Abstract approved: _____________________________________________________

Dorthe Wildenschild       Brian D. Wood

This work focuses primarily the development of methods for imaging microbial biofilms in opaque porous media using x-ray computed microtomography (CT). Two methods for evaluating biofilms in porous media are presented. The first focuses on the addition of silver-coated, hollow glass microspheres to a biofilm-containing micro-model. The silver-coated microspheres affix to the surface of biofilm present in the hydraulically available pore space providing a surface coating at the biofilm-aqueous phase interface which is detectable using synchrotron-based CT scanning. Through image processing, the silver microspheres were able to be isolated and a triangulated mesh representing the biofilm surface was able to be reconstructed and quantified. The second method focuses on the addition of a pore-filling barium sulfate contrast agent to biofilm column growth reactors. Methods for analyzing and quantifying data sets collected using both polychromatic and monochromatic (synchrotron-based) CT are presented. Finally, the barium sulfate method for imaging biofilm using synchrotron-based x-ray CT is applied to a biofilm growth experiment evaluating growth of Shewanella oneidensis under hydraulic loading rates corresponding to Reynolds numbers of 0.1, 1.0 and 10. Results from this study show good agreement between laboratory measured changes in hydraulic
conductivity and hydraulic conductivity estimates generated using the segmented CT data sets and the Kozeny-Carman model for estimating hydraulic conductivity using porosity measures.
Visualization and Characterization of Biofilm Spatial Distribution in Porous Media using X-ray Computed Microtomography.

by

Gabriel C. Iltis

A DISSERTATION

Submitted to

Oregon State University

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Presented September 12, 2013
Commencement June 2014
Doctor of Philosophy dissertation of Gabriel C. Iltis presented on September 12, 2013.

APPROVED:

_______________________________________
Co-Major Professor, representing Environmental Engineering

_______________________________________
Co-Major Professor, representing Environmental Engineering

_______________________________________
Head of the School of Chemical, Biological, and Environmental Engineering

_______________________________________
Dean of the Graduate School

I understand that my dissertation will become a part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

_______________________________________
Gabriel C. Iltis
ACKNOWLEDGEMENTS

The author expresses sincere appreciation to his parents, Robert and Snick Iltis, for their unwavering support and encouragement during this process. To Ryan Armstrong, Mark Porter, and Kristin Sample for helping me to stay sane, at least for a while. To Mark Dolan, for always encouraging me to explore new topics. To Monte Christie and Marc Orman for enabling my return to OSU. And finally, to Dorthe Wildenschild for her support, encouragement and direction over these last four years.
CONTRIBUTION OF AUTHORS

Dr. Ryan Armstrong, and Dr. Danielle Jansik conducted the experiments and contributed the raw CT data for Chapter 2. Dr. Yohan Davit provided the polychromatic CT data that is analyzed and discussed in Chapter 3. He also provided technical assistance and aided in conducting some of the experimental work addressed in Chapter 4. James Connolly assisted with experiments addressed in Chapter 4 as well. James also provided the SEM image of the barium sulfate contrast agent. Dr. Robin Gerlach provided material and technical assistance for experiments addressed in Chapter 4. Dr. Brian Wood provided technical assistance for experiments addressed in Chapter 1. He also provided input for the design of experiments detailed in Chapters 4 and 5.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 General Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2 Imaging biofilm architecture within porous media using synchrotron based x-ray computed microtomography</td>
<td>11</td>
</tr>
<tr>
<td>2.1 Abstract</td>
<td>11</td>
</tr>
<tr>
<td>2.2 Introduction</td>
<td>11</td>
</tr>
<tr>
<td>2.3 Methods</td>
<td>14</td>
</tr>
<tr>
<td>2.4 Results</td>
<td>17</td>
</tr>
<tr>
<td>2.5 Discussion</td>
<td>18</td>
</tr>
<tr>
<td>2.6 Acknowledgements</td>
<td>21</td>
</tr>
<tr>
<td>2.7 Bibliography</td>
<td>22</td>
</tr>
<tr>
<td>3 Quantitative analysis of three dimensional biofilm architecture in porous media using a barium sulfate contrast agent and polychromatic x-ray computed microtomography</td>
<td>31</td>
</tr>
<tr>
<td>3.1 Abstract</td>
<td>31</td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>32</td>
</tr>
<tr>
<td>3.3 Methods and materials:</td>
<td>36</td>
</tr>
<tr>
<td>3.3.1 Growth reactor design:</td>
<td>37</td>
</tr>
<tr>
<td>3.3.2 Bacterial species and growth conditions:</td>
<td>37</td>
</tr>
<tr>
<td>3.3.3 Contrast agent addition:</td>
<td>38</td>
</tr>
<tr>
<td>3.3.4 Three-dimensional imaging:</td>
<td>39</td>
</tr>
<tr>
<td>3.3.5 Image segmentation:</td>
<td>39</td>
</tr>
<tr>
<td>3.4 Results and discussion:</td>
<td>44</td>
</tr>
<tr>
<td>3.4.1 Demonstration column, 100% BaSO₄ suspension, no iodide</td>
<td>44</td>
</tr>
<tr>
<td>3.4.2 Porous media packing, 50% BaSO₄, with iodide, low and high Re</td>
<td>46</td>
</tr>
<tr>
<td>3.5 Conclusions</td>
<td>52</td>
</tr>
<tr>
<td>3.6 Bibliography</td>
<td>56</td>
</tr>
<tr>
<td>4 On the detailed analysis of three dimensional biofilm architecture in porous media using synchrotron based x-ray computed microtomography</td>
<td>67</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS (Continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Abstract</td>
<td>67</td>
</tr>
<tr>
<td>4.2 Introduction</td>
<td>68</td>
</tr>
<tr>
<td>4.3 Experimental methods</td>
<td>76</td>
</tr>
<tr>
<td>4.3.1 Growth reactor design:</td>
<td>76</td>
</tr>
<tr>
<td>4.3.2 Microbial species:</td>
<td>76</td>
</tr>
<tr>
<td>4.3.3 Contrast agent:</td>
<td>77</td>
</tr>
<tr>
<td>4.3.4 X-ray computed microtomography</td>
<td>78</td>
</tr>
<tr>
<td>4.3.5 Image processing</td>
<td>79</td>
</tr>
<tr>
<td>4.4 Barium sulfate concentration - sensitivity analysis results</td>
<td>80</td>
</tr>
<tr>
<td>4.5 Grayscale filtering</td>
<td>85</td>
</tr>
<tr>
<td>4.5.1 Grayscale filtering Results</td>
<td>87</td>
</tr>
<tr>
<td>4.6 Thresholding</td>
<td>88</td>
</tr>
<tr>
<td>4.6.1 Thresholding sensitivity analysis</td>
<td>96</td>
</tr>
<tr>
<td>4.6.2 Final segmentation</td>
<td>99</td>
</tr>
<tr>
<td>4.7 Quantitative analysis and Recommendations:</td>
<td>101</td>
</tr>
<tr>
<td>4.8 Conclusions</td>
<td>105</td>
</tr>
<tr>
<td>4.9 Acknowledgements</td>
<td>107</td>
</tr>
<tr>
<td>4.10 Bibliography</td>
<td>108</td>
</tr>
<tr>
<td>5 Resolving the influence of Flow Rate on Biofilm growth in Three Dimensions using microimaging</td>
<td>123</td>
</tr>
<tr>
<td>5.1 Abstract</td>
<td>123</td>
</tr>
<tr>
<td>5.2 Introduction</td>
<td>124</td>
</tr>
<tr>
<td>5.3 Experimental Methods</td>
<td>127</td>
</tr>
<tr>
<td>5.3.1 Packed bead column reactors</td>
<td>127</td>
</tr>
<tr>
<td>5.3.2 Microbial species</td>
<td>128</td>
</tr>
<tr>
<td>5.3.3 Experimental growth conditions</td>
<td>128</td>
</tr>
<tr>
<td>5.3.4 Differential Pressure measurement</td>
<td>130</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3.5 DO measurement</td>
<td>130</td>
</tr>
<tr>
<td>5.3.6 CT imaging</td>
<td>131</td>
</tr>
<tr>
<td>5.3.7 Image Processing and Quantitative Analysis</td>
<td>132</td>
</tr>
<tr>
<td>5.4 Results</td>
<td>134</td>
</tr>
<tr>
<td>5.4.1 Laboratory investigation</td>
<td>134</td>
</tr>
<tr>
<td>5.4.2 Quantitative Image Analysis</td>
<td>136</td>
</tr>
<tr>
<td>5.5 Conclusions:</td>
<td>143</td>
</tr>
<tr>
<td>5.6 Bibliography</td>
<td>145</td>
</tr>
<tr>
<td>6 bibliography</td>
<td>161</td>
</tr>
<tr>
<td>7 Appendix 1</td>
<td>169</td>
</tr>
<tr>
<td>7.1 Summary</td>
<td>172</td>
</tr>
<tr>
<td>7.2 Introduction</td>
<td>173</td>
</tr>
<tr>
<td>7.3 Material and methods</td>
<td>179</td>
</tr>
<tr>
<td>7.3.1 The porous models</td>
<td>179</td>
</tr>
<tr>
<td>7.3.2 Growing biofilms</td>
<td>180</td>
</tr>
<tr>
<td>7.3.3 Contrast agent</td>
<td>180</td>
</tr>
<tr>
<td>7.3.4 Imaging protocols</td>
<td>182</td>
</tr>
<tr>
<td>7.3.5 Data analysis</td>
<td>184</td>
</tr>
<tr>
<td>7.4 Results</td>
<td>185</td>
</tr>
<tr>
<td>7.4.1 Two-dimensional experiments</td>
<td>185</td>
</tr>
<tr>
<td>7.4.2 Results of the 3-D tomography and discussion</td>
<td>189</td>
</tr>
<tr>
<td>7.5 Conclusions:</td>
<td>192</td>
</tr>
<tr>
<td>7.6 References</td>
<td>Error! Bookmark not defined.</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2-1: Preliminary three-dimensional CT biofilm imaging results of biofilm (green) grown in a glass bead pack (gold).</td>
<td>24</td>
</tr>
<tr>
<td>Figure 2-2: Comparison of light microscopy images to representative CT images for two unique biofilm features at the grayscale, binary and PointWrap stages of image processing.</td>
<td>25</td>
</tr>
<tr>
<td>Figure 2-3: Supporting material Figure SM-1. Micro-model flow cell detail.</td>
<td>26</td>
</tr>
<tr>
<td>Figure 2-4: Supporting material Figure SM-2. Volume renderings of biofilm growth (light blue mesh) at two unique locations within the micro-model flow cell captured using synchrotron based CT.</td>
<td>27</td>
</tr>
<tr>
<td>Figure 2-5: Supporting material Figure SM-3. Light microscopy image detailing the distribution and adherence of silver-coated hollow glass micro-spheres to the biofilm surface.</td>
<td>28</td>
</tr>
<tr>
<td>Figure 2-6: Supporting material Movie 1-1. Three-dimensional rendering of the preliminary investigation results.</td>
<td>29</td>
</tr>
<tr>
<td>Figure 3-1: Scanning electron microscopy (SEM) image of the barium sulfate particles contained within the barium sulfate contrast agent suspension.</td>
<td>58</td>
</tr>
<tr>
<td>Figure 3-2: (a) Histogram plot for the barium sulfate demonstration column illustrating the various peaks used for thresholding segmentation.</td>
<td>59</td>
</tr>
<tr>
<td>Figure 3-3: Surface Reconstructions of the 100% BaSO4 demonstration column.</td>
<td>60</td>
</tr>
<tr>
<td>Figure 3-4: Volume histograms and representative cross-sectional gray-scale slice images for (a) the low Re and (b) high Re biofilm growth reactors.</td>
<td>61</td>
</tr>
<tr>
<td>Figure 3-5: (a) Original gray-scale cross-section of the high Re biofilm growth reactor.</td>
<td>62</td>
</tr>
<tr>
<td>Figure 3-6: Surface reconstructions of the plastic bead solid matrix (yellow), biofilm (red), and plastic beads overlain by biofilm for (a) the low Re volume and (b) the high Re volume.</td>
<td>63</td>
</tr>
<tr>
<td>Figure 3-7: Graphical representations of the axial spatial distribution of (a) biofilm volume, and calculated porosity for (b) the low Re column, and (c) the high Re column.</td>
<td>64</td>
</tr>
<tr>
<td>Figure 4-1: Histogram comparison of control column sections containing various concentrations of the barium sulfate contrast agent.</td>
<td>112</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 4-2: Volume subtraction concentration comparison for control columns and columns containing biofilm for barium sulfate suspension dilutions ranging from 25% to 50% stock solution.</td>
<td>113</td>
</tr>
<tr>
<td>Figure 4-3: Histogram comparison of three filtering methods applied to a biofilm-containing column section.</td>
<td>114</td>
</tr>
<tr>
<td>Figure 4-4: Visual comparison of original grayscale image to filtering results using a Median Filter (center column) and a Non-Local Means filter (right column).</td>
<td>115</td>
</tr>
<tr>
<td>Figure 4-5: Histogram-based sensitivity analysis for the 50% barium sulfate (a) control column subtraction volume, (b) the control column Below Edge volume, (c) the biofilm subtraction volume and (d) the biofilm Below Edge volume.</td>
<td>116</td>
</tr>
<tr>
<td>Figure 4-6: Histogram-based sensitivity analysis for the 33% barium sulfate (a) control column subtraction volume, (b) the control column Below Edge volume, (c) the biofilm subtraction volume and (d) the biofilm Below Edge volume.</td>
<td>117</td>
</tr>
<tr>
<td>Figure 4-7: Subtraction volume sensitivity analysis quantified results.</td>
<td>118</td>
</tr>
<tr>
<td>Figure 4-8: Below Edge volume sensitivity analysis quantified results.</td>
<td>119</td>
</tr>
<tr>
<td>Figure 4-9: Flowchart depiction of the final segmentation for phase quantification.</td>
<td>120</td>
</tr>
<tr>
<td>Figure 4-10: Illustrative comparison of (a) biofilm grown within the sample column to (b) the final CT data segmentation and surface reconstructions.</td>
<td>121</td>
</tr>
<tr>
<td>Figure 4-11: Summary of quantified volumes measured from surface reconstructions of the final segmented data sets.</td>
<td>122</td>
</tr>
<tr>
<td>Figure 5-1: Continuous flow experimental column setup schematic.</td>
<td>147</td>
</tr>
<tr>
<td>Figure 5-2: Comparison of grayscale cross-sections to final segmented results generated using the factorization segmentation method vs. the above edge peak subdivision method.</td>
<td>148</td>
</tr>
<tr>
<td>Figure 5-3: Summary of the average temporal differential pressure record according to initial column Reynolds number (and associated flow rate).</td>
<td>149</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 5-4: Summary comparison of initial and final hydraulic conductivity for each experimental column.</td>
<td>150</td>
</tr>
<tr>
<td>Figure 5-5: Temporal experimental dissolved oxygen record.</td>
<td>151</td>
</tr>
<tr>
<td>Figure 5-6: Summary comparison of spatial variation in column porosity in the axial direction as calculated using results from both segmentation methods.</td>
<td>152</td>
</tr>
<tr>
<td>Figure 5-7: Direct comparison of calculated spatial changes in porosity calculated using the factorization segmentation routine as opposed to the alternate, peak subdivision, segmentation routine.</td>
<td>153</td>
</tr>
<tr>
<td>Figure 5-8: Hydraulic conductivity (K) values estimated using the Kozeny-Carman equation, and direct comparison to K to experimental K values calculated using the terminal differential pressure measurements from the biofilm growth experiment.</td>
<td>154</td>
</tr>
<tr>
<td>Figure 5-9: Hydraulic conductivity sensitivity analysis comparing Kozeny-Carman estimates calculated using various integration heights.</td>
<td>155</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2-1: Quantitative comparison of the light microscopy and representative two-dimensional CT images for two unique biofilm features using the Avizo® PointWrap algorithm.</td>
<td>30</td>
</tr>
<tr>
<td>Table 3-1: Summary of column characteristics and relevant parameters for quantitative image analysis.</td>
<td>65</td>
</tr>
<tr>
<td>Table 3-2: Quantified results for biofilm segmentation using barium sulfate as a contrast agent.</td>
<td>65</td>
</tr>
<tr>
<td>Table 3-3: Quantitative summary of results for all three segmented volumes.</td>
<td>66</td>
</tr>
</tbody>
</table>
Visualization and Characterization of Biofilm Spatial Distribution in Porous Media using X-ray Computed Microtomography.
1 GENERAL INTRODUCTION

Microbes, including bacteria and fungi are ubiquitous in nature. According to some estimates, more than 1 billion microbes exist per gram of soil (Whitman et al. 1998). Bacteria, specifically, are common to everything from skin to acid heap leach mining pads. Often, bacteria fare poorly as single, isolated organisms; instead they tend to exist in multicellular communities, termed biofilms, where an individual bacterium exists and functions in tandem with other unicellular organisms, which can be of the same species or not. The cells are interconnected by an extracellular polymeric substance (EPS) layer (Costerton et al., 1995). The presence of biofilms can be beneficial, negative or neither, often depending on the environment in which they are found. In nature, biofilms play a vital role in global-scale biogeochemical processes such as nutrient cycling (e.g. nitrogen fixation and metal reduction (Lovley, 1993)). In engineered systems, biofilms are involved in a variety of processes from wastewater treatment to bio-processing (Liu and Tay, 2004). In porous media applications, biofilms have been utilized in a variety of ways. Bioremediation, the use of biological agents to treat soil or groundwater contamination, has been studied extensively for applications such as industrial chemical spill remediation or the treatment of contamination by heavy metals such as uranium (Bielefeldt et al., 2004; Marsili et al., 2007, Li et al., 2009). Biomediated mineral precipitation has been used to help stabilize weak soil structures for geotechnical applications (Mitchell and Santamarina, 1995). Biofilms are even utilized in heap leach gold mining in order to increase production of winnable metals (Rawlings and Johnson, 2007).
The growth of biofilm in porous media results in filling of the pore space, and has come to be termed bioclogging. This attribute of biofilms in soil is of prime importance as it can directly impact soil structural or mechanical properties including fluid transport through porous materials. Due to the prevalence of biofilms in both natural and engineered porous media systems, a significant effort has been undertaken to characterize biofilms experimentally in systems including fabricated two-dimensional, or pseudo-three dimensional micro-models and porous media packed columns. Characterization of biofilms in porous media, and of the impact that biofilms have on porous media, relies primarily on bulk parameter measurements such as mass balances, quantitative cell counts, and differential pressure. Mass balances can be used to evaluate biofilm growth rates and pollutant removal or conversion kinetics (Rodriguez and Bishop, 2008; Li et al., 2009). Cell counts can provide information on the quantity of bacteria cells exiting the growth reactor over the course of an experiment, or present in the matrix affixed biofilm at the end of an experiment for inclusion in a calculated mass balance (Marsili et al., 2007). The measurement of differential pressure across a porous media reactor enables the calculation of changes in hydraulic conductivity (K) due to bioclogging; a measure indicative of how biofilm growth is altering pore size and pore morphology, which directly affect fluid flow and transport. Bielefeldt et al. (2004) studied bioclogging in saturated sand columns and reported decreases in K of 1.5 to 3 orders of magnitude associated with bioclogging, as calculated using differential pressure measurements. This decrease in K compares well with numerous other studies, and the general conclusion has been that bioclogging can reduce measured K values by 2 to 4 orders of magnitude
(Cunningham et al., 1991; Vandevivere et al., 1992a, 1992b; Hand et al., 2008; Thullner, 2010). One thing that Bielefeldt makes sure to point out is that the reported changes in $K$ are bulk average parameters for an entire column section between sampling ports, and may not actually be the result of a uniformly distributed reduction in porosity. Furthermore, they acknowledge that the change in $K$ is not necessarily solely associated with biofilm or accumulated biomass, and that byproducts of biofilm growth, such as biogenic gas, can play a role in $K$ reduction without being readily apparent to the experimenter. This illustrates one of the main limitations associated with the study of biofilms and bioclogging in porous media using packed column reactors. Resolution of this limitation has focused on the coupling of bulk parameter measurement with direct visualization and imaging of biofilms in porous reactors. The coupling of quantitative imaging and visualization to bulk parameter measurement is required in order to assess how, or why, biofilm growth in the porous medium is effecting change in the measured bulk parameters. Direct visualization allows for detailed evaluation of changes to pore morphology resulting from biofilm growth as well as potential variation in the spatial distribution of morphological changes. Generally, the objective of most porous media-associated biofilm growth studies is to link (a) how changes in environmental or genetic conditions affect (b) biofilm growth and pore morphology, resulting in (c) the measured changes in a bulk parameters. The relationship between these links (a $\rightarrow$ b $\rightarrow$ c), once determined, can then, be incorporated into a characteristic model for application to other biofilm growth, or bioclogging scenarios. Experimental investigations which couple
visualization with bulk parameter measurement end up being restricted by the limitations of the selected imaging technique.

Conventional two-dimensional (2D or pseudo-3D) techniques for imaging biofilm include

- Light microscopy, which captures 2D results, including periodic temporal results, for thin, optically transparent, micro-model growth reactors. Light microscopy has been used to evaluate temporal changes in biofilm spatial distribution internal to micro-models (e.g. Paulsen et al., 1997; Yang et al., 2000; Sharp et al., 2005).
- Environmental Scanning Electron Microscopy (ESEM) (e.g. Davis et al., 2009), and Scanning Electron Microscopy (SEM) (e.g. Vivandevivere et al., 1992a; 1992b; Mitchell et al., 2009) can be used to image a wide variety of systems at micron or sub-micron resolutions, though samples must be extracted and prepared for imaging, thereby disrupting the pore scale structure.
- Confocal Laser Scanning Microscopy (CLSM) (e.g. Heydorn et al., 2000; Leis et al., 2005; Rodriguez and Bishop, 2007; 2008) requires thin, plate-like, optically transparent reactors and can provide high resolution 2D imaging results, including temporal series as the output. An added benefit to using CLSM is that multiple 2D images can be captured at fixed increments in the z-direction thereby enabling 3D reconstruction of biofilm structures. However, the focal distance for these 3D reconstructions is only around 250 \( \mu \text{m} \). CLSM has become one of the most frequently used techniques for biofilm characterization because of the wide variety of selective dyes or stains available for this type of imaging which has broadened the capabilities of this technique.

Thus the use of conventional imaging techniques for studying biofilm in porous media requires that experimental growth reactors be (a) optically transparent and limited in size in at least one dimension, or (b) that imaging specimens be collected via destructive sampling of reactors. These limitations, along with the simplification that come with analyzing and modeling experimental systems in 2D, as opposed to 3D, have
resulted in the prominence of fabricated micro-models in experimental bioclogging research. However, since bioclogging is inherently a 3D, volumetric, process, the use of quantitative parameters derived from 2D systems to describe or model 3D systems is questionable owing to the significant difference in system complexity. In order to confirm that parameters and biofilm growth models developed using 2D micro-models and conventional imaging techniques are applicable to natural or engineered 3D porous systems, techniques for imaging biofilm internal to opaque, 3D, porous media are required.

Given that the majority of the porous materials of interest in bioclogging applications are opaque in nature, evaluating biofilm spatial distribution, structure, and variation to pore morphology is a challenging problem. Several approaches addressing this issue have been presented in the literature. One solution has focused on the sectioning of growth reactors at the conclusion of experiments. Sectioning of the column reactors can be accomplished with the wet columns (if prefabricated, removable, sections are included in the column construction), or by addition of an epoxy or other such hard resin to solidify the experimental reactor prior to slicing. Supplemental microscopy of the sections and measurements of parameters such as bulk and dry weight can provide estimates of biofilm mass and volume in each section. However, the similarity in density between water and biofilm makes mass measurements tenuous, and destructive sampling of column specimens can result in severe modification to the biofilm structure either during sectioning, or simply by adding the resin solution.
Alternately, magnetic resonance microscopy (MRM), and nuclear magnetic resonance imaging (NMR) have been utilized by a number of groups to measure the three-dimensional features of biofilms, as well as properties such as effective diffusion coefficients in developed biofilms (Manz et al., 2003; Seymour et al., 2004; Seymour et al., 2007; McLean et al., 2008; Renslow et al., 2010). In terms of gross structural characterization, these techniques have generally been successful. However, thus far, the method has been limited in resolution (resolving features on the order of 20-100 µm, usually with anisotropic discretization on the order of centimeters), and the acquisition time is very large, which could severely limit the method for some applications (Fridjonsson et al., 2011).

X-ray computed microtomography (CT) is a technique that has been used for decades to characterize a variety of material, including geomaterials such as rock cores and soil. The benefits to CT imaging include that the resolution of resulting data sets can be much higher (currently limited to 1-2 microns for newer conventional CT systems) than what is achievable using MRM techniques, and the results have isotropic spatial resolutions. Scan times can be much more rapid than for MRM, however, the actual scan time is dependent on the materials to be scanned and the particular tomograph used for scanning. In addition, a wide variety of materials can be imaged, and if monochromatic x-rays are used in imaging, element specific scanning is possible. Differentiation of specific materials, or phases, in a sample specimen can be accomplished based on differences in material densities, so long as the x-ray attenuation coefficient for each material is different. Materials can also be differentiated through the addition of contrast agents,
which are chemicals, or materials that can be added to a specific phase, present in the scanned object, in order to enhance the x-ray absorption properties of a particular phase, or material, and aid in differentiating it from the other materials present in the sample. If a monochromatic x-ray source is used for imaging, element specific x-ray scanning is possible as well. One example, presented in Armstrong and Wildenschild (2012) is the addition of cesium chloride to a brine solution to aid in differentiating brine from oil and glass beads in a packed bead column. For a detailed review and discussion of CT techniques and equipment I refer to Wildenschild and Sheppard (2013). The major limitations to imaging biofilm and bioclogging in porous media using CT techniques are (1) that the material densities of biofilm and water are similar, and (2) that conventional contrast agents, such as potassium iodide, or cesium chloride, diffuse readily into biofilm. In order to image biofilm using x-ray CT new methodologies are required.

The development and application of new and novel contrast agents for imaging biofilm in opaque porous media is the subject of this dissertation. In Chapter 2 the first method that has been developed for identifying biofilm in porous media is presented. It was published as Iltis et al. (2011). This method utilizes silver-coated, hollow glass microspheres as a surface coating material, used to delineate the interface between biofilm and the aqueous phase. Verification and validation of the method is accomplished through a comparison of CT scanning results to light microscopy images of the same area.
Concurrent to the development of the silver microsphere method for identifying biofilm using x-ray CT, we also got involved in the development of a similar method which utilizes a barium sulfate suspension to fill the hydraulically available aqueous phase, while being size excluded from the developed biofilm. The method was initially presented in Davit et al., (2011), which is provided as Appendix 1. Davit et al. (2011) presented the methodology for using barium sulfate to differentiate biofilm using polychromatic x-ray sources. Chapter 3 presents a method for quantitatively evaluating biofilm inside packed bead columns if imaged using the method of Davit et al. (2011). Using polychromatic x-ray tomography to image biofilm with this method presents a number of issues, such as beam hardening artifacts. These issues are addressed in Chapter 3, adjustments to the method presented in Davit et al. (2011) are suggested for future users, and a procedure for segmenting polychromatic data sets for quantitative analysis is provided as well.

Chapter 4 takes the barium sulfate-based method into the synchrotron realm; Monochromatic CT enables element specific imaging, and Chapter 4 first investigates the useful range of barium sulfate concentrations for monochromatic imaging, followed by an analysis and comparison of available options for the filtering and segmentation of resulting data sets. Recommendations for using barium sulfate as a contrast agent for monochromatic imaging in the future as well as proposed method for data segmentation and quantitative analysis is provided as well.
Chapter 5 focuses on the application of the barium sulfate-based imaging method in an experiment evaluating biofilm development and bioclogging in packed bead columns subjected to hydraulic loading rates which correspond to Reynolds numbers spanning three orders of magnitude. During the growth phase of the experiment the measured bulk parameters include differential pressure and dissolved oxygen. Quantified CT data sets are compared to the measured bulk parameters and demonstrate the added value that CT imaging of biofilm-containing columns contributes to bioclogging experimental investigations.

Finally, Chapter 6 provides a comparison of both the silver microsphere and barium sulfate methods. A summary description of the relative advantages and limitations of each method is provided, and recommendations for future research and study are provided.
IMAGING BIOFILM ARCHITECTURE WITHIN POROUS MEDIA USING SYNCHROTRON BASED X-RAY COMPUTED MICROTMOMOGRAPHY

Gabriel C. Iltis, Ryan T. Armstrong, Danielle P. Jansik, Brian D. Wood, and Dorthe Wildenschild

Water Resources Research

John Wiley & Sons Inc

350 Main Street

Malden MA 02148, USA

Issue 47
2 IMAGING BIOFILM ARCHITECTURE WITHIN POROUS MEDIA USING SYNCHROTRON BASED X-RAY COMPUTED MICROTMOTOMOGRAPHY

2.1 Abstract
A new method to resolve biofilms in three dimensions in porous media using high-resolution synchrotron-based x-ray computed microtomography (CT) has been developed. Imaging biofilms in porous media without disturbing the natural spatial arrangement of the porous medium and associated biofilm has been a challenging task, primarily because porous media generally preclude conventional imaging via optical microscopy; x-ray tomography offers a potential alternative. Using silver-coated microspheres for contrast, we were able to differentiate between the biomass and fluid-filled pore spaces. The method was validated using a two-dimensional micro-model flow cell where both light microscopy and CT imaging were used to image the biofilm.

2.2 Introduction
Biofilms are observed in both natural and engineered systems, and are believed to be the primary habitat for most microorganisms (Costerton et al., 1995). In porous media, biofilm growth and development occurs over a continuum of scales ranging from nanometers to millimeters or more and has been a topic of interest in applications and industries including mining, filtration, water and wastewater treatment, and bioremediation (e.g. Fitch et al., 1998; Rawlings and Johnson, 2007; Rodríquez and Bishop, 2008). Due to the prevalence of biofilm in both natural and engineered porous media systems, a significant effort has been undertaken to understand biofilm growth and
development in porous media, yet much of the information about the three-dimensional architecture of biofilms in porous media is based on the results of mathematical models rather than on direct experimental data. In order to verify and validate existing and new models, experimental data quantitatively detailing the structural arrangement and distribution of biofilm in porous media is required.

Visualization of the biological phase in porous media is a useful means for increasing our understanding of microbial-soil structure interactions and for developing quantitative experimental data sets for mathematical model validation. Conventional techniques for imaging biofilm include light microscopy (e.g. Paulsen et al., 1997; Yang et al., 2000; Sharp et al., 2005), Environmental Scanning Electron Microscopy (ESEM) (e.g. Davis et al., 2009), and Confocal Laser Scanning Microscopy (CLSM) (e.g. Leis et al., 2005; Rodriguez and Bishop, 2007), all of which are useful for examining biofilm on surfaces or in two-dimensional or quasi-two-dimensional porous systems. While the aforementioned imaging techniques are capable of providing significant insight regarding microbial interaction and biofilm structural formation, imaging porous-media-associated biofilm using these techniques requires that model porous media systems be constrained to a few particle diameters, that the porous medium and fluid be index-matched, or that samples be extracted and prepared, thereby disrupting the pore scale structure. Thus, new techniques that allow for direct visualization of biofilm in situ are required in order to characterize biofilm growth, surface architecture, and three-dimensional spatial distribution within porous media to provide relevant experimental data for the verification and validation of porous media associated biofilm growth models. One such
A new methodology using synchrotron based X-ray computed microtomography (CT) to render high-resolution measurements of the spatial distribution of biofilms in porous media is presented in this work. This methodology has the advantages that (i) a variety of solid substrates can be used; and (ii) the method is non-destructive to the porous medium allowing for three-dimensional \textit{in situ} visualization of biofilm. CT has been available for more than three decades and has been a powerful tool for studying a wide array of processes in porous media systems (e.g. Nunan et al., 2006; Wildenschild et al., 2002; Werth et al., 2010). However, synchrotron-based tomographic imaging of biofilms has yet to be accomplished, primarily due to the fact that obtaining x-ray contrast between the biomass and water has posed a significant challenge since conventional contrast agents, such as potassium iodide, diffuse readily into biofilm when present in the fluid phase. To address the contrast issue, we used a silver-based fluid-phase contrast agent that provides x-ray absorption contrast between the fluid and biofilm phases by adsorbing to the surface of the biofilm, and thereby delineating the biofilm/aqueous phase interface. To our knowledge, we report the first use of synchrotron
based CT to image biofilm within porous media as well as method validation using a separate imaging technique.

2.3 Methods

Two-dimensional micro-model flow cells were constructed from PDMS (polydimethyl siloxane). The inner dimensions of the flow chamber were 9.8 x 30.0 x 3.4 mm with 1 mm diameter cylindrical columns spaced 1 mm apart, see supporting material Figure SM-1 (included as Figure 2-3) for details. *Shewanella oneidensis* MR-1 was used in all experiments. *S. oneidensis* MR-1 is a gram negative, facultatively anaerobic, polarly flagellated bacteria capable of dissimilatory metal reduction and biofilm formation (Venkateswaran et al., 1999; Majors et al, 2005). Cultures were grown for 24 hrs in a 10% Tryptic Soy Broth (TSB) solution, concentrated at 6000 rpm for 10 minutes, resuspended in 5 ml of 10% TSB followed by inoculation of the micro-model flow cell. The injected culture sat stagnant for 24 hrs allowing for microbial attachment to the surfaces, followed by flow of 10% TSB solution at 0.01 ml/min for 12 days. Neutrally buoyant hollow silver-coated microspheres with an average diameter of 10 µm (particle range: 5-15µm based on coulter counter measurements, Microsphere Technology Ltd, Pentlands Science Park, Edinburgh EH26 0PZ, UK) were deposited at the biofilm surface by flowing a concentrated solution (approximately 4 mg microspheres/mL) through the micro-model, followed by subsequent rinsing with 10% TSB to remove spheres not attached to the biomass. Imaging commenced following the micro-model rinse in order to limit the potential for biofilm rearrangement due to the antimicrobial nature of the silver microspheres.
A Leica Z16 APO light microscope was used to acquire light microscopy images of the micro-model at a resolution of 1.4 µm/pixel. Regions of the micro-model containing unique, identifiable, biofilm features were observed using digital microscopy; the locations of the biofilm features were measured with a stage micrometer using the micro-model sidewall as a datum. Biofilm features were mapped in all three principal dimensions to enable alignment with the CT data. The entire micro-model flow cell was imaged using CT and regions of interest were located in the reconstructed volume using the microscope coordinate measurements, see supporting material (Figure SM-1, included as Figure 2-3).

Microtomographic imaging was performed at beamline 8.3.2 at the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory. Preliminary experiments were performed at beamline 13-BMD, GSECARS at the Advanced Photon Source (APS/GSECARS), Argonne National Laboratory. The data collected at the ALS and APS was acquired at resolutions of 4.5 µm/pixel and 11.8 µm/pixel respectively. An energy level slightly above the K-shell absorption edge for silver (25.5 keV) was used for imaging to optimize x-ray absorption and thus contrast.

Image reconstruction, post-processing, and segmentation were done with scripts written in Interactive Data Language (IDL®), and using the commercially available visualization software Avizo®. The grayscale images were coarsened to a 4.5 µm/pixel resolution corresponding to the resolution of the CT data. Raw CT data was pre-processed by integrating (or collapsing) volume data in the light microscopy image region of interest into a single slice for direct comparison of representative images. All
images were then processed using a standard Sobel edge detection algorithm followed by segmentation using a simple histogram threshold. For additional information on the Sobel edge detection algorithm we refer to the IDL software documentation as well as to Duda and Hart (1973). The PointWrap surface generating algorithm in Avizo® was then applied to the resulting binary images to generate surfaces for quantification of the spatial distribution of biofilm. The PointWrap algorithm generates a surface reconstruction from a cloud of unorganized points by simulating the path of a sphere through a cloud of points or along a surface. A triangulated mesh is generated by assuming that a surface exists every time a set number of points are in contact with the simulated sphere body simultaneously. This algorithm allows for the association and interconnection of a cloud of points, which corresponds to the individual silver-coated microspheres adhering to the surface of the biofilm. For additional information on the PointWrap algorithm, we refer to the Avizo® software documentation as well as to Sander and Runge (2000). The PointWrap analysis was also verified using a standard marching cubes surface-generating algorithm. Surface area error estimates generated using the two methods varied by less than one percent.

Volume rendering of the CT data for three-dimensional evaluation of the biofilm was also done using the Avizo® software package. The volume files were segmented to differentiate between the solid (PDMS) and silver particles. A Sobel edge detection filter was applied in order to segment the silver particles. Volumetric quantitative analysis using both the PDMS (solid-phase) as well as the silver micro-sphere (biofilm) surfaces
as indices was performed using the triangulated mesh created using the PointWrap algorithm in Avizo®.

2.4 Results

Preliminary experimentation using the new imaging approach was conducted at the APS and provided compelling evidence that our choice of contrast agent and CT imaging method produces realistic three-dimensional representations of biofilm present in the experimental packed bead column system. A visualization of the preliminary results is provided in Figure 2-1 as well as in Movie 1-1 of the Dynamic Content (included as Figure 2-6). Figure 2-1 indicates that biofilm (green) forms both on the surfaces of the glass beads within the column (gold) as well as within the column pore space bridging multiple beads together. In order to validate our preliminary findings, we developed the previously mentioned system in which the distribution of biofilm could be visualized using both digital microscopy and CT.

This validation of the CT imaging technique for visualizing biofilm in situ in porous media is the focus of this work.

In the two-dimensional micro-model used for validation, two distinct regions of the micro-model flow cell containing \textit{S. oneidensis} biofilm were selected for analysis (Features A and B). Visualizations of the light microscopy and composite CT images of both features are depicted in Figure 2-2 along with the processed binary and PointWrap biofilm representations. The flow cell column posts have been superimposed as gray circles in the grayscale images for ease of interpretation. Visual comparison of the light microscopy images to the CT composite images indicate very good agreement between
the imaging methods and suggest that the silver particles detected using CT do, in fact, allow for the delineation of biofilm within the pore space. The correlation between the two imaging techniques for Features A and B was quantified, via surface area, using the PointWrap surface generating algorithm on the binary images resulting in a percent error for the CT images, normalized to the light microscopy images, of between 1-5%. Results of the comparison are detailed in Table 2-1.

Three-dimensional renderings that illustrate the spatial distribution of biofilm within the porous medium for Features A and B are provided in the supporting material (Figure SM-2, included as Figure 2-4). Through the use of the PointWrap algorithm, the volume change and change in macroporosity associated with biofilm growth were quantified for both features. The volume of biofilm associated with Features A and B were calculated to be 1.67 mm³ and 2.44 mm³, which corresponds to decreases in macroporosity from 63% to 48% for Feature A and from 68% to 50% for Feature B. This three-dimensional analysis is included to illustrate the potential for quantification that the technique allows for.

2.5 Discussion

Validation of CT imaging of biofilm in porous media using silver-coated glass microspheres as a contrast agent to outline a biofilm surface was successful. The microspheres adhere to the S. oneidensis biofilm-aqueous phase interface providing the necessary contrast needed for CT imaging. Light microscopy images showed good correlation with representative CT images, and biofilm volume renderings could be created from a cloud of points generated by the distribution of silver particles across the
biofilm interface with the aqueous phase. Currently, the technique facilitates three-dimensional imaging of biofilm within porous media and accurately represents the solid-biofilm-aqueous phase spatial arrangement. This method is particularly suited for pore-scale investigations where a triangulated mesh can be generated which provides a convenient platform for additional analysis (for example, fluid and solute transport within the biofilm via a finite element or finite volume numerical simulator). The proposed CT-based method can image biofilm within porous media with high fidelity (see Movie 1-1 of the Dynamic Content, included as Figure 2-6) and also allows measurement in situ to a biologically-relevant environment. Secondly, CT can image at the micron scale and still provide spatial information at the centimeter scale. The beamline setup (beam width) ultimately determines the maximum sample diameter that can be imaged. The maximum specimen diameter at the beamlines utilized in this manuscript are approximately 4 to 5 cm. However, the image resolution is dependent on the magnification of the lens attached to the detector which decreases the applicable sample diameter. In general, the resulting image resolution is 1/1000 of the specimen diameter. The sample length (axial length) is effectively limited by the acceptable data acquisition time for the collection of multiple scans at different heights. Thus, CT can provide biofilm spatial distribution over many centimeters in the sample axial direction and resolve interfacial architecture at the micron resolution. A key benefit to using CT to image biofilm in porous media is that no limitation to direct surface access or visualization depth exists, as in the case of CLSM. Additionally, refractive index matching of the porous medium is not required since attenuation and optical transparency are not significant issues associated with the
presented method. As a result, biofilms within their natural environments (e.g. soil and rock) can potentially be imaged \textit{in situ}.

The key limitations to the method include 1) x-ray exposure associated with CT imaging is expected to kill or severely retard microbial growth; 2) silver is a biocide and, for this reason, must be added to sample specimens immediately prior to imaging; 3) the ability to visualize biofilm is dependent on the extent of silver coverage on the biofilm surface; 4) the presented method, at this point, is limited in utility to imaging changes to the macropore structure of the porous medium, as a result the internal porosity of the biofilm is not imaged using this method. The effect of x-ray exposure as well as the introduction of silver microspheres is expected to have a negative impact on biofilm growth. As a result, the imaging of biofilm using the presented method should be considered the terminal step for particular specimens. The quality of coverage by silver particles on the surface of the biofilm is key to successful use of the presented method. Since the silver addition occurs immediately prior to imaging, surface attachment will be limited to biofilm surfaces exposed to flow channels through the micro-model or porous medium column. Dead end pores containing biofilm have the potential to be interpreted as fluid space if silver particles do not come into contact with the biofilm. Regions that are occluded from flow also have the potential to be attributed to biofilm if the region is surrounded by silver coated biofilm. Since the method relies on the deposition of silver coated microspheres on the biofilm surface, the method is limited to providing insight into changes in macropore morphology external to the biofilm, however, the ability to acquire quantitative experimental data detailing the changes in macropore morphology
can potentially provide insight into biofilm associated changes in porous media hydrodynamics as well as solute transport.

To our knowledge, this is the first successful attempt using high resolution CT to image three-dimensional biofilms in situ within intact porous media. Both spatial distribution of biofilm and change in porosity are important parameters for investigating the impact of biofilm on porous media hydrodynamics, and on the mass transport and reaction processes that occur during bioremediation. Admittedly, we present one working approach for applying a contrast agent to biofilm for imaging using CT. The method is potentially applicable using microspheres coated in a variety of elements, however, additional research into the efficacy of other contrast agents as well as the effect of varying particle size and density is ongoing.

2.6 Acknowledgements

This work was supported by the Environmental Remediation Science Program (DE-FG02-09ER64734) under the Department of Energy, Office of Biological and Environmental Research (BER), Grant No. ER64734-1032845-0014978. The work was performed at beam lines 8.3.2 at the Advanced Light Source (ALS), LBNL, and GeoSoilEnviroCARS (Sector 13), Advanced Photon Source (APS), ANL. The APS is supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Energy Research, under Contract No. 248 W-31-109-Eng-38. We would like to thank Alastair MacDowell and James Nasiatka at Beamline 8.3.2, as well as Mark Rivers at GSECARS for assistance with CT imaging.
2.7 Bibliography


Bibliography (Continued)


Figure 2-1: Preliminary three-dimensional CT biofilm imaging results of biofilm (green) grown in a glass bead pack (gold). The visualization experiment was performed at the Advanced Photon Source (APS), Argonne National Laboratory, using neutrally-buoyant, silver-coated hollow glass spheres (10 μm diameter) as an x-ray contrast agent. The spatial arrangement of the silver particles is interpreted as being attached to the biomass grown within the bead pack.
Figure 2-2: Comparison of light microscopy images to representative CT images for two unique biofilm features at the grayscale, binary and PointWrap stages of image processing. Dark areas correspond to fluid, light/grey areas show silver particles attached to biofilm. The light blue regions in the PointWrap images correspond to the representative region containing biofilm. (a) Light microscopy images of Feature A, (b) CT images of Feature A, (c) Light microscopy images of Feature B, (d) CT images of Feature B.
Figure 2-3: Supporting material Figure SM-1. Micro-model flow cell detail: (a) Example two-dimensional micro-model flow cell constructed from PDMS (polydimethyl siloxane). The inner dimensions of the flow chamber are 9.8 X 30.0 X 3.4 mm with 1 mm diameter cylindrical columns spaced 1 mm apart. (b) Close up detail of the cylindrical columns molded into the flow cell. (c) Plan view schematic of the flow cell. The arrows indicate the flow direction. Details A and B correspond to the locations of Features A and B. The shaded area corresponds to the region of porous matrix. The red lines correspond to the X and Y datum from which spatial measurements for locating the feature regions were taken.
Figure 2-4: Supporting material Figure SM-2. Volume renderings of biofilm growth (light blue mesh) at two unique locations within the micro-model flow cell captured using synchrotron based CT. The PDMS solid phase (gold) has a measured volume of 4.26 mm$^3$ and 4.31 mm$^3$ for Features A and B, corresponding to initial porosities of 63% and 68% respectively. The measured volume of the biofilm for Features A and B are 1.67 mm$^3$ and 2.44 mm$^3$ resulting in a decrease in macroporosity of 15% and 18%, respectively. The volume of biofilm and associated change in macroporosity was quantified after applying the PointWrap algorithm to the imaged silver particles (dark blue).
Figure 2-5: Supporting material Figure SM-3. Light microscopy image detailing the distribution and adherence of silver-coated hollow glass micro-spheres to the biofilm surface. The silver particles can be seen to adhere solely to the biofilm (a) as opposed to the PDMS column posts (b) indicating that spatial distribution of silver particles within the x-ray tomography data corresponds to the location of biofilm.
Dynamic Content

Located at:


Figure 2-6: Supporting material Movie 1-1. Three-dimensional rendering of the preliminary investigation results. The data has been segmented to differentiate biofilm (green) from the glass beads (gold) within the sample column. Biofilm appears both as surface associated cell clusters on the surface of glass beads as well as within the pore space bridging multiple glass beads.
Table 2-1: Quantitative comparison of the light microscopy and representative two-dimensional CT images for two unique biofilm features using the Avizo® PointWrap algorithm.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Light Microscopy</th>
<th>Computed Microtomography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Area</td>
<td>1.595</td>
<td>1.529</td>
</tr>
<tr>
<td>Absolute Error</td>
<td></td>
<td>4.13</td>
</tr>
<tr>
<td>Normalized</td>
<td></td>
<td>1.82</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Feature</th>
<th>Light Microscopy</th>
<th>Computed Microtomography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Area</td>
<td>1.998</td>
<td>2.034</td>
</tr>
<tr>
<td>Absolute Error</td>
<td></td>
<td>1.82</td>
</tr>
<tr>
<td>Normalized</td>
<td></td>
<td>1.82</td>
</tr>
</tbody>
</table>
3 QUANTITATIVE ANALYSIS OF THREE DIMENSIONAL BIOFILM ARCHITECTURE IN POROUS MEDIA USING A BARIUM SULFATE CONTRAST AGENT AND POLYCHROMATIC X-RAY COMPUTED MICROTMOTOGRAPHY.

Gabriel C. Iltis, Yohan Davit, and Dorthe Wildenschild

3.1 Abstract

Biofilms are of importance in porous media applications ranging from groundwater bioremediation to the clogging of filters and drainage systems. To date, visualization and quantification of biofilm in porous media has relied upon optically transparent two-dimensional and pseudo-three-dimensional micromodels and growth reactors. Results from these studies have then been extrapolated to three-dimensional systems. In order to more fully understand biofilm growth, and the impact of biofilm growth on hydrodynamic processes in three-dimensional systems, techniques for visualizing biofilm in opaque porous media are required. One such method has been presented by Davit et al. (2011) who utilized a barium sulfate suspension as a contrast agent for visualizing biofilm in opaque porous media using polychromatic x-ray computed microtomography. Davit et al. (2011) presented the operational methodology for using the barium contrast agent and demonstrated, qualitatively, that suitable contrast was provided by the barium sulfate suspension in order to delineate biofilm from the aqueous phase in sample reactors. This communication extends the work presented in Davit et al (2011) by presenting a methodology for segmentation and quantitative analysis of biofilm grown in
three-dimensional growth reactors, and collected using a conventional bench-top polychromatic x-ray tomography system and a barium sulfate contrast agent. Three column data sets are presented in order to demonstrate the potential of this method for directly measuring parameters such as biofilm volume and change in column porosity both spatially and as bulk column parameters, without having to destructively sample experimental growth reactors. Evaluation of biofilm formation as a function of flow rate, and associated Reynolds number (Re) is presented as well. Quantitative results suggest that a threshold Reynolds number exists between Re=10 and Re=100 at which point hydrodynamic shear forces limit biofilm growth to thin, approximately 15 µm thick, surface-associated colonies.

3.2 Introduction

Microbes are found in nearly every environment regardless of whether the environment is man-made or natural. Typically, microbes form, and exist in communities where single cells grow and persist in tandem with other cells, while being joined together in an extracellular polymeric substance, or EPS layer. These communities have come to be known, collectively, as biofilms (Costerton et al., 1995). In nature, biofilms play a vital role in global-scale biogeochemical processes such as nutrient cycling (e.g. nitrogen fixation and metal reduction (Lovley, 1993)). In engineered systems, biofilms are involved in a variety of processes from wastewater treatment to bio-processing. In porous media, biofilm growth and development has been a topic of interest in a number of applications and industries including mining, solid waste management, filtration, water and wastewater treatment, and bioremediation (e.g. Fitch et al., 1998; Palmeira et al.,
Biofilm growth occurs in the pore space available to fluid transport, and therefore results in the clogging of porous material (termed bioclogging), an attribute which can be positive or negative depending on the application. A significant effort has been undertaken to characterize biofilms experimentally in systems including packed columns and fabricated two-dimensional micro-models. The objective of these studies has typically been to evaluate the potential for bioremediation of contaminated soil and groundwater, or to evaluate the impact of biofilm growth on bulk fluid transport parameters such as change in porosity or hydraulic conductivity (Rittman, 1982; Cunningham et al., 1991; Kim et al., 2013).

As both Thullner (2010) and Baveye (2010) state, a significant limitation in current experimental efforts to monitor and evaluate biofilm formation and bioclogging in porous media is the fact that experimental studies generally rely on macroscale parameters (e.g. differential pressure across the column, nutrient mass balance, sectional cell counts, or carbon quantification) to draw conclusions about the impact of biofilm in porous media on hydraulic parameters (such as hydraulic conductivity). The generation and persistence of preferential flow paths, pore clogging, spatial arrangement of, and types of biofilm (aggregation, pore coating and filling, filament or stringer formation) can only be monitored and evaluated in one type of experimental system, namely optically transparent micromodels (e.g. Heydorn, 2000; Kim and Fogler, 2000; and Kim et al., 2013). The application of results and conclusions generated from one- and two-dimensional experimental systems and models to three-dimensional systems is less than ideal. Thus, new techniques that allow for direct visualization of biofilm in situ are
required in order to characterize biofilm growth, surface architecture, and spatial
distribution in porous media to provide relevant experimental data, ultimately for the
verification and validation of porous media associated biofilm growth models.

X-ray transmission computed microtomography (CT) has proved to be a useful tool
for characterizing porous media. A number of types of CT systems are available for
imaging porous media ranging from commercially available medical, industrial or
laboratory scanners to synchrotron-based imaging systems. A thorough review of CT
systems and imaging approaches are detailed in Wildenschild and Sheppard (2013). In
summary, all CT systems function in essentially the same manner. X-rays are emitted
from a source and pass through the sample, where they attenuate, to a detector. Using a
series of radiographs of a sample, collected at fixed angular increments during sample
rotation, a three-dimensional representation of the sample can be reconstructed.
Applications presented in the literature range from structural characterization of soil and
rock (e.g. Nunan et al., 2006; Prodanovic, et al., 2007) to single- and multi-phase fluid
transport investigations (e.g. Culligan et al., 2004, Porter et al., 2010). The key
advantages to using x-ray tomographic imaging for porous media experimental
investigations are that (1) a wide variety of porous materials, ranging from plastic or
glass beads to natural sand or rock can be imaged, (2) imaging results are complete three-
dimensional reconstructions of the experimental system acquired without requiring
destructive sampling, and (3) the images can be high resolution, on the order of 5 to 10
µm per pixel for REV-sized porous media samples.
Methods for imaging biofilm in porous media using x-ray CT have only recently started being developed. The main hindrance has been the similarity in density between water and biofilm. In addition, conventional contrast agents such as potassium iodide or cesium chloride diffuse readily into the biofilm “phase.” To date, two approaches have been taken in order to evaluate the impact of biofilm in porous media investigations. Iltis et al. (2011) relied upon a surface coating technique using silver-coated, neutrally buoyant, hollow glass microspheres. This method focused on the ability of silver to attach to the biofilm surface. Through the use of monochromatic, synchrotron-based x-ray CT, scans were collected above the k-shell absorption edge for silver, enabling the isolation of the silver microspheres during image segmentation. So long as the biofilm surface was sufficiently covered by silver microspheres, a triangulated mesh connecting the microspheres was generated and used to quantitatively evaluate biofilm structure in the pore space, as well as the overall change in porosity for the porous medium. Davit et al. (2011) utilized a barium sulfate suspension as a pore filling contrast agent. The suspension contained barium sulfate particles roughly 1µm in diameter, thus the particles are size-excluded from the biofilm matrix. As a result, only the pore space available to fluid transport is filled with the barium suspension. Davit et al. (2011) demonstrated that barium sulfate addition provides sufficient contrast for qualitative, visual, differentiation of biofilm from the open pore space utilizing a polychromatic x-ray CT scanner, and introduced the operational technique for imaging biofilm using polychromatic tomographic imaging and a barium sulfate suspension as a contrast agent. We are now able to demonstrate that quantitative analysis is also possible.
The work contained herein extends from the work previously presented in Davit et al. (2011) and addresses the quantitative analysis of biofilm imaging data. With this in mind, this manuscript has three objectives: (1) to illustrate the quantitative potential of the technique developed by Davit et al. (2011), (2) to address experimental challenges and opportunities that arise when using polychromatic systems for imaging biofilms in porous media, including the effect of BaSO₄ concentration, the need for an additional contrast agent (iodide) for soft porous media, and artifacts caused by beam-hardening, and (3) to present an example quantitative analysis that evaluates the effect of flow regime on biofilm formation.

3.3 Methods and materials: Quantitative image processing was conducted on a total of three sample specimens:

(1) Demonstration column, 100% BaSO₄, no iodide

(2) Porous media packing, 50% BaSO₄, with iodide, low Re

(3) Porous media packing, 50% BaSO₄, with iodide, high Re

Detailed descriptions of the experimental conditions and tomographic scanning parameters are provided in Davit et al. (2011). Brief descriptions of the experimental setup, growth conditions, contrast agent and polychromatic scanning are provided below. In addition, a summary of the column-specific experimental parameters are provided in Table 3-1.
3.3.1 Growth reactor design:
A detailed description of the original experimental setup is provided in Davit et al. (2011). In summary, polystyrene column reactors, measuring 3.5 mm in diameter, were filled with either 3 mm diameter polyamide beads or polystyrene beads with diameters ranging from 0.5 to 1.5 mm. As noted in Davit et al. (2011) polystyrene and polyamide beads were used for experimentation and imaging due to the low x-ray absorption coefficients inherent to these types of plastic materials. Selection of materials having low x-ray absorption coefficients reduces the required time for tomographic image collection; thereby minimizing the risk of biofilm movement or barium sulfate settling during image acquisition.

3.3.2 Bacterial species and growth conditions:
The natural microbial flora from filtered (500 micron screen) river water from the river Garonne (France) was used for all of the biofilm growth experiments. The filtered river water was amended using CH₃COONa; 3H₂O (additional carbon source), and KNO₃ (electron acceptor). Amended river water was then pumped through the experimental growth reactors for time periods ranging from 7 to 9 days. The source reservoir was continuously aerated to help ensure aerobic conditions within the growth reactors. The natural microbial flora was capable of biofilm formation, and sufficient microbial attachment and biofilm formation was achieved during the growth period for CT imaging to commence. Additional details are provided in Davit et al. (2011).

The applied flow rate for the packed bead column containing 3 mm diameter beads was 6 mL per min, corresponding to an approximate initial Re of 67. The applied flow
rate for the packed bead columns containing smaller diameter beads, ranging from 0.5 to 1.5 mm in size, was either 4.2 mL per min, or 30 mL per min. The corresponding initial Re for the smaller bead diameter columns are 14, and 84 respectively. At the conclusion of the experimental growth phase, each growth reactor was disconnected and prepared for contrast agent injection.

3.3.3 Contrast agent addition:
The contrast agent utilized in the presented investigations consists of a commercially available, medical-grade barium sulfate suspension (Micropaque, Guerbet) with a stock concentration of 1.0 g per mL barium sulfate. Barium sulfate is insoluble in water and the stock product contains barium sulfate particles on the order of 1 µm in size. An SEM image of the actual barium sulfate particles is included in Figure 3-1. Columns were imaged using concentrations of 100% (1.0 g per mL) and 50% (0.5 g per mL) to evaluate the effect of BaSO₄ concentration on image quality. Since plastic beads have been used as the solid matrix, the x-ray absorption properties for the solid matrix are roughly equivalent to the x-ray absorption parameters for biofilm, as can be seen in the results for the barium sulfate demonstration column presented in Section 3.4.1. Due to the fact that plastic beads were required for imaging using the polychromatic x-ray source, and that the x-ray absorption properties for the plastic solid matrix are similar to that of the biofilm, an additional contrast agent, potassium iodide, was deemed to be necessary for polychromatic imaging, and was added to some of the experiments (50% barium sulfate contrast agent mixtures at a concentration of 100 mg per mL) to differentiate biofilm from the plastic bead solid matrix. Potassium iodide diffuses readily into the biofilm, thus
altering the x-ray absorption properties for the biofilm and allowing for segmentation. Results for the experimental columns containing both BaSO₄ and KI contrast agents are provided in Section 3.4.2.

Addition of the contrast agent to prepared specimen columns was accomplished via direct, manual, injection of the prepared barium sulfate suspension to the influent side of the growth columns, immediately prior to imaging.

3.3.4 Three-dimensional imaging:
As described in Davit et al. (2011), three dimensional imaging was accomplished using a Skyscan 1174 tomograph, with a tension voltage of approximately 50 kV and a current of 800 µA. The final resolution of the collected data sets ranges from 7 µm per pixel to 9 µm per pixel. Tomographic imaging was conducted using a full 360 degree rotation with radiographs being collected every 0.5 degrees, producing 720 projections. The approximate total scan time for each individual sample is 1.5 hrs. Volume reconstruction using the original 12-bit absorption projection images was completed using the commercial software package NRecon (Skyscan), with the final output being a z-stack of gray-scale cross-sectional tiff images.

3.3.5 Image segmentation:
Volume segmentation and quantitative analysis was completed using the commercial software package Avizo Fire (version 7.1, www.vsg3d.com). The initial step in segmenting all columns was to apply a non-local means filter to the original gray-scale volume data sets. The non-local means filter minimizes errant voxel intensities or noise by evaluating the entire gray-scale data set, and identifies all regions with equivalent
Gaussian neighborhoods. Once all of the equivalent regions are identified, the mean value of all of the equivalent neighborhoods is used as the denoising parameter for each individual neighborhood. Additional details of the non-local means filtering method can be found in Buades et al. (2005). Segmentation was then achieved by thresholding volume histograms by bounding the predominant regions, or peaks associated with each phase, see Figure 3-2. This was accomplished by selecting intensity values within the valleys surrounding each peak, followed by direct visual comparison of the identified pixels within the bounded region to the original gray-scale data set. Thresholding and generation of representative binary data sets was accomplished using an interactive thresholding algorithm specifically configured to identify and isolate all voxels within the specified intensity range. For the demonstration column (without KI), it was necessary to separate three phases in the images, while for the porous media packings which included KI, four phases needed separation. A plot of the volume histogram for the 100% barium demonstration column is provided in Figure 3-2a. The histogram has been broken up into each of the four regions corresponding to individual peaks in the histogram data. Figure 3-2c consists of a two-dimensional cross section illustrating the regions to which each of the histogram peaks correspond. In general, the lowest intensity peak (at or around 0) corresponds to the column exterior, as well as to the lowest intensity regions of the plastic beads. The bulk of the bead mass is either lumped into the histogram peak local to zero intensity, or presents as a second peak distributed somewhere in the first quarter of the measured intensities. In Figure 3-2, the first peak (local to zero) predominantly corresponds to the column exterior, and is highlighted in light blue. The second peak is
identified in dark blue, and contains most of the plastic bead and biofilm voxels within the data set. In the case of the KI containing volumes, the middle-intensity peak generally corresponds to the biofilm phase. The highest intensity peak(s) correspond to the barium sulfate contrast agent. In some cases the barium sulfate will differentiate into more than one peak on the high end of the histogram intensity spectrum as can be seen in Figure 3-2 where the third peak contains most of the barium sulfate voxels, and is identified in red, while the fourth peak contains all of the highest intensity barium voxels, as well as voxels associated with beam hardening artifacts. This data is highlighted in green in both the histogram (Figure 3-2a) and in Figure 3-2c. The presence of the fourth peak is the result of beam hardening, which occurs when lower energy x-rays are absorbed by a sample while higher energy x-rays are not. Beam hardening results in intensity variations both within materials and at material transition zones and can also manifest as streaks between points of high intensity. Spatial variations in density within the injected barium sulfate suspension resulting from settling or accumulation may also have contributed to this variation in voxel intensity.

Ideally, each of the described peaks would be used to isolate particular, distinct, phases or materials within the images. Unfortunately, the intensity ranges within each of these materials (exterior, plastic beads, and biofilm) vary, as can be seen in both the gray-scale cross sectional image (Figure 3-2b) as well as in the labeled image (Figure 3-2c). The end result is that selection of distinct thresholding values results in the persistence of miss-labeled voxels (noise) which require additional processing in order to resolve properly. An example of the potential for mislabeling due to threshold selection can be
seen either in the outer edge of the column in the transition region from column exterior to barium sulfate (dark blue ring in Figure 3-2c) or in the central region of the plastic bead, where a ring artifact contains intensity values associated with the exterior histogram peak.

As mentioned previously, when iodide is added to the contrast agent mixture, a total of 4 binary representations (or isolated phases) are needed in order to complete segmentation: (1) barium sulfate (or open pore space), (2) the plastic beads (solid matrix), (3) the biofilm, and (4) all voxels exterior to the column growth reactor. Once all of the required binary volumes are generated, the volumes are recombined into a single label field where each phase is assigned a distinct value (ranging from 1 to 4). Any voxel unassigned to a particular phase has a value of 0. Through the use of catch-basin/dilate-basin algorithms included in the Avizo software package, all unassigned voxels are assigned a value corresponding to the most likely phase with which they are associated, using a gradient magnitude map (generated from the original gray-scale volume) as a reference. Specific segmentation details for each column are described in the results and discussion section.

Once the final segmented volume has been generated, each of the phases is quantified on the basis of total volume, total surface area and interfacial area between phases. Quantification was performed both on the volume as a whole, as well as spatially, in the axial direction, on a slice by slice basis (each slice has a thickness of 1 voxel). Once the image segmentation is complete, a number of different measured can be established,
including the average biofilm thickness and initial and final porosity. The average biofilm thickness can be calculated as

\[
L_{Biofilm} = \frac{V_{Biofilm}}{S_{A_{Biofilm}}}
\]

Equation 1

where \(L_{Biofilm}\) is the average biofilm thickness, \(V_{Biofilm}\) is the total volume of biofilm for the column, and \(S_{A_{Biofilm}}\) is the total surface area of the biofilm. The initial and final porosity for the columns can be calculated as

\[
\varphi = \frac{V_{Void}}{V_{Total}}
\]

Equation 2

where \(V_{Total}\) is the total volume of all phases present in the column interior, calculated as

\[
V_{Total} = V_{Beads} + V_{Barium} + V_{Biofilm}
\]

Equation 3

and \(V_{Void}\) is the volume of the pore space

\[
V_{void} = V_{barium} + V_{biofilm}
\]

Equation 4

The calculated biofilm volume (\(V_{Biofilm}\)) is included in the \(V_{Void}\) parameter when calculating the initial porosity (\(\varphi_1\)), and is excluded from \(V_{Void}\) when calculating the final column porosity (\(\varphi_f\)). The parameters \(V_{Beads}\) and \(V_{Barium}\) correspond to the measured volume of beads and barium as determined from the quantitative image analysis. In addition, measures of interfacial area are easily quantifiable. From the interfacial area data we can quantify parameters such as the surface area of attachment, which corresponds to the interfacial area between the beads and biofilm, and the reactive surface area, which corresponds to the interfacial area between the barium sulfate (pore space available to flow) and the biofilm.
3.4 Results and discussion:
Image results for a total of three distinct experimental columns have been used for quantitative analysis of biofilm formation. This discussion will first focus on results demonstrating the efficacy of using barium sulfate as a contrast agent for imaging biofilm formation using polychromatic tomographs.

3.4.1 Demonstration column, 100% BaSO₄ suspension, no iodide
The presented demonstration column illustrates the most important aspect of barium sulfate as an x-ray CT contrast agent, namely that addition of the barium sulfate provides distinct delineation of the available pore space from the developed biofilm.

Because the plastic beads and biofilm have very similar x-ray absorption properties, the two materials end up having similar voxel intensities in the reconstructed volume. To demonstrate that barium sulfate provides adequate contrast for volume segmentation, the final segmented product is compared directly to the original gray-scale source data. Segmenting the volume as a 2-phase system with biofilm and plastic beads combined as a single phase, and barium sulfate as the second phase provides an adequate result for this test case since the biofilm and bead phases are not directly distinguishable. The most significant obstacles in the segmentation of the demonstration column were (1) beam hardening, and (2) that the barium sulfate in suspension varies in density within the column.

Global thresholding for volume segmentation results in the mislabeling of regions as is demonstrated in the 2-phase isolation presented in Figure 3-2d. Comparison of the gray-scale image (Figure 3-2b) to the 2-phase image (Figure 3-2d) demonstrates that
biofilm attached to the column wall, which is easily discernible visually, is not present in Figure 3-2d. This is due to the two distinct peaks present in the barium sulfate portion of the histogram. This is likely the result of beam hardening, as was described previously. By separating the volume into two regions, an inner core, and an outer ring proper identification and isolation of biofilm was accomplished, as can be seen in Figure 3-2e. Thresholding of the inner and outer ring was accomplished using manual thresholding; a technique that is less than ideal, but necessary due to the variation in voxel intensity (beam hardening artifacts) in the barium sulfate portion of the data set. Direct comparison of the original gray-scale cross-sectional image (Figure 3-2b) to the modified 2-phase segmented image demonstrates good agreement at the conclusion of segmentation. Thus, even though the use of barium sulfate, at the 100% stock concentration, was prone to issues such as beam hardening, reasonable segmentation is achievable.

An approximate reconstruction of the 3 mm diameter plastic beads was generated in order to estimate additional parameters such as initial and final porosity. Evaluation of the spatial distribution of biofilm within the column, and on the surfaces of the plastic beads is also possible. A cross-sectional image of the 3-phase segmentation is provided as Figure 3-2f. Surface reconstructions of (a) the biofilm/bead material, (b) the isolated plastic beads, and (c) the isolated plastic beads overlain by the isolated biofilm are provided in Figure 3-3. Table 3-2 provides a summary of quantitative parameters measured based on the final surface reconstructions.
The surface reconstruction indicates that biofilm growth within this demonstration column is minimal. The biofilm has predominantly attached to the plastic beads and column wall and developed in small, surface coating clusters and is measured to have a volume of 2.5 mm$^3$. Using the reconstructed plastic beads, the initial porosity for the column is 53.6% and the porosity after biofilm growth is 50%, corresponding to a change in column porosity of 3.6%. The average biofilm thickness is 16.8 µm, and the measured interfacial area between the barium sulfate and biofilm is 81.4 mm$^2$. The latter represents the total interfacial area between the biofilm and the pore space available to flow and is therefore relevant to biofilm studies where the reactive surface area is of interest, i.e., in nutrient or contaminant transport research.

The main issues presented by the barium sulfate demonstration column are that (1) as expected, the plastic beads and biofilm have similar x-ray absorption properties and thus are indistinguishable in the CT scan results, and (2) that beam hardening artifacts are significant when using a 100% stock concentration of the barium sulfate suspension. Refinement of the method for use, specifically, with low x-ray absorbance porous media and polychromatic x-ray tomography focused on (1) diluting the barium sulfate to a 50% (0.5 g per mL) concentration in an effort to reduce beam hardening associated with barium sulfate, and (2) addition of potassium iodide (KI) into the contrast agent solution. Experimental results utilizing these modifications are presented in the following section.

### 3.4.2 Porous media packing, 50% BaSO$_4$, with iodide, low and high Re

To address the beam hardening issues associated with using barium sulfate at the stock concentration, as well as to facilitate better contrast between beads and biofilm a
new contrast agent mixture was prepared that consisted of 50% (0.5 g per mL) BaSO₄ and 100 mg per mL KI. The KI in this mixture will diffuse into the biofilm and provide contrast. Two columns were prepared, each at distinctly different flow rates as introduced earlier.

As Davit et al. (2011) demonstrated, the combination of lower barium sulfate concentration and addition of KI to the injected solution aided significantly in **qualitative** delineation of the plastic bead material from the biofilm material in reconstructed volumes. However, examination of the histograms for both the high and low Re columns, shown in Figure 3-4, illustrates that the KI addition can in certain cases be a hindrance to quantitative segmentation. In the gray-scale images in Figure 3-4, bright regions correspond to areas of high barium concentration, black regions are the beads, and the grey regions are biofilm saturated with iodine. In the low Re histogram, the addition of KI to the contrast agent mixture can be seen to produce a distinguishable second peak, while in the high Re histogram, no such second peak exists. Examination of the gray-scale cross-sectional images of each respective column provides some insight into the reasoning for this. Essentially, the low Re data set contains a much greater amount of biofilm, which corresponds to a higher volume in which only KI is present. As the physical amount of biofilm decreases, so does the amount of distinguishable KI. In the high Re experiment, the quantity of biofilm, and thus of diffused KI, is insufficient to produce a second peak in the volume histogram. For both the low and the high Re data sets, visual comparison of threshold boundaries to the corresponding highlighted regions within the data sets was used in order to select the thresholding intensity limits for
material isolation. Manual thresholding is less than ideal due to the fact that the method is inherently subjective. Application of thresholding methods that are more objective such as moment preserving thresholding or factorization failed to isolate appropriate threshold values. One of the main complicating factors for volume thresholding is that KI addition can result in intensity variation within the biofilm regions. Blurring at the transition regions also occurred to varying degrees, an example of which is provided in Figure 3-5. Some of the streaking (shown in Figure 3-5b) occurs when a high concentration of contrast agent is used, in which case a mottled intensity variation expresses in the plastic bead regions of the volume data set. As a result, some overlap in threshold ranges was required for volume segmentation. One technique which is often helpful when imaging solid and fluid materials with similar x-ray absorption properties is collection of a dry image, allowing for isolation of the solid matrix independent of all other materials. The main result of the various beam-hardening effects is that portions of each phase are wrongly assigned. A high contrast gray-scale image that demonstrates the beam-hardening artifacts affecting the plastic bead regions in particular is provided as Figure 3-5b. Figure 3-5c depicts the same image after thresholding to isolate biofilm, and highlights the fact that portions of the plastic bead regions get erroneously assigned as biofilm. In the absence of a dry image, final isolation of the plastic beads was accomplished by generating a gradient magnitude mask which was used to identify the exterior limit to each of the plastic beads, and a blob removal filter and subsequent large kernel erosion/dilation operation was then applied to (a) fill any holes remaining in the plastic bead regions and (b) remove extraneous material not associated with the beads.
Once each of the relevant phases was isolated, the volume was reconstructed as a label field for quantitative analysis. Cross sections of the final segmented volume are included in Figure 3-5d to demonstrate that the segmentation routine outlined herein does, in fact, resolve each of the phases accurately.

As expected, the quantified initial porosity for the low Re and high Re columns are 46.5% and 38.6%. The final porosity for the columns are quite different with values of 18.6% for the low Re volume and 34.4% for the high Re volume. Measures of the total biofilm volume and surface area are reported in Table 3-3 along with the quantified interfacial area and porosity measures. Based on the measured volume and surface area values, the resulting average biofilm thickness for the two volumes is 70.5 µm for the low and 13.0 µm for the high Re volume. Surface reconstructions of the plastic beads, biofilm, and plastic beads overlain with biofilm are provided in Figure 3-6.

As can be seen from the surface reconstructions provided in Figure 3-6, the accumulated biofilm growth varies significantly between the low and high Re experiments. Quantitatively this is most apparent in the variation in final column porosity, as well as the variation in measured biofilm volume between the two columns. Comparison of the average biofilm thicknesses (70.5 µm vs 13.0 µm) indicates a difference in biofilm structure as well. The higher value of average biofilm thickness in the low Re experiment suggests that not only was biofilm able to grow in larger clumps within the column, but pore filling and fluid channeling also occurred. The smaller average biofilm thickness obtained under high Re conditions suggests that biofilm...
formation was forced to be predominantly surface associated, leaving the bulk of the pore channels available for fluid transport. This conclusion is reinforced by the reported interfacial area measurements. The surface area of attachment for the quantified biofilm in the low and high Re volumes (140.4 mm$^2$ and 135.4 mm$^2$) are comparable. Comparison of the biofilm “reactive surface area,” or the interfacial area between the biofilm and pore space available to flow (barium) for the low and high Re volumes (67.0 mm$^2$ and 130.3 mm$^2$) indicates that even though a greater surface area was available for nutrient uptake in the high Re volume, biofilm growth remained surface associated, rather than pore filling. This suggests that hydrodynamic conditions to a large degree controlled biofilm growth and development in the high Re experiment, while they play a lesser role in the low Re experiment.

In addition to the bulk system quantification, segmentation allows for analysis of the spatial distribution of phases within the imaged column section. A direct comparison of axial biofilm volume distribution throughout the column for both the low and high Re columns is provided in Figure 3-7a, and shows that the largest reduction in pore space associated with biofilm growth occurs within the initial 2 mm of the low Re column. The biofilm volume then decreases, in general, with column length. Axial variation in biofilm volume may be associated with growth conditions, such as nutrient limitations and oxygen depletion, however, additional testing and experimentation is required to verify this supposition. Regardless, a direct impact of this variation in biofilm volume distribution is that the pore space available for fluid transport is least in the region near the base of the column, where biofilm volume is greatest. Evaluation of changes in
porosity with length in the low Re column section, provided in Figure 3-7b, shows that from a height of approximately 1.5 to 2.5 mm the column porosity decreases to a minimum of 12.4%, further reinforcing the fact that the increase in biofilm volume near the base of the column acts to constrict fluid flow. Conversely, the measured biofilm volume and associated change in porosity for the high Re column (Figure 3-7c) is relatively uniform along the entire length. As the only variable that has changed between the two columns is the applied flow rate the overall conclusion from this study is that a threshold point has been reached at which point the shear forces imparted on the biofilm become great enough that biofilm accumulation is no longer possible. As a result, the biofilm present in the high Re volumes remains closely associated with solid surfaces. A comparison of the high Re column surface reconstruction (Figure 3-6b) to the demonstration column (with Re =67 and presented earlier in Figure 3-3), shows similar surface associated biofilm growth with average biofilm thicknesses of 16.8 µm for the demonstration column and 13.0 µm for the high Re column. This result suggests that biofilm growth in both columns was limited due to fluid transport and interfacial shear rates. Finally, a direct comparison of the initial Re for all three columns, Re=14 for the low Re column, Re=67 for the demonstration column, and Re=84 for the high Re column, indicates that the threshold point at which fluid velocity and interfacial shear prevent biofilm accumulation is located somewhere between 10 and 67 for a packed bead column growth reactor.
3.5 Conclusions:
The biofilm imaging method presented in Davit et al. (2011) as well as the segmentation methodology and quantitative analysis presented herein focuses on biofilm imaging using conventional, *polychromatic* x-ray tomography. These types of x-ray CT systems exist in many laboratories and facilities and imaging can thus be achieved without the need for traveling to a synchrotron facility. However, as we have shown, there are some challenges to overcome when using polychromatic radiation for biofilm imaging.

Successful quantitative imaging was accomplished using a barium sulfate concentration of 0.5 g per mL, and this is the recommended concentration for future experimentation using polychromatic x-ray CT. The addition of potassium iodide allowed us to successfully differentiate biofilm from both the barium sulfate and the plastic bead solid matrix; however, the resulting data sets were affected by moderate interfacial blurring from beam-hardening, which complicated quantitative segmentation. The ease of quantitative segmentation may be improved by eliminating the KI addition, and focusing on increasing the x-ray absorption of the solid matrix instead by using a denser porous medium, or through the incorporation of a dry scan collected prior to experimentation. Though, one issue that needs to be considered when planning to utilize a dry scan in image processing is the potential for bead movement during the biofilm growth portion of a given experiment. Changes in the porous structure associated with movement can hinder volume alignment and registration.
The methodology that provided the best results for quantitative analysis of the CT data sets is as follows:

(1) Application of a grayscale filter. For all of the data sets presented in this manuscript, a non-local means filter was used to reduce intra-material voxel intensity variations.

(2) Measure the volume histogram.

(3) Identify each peak or region within the histogram and the associated material in the grayscale data set.

(4) Select threshold values for material isolation.

(5) Manually evaluate the resulting binary volumes to assess whether material mislabeling has occurred.

(6) If necessary, isolate and reassign mislabeled regions.
   a. Tools such as the gradient magnitude mask can aid in this task by helping to identify material boundaries which may have been distorted due to issues such as beam hardening.

(7) Once each material has been defined, recombine the materials (solid phase, biofilm, and pore space) into a single label field for quantification.

Quantitative analysis of biofilm volume, spatial distribution of biofilm, change in porosity, and biofilm thickness from three-dimensional CT scans was successfully accomplished using the presented segmentation methodology. The preliminary investigation of the impact of flow rate, or Re, on biofilm formation in packed bead columns indicates that a hydrodynamic threshold exists between the initial Re of 10 and 65 at which point hydrodynamic shear stresses dominate biofilm formation. Additional research is needed to confirm this hypothesis and will be addressed in upcoming research by this group.

The main benefits to using polychromatic x-ray tomography are that (1) they are readily available, and can be purchased and implemented by individual labs, organizations or universities. (2) with these systems, high resolution data sets with pixel resolutions of 5-10 microns per pixel for sample column diameters on the order of 5 mm
can reliably be expected, (3) visualization of experimental specimens can be collected without requiring destructive sampling or optically transparent apparatuses.

The key limitations to using polychromatic x-ray tomography for biofilm imaging are as follows:

1. Beam-hardening and the effects resulting from the non-uniform absorption of x-rays. Often so-called cupping is observed (e.g. Figure 3-2(b and c)) where artificially higher intensities are measured around the perimeter of the object, but as shown here, can also result in spatially varying effects (streaking and blurring) that are more difficult to deal with during quantitative analysis. The most common method for eliminating, or reducing the impact of beam hardening artifacts is to filter x-rays emitted by the source before they pass through the sample specimen using a stainless steel or other metal screen, or by applying software corrections to the data during image processing.

2. Most commercially available systems are limited by the photon flux emitted by the x-ray source, resulting in long exposure times for natural granular materials. Thus, the density of the solid matrix to be imaged requires special consideration. Plastic beads were used as the solid matrix for the presented data sets in order to limit the x-ray exposure time for each scan to approximately 1.5 hrs. The use of a natural porous medium with higher density could potentially increase scan times to 24 hrs or more. As the scan time for an individual sample increases, so too does the potential for biofilm movement or decay, particularly in the prolonged presence of x-rays or biocides such as iodide. An additional reason for minimizing CT scanning time is the fact that since the barium contrast agent consists of suspended particles of BaSO₄, particle settling can be expected within samples and the impact that settling has on volume reconstruction is directly related to the total scan time for each experimental column.

3. As with any CT system, contrast in the resulting reconstructed volume relies, primarily, on variations in density. As a result, each phase to be isolated needs to have a different density, or must include phase-specific contrast agents which aid in differentiating materials based on x-ray absorption. A mixture of two contrast agents (barium sulfate and potassium iodide) was utilized to differentiate open pore space from biofilm, and biofilm from plastic beads. This approach produced results that are quantifiable, though less than ideal because manual segmentation was required. The presence of interfacial and inter-phase blurring within the original gray-scale volumes complicated volume segmentation to the point that each volume needed to be segmented individually, and required varying amounts of post-threshold
processing in order to isolate each individual phase. Implementation of
techniques for reducing beam hardening has the potential to reduce the
dependence on dual contrast agent addition, thereby simplifying phase
segmentation, and potentially facilitating automated, or batch, segmentation
and quantification of collected data sets.

One potential method for improving biofilm imaging is to use synchrotron-based x-
ray CT. Synchrotron light sources produce x-rays with a photon flux that is sufficiently
high that the beam can be filtered to a specific wavelength or range of wave lengths using
a monochromator. The increased photon flux has several distinct advantages over
polychromatic imaging. Firstly, the increased photon flux decreases the required image
acquisition time, and secondly, the monochromatic radiation allows for the detection of
specific elements based on the electron-shell absorption edge for the element. This
specificity enables the use of element specific contrast agents (such as barium, iodine or
silver) and facilitates digital subtraction tomography which would allow for direct
isolation of contrasting phases. We expect that the direct isolation of contrasting phases
will enable more objective, and possibly more automated, thresholding and volume
segmentation of biofilm containing volumes. Adaptation of the barium sulfate imaging
technique for three dimensional quantification of biofilm in situ to opaque porous media
using synchrotron-based x-ray CT is a current focus of research by our group.
3.6 Bibliography


Bibliography (Continued)


Figure 3-1: Scanning electron microscopy (SEM) image of the barium sulfate particles contained within the barium sulfate contrast agent suspension (Image courtesy of James Connolly and Adrienne Phillips, Montana State University).
Figure 3-2: (a) Histogram plot for the barium sulfate demonstration column illustrating the various peaks used for thresholding segmentation. (b) gray-scale orthoslice of the original data, provided for comparison. (c) labeled depiction of the regions of the image to which each histogram peak corresponds. The histogram has been color coded to match the label field images (c-f). (d) global thresholding result (T=77, value determined using a factorization automatic thresholding algorithm) resulting in a binary segmentation of barium (red) and not-barium (blue). (e) The resulting 2-phase segmentation when the beam hardened outer ring is thresholded separately from the inner core, (f) 3-phase segmentation result. The assigned colors represent specific materials as follows: light blue=exterior, dark blue=beads combined with biofilm, with the exception of (f) where dark blue identifies the isolated bead material, red=barium, green=beam hardening artifacts, and yellow=isolated biofilm specifically identified in (f).
Figure 3-3: Surface Reconstructions of the 100% BaSO4 demonstration column including: (a) the segmented non-barium sulfate phase within the trial column, (b) surface reconstruction of the assumed bead location within the column, (c) isolated biofilm (blue) overlaying the segmented beads (yellow) in the trial column.
Figure 3-4: Volume histograms and representative cross-sectional gray-scale slice images for (a) the low Re and (b) high Re biofilm growth reactors.
Figure 3-5: (a) Original gray-scale cross-section of the high Re biofilm growth reactor. (b) High contrast gray-scale axial cross-section of the high Re biofilm growth reactor. The boxed in regions highlight areas of the plastic beads where intensity variations directly impact threshold segmentation. (c) A representative cross section of the biofilm thresholding result. Note the mislabeling of portions of the plastic beads as biofilm. (d) The final segmented volume cross sections where biofilm has been colored green, plastic beads are colored red, the barium sulfate or available pore space is dark blue, and the column exterior is light blue.
Figure 3-6: Surface reconstructions of the plastic bead solid matrix (yellow), biofilm (red), and plastic beads overlain by biofilm for (a) the low Re volume and (b) the high Re volume.
Figure 3-7: Graphical representations of the axial spatial distribution of (a) biofilm volume, and calculated porosity for (b) the low Re column, and (c) the high Re column.
Table 3-1: Summary of column characteristics and relevant parameters for quantitative image analysis.

<table>
<thead>
<tr>
<th></th>
<th>Barium sulfate demonstration column</th>
<th>Low Reynolds number test column</th>
<th>High Reynolds number test column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bead material</td>
<td>Polyamide</td>
<td>Polystyrene</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>Bead diameter</td>
<td>3 mm</td>
<td>0.5 to 1.5 mm</td>
<td>0.5 to 1.5 mm</td>
</tr>
<tr>
<td>Applied flow rate</td>
<td>6 mL per min</td>
<td>4.2 mL per min</td>
<td>30 mL per min</td>
</tr>
<tr>
<td>Estimated initial Reynolds number</td>
<td>67</td>
<td>14</td>
<td>84</td>
</tr>
</tbody>
</table>

Table 3-2: Quantified results for biofilm segmentation using barium sulfate as a contrast agent.

<table>
<thead>
<tr>
<th>Segmented phase</th>
<th>Surface area mm²</th>
<th>Volume mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>barium</td>
<td>175.9</td>
<td>34.3</td>
</tr>
<tr>
<td>beads</td>
<td>76.5</td>
<td>31.8</td>
</tr>
<tr>
<td>biofilm</td>
<td>147.8</td>
<td>2.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inner phase</th>
<th>Outer phase</th>
<th>Interfacial area, mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>beads</td>
<td>barium</td>
<td>9.5</td>
</tr>
<tr>
<td>biofilm</td>
<td>barium</td>
<td>81.4</td>
</tr>
<tr>
<td>biofilm</td>
<td>beads</td>
<td>59.4</td>
</tr>
</tbody>
</table>
Table 3-3: Quantitative summary of results for all three segmented volumes.

<table>
<thead>
<tr>
<th>Volume label</th>
<th>Total volume, mm$^3$</th>
<th>Total surface area, mm$^2$</th>
<th>Measured porosity, %</th>
<th>Interfacial area, mm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Demonstration column</td>
<td>Low Re column</td>
<td>High Re column</td>
<td></td>
</tr>
<tr>
<td>plastic beads</td>
<td>31.8</td>
<td>33.7</td>
<td>52.3</td>
<td></td>
</tr>
<tr>
<td>biofilm</td>
<td>2.5</td>
<td>17.5</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>barium</td>
<td>34.3</td>
<td>11.7</td>
<td>29.3</td>
<td></td>
</tr>
<tr>
<td>plastic beads</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>biofilm</td>
<td>76.5</td>
<td>174.7</td>
<td>256.6</td>
<td></td>
</tr>
<tr>
<td>biofilm</td>
<td>147.8</td>
<td>248.5</td>
<td>277.6</td>
<td></td>
</tr>
<tr>
<td>initial porosity</td>
<td>53.6%</td>
<td>46.5%</td>
<td>38.6</td>
<td></td>
</tr>
<tr>
<td>final porosity</td>
<td>50.0%</td>
<td>18.6%</td>
<td>34.4</td>
<td></td>
</tr>
<tr>
<td>biofilm/barium</td>
<td>81.4</td>
<td>67.0</td>
<td>130.3</td>
<td></td>
</tr>
<tr>
<td>biofilm/beads</td>
<td>59.4</td>
<td>140.4</td>
<td>135.4</td>
<td></td>
</tr>
</tbody>
</table>
4 ON THE DETAILED ANALYSIS OF THREE DIMENSIONAL BIOFILM ARCHITECTURE IN POROUS MEDIA USING SYNCHROTRON BASED X-RAY COMPUTED MICROTMOMOGRAPHY.

Gabriel C. Itis, Yohan Davit, James Connolly, Robin Gerlach, and Dorthe Wildenschild

4.1 Abstract
This communication continues to develop the method, first presented in Davit et al., (2011) for imaging biofilm in porous media using a barium sulfate contrast agent and x-ray computed microtomography (CT). The method has now been modified for imaging biofilm-containing packed bead columns using monochromatic synchrotron-based x-ray CT. Monochromatic CT contributes the added benefit of element specific imaging. First we investigate the useful range of barium sulfate concentrations for monochromatic imaging, followed by an analysis and comparison of available options for the filtering and segmentation of resulting data sets. Recommendations for using barium sulfate as a contrast agent for monochromatic imaging in the future as well as proposed method for data segmentation and quantitative analysis is provided.

The final recommendation for using barium sulfate in monochromatic CT imaging studies is that the contrast agent concentration be limited to a range of 0.33 g per mL to 0.5 g per mL. Our recommendations for quantitative segmentation of resulting data sets include (1) filtering grayscale volumes using a non-local means filter, followed by (2) using digital image subtraction (subtraction of the below edge volume from the above edge volume) to isolate the barium sulfate contrast agent and associated hydraulically
available pore space. (3) Both the resulting subtraction volume and below edge volume are thresholded using a factorization automatic thresholding algorithm, thus isolating (a) the hydraulically available pore space (barium sulfate), and (b) the combined glass beads and the hydraulically available pore space (barium sulfate). (4) Application of a logical-not operator on (b) the thresholded below edge volume isolates (c) a combination of the biofilm present in the columns and the column exterior. (5) Recombination of (a) and (c) isolates (d) the entire volumetric pore space (and column exterior). (6) Logical subtraction of (d) from (b) isolates (e) the glass bead solid phase. (7) Recombination of (c), (e), and (a) results in (f) a segmented representation of the original volume, which then requires application of a cylindrical mask to remove the column exterior, thus resulting in (g) the final quantifiable result.

4.2 Introduction

The investigation of biofilms has been of interest to the scientific community since the phrase was coined in the mid 1970’s to describe communities of microorganisms populating the same area or surface, and joined together by an extracellular polymeric substance (EPS) (Costerton et al., 1995; Hall-Stoodley et al., 2004; Stoodley et al., 2002). Biofilms have been found, and studied, in most habitats and environments. Coincidentally, the presence of biofilms can be harmful, beneficial, or neither -- depending on the application, environment, or research question being asked. For example, biofilm growth on catheters, eye contact lenses, or medical implants can cause serious infections in medical patients and the general populace. Biofilm growth in streams and rivers can alter stream flow mechanics, while similar biofilms in water
transport pipes can facilitate and increase corrosion and decomposition of the pipes. In porous media, biofilms can act beneficially to help remediate contaminated groundwater, in water filtration, or even to aid in soil improvement and stabilization (e.g. Fitch et al., 1998; Mitchell and Santamarina, 2005; Palmeira et al., 2007; Rawlings and Johnson, 2007; Rodriguez and Bishop, 2008)). Biofilms can also induce clogging and reduction in hydraulic conductivity. For a summary of column testing applications and results pertaining to bioclogging in porous media, we refer to Thullner (2010). In general, bioclogging has been seen to result in reductions of hydraulic conductivity on a scale of 2 to 4 orders of magnitude (Cunningham et al., 1991; Vandevivere et al., 1992a, 1992b; Bielefeldt et al. 2004; Hand et al., 2008; Thullner, 2010). Historically, investigations into biofilm growth in porous media have focused on either bulk column testing (3D porous media investigations) or in 2D or pseudo 3D micromodel experiments. Three-dimensional bulk column experimental results typically consist of bulk parameter measurements such as differential pressure and substrate/byproduct measurement and analysis at the inlet and outlet, and perhaps periodically along the length of experimental columns. Unfortunately, direct evaluation of biofilm spatial distribution, structure, and alteration of pore morphology internal to a growth reactor has been a challenging problem, particularly when using opaque porous media. One solution has focused on the sectioning of growth reactors at the conclusion of experiments. Supplemental microscopy of the sections or measurement of parameters such as bulk and dry weight can provide estimates of biofilm mass and volume in each section. However, the similarity in density between water and biofilm makes mass measurements tenuous, and destructive sampling
of column specimens can result in severe modification to the biofilm structure either during sectioning, or simply by adding the resin solution. Recent advances in three-dimensional imaging have been shown to provide quantitative measurements of biofilm and biofilm structure, internal to the porous medium, without requiring destructive sampling of the specimen. Non-destructive, three-dimensional imaging of opaque porous media has been accomplished using Magnetic Resonance Microscopy (MRM), Nuclear Magnetic Resonance imaging (NMR), and x-ray computed microtomography (CT). Several groups have utilized MRM or NRM successfully to examine the gross structural characteristics of biofilm within column growth reactors (Manz et al., 2003; Seymour et al., 2004; Seymour et al., 2007). Unfortunately, these investigations have typically been severely limited in imaging resolution, resolving features in two dimensions at resolutions on the order of 20 to 100 μm, but suffering from dimensional anisotropy and thus resulting in resolutions on the order of mm or cm in the third dimension (Fridjonsson et al., 2011). As a result, magnetic imaging results in largely qualitative indications of growth and alteration of fluid flow and transport internal to the porous medium. However, through the use of CT imaging it is possible to image samples at much higher resolutions than MRM, and the spatial resolution of CT data sets are isotropic, facilitating improved three-dimensional spatial reconstructions for quantitative analysis.

CT systems range in size from bench-top sized scanners to synchrotron particle accelerators. The x-ray sources used can be polychromatic, emitting a wide range of x-ray wavelengths, or monochromatic, the latter being generally limited to synchrotron-based systems because of the limited photon flux in smaller x-ray tube-based systems.
For a detailed description and review of CT equipment and techniques, we refer to Wildenschild and Sheppard (2013). The imaging of biofilms in porous media using x-ray CT has been hindered for two reasons. Firstly, imaging biofilm in an aqueous porous media environment using CT suffers the complication that biofilm and water have very similar densities and x-ray absorption properties; moreover, one is attempting to quantify a very soft substance (biofilm and water) embedded in a much denser matrix (the porous medium). This predicament requires creative use of contrast agents, which brings us to the second reason, which is that traditional contrast agents such as potassium iodide or cesium chloride diffuse readily into both the aqueous phase and biofilm.

Two novel contrast agents have been proposed, thus far, in the literature as ways to resolve the difficulty associated with differentiating biofilm from the aqueous phase in a porous matrix. Iltis et al. (2011) implemented a biofilm surface coating technique using silver coated, neutrally buoyant, hollow, glass microspheres whereby synchrotron based x-ray CT was utilized to identify the silver-coated biofilm surface. By connecting the silver microspheres, which coat the biofilm, with a triangulated mesh, biofilm was reconstructed and quantitative measures such as biofilm volume, surface area and change in porosity of the porous medium were calculated. The main limitations to this method include that (1) the size of the structural details which can be identified ends up being limited by the particle size of the silver microspheres rather than the actual image resolution, (2) the method is capable of identifying the biofilm/aqueous phase interface but doesn’t conclusively indicate which side of the interface corresponds to biofilm, and (3) the triangulated mesh reconstruction requires thorough silver coverage of the biofilm.
throughout the imaged region and continuous connectivity. The combination of these limitations makes the silver microsphere particularly useful for examining particle transport through biofilm-containing columns, though potentially less reliable for quantifying parameters such as biofilm volume and change in porosity. However, Davit et al. (2011) implemented an aqueous phase-filling technique using a barium sulfate suspension to demonstrate, qualitatively, that biofilm can be differentiated from the aqueous phase. Image acquisition for the Davit et al. (2011) study was accomplished using a conventional, polychromatic, bench-top CT scanner. Use of the polychromatic scanner presented several issues, including (1) the length of time required for scanning of column specimens, and (2) the prevalence of artifacts in the resulting data sets attributed to phenomena such as beam hardening which occurs as the lower (softer) energy range of the emitted polychromatic x-ray spectrum is absorbed, resulting in an overall hardening effect on the energy spectrum.

In order to keep scanning times on the order of one to two hours, plastic (polyamide and polyacrylamide) beads were used as the porous medium due to the low attenuation coefficient of these materials. Initial scans performed on biofilm-containing column reactors, using only the barium sulfate suspension resulted in image data sets where the hydraulically available pore space was clearly delineated by the barium sulfate, but the biofilm and plastic beads were not directly distinguishable, owing to their similar x-ray absorption properties. To counter this, potassium iodide was added to the barium sulfate solution resulting in a dual contrast agent system where the potassium iodide diffused readily into the biofilm, increasing the x-ray absorption capacity of the biofilm and
allowing for delineation of biofilm and plastic beads in the column reactor. However, column samples containing barium sulfate or barium sulfate and potassium iodide suffered significant blurring at the transition regions between phases due to beam hardening. The results presented in Davit et al. (2011) were therefore of a largely qualitative nature. However, through the use of a fairly extensive image processing effort reliable quantitative analysis of these initial scans was shown to be possible, the results of which were presented in Chapter 3. The most cumbersome, and crucial, aspect of the quantitative analysis was the selection of appropriate threshold values for material isolation and quantification. Manual thresholding of the data sets was required in order to isolate the barium sulfate, biofilm and plastic bead solid matrix. This subjective approach to thresholding was required due to a combination of variability in material intensity values between data sets and beam hardening artifacts which blurred transition regions between materials. In addition to manual thresholding being required, individual approaches to isolating and reassigning mislabeled voxels were required prior to quantitative analysis in order to verify and ensure that the final quantitative results were as accurate as possible. The inherently subjective nature of the segmentation and quantitative analysis leaves much room for method improvement. The main message from both Davit et al. (2011) and the quantitative analysis presented in Chapter 3 is that biofilm investigations using polychromatic sources and a barium sulfate contrast agent are possible, but that the method might be more optimally implemented using synchrotron-based CT. The application of synchrotron-based x-ray CT has three main objectives. The first objective is to allow for rapid scanning of more natural or realistic
porous media. The second objective is to reduce or eliminate the beam hardening artifacts that were prominent in the polychromatic data sets, and the third objective is to develop an objective segmentation routine for quantitative analysis of the resulting data sets.

The main benefits to incorporating synchrotron-based CT into biofilm imaging studies using the barium sulfate contrast agent include the fact that synchrotron light sources produce x-rays with a sufficient photon flux that the beam can be filtered to a specific wavelength or range of wavelengths using a monochromator. The monochromatization allows for the detection of specific elements based on the K-shell absorption edge for the element, and thus the use of element specific contrast agents. This aspect of monochromatic CT imaging facilitates digital subtraction tomography which is important for simplifying segmentation. In addition, the increased photon flux decreases the required image acquisition time from hours to minutes, even for dense porous media. Thus, synchrotron-based CT imaging enables both rapid image acquisition in more natural, opaque, porous media and enables dual energy scanning immediately above and below the absorption edge for barium sulfate, thereby enabling the isolation of barium without the need for an additional contrast agent (potassium iodide).

While dual edge scanning can aid in the isolation of distinct materials, image processing and quantitative analysis remains nontrivial. The three main variables which need to be considered in developing a more objective method for biofilm quantification are (1) identification of consistent, reliable, barium sulfate concentrations to be used for imaging, (2) the selection of an initial grayscale filter can aid in reducing voxel intensity variations within materials, a result that can aid in volume segmentation, however,
application of an inappropriate grayscale filter can also blur or eliminate boundaries between materials. As a result, care must be taken in selecting a grayscale filter since numerous options for filtering have been presented in the literature and incorporated into image processing software packages. The third variable is the selection and application of an objective method for threshold value selection. Again, numerous strategies and algorithms have been proposed and implemented in both the literature and in image processing software packages. Identification of an optimal, objective, method for threshold value determination should be based on a given method’s ability to consistently identify appropriate threshold values for material isolation and quantification.

Thus, as a natural step forward we have used barium sulfate with synchrotron-based CT and the results of this advance are presented in this communication via (1) an investigation of the effective concentration range for the barium sulfate contrast agent when using monochromatic synchrotron-based CT, which is presented in Section 4.4, (2) by further refining the method for application of barium sulfate to experimental columns and addressing potential issues associated with CT scanning using this contrast agent, (3) by evaluating various image processing approaches, including grayscale filtering (addressed in Section 4.5) and threshold value selection methods (found in Section 4.6), that allow for objective quantitative analyses and, potentially, batch, or automated volume segmentation, and (4) by presenting the first quantitative measures of biofilm obtained using this technique.
4.3 Experimental methods

The presented experimental methods cover two separate imaging sessions at Sector 13 BMD, GSECARS, Argonne National Laboratory, where two separate types of biofilm were evaluated. The experiments were conducted under roughly equivalent conditions and additional details are provided below on both the physical experimental methods, and the variables assessed in image processing.

4.3.1 Growth reactor design:

Cylindrical flow cells were constructed of polycarbonate tubing (mean inner diameter of 6.3 mm and a length of either 25 mm, for the *S. oneidensis* trial, or 65 mm, for the *E. coli* trial. The flow cells were packed with soda-lime silica glass beads (particle size range: 1.4 to 1.7 mm) to achieve an average initial porosity of 42%. Prior to inoculation, all columns and tubing were sterilized using a 90% ethanol flush for a minimum of 10 minutes. After the ethanol flush, growth media flow was initiated as the final step in preparing the columns for experimentation. Growth media was flushed through the columns for a minimum of 30 minutes prior to inoculation in order to ensure that no ethanol remained in the growth reactor columns at the time of inoculation.

4.3.2 Microbial species:

Two distinct species of bacteria, known to form biofilm, were used for experimentation. The species used are *Escherichia coli* K-12, MG1655 AF504 *gfp*, pOX38km, and *Shewanella oneidensis* MR-1. *E. coli* K-12, MG1655 AF504 *gfp*, pOX38km is resistant to ampicillin and is capable of producing a biofilm similar to that of *Pseudomonas aeruginosa* (Folkesson et al., 2008). *S. oneidensis* MR-1 is a gram-negative, facultative anaerobe capable of biofilm formation, and known for its iron-
reducing potential. For additional description of *S. oneidensis* we refer to Iltis et al. (2011), Majors et al. (2005), and Venkatesweran et al. (1999). After inoculation, columns sat stagnant for between 4 hours (*E. coli*) and 24 hrs (*S. oneidensis*) to enable preliminary attachment to the porous medium. Flow was induced at a rate of between 40 and 45 mL hr⁻¹ for the duration of the 11 to 12 day column growth period. At the conclusion of the growth phase, columns were disconnected from the pump and prepped for contrast agent addition.

### 4.3.3 Contrast agent:

The contrast agent utilized in the presented investigations consists of a commercially available, medical-grade barium sulfate suspension (Micropaque, Guerbet) with a stock concentration of 1.0 g per mL barium sulfate. Barium sulfate is insoluble in water and the stock product contains barium sulfate particles on the order of 1 µm in size. As discussed in Davit et al. (2011), medical grade contrast agents are designed to have characteristics such as high density to facilitate x-ray contrast, and low viscosity to enable the suspension medium to move freely into, and around, objects to be imaged. Moreover, formulations focus on maintaining a largely uniform particle size, as well as on preventing flocculation and particle settling. In order to assess the impact of barium concentration on the quality of the final CT product, barium concentrations ranging from approximately 0.25 g per mL (25%) to 0.5 g per mL (50%) were evaluated in control columns. Fractional barium concentrations were mixed by diluting barium sulfate suspension stock using growth media appropriate for each column, specific to the
microbial species being evaluated. Results from the barium sulfate concentration investigation are provided in Section 4.4.

In order to prevent biofilm detachment and flushing during the injection of barium sulfate due to density differences, injection flow rates were decreased significantly from the experimental flow rates under which biofilm growth occurred to an injection rate of 1 mL per hr. Injection at this flow rate resulted in little to no biofilm detachment in the experimental systems evaluated. In total, approximately 4 pore volume of the contrast agent was added to each column prior to CT imaging.

4.3.4 X-ray computed microtomography
Synchrotron-based x-ray computed microtomographic (CT) imaging was conducted at Beamline 13-BMD, GeoSoilEnviroCARS (GSECARS), at the Advanced Photon Source (APS), Argonne National Laboratory. CT image data sets were collected at x-ray energies slightly above and below the K-shell absorption edge for barium (37.44 keV±0.5) to allow for digital subtraction of the above and below scans, which aids in segmentation of the element-specific contrast agent. Volume reconstruction was completed using software developed by Rivers (2010) for use with Interactive Data Language (IDL). The final 16-bit, grayscale imaged volumes have dimensions of 695x695x520 with isotropic pixel resolutions ranging from 10.5 to 11.3 μm per pixel. Image data sets were acquired using 2x2 binning in order to reduce both scanning time and total volume size. The individual scan time for the imaging system was approximately 5 minutes, thus scanning each column section, both above and below the barium edge, took approximately 10 minutes (2 scans total). One of the key advantages to
this method of imaging is the ability to scan multiple column sections successively, thus enabling image acquisition for entire column growth reactors. However, for the purpose of this manuscript we will be evaluating and comparing individual sections from each of the demonstration columns.

4.3.5 Image processing

For the purposes of developing an optimal segmentation method and image processing routine, variables including selection of an initial grayscale volume filtering method, and threshold selection (thresholding) method are evaluated. The objective of initial grayscale filtering is to reduce intra-material voxel intensity variation (noise) while maintaining, or maximizing, inter-material voxel intensity differences. A total of three grayscale filtering cases are evaluated in this manuscript including (1) the original reconstructed raw data, (2) application of a median filter, and (3) application of a non-local means filter. Results from the comparison of grayscale filtering methods are provided in Section 4.5.

A total of five thresholding methods have been implemented and compared to original grayscale data sets. Each of these methods are discussed and the results from a comparative analysis of the evaluated thresholding methods are provided in Section 4.6. Evaluation of the thresholding methods is based upon a comparison of selected threshold points in reference to the volume histograms. All of the additional steps required in the segmentation and quantitative analysis of the presented data sets are presented in Section 4.7. All image processing was completed using the commercially available software package Avizo Fire.
4.4 Barium sulfate concentration - sensitivity analysis results

The quality of CT imaging results is highly dependent on a number of factors including distinguishable differences in density between phases in an experimental sample, the concentration of contrast agents used to identify particular phases, and lack of movement internal to an imaging specimen during tomographic scanning. The initial step in characterizing barium sulfate suspensions as a contrast agent for imaging biofilm, using synchrotron-based x-ray CT, was to identify the useful range of barium sulfate mixture concentrations that are capable of resulting in reasonable segmentation of reconstructed volume data sets. Saturated, abiotic, bead packed control columns were used in this analysis. Barium sulfate mixtures with concentrations of 25%, 33%, and 50% were added to separate control columns and Above Edge (AE) and Below Edge (BE) data sets were collected for each control column. Representative cross-sections for each concentration, along with the corresponding volume histograms are provided in Figure 4-1.

A direct, quantitative and visual comparison of how barium sulfate concentration changes impact both the AE and BE volumes is presented in Figure 4-1. This comparison of the measured histograms for each data set, along with the illustrative cross-sectional images of each data set helps to illustrate several key characteristics in the raw data. The AE histograms in Figure 4-1 present three distinct peaks regardless of contrast agent concentration. The lowest intensity peak corresponds to the exterior, the column wall, as well as to the biofilm or air that has grown or been entrapped inside the sample columns. The middle peak typically corresponds to the glass bead/solid phase and the highest peak is attributed to the bulk of the barium sulfate contrast agent. Comparison of the AE
histograms in Figure 4-1 demonstrates that as the barium sulfate concentration increases the separation of the middle and highest intensity peaks increases. The BE scans generate histograms which are distinctly different from the AE histograms. The BE histograms typically contain two peaks with the first peak consisting of pixels associated with (a) regions exterior to the sample column, (b) the plastic column wall, (c) entrapped air, or (d) biofilm and the second peak containing a combination of the glass bead porous medium and the barium sulfate suspension. The most notable difference between the AE and BE histograms is the fact that even as the barium concentration range is varied from 25% to 50% the changes between the BE histograms are marginal, while the separation of the middle and high intensity peaks in the AE histograms vary significantly.

In both the AE and BE histogram comparisons the 25% barium concentration presents problematic characteristics. In the AE histogram the barium and glass bead phase peaks begin to blend together, as can be seen by the increasing minima in the valley between the middle and high intensity peaks. This merging of peaks is undesirable, and can result in the mislabeling of regions during volume thresholding and segmentation, if histogram-based thresholding of the AE data set were to be used to isolate barium from the glass bead phase. This mislabeling occurs because portions of both the glass bead phase and the barium sulfate phase have equivalent absorption intensities at this concentration. In the BE histogram, a concentration of 25% barium sulfate solution causes the photon absorption of the barium sulfate solution to be less than the glass beads. Visually, the glass beads appear as higher intensity regions in Figure 4-1f, than the barium sulfate filled pore space. This is likely due to the glass beads having
a higher physical density than the barium sulfate solution at this concentration. Quantitatively this expresses in the 25% barium BE histogram as an additional peak in the histogram, with the middle peak now being associated with the barium sulfate associated pore space. In addition, the 25% barium concentration AE and BE cross sections (Figure 4-1c and Figure 4-1f) show bright rings surrounding several of the glass beads in both images. At a concentration of 25% barium sulfate the concentration of barium sulfate particles in solution appears to have crossed a threshold so that particles begin settling out of the open pore space and on to available bead surfaces. Gravitational particle settling has the potential to (1) cause problematic intensity gradients inside the pore space and (2) particle movement during sample scanning inhibits volume reconstruction, registration, as well as the final quantitative analysis of the samples. As a result, the 25% (0.25 g per mL) barium sulfate concentration is not recommended for quantitative analysis of biofilm in porous media using synchrotron-based x-ray CT.

At barium sulfate concentrations at or above 33% distinct separation of the middle and high intensity peaks exists in the AE histograms, and the BE histogram differentiates into two distinct peaks. At this concentration the absorption constant for the barium sulfate solution is approximately equal to that of the glass beads and the two phases become almost indistinguishable (Figure 4-1e). This suggests that the minimum usable concentration of barium sulfate is approximately 33%, or 0.33 g per mL. A contrast agent concentration of 50% begins to broaden the high intensity histogram peak indicating that the barium sulfate (pore space) absorption is higher than the absorption of the glass beads. Visually the 50% barium concentration begins to show up as the brightest phase in
the BE scans (Figure 4-1d). At this point, sample columns begin to run the risk of being too dense for rapid scanning, and careful attention needs to be paid to the photon count passing through column specimens, as the density is reaching the point that reconstruction quality may begin to be negatively impacted. Increasing the barium sulfate concentration beyond 50% is not advised, especially when sampling experimental columns that are 6 to 7 mm in diameter.

One issue that was previously identified in Chapter 3 is the potential for particle settling to cause concentration gradients within the injected barium sulfate phase, regardless of the initially injected concentration. One major benefit to using monochromatic, dual edge scanning is the ability to specifically isolate barium sulfate, on a voxel-by-voxel basis, based on the change in x-ray absorption for each voxel between the AE and BE data sets. This change in x-ray absorption, and by association, voxel intensity is evaluated by implementing digital image subtraction where the intensity of each voxel in the BE volume is subtracted from the corresponding voxel in the AE volume, resulting in the creation of the Subtraction Volume (SV). Isolation of the contrast agent using this method helps to prevent the mislabeling of voxels which can potentially occur if global thresholding of the the AE or BE volumes were used as the sole method for isolating each phase within the volumetric data sets.

Summary histograms for the resulting control column SV data sets are provided in Figure 4-2, from which we see that increases in the barium sulfate concentration translates directly into increased separation between the two peaks present in the histograms. Broad separation of peaks in the SV data set allows for binary segmentation
of (1) barium sulfate and (2) not barium sulfate, thus enabling the isolation of the pore space internal to the scanned column specimens. While the control column SV histograms all appear to contain two distinct peaks for barium isolation, comparison of the SV histograms to the cross-sectional images of each column (Figure 4-2a-c) demonstrates that the distribution of barium sulfate is poor and non-uniform in the 25% SV control column. In addition to the control columns, a set of columns containing biofilm were imaged at each of the evaluated concentrations and SV histograms and cross-sectional images are also provided in Figure 4-2(d-f). For all three barium concentrations evaluated, the histogram peak corresponding to barium sulfate is becomes less pronounced when biofilm exists in the column pore space. In biofilm-containing volumes, a concentration of 25% barium sulfate results in an SV histogram with little to no distinguishable barium sulfate peak. Visually (Figure 4-2f), the 25% column cross section can be seen to suffer from severe concentration gradients, due to settling, making the volume less than optimal for quantitative segmentation. At a concentration of 33% barium sulfate a measureable peak is present in the volume histogram. The cross section at 33% shows some evidence of concentration variation, suggesting some clumping or settling may have occurred, but in general, the barium concentration in the pore space is uniformly distinguishable (Figure 4-2e). Use of a 50% barium sulfate suspension generates the most easily distinguishable histogram peak, as well as the most uniformly distributed concentration in the pore space (Figure 4-2d).
In order to optimize the use of barium sulfate as a contrast agent the separation of peaks in both the AE and BE histograms needs to be maximized while, at the same time, avoiding making sample specimens too absorptive in the AE case. In addition, the barium concentration must be high enough that settling and variation in concentration and density are minimized or avoided all together. Keeping these factors in mind, the recommended concentration range for using barium sulfate suspensions as a contrast agent is 33% (0.33 g per mL) to 50% (0.5 g per mL).

4.5 Grayscale filtering

The incorporation and application of grayscale image filtering aids in minimizing voxel intensity variations (noise) within specific materials. In addition, grayscale filtering helps to maximize voxel intensity differences between materials. A total of three test cases were used in the evaluation of grayscale filtering options for the optimization of the segmentation routine for quantitative analysis of biofilm-containing x-ray CT data sets. The three test cases include (1) the original grayscale data, (2) application of a median filter, and (3) application of a non-local means filter.

The median filter applies a 3x3x3 kernel to the grayscale volume and for each voxel the median intensity value for the kernel cube is applied to the central voxel. This operation is performed throughout the image volume, and only original voxel intensities are used to generate the new filtered volume. This method is described in more detail by Russ (2011) and Huang, Yang, and Tang (1979) and has become a standard filtering option due to the fact that median filtering is capable of removing large amounts of noise from a data set without succumbing to intensity shifts which might occur if a simple
averaging approach were to be taken. Moreover, edge blurring is only marginally impacted. The more recent non-local means filter applies non-local averaging of all voxels in an image data set using the algorithm defined by Buades et al. (2005) as

\[ NL[u](x) = \frac{1}{C(x)} \int_{\Omega} e^{-\frac{(G_a * |u(x+) - u(y+)|^2)}{h^2}u(y)}dy \]

Equation 5

where \( x \) is an element of \( \Omega \), \( C(x) = \int_{\Omega} e^{-\frac{(G_a * |u(x+) - u(z+)|^2)}{h^2}u(y)}dz \) serves as a normalizing constant, \( G_a \) is a Gaussian kernel, and \( h \) is the filtering parameter. In essence, the non-local means filter identifies all of the regions within a data set that have equivalent Gaussian neighborhoods to the element being examined (\( x \)) and determines the denoised value of \( x \) as the average value of all of the values with equivalent Gaussian neighborhoods. The main difference between non-local means filtering and local filtering operations is the incorporation and inclusion of all equivalent regions in each point operation (Buades et al., 2005). According to Buades et al. (2005), the universal inclusion of all possible self-predictions reduces the potential for local skewness associated with variations in noise within an image. An evaluation of each of the filtering methods, after application to CT data sets, is provided below along with a comparison of each of the methods to original, raw, data as well as to each other. The objective of this comparison is to select a grayscale filter for general implementation in the a standardized segmentation routine which aids in reducing noise that would otherwise result in the mislabeling of voxels during volume segmentation.
4.5.1 Grayscale filtering Results

Initial filtering of grayscale data can aid in reducing noise, or speckling, in data sets that can skew final quantification and segmentation quality significantly. Evaluation of the filtered volume histograms is one key method for investigating the impact that grayscale filtering can have on image quality and segmentation. Figure 4-3 depicts the volume histograms for a single, biofilm-containing, column section imaged using a 50% barium sulfate suspension.

Review of the histogram plots for the single volume, before and after application of the relevant filtering algorithm demonstrates that initial grayscale filtering results in a marked improvement in the separation of peaks in both the AE and BE volume histograms. The increase in maximum pixel count combined with the decrease in peak width indicates that filtering has, in fact, removed noise or erratic fluctuations within the grayscale material phases. Based on the histogram representations of the filtered data sets, very little difference can be seen between the median filtered data and the non-local means filtered data. In fact, the histograms for both filtering methods overlay each other with only minor differences. Figure 4-4 demonstrates the visual impact that initial grayscale filtering has on image quality. Comparison of the grayscale image cross-sections (Figure 4-4a to Figure 4-4c) to the median filtering results (Figure 4-4d to Figure 4-4f) shows that local variability (noise) within the respective regions (barium, biofilm, or glass beads) decreases, however, transitional regions between phases suffer from blurring, and regions containing small amounts of biofilm blur to the point that they can potentially be mislabeled as portions of the glass bead phase. Incorporating the non-
local means filtered data into the comparison shows that, while the median filtered and non-local means filtered data sets have histograms that are very similar, the fine regions (e.g. thin sections of biofilm, in between glass beads and barium sulfate) remain distinct in the non-local means filtered data set, while in the median filtered image the thin biofilm section has been blurred into the nearest glass bead. Extending the comparison to the final segmented result, we see some variation between all three of the presented sets of data. In the median filtered volume cross-section we see that some of the biofilm in the cross-section has been assigned to be part of the glass bead (upper left hand corner), and that isolated regions of biofilm (three examples on the right hand side of the images) are reduced in size, indicating that median filtering may underestimate the amount of biofilm present within column sections. Comparison of the segmented raw data set (Figure 4-4c) to the segmented non-local means data set (Figure 4-4i) shows good agreement, based on a visual comparison. Additional quantitative comparisons are presented in the thresholding discussion (Section 4.6), however, application of the non-local means filter to the biofilm-containing CT data sets results in quality homogenization within material regions, while maintaining fine details and boundaries between materials. As a result, the non-local means filter is the preferred grayscale filter for preparing CT data sets for segmentation.

4.6 Thresholding

Image thresholding is a segmentation tool used to isolate objects of interest in an image data set based on characteristic intensity values associated with the objects of interest. In essence, all pixels (or voxels) above a selected intensity value are assigned a
value of one, and all pixels below the selected intensity value are assigned a value of zero. The threshold ranges and values are often selected on the basis of histogram analysis or other such quantitative evaluation of the image data set. Thresholding and subsequent segmentation is arguably the most crucial step in quantitative image analysis. The ideal goal is to objectively determine thresholding limits for the isolation of each phase of interest; however, this step often ends up being subjective in nature and therefore dependent on the visual perception of the person performing the task.

Numerous methods and algorithms are available for volume thresholding, and selection of a thresholding method often depends on the data, and the shape of the associated histograms.

Dual energy CT image acquisition results in two distinct data sets: one collected above the K-shell absorption edge for barium (the AE volume), the other collected below the K-shell absorption edge for barium (the BE volume). As mentioned previously, a third data set is obtained through digital image subtraction referred to as the subtraction volume (SV). Segmentation of the biofilm containing data sets requires isolation of three phases, a biofilm phase, a solid matrix phase, and an aqueous phase. In order to isolate all three of these phases, two threshold limits are required. The first applies to the SV in order to isolate the barium sulfate, or hydraulically available pore space, and the second threshold value applies to the BE volume resulting in a binary volume containing a combination of the solid matrix (glass bead) phase and the barium sulfate. Several additional steps are required in order to generate the final, quantifiable, segmented
volume, however, the threshold selection for these two data sets acts as the first step in
the segmentation process.

Several objective thresholding methods have been evaluated for comparison based on
(a) the location of the threshold within the histogram, and (b) variation in the resulting
calculated volume, quantified using the generated binary data sets. The thresholding
methods evaluated include (1) moment-preserving thresholding, (2) factorization
thresholding using the Otsu method (Otsu, 1979), and (3) entropic thresholding, as
presented by Pun (1981) and refined by Kapur et al. (1985). In addition, threshold
determination using (4) histogram 1st and 2nd derivatives, and (5) an alternate 1st
derivative method termed the “slope-hinge” method are evaluated as easily accessible
thresholding options, that can be implemented using a simple spreadsheet software
package. Each of these methods are described in more detail below and the thresholding
results are compared using two 50% barium sulfate data sets and two 33% barium sulfate
data sets. Each pair of concentration volumes contains one abiotic control and one
biofilm containing volume. Comparative results are provided in Section 4.6.1.

(1) Moment preserving thresholding

In general, moment preserving threshold values are isolated, as detailed by Tsai
(1985), by determining the moments for an image data set using the following equation,

\[ m_j = \sum_{l=0}^{n} p_l [z_l]^j \]  

Equation 6
where \( m_j \) is the \( j \)th moment for the data set, \( z_i \) is the grayscale value for the pixel being examined, and \( p_i \) corresponds to the probability of occurrence for the grayscale level \( z_i \). The probability of occurrence is defined as,

\[
p_i = \frac{n_i}{n}
\]

Equation 7

where \( n \) is the total number of pixels, or voxels, in the image data set, and \( n_i \) is the total number of pixels in the data set with a grayscale value of \( z_i \). The ideal threshold value for an image data set is acquired by identifying a threshold value for which the first three moments of the final binarized image equal the first three moments of the grayscale original. Additional details on the formulation, refinement, and application of this method of thresholding can be found in Tsai (1985). In previous comparisons of thresholding methods, Sahoo et al. (1988) found that moment preserving thresholding is most applicable to data sets containing a bimodal histogram distribution, and even performs well when the bimodal distribution of the histogram is uneven. However, the method falters when the histogram distribution is unimodal in nature.

(2) **Factorisation thresholding**

The factorization thresholding method was first presented by Otsu (1979). As a summary of the method, factorization thresholding estimates the variance for two classes (\( C_0 \) and \( C_1 \)) of voxels within a specified bounding range. The two classes correspond to pixels belonging to the background (\( C_0 \)) and the material of interest (\( C_1 \)). The point of differentiation between the two classes corresponds to the selected thresholding value, \( t \).
Using the same probability of occurrence \((p_i)\) as was defined in the Moment thresholding discussion above, the probabilities of class occurrence \((w_i)\) are defined as

\[
\begin{align*}
    w_0 &= \sum_{i=I_{\text{min}}}^{t} p_i \\
    w_1 &= \sum_{i=t+1}^{I_{\text{max}}} p_i
\end{align*}
\]  

Equation 8

where \(I_{\text{min}}\) and \(I_{\text{max}}\) are the minimum and maximum intensity values for a given data set.

The class mean levels \((\mu)\) are then defined as

\[
\begin{align*}
    \mu_0 &= \frac{\sum_{i=I_{\text{min}}}^{t} ip_i}{w_0} \\
    \mu_1 &= \frac{\sum_{i=t+1}^{I_{\text{max}}} ip_i}{w_1}
\end{align*}
\]  

Equation 10

and

\[
\begin{align*}
    \mu_0 &= \sum_{i=I_{\text{min}}}^{t} \frac{ip_i}{w_0} \\
    \mu_1 &= \sum_{i=t+1}^{I_{\text{max}}} \frac{ip_i}{w_1}
\end{align*}
\]  

Equation 11

and the between class variance is defined as

\[
\sigma_B^2[\tau] = w_0[\tau] * w_1[\tau] * (\mu_0[\tau] - \mu_1[\tau])^2
\]  

Equation 12

The threshold value \((\tau)\) is selected so that the between class variance is maximized.

Historically, the factorization method has been found to perform well on data sets containing a bimodal histogram. However, several groups have found that the factorization method performs well for data sets containing distinctly unequal biomodal histogram distributions, and even unimodal histogram distributions (Sahoo et al, 1988; Gonzales-Barron and Butler, 2006). According to Sezgin and Sankur (2004) this method of threshold selection performs well when the number of pixels assigned to each class is comparable. In an investigation directly comparing thresholding methods Sezgin and Sankur (2004) found that factorization thresholding ended up being one of the top 10 most reliable thresholding methods for identifying flaws in non-destructive testing image
data sets. One of the main critiques of the method is that threshold value selection can, potentially, be computationally expensive, particularly in multithresholding scenarios (Lee and Park, 1995). However, each of the applications presented in this manuscript seek singular global thresholds for both the BE and SV data sets.

(3) Entropic thresholding

Entropic thresholding allows you to measure the disorder of the system between user specified bounding ranges. A number of variations on entropic thresholding have been presented in the literature (e.g. Lan and Zeng, 2013 and Yimit et al., 2012), however, our application focuses on the method presented by Pun (1981), and improved upon by Kapur et al. (1985). Entropic thresholding focuses on isolating, and minimizing the sum of, two partial entropies: above and below the threshold level and defined as,

\[
H_{\text{Below}}[t] = - \sum_{i \leq t} p_1[i] \log_2(p_1[i])
\]

\[
H_{\text{Above}}[t] = - \sum_{i > t} p_2[i] \log_2(p_2[i])
\]

where \(p[i]\) is the probability of occurrence of grayscale value \(i\) in the bounding range defined by the summation, and the final threshold value corresponds to the threshold value \(t\) at which the summation of \(H_{\text{Above}}\) and \(H_{\text{Below}}\) are at a minimum. As Kapur et al. (1985) states, the methods for entropic thresholding have been designed specifically to address situations where the grayscale histogram for a given data set is not bimodal in nature. Results from a comparison of available thresholding techniques conducted by Sahoo et al. (1988) agreed with Kapur et al. (1985) finding that the entropic thresholding
methods perform best, and are most applicable, when histogram distributions are not bimodal.

(4) Thresholding using first derivative analysis

Threshold selection using a first derivative analysis inherently requires a bimodally distributed histogram. This method focuses on finding the grayscale value corresponding to the minimum pixel count in the “valley” between two peaks. Isolation of these valley minima requires analysis of both the first derivative and the change in sign of the first derivative for the image data set being evaluated; a determination which can be achieved using the 2\textsuperscript{nd} derivative of the histogram. The first derivative is generally implemented as

\[ H'(z) = \frac{dp_c(z)}{dz} \]

Equation 15

where \( p_c \) is the pixel count at the grayscale value \( z \). Isolation of the histogram maxima and minima requires examination of the changes in sign of the first derivative function. A change in sign (slope) from positive to negative indicates a maximum, or peak, while a change from negative to positive corresponds to a minimum, and the latter are the values used in thresholding the image data sets. There are several key limitations to using first derivative analysis to isolate threshold values. Firstly, this method requires that a given data set have a bimodal distribution. If two or more peaks do not exist in the histogram, then a first derivative threshold point cannot be determined. Secondly, the method is highly susceptible to error associated with noise in the histogram such as nominal variation and fluctuation in pixel count values. This issue can be addressed by varying the
size of \( dz \), as well as by bounding the region of interest using the isolated peak values of
the histogram. This method also suffers when valleys are broad and flat.

**5) Slope-hinge threshold determination**

The slope-hinge thresholding method is a variation on the first derivative method and
was developed specifically for thresholding of image data sets that contain a unimodal
histogram distribution, those data sets which have broad and flat valleys between peaks,
and/or data sets containing extremely disproportionate peaks.

This thresholding method is termed “slope-hinge” because it focuses on the measured
difference between the average slope of the points leading \( a_{\text{leading}} \) the point being
examined, and the average slope of the points trailing \( a_{\text{trailing}} \) the point being examined.
In general this takes the form of:

\[
\begin{align*}
    a_{\text{Trailing}} &= \frac{\sum_{i=L}^{i-1} H'(z_i)}{L} \\
    a_{\text{Leading}} &= \frac{\sum_{i=L}^{i+L} H'(z_i)}{L}
\end{align*}
\]

where \( z_i \) corresponds to the grayscale value being examined, \( H'(z_i) \) is the slope of the
histogram at grayscale intensity value \( z_i \), and \( L \) corresponds to the arm length, or number
of grayscale values being included in the individual point analysis. The slope-hinge value
for each grayscale intensity \( sH \) then equals

\[
sH_i = a_{\text{Trailing}} - a_{\text{Leading}}
\]

The first derivative of the slope-hinge data set allows for examination of the rate of
change in the calculated slope-hinge data sets. Threshold selection based on changes in
the sign of $s_H$ enables the identification of the potential threshold values corresponding to
changes in histogram curvature without requiring the presence of a valley feature.

Utilization of the slope-hinge method for binary segmentation of data sets with poor
contrast, i.e., that contain only a single histogram peak enables threshold selection near
the base of the histogram peak, thus facilitating the isolation of the high intensity objects
(barium sulfate, or glass beads and barium sulfate) even when other methods fail.

The most significant variable, as well as the most significant limitation to this method
is the selection of an arm length for analysis. The arm length consists of the number of
intensity values ($z$) either leading or trailing the point being examined that will be used to
determine an average slope for the slope difference calculation. An additional limitation
is the fact that the slope hinge method will identify all potential thresholding points that
correspond to large changes in the histogram slope. As a result, the method will only
provide potential thresholding points, and still requires visual comparison of the binary
result to the original grayscale image in order to verify that the selected threshold value is
appropriate.

4.6.1 Thresholding sensitivity analysis

A sensitivity analysis has been conducted in order to allow direct comparison of each
of the thresholding methods. In addition, variation of the arm length variable in the
proposed slope-hinge thresholding method is evaluated to examine how this variable
impacts threshold value selection. A total of four volumes are included in the analysis in
order to comprehensively evaluate the usable barium sulfate concentration range (33% to
50%) as well as to confirm that trends hold regardless of whether biofilm is present or
not. The objective of this sensitivity analysis is to provide recommendations on thresholding methods for future biofilm research using the barium sulfate contrast agent. A summary of the sensitivity analysis results for the 50% barium sulfate volumes are provided in Figure 4-5. Sensitivity analysis results for the 33% barium sulfate volume are provided in Figure 4-6. Both Figure 4-5 and Figure 4-6 illustrate the range of potential threshold points that are possible using the objective selection methods described previously. In addition, the quantified volume measures for each selected threshold are presented for direct comparison as well.

From Figure 4-5 and Figure 4-6 we see that the entropic thresholding method fails consistently in all four column sections evaluated. The identified threshold value is consistently higher in intensity than the second histogram peak (Figure 4-5a to c and Figure 4-6a and b), lower than the first histogram peak (Figure 4-5d and Figure 4-6d) or, in the case of the histogram with no second peak, at a higher intensity than all other isolated threshold values (Figure 4-6c). A comparison of the isolated threshold values to quantified volume, using Figure 4-7 and Figure 4-8, further confirms that the threshold values identified using entropic thresholding generate either erroneously large binarized volumes, or binarized volumes that are negligible. Moment-preserving threshold isolation is a bit more reliable than entropic thresholding, however application of the moment-preserving thresholding method to Below Edge volume data sets isolates threshold values that are well out of the histogram valley, and approaching the pinnacle of the second histogram peak (see Figure 4-5b and Figure 4-6d). This inconsistency makes the moment-preserving thresholding method questionable for threshold selection of barium
sulfate containing image data sets. However, further comparison of the select threshold
values to the quantified binary volumes shows that even through the threshold values
selected using moment-preserving thresholding vary in reliability when compared to the
volume histograms, the actual quantified volumes end up being consistent to volumes
ascertained using the factorization method, the histogram 1st derivative or the slope-hinge
method.

Inspection of Figure 4-8 indicates that for the BE volume data sets, thresholding using
factorization, the histogram 1st derivative as well as the slope-hinge method all return
similar quantified volumes, particularly when the slope-hinge arm is limited to values of
800 and 1000 (8 to 10 bins). The volume subtraction data sets show the most variability
depending on threshold method. In general, the factorization and 1st derivative
thresholding methods generate similar thresholding values and quantified binary
volumes. The key limitation to the 1st derivative method is illustrated in Figure 4-6c. The
33% barium sulfate, biofilm-containing data set did not present a second histogram peak,
and therefore the 1st derivative threshold method was unable to isolate a threshold value.
This data set also presents a limitation to the slope-hinge method in that the slope-hinge
method isolated two sets of thresholding values: one near the base of the peak, and one in
the higher intensity range as the histogram tail levels out. As illustrated in Figure 4-7, the
quantified volumes ascertained using the first set of slope-hinge threshold values (avg.
54.4 mm³) are approximately twice as large as the second set of threshold values (avg.
26.0 mm³), though the second set agree with the quantified volume determined using the
factorization method (22.7 mm³). Within the second set of threshold values for the slope-
hinge method, variability in the quantified volume between the arm lengths investigated is minimal, with a range of 28.6 mm$^3$ (Arm Length: 600) to 23.7 mm$^3$ (Arm Length: 1500). Narrowing the arm length variable to a range of 800 to 1000, and focusing on the second set of threshold values, when two sets of threshold values are identified, provides consistent agreement to both the factorization and 1st derivative threshold values (when applicable) and corresponding calculated volumes.

The most consistent and reliable thresholding method for both the SV and BE volumes in the presented data sets is the factorization method. This method was capable of identifying appropriate threshold values for data sets regardless of whether the associated volume histogram had a classical bimodal distribution (Figure 4-6b), a bimodal distribution with uneven peaks (Figure 4-6a), a multi-modal distribution (Figure 4-5b), or a unimodal distribution (Figure 4-6c). The two most reliable alternate thresholding methods are 1st derivative thresholding, and use of the slope-hinge method. As was noted previously, there are situations where the use of the 1st derivative will not be possible, in those cases the slope-hinge method should be used as the alternative to the factorization method when thresholding the BE and SV data sets for quantitative analysis of biofilm-containing CT data sets, imaged using the barium sulfate contrast agent.

4.6.2 Final segmentation:

Final segmentation of the data sets are possible once threshold values for the SV and BE data sets has been selected. Demonstration of the segmentation routine will focus on results derived using threshold values determined using the factorization threshold method. Binarization of the SV data set using the identified threshold value isolates the
barium sulfate and represents regions of the column where pore space is available for fluid transport (Barium_Iso). The binary volume generated by thresholding the BE volume contains a combination of the pore space occupied by barium sulfate and the glass beads, we’ll term this data set Binary_BE. Two basic methods are available for isolating the glass bead solid matrix. The first method isolates the glass beads by subtracting the isolated barium sulfate volume (Barium_Iso) from Binary_BE. This subtraction removes the barium sulfate/pore space from the binary volume containing the combination of barium sulfate and glass beads. The second method, which we have found to be more effective, focuses first on isolating the entire pore space, which consists of a combination of the existing biofilm and barium sulfate. In order to accomplish this, the inverse of the Binary_BE is generated through application of a logical-not operation. The binary result of this operation consists of the biofilm present in the data set as well as all regions exterior to the actual experimental specimen (Biofilm-Ext_Iso). By merging this new volume with Barium_Iso a new binary volume is created that has all voxels associated with biofilm, barium sulfate or the column exterior set equal to 1, and all voxels associated with the glass bead solid matrix set to 0 (Pore-Sp_Iso). In order to make sure that the isolated total pore space is completely connected and closed, a union-closing operation is performed, which preserves large circular or spherical objects while eliminating small gaps or holes in the data set. By inverting Pore-Sp_Iso, again using the logical-not operation, we are then able to isolate the glass bead phase of each data set (GlassBd_Iso). The latter method for isolating the glass beads is outlined in the flowchart depicted in Figure 4-9. The key benefit to this second method for glass bead isolation is a
drastic reduction in the number of small blobs, not associated with the glass beads, which remain to be removed from the glass bead phase binary volume. In addition, voids within the isolated glass beads (owing to defects inside the glass beads) are removed as well. Now that the three key materials (biofilm – Biofilm-Ext_Iso, glass beads – GlassBd_Iso, and barium sulfate – Barium_Iso) have been isolated, all of the phases are recombined into a single segmented label field. The final step is to remove the column exterior, which was originally included in the isolated biofilm data set. Cross-sections through a typical segmented volume are depicted in Figure 4-10a and Figure 4-10b. However, surface reconstructions of each of the segmented phases are often more impactful for qualitative visual analysis (Figure 4-10c and Figure 4-10d). Surface generation is achieved using a traditional marching cubes algorithm (Lorenson and Cline, 1987) in the commercial software package Avizo Fire. Quantitative measures of each of the segmented phases are made based on the final surface reconstructions generated from the segmented volume. A summary depiction of segmented results is provided in Figure 4-10e. This figure illustrates the types of results possible through volume segmentation in reference to an original light microscopy image of the sample volume.

4.7 Quantitative analysis and Recommendations:
Once volume segmentation has been completed, a three dimensional surface reconstruction of the final segmented volume is generated from which material quantification can be completed. Parameters such as material volume, surface area, and interfacial area can be quantified from the segmented results. From these measures additional quantities such as the change in column porosity due to biofilm growth can be
calculated (along with the CT estimated initial and final porosity). In addition, estimates of the average biofilm thickness throughout the column section can be calculated as

\[
L_{Biofilm} = \frac{V_{Biofilm}}{S_{A_{Biofilm}}}
\]

Equation 19

where \( L_{Biofilm} \) is the average biofilm thickness, \( V_{Biofilm} \) is the total volume of biofilm for the column, and \( S_{A_{Biofilm}} \) is the total surface area of the biofilm. In addition, measures of the interfacial area between phases within the column sections are possible. Measures of interfacial area in biofilm growth reactors have the potential to elucidate parameters such as reactive surface area and surface area of attachment. The reactive surface area corresponds to the interfacial area between the biofilm and barium sulfate. The surface area of attachment corresponds to the combination of the biofilm/glass bead interfacial area and the biofilm/exterior (column wall) interfacial area. Even though the factorization thresholding method was determined to be the best choice for thresholding the SV and BE volumes for quantitative segmentation, quantification was performed using each of the presented thresholding methods. The only method that has been excluded from final quantitative analysis is the entropic thresholding method since the threshold values presented in the previous section for entropic thresholding were extremely skewed. In addition, only results from the second set of slope-hinge threshold values are presented.

A graphical summary comparison of the calculated volume of each phase for the column sections presented in this manuscript is provided in Figure 4-11. In general, the investigated methods result in similar quantified values. An exception to this statement is that the method of moments results in quantified biofilm volumes that are significantly
higher than those determined using other methods in the 50% barium control section, as well as the 33% biofilm containing column section. While some small air bubbles were present in the control columns the moments thresholding method reports an air/biofilm volume of 8.4 mm$^3$ and all other methods report volumes of less than 1 mm$^3$. In the biofilm column imaged using a 33% barium concentration, the BE threshold value identified using the moment-preserving thresholding method corresponds to an intensity value that almost at the pinnacle of the second peak in the histogram (Figure 4-6d). The quantitative impact of this error in thresholding is a gross under estimate of the barium sulfate/open pore space phase within the column section, as can be seen in Figure 4-11d. Also, as demonstrated in the previous section, the 1st derivative method is unable to generate threshold values in the biofilm-containing column imaged using a 33% barium concentration, due to the fact that the volume subtraction histogram for this column does not exhibit a second peak. With the exception of these aforementioned cases, the results presented in Figure 4-11 show that even though the identified threshold values varied depending on the thresholding method used for initial binarization of the SV and BE data sets, the final quantified volumetric results are generally consistent and equivalent regardless of whether the factorization, 1st derivative, or slope-hinge methods are used for initial volume thresholding.

The measured initial porosity for the 50% and 33% barium control columns ranged from 0.36 to 0.39 and from 0.35 to 0.40, with average glass bead volumes of 108.8±1.8 mm$^3$ and 109.6±3.85 mm$^3$, respectively. The average volume of air entrapped in the two control columns was 1.5±0.07 mm$^3$ and 0.7±0.01 mm$^3$. For the biofilm-containing
volumes imaged using 50% and 33% barium sulfate concentrations, the measured initial porosities ranged from 0.35 to 0.39 and from 0.4 to 0.43. Measured final porosities ranged from 0.3 to 0.33 and from 0.15 to 0.2 respectively. The average measured glass bead volume for the biofilm-containing data sets were 108.7±2 mm$^3$ for the 50% barium volume and 89.3±2 mm$^3$ for the 33% barium column. The calculated volume of biofilm within the two column sections is 8.6±1.4 mm$^3$ and 37.5±4.5 mm$^3$. The initial porosity values calculated from the segmented data sets compare favorably to the initial porosity for the columns, measured by mass, which was 0.42 and 0.44 for the 50% and 33% barium control columns and 0.41 and 0.40 for the biofilm containing columns imaged using 50% and 33% barium concentrations, respectively. The difference in measured biofilm between the 50% and 33% biofilm-containing column sections is attributed to the fact that these two columns contained different species of bacteria (E. coli vs. S. oneidensis). Even though the variability in quantitative measurement between the factorization, slope-hinge, and 1st derivative (when applicable) methods of thresholding was small, the factorization method is the only method that we evaluated that identified reliable thresholding values for all four test cases presented in this study. The quantitative results after volume segmentation serve to reinforce our earlier conclusion and we recommend that the factorization method be utilized for thresholding CT data sets acquired using a barium sulfate contrast agent for the imaging of biofilm in future experimental investigations.
4.8 Conclusions

To date, the imaging of biofilms in experimental systems has been largely limited to optically transparent two-dimensional or pseudo-three-dimensional growth reactors. The method for imaging biofilm in opaque porous media using x-ray CT was first presented by Davit et al. (2011) and has now been modified for use in synchrotron-based CT experiments. The main advantages to using synchrotron-based x-ray CT rather than polychromatic CT systems are that imaging times are reduced to between 5 and 10 minutes, that the use of a monochromatic x-ray source facilitates three-dimensional imaging of biofilm in more natural porous media without the need of the supplemental KI contrast agent that was required for polychromatic scanning, and that the beam hardening prevalent in polychromatic results is greatly reduced in the synchrotron-based CT results.

The main recommendations for segmenting synchrotron based CT data sets are that (1) a non-local means filter be applied to the original grayscale volumes as an initial step in the segmentation routine, and that (2) the factorization thresholding method be utilized for thresholding both the Below Edge and Volume Subtraction data sets in order to isolate the combined solid matrix and barium binary volume and the isolated barium volume, both of which are required as part of the segmentation routine presented in this communication.

One consideration that needs to be made in selecting a grayscale filtering method is the computational resources required for processing. Application of a median filter to a single CT volume takes approximately 30 seconds to process and the application requires only a single processor on a dual hex-core xeon processing computer. When applying the non-local means filter to the same volume, the application requires 1 to 3 hours
depending on how many processing cores are allocated for the filtering application (we used a maximum of 8 processors for the filtering application). Since the two filtering methods require vastly different computation requirements, the final filtered result should be examined to determine which filter (median or non-local means) is most applicable for batch processing of CT data sets. We demonstrate that the result achieved using the non-local means filter contains improved delineation of phases between biofilm and the solid matrix, however, this level of detail may not be necessary in all applications. For this reason, median filtering should be considered as a viable alternative to non-local means filtering if computational resources are limited.

Two biofilm-containing data sets have been presented within which different species of biofilm were grown under equivalent hydraulic loading conditions. The two column sections were presented in order to demonstrate that the segmentation routine developed and presented in this communication works for the utile concentration range of barium sulfate. Comparing the results for the two column sections indicates that significant variation in biofilm formation can be expected depending on the particular species, or, perhaps, even each particular strain of bacteria. The results presented in Davit et al. (2011) and Chapter 3 indicate that variation of hydraulic loading conditions has a direct impact on biofilm growth and accumulation. Thus, additional work is required in order to further characterize additional species of biofilm forming microbes, as well as to further investigate the impact of environmental conditions on biofilm growth and development. However, methods are now available to facilitate this work using both polychromatic and monochromatic x-ray CT. The ability to image biofilm in three-dimensional, opaque,
porous media systems enables the acquisition of truly three-dimensional experimental data sets which can be used to further our understanding of biofilm growth and development in porous media as well as to aid in the development and validation of porous media-associated biofilm growth models.

4.9 Acknowledgements:

This work was supported by The Department of Energy, Office of Biological and Environmental Research (BER), Grant No. DE-FG02-07ER64417 and The Department of Energy, Environmental Remediation Science Program (ERSP), Grant No. DE-FG02-09ER64734.

The work was performed at GeoSoilEnviroCARS (Sector 13), Advanced Photon Source (APS), Argonne National Laboratory. GeoSoilEnviroCARS is supported by the National Science Foundation-Earth Sciences (EAR-0217473), Dept. of Energy-Geosciences (DE-FG01-94ER14466) and the State of Illinois. Use of the APS was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Energy Research, under Contract No. 248 W-31-109-Eng-38. We would like to thank Mark Rivers at GSECARS at the APS for assistance with CT imaging.
4.10 Bibliography


Bibliography (Continued)


Iltis, G.C., Davit, Y., Wildenschild, D. (2013) Quantitative analysis of three dimensional biofilm architecture in porous media using a barium sulfate contrast agent and polychromatic x-ray computed microtomography.prepared for submission to Biotechnology and Bioengineering. Included as Chapter 3 of this thesis.


Bibliography (Continued)


Bibliography (Continued)


Figure 4-1: Histogram comparison of control column sections containing various concentrations of the barium sulfate contrast agent. The concentrations evaluated include a 50% mixture, a 33% mixture, and a 25% mixture. Scans were completed both above (top) and below (bottom) the k-shell absorption edge in order to aid in isolating the barium sulfate contrast agent through the use of digital image subtraction. Illustrative cross-sections at all three concentrations are provided for the Above Edge scans (a-c), and Below Edge scans (d-e).
Figure 4-2: Volume subtraction concentration comparison for control columns and columns containing biofilm for barium sulfate suspension dilutions ranging from 25% to 50% stock solution. The subtraction volumes are the result of digital image subtraction of the Below Edge volume from the Above Edge volume. The difference indicates the presence and location of barium sulfate in the hydraulically available pore space. Data for the control column scans is provided in the top set of histograms for all three concentrations with cross sectional images of representative volume data sets at each concentration are provided as images a to c. Equivalent histograms and cross sectional images for biofilm containing scans are provided as well (bottom) with the images labeled as d to f.
Figure 4-3: Histogram comparison of three filtering methods applied to a biofilm-containing column section. Histogram plots for the Above Edge volume data set (top), Below Edge volume data set (middle) and volume subtraction data set (bottom) are included. The filtering applications included in the histograms include (a) no filtering, (b) Median Filtering, and (c) application of the Non-Local Means filter.
Figure 4-4: Visual comparison of original grayscale image to filtering results using a Median Filter (center column) and a Non-Local Means filter (right column). Comparative image sets include full cross-sections (top row), zoomed in regions focusing on an area containing all three materials of interest (biofilm, barium sulfate, and glass beads), and segmented representations of the zoomed in regions (bottom row), where biofilm is labeled in red, barium sulfate in light blue and glass beads are labeled using dark blue.
Figure 4-5: Histogram-based sensitivity analysis for the 50% barium sulfate (a) control column subtraction volume, (b) the control column Below Edge volume, (c) the biofilm subtraction volume and (d) the biofilm Below Edge volume. Individual threshold values are juxtaposed with the quantified binary volume for each threshold value.
Figure 4-6: Histogram-based sensitivity analysis for the 33% barium sulfate (a) control column subtraction volume, (b) the control column Below Edge volume, (c) the biofilm subtraction volume and (d) the biofilm Below Edge volume. Individual threshold values are juxtaposed with the quantified binary volume for each threshold value.
Figure 4-7: Subtraction volume sensitivity analysis quantified results. Direct comparison of (a) the isolated threshold values using each of the investigated thresholding methods to (b) the quantified binary volumes generated by thresholding at the specified value.
Figure 4-8: Below Edge volume sensitivity analysis quantified results. Direct comparison of (a) the isolated threshold values using each of the investigated thresholding methods to (b) the quantified binary volumes generated by thresholding at the specified value.
Figure 4-9: Flowchart depiction of the final segmentation for phase quantification.

1. **below edge (BE) volume**
2. **threshold value selection and volume binarization**
3. **barium and glass beads**
4. **isolated biofilm and column exterior (Biofilm-Ext_Iso)**
5. **total pore space and exterior**
6. **logical and**
7. **logical not**
8. **logical or**
9. **final segmentation**
10. **subtraction volume (SV)**
11. **threshold value selection and volume binarization**
12. **isolated barium sulfate (Barium_Iso)**
13. **Pixel Count**
14. **Pixel Intensity**
15. **Pixel Count**
16. **Pixel Intensity**
17. **Pixel Count**
18. **Pixel Intensity**
Figure 4-10: Illustrative comparison of (a) biofilm grown within the sample column to (b) the final CT data segmentation and surface reconstructions from which quantification is possible. Section (c) shows the biofilm reconstruction with a reference slice of the segmented volume, and (d) a surface reconstruction containing both the isolated biofilm and the glass bead solid matrix.
Figure 4-11: Summary of quantified volumes measured from surface reconstructions of the final segmented data sets. Note that the 1st Derivative measurements in the 33% Barium Sulfate Biofilm Column are all set to 0, this is due to the fact that the 1st Derivative method of thresholding is not applicable to this volume as there is no second peak in the volume histogram.
5 RESOLVING THE INFLUENCE OF FLOW RATE ON BIOFILM GROWTH IN THREE DIMENSIONS USING MICROIMAGING

Gabriel C. Iltis, Brian Wood, and Dorthe Wildenschild

5.1 Abstract

This study presents the first application of synchrotron-based x-ray computed microtomography in the systematic investigation of the impact of hydraulic loading rate on biofilm growth in opaque porous media. A total of three flow rates, corresponding to initial Reynolds numbers of 0.1, 1.0 and 10 were evaluated. Biofilm growth was monitored and evaluated during an 11 day growth phase using a combination of differential pressure measurements and dissolved oxygen readings. At the end of the growth period, the growth media was replaced with a barium sulfate contrast agent to allow for x-ray CT imaging. Quantitative analysis of the resulting image data sets focuses on quantifying the spatial distribution of biofilm internal to the porous medium for direct comparison to the laboratory measured change in hydraulic conductivity. The comparison of CT data to laboratory measurements is accomplished using the Kozeny-Carmen model for relating porosity to hydraulic conductivity. All columns showed a decrease in hydraulic conductivity of 1 to 3 orders of magnitude. Final results showed poor agreement when using bulk average final porosity measurements to calculate hydraulic conductivity estimates. However, CT imaging enables the investigation and evaluation of the spatial distribution of parameters such as the porosity of a porous medium. CT results were used to isolate and identify regions where bioclogging had maximized the reduction in porosity. Recalculation of the hydraulic conductivity estimates improve the overall results. In general,
columns with an initial Re of 0.1 had the lowest measured change in hydraulic conductivity; a result that was difficult to replicate using the Kozeny-Carman relationship. Measured hydraulic conductivity values increased with flow rate, and reasonable agreement between laboratory and CT data sets for the middle and high flow rate columns using the Kozeny-Carman model were achievable.

5.2 Introduction
The presence of biofilms in porous media can impact any number of properties including porosity, permeability, dispersion, diffusion, and mass transport of solutes. A recent focus of scientific investigation of biofilms in porous media is the linking of experimental porous media studies to computational models. However, a key limitation in coupling experimental results and modeling efforts has been that traditional column studies and field site evaluations rely on bulk parameter measurements such as mass balances and differential pressure measurement to obtain aggregate properties for biofilm substrate utilization and growth internal to a porous medium (Tullner, 2010). Direct visualization of biofilm growth has, historically, relied on the use of optically transparent porous media (Leis et al., 2005), and/or two-dimensional micro models (e.g. Stoodley et al., 1999; Kim and Fogler, 2000). Visualization of biofilm within these optically transparent systems has traditionally been achieved using techniques such as confocal laser scanning microscopy (CLSM) whereby biofilm growth can be monitored through the use of fluorescent stains or even particle tracking. The key advantage to this method is that temporal studies of biofilm growth are possible. Conversely, a major limitation is that experimental systems must be optically transparent, small enough to fit onto the microscope stage, and be thin
enough to fit within the focal range of the CLSM. With these restrictions in mind, CLSM has been used extensively to evaluate biofilm growth in 2D and pseudo-3D systems (e.g. Beyenal et al., 2004; Rodriguez and Bishop, 2007). Yet, linking processes and parameters derived from 2D or pseudo-3D flow-through systems to fully three-dimensional systems is tenuous, owing to the difference in complexity between 2D and 3D systems. As Baveye (2010) suggests, we need to be able to characterize experimental systems, such as 3D column growth reactors, to advance our understanding of bioclogging in porous media.

One of the key parameters, linked to bioclogging, that is relatively easy to evaluate experimentally is variation in hydraulic conductivity. Numerous studies have been conducted under varying conditions (e.g. Cunningham et al., 1991; Seifert and Engesgaard, 2007), and the overall conclusion has been that bioclogging can reduce hydraulic conductivity by 2 to 3 orders of magnitude (Tullner, 2010). However only a few studies have been conducted attempting to associate changes in porosity with changes in measured hydraulic conductivity. Bielefeldt et al. (2002) reported hydraulic conductivity to decrease by 2 to 2.5 orders of magnitude due to bioclogging, and determined that a majority of the biomass, and associated clogging occurred in the first several centimeters of their growth reactors. Seifert and Engesgaard (2007) reported decreases in hydraulic conductivities of up to four orders of magnitude, and correlated the reduction to an immobile biomass porosity. To this point, our inability to visualize and quantify biofilm volumetrically in porous media has been the main limitation in relating porosity changes associated with biofilm growth to changes in hydraulic conductivity. However, visualization of biofilm growth, internal to opaque porous media has recently been achieved by both Iltis et al. (2011) and Davit et al. (2011) using x-ray computed microtomography (CT). X-ray CT can be
used to scan experimental specimens in packed bead columns or other types of flow cells with resolutions as low as ~5 μm per pixel for 5 to 10 mm diameter specimens. Yet, the application of CT imaging to quantitative biofilm studies in porous media is still in its infancy. Davit et al. (2011) used a barium sulfate suspension as an x-ray contrast agent that fills the available pore space (aqueous phase) within a porous medium, while being size excluded from the biofilm phase. This method has now been tailored for use with both polychromatic x-ray CT (Davit et al., 2011; Iltis et al., 2013a) and monochromatic, synchrotron-based x-ray CT (Iltis et al. 2013b) investigations. Yet, in these existing studies, a link between quantitative image analysis of CT data and physical measurements of biofilm growth is yet to be accomplished. The quantitative image analysis presented in Iltis et al. (2013a) came close, by linking calculated initial Reynolds number values for experimental columns to biofilm measures quantified based upon CT image analysis. However, bulk measurements of differential pressure across the experimental columns were not collected. This prevents thorough comparison and validation of the image processing results. The general conclusion from the initial Reynolds number (Re) vs. image analysis study was that the range of flow rates applied to the experimental columns (initial Re values ranging from 10 to 67) span a hydrodynamic threshold below which, biofilm forms as a pore-filling mass, affecting bulk channel flow paths (at or around an Re of 10), and above which, surface associated biofilm forms, and large, pore-filling, masses do not develop due to hydrodynamic shear effects (Re greater than 50).

The work presented in this communication builds on the work of Davit et al. (2011), Iltis et al. (2013a), and Iltis et al. (2013b) by presenting the first documented analysis coupling bulk parameter measurement of biofilm growth in porous media to CT imaging. The selected initial
Re values span three orders of magnitude (Re=0.1, 1.0 and 10), and correspond to flow conditions ranging from laminar flow into the transition flow regime between laminar and turbulent. This study aims to characterize changes in biofilm structure under the stipulated flow rate conditions and compare these changes to bulk flow measurements. This work introduces a truly three-dimensional analysis of biofilm growth within opaque porous media along with correlation to (1) differential pressure measurements (for evaluation of bioclogging) and (2) dissolved oxygen to evaluate potential nutrient limitations imparted by the applied flow rates.

5.3 Experimental Methods

5.3.1 Packed bead column reactors

Packed bead column reactors measuring 6.3 mm diameter, and 25 mm long were constructed of polycarbonate tubing. The porous medium consisted of soda-lime silica glass beads (particle size range of 1.4 to 1.7 mm, specific gravity of 2.5). A total of 6 growth reactors were used in this study, two at each flow rate. Each column is identified based on its initial prescribed Reynolds number as well as the order (either A or B) in each set of columns to which a particular column was assigned (e.g. Re=1.0, A identifies the first column in the 45 mL per hr flowrate group) The average initial porosity for the experimental growth reactors is 39.5%, by mass. Prior to column inoculation, each growth reactor and pressure transducer setup was tested under flow conditions ranging from 0 mL per hour to 2500 mL per hour. Differential pressure measurements were recorded for the duration of the flow load application and initial hydraulic conductivity was calculated graphically for each column by determining the slope of the rearranged form of Darcy’s Law.
where $K$ is the evaluated hydraulic conductivity (cm per sec), $Q$ is the applied flow rate (mL per sec), $L$ is the measured length of the column (cm), $A$ is the cross-sectional area of the column (cm$^2$), and $\Delta H$ is the measured difference in hydraulic head (cm H$_2$O), as read by the differential pressure transducers. The average initial hydraulic conductivity for the experimental columns is 0.41±0.09 cm per sec. All column growth reactors and tubing were sterilized prior to inoculation by flushing a 90% ethanol solution through the test apparatus for 30 min, at which point a growth media flush commenced to ensure that all ethanol was removed from the system prior to inoculation.

5.3.2 Microbial species
The bacterial species used in this study is *Shewanella oneidensis* MR-1; a metal reducing bacterium originally isolated from sediment from Lake Oneida in New York. Fully characterized by Venkateswaran et al. (1999), *S. oneidensis* MR-1 is a gram-negative, polarly flagellated, facultative anaerobe capable of dissimilatory metal reduction and biofilm formation (Majors et al., 2005; Venkateswaran et al., 1999). This bacterium has been used successfully in previous biofilm imaging studies conducted by our group (Iltis et al., 2011).

5.3.3 Experimental growth conditions
Inoculum was grown from frozen stock for two growth cycles. Initially, frozen stock was thawed and 0.5 mL stock culture was added to 30 mL sterile 100% (30 g per L) Tryptic Soy Broth (TSB). Batch cultures were grown on an incubated shaker table at 250 rpm and 30C for 20 hours at which point the second growth cycle was started by adding 1 mL of batch culture from the first growth cycle to 30 mL fresh, sterile 100% TSB growth media. Batch cultures were then
incubated for another 20 hour cycle. At the conclusion of the second growth cycle, batch cultures were centrifuged at 6000 rpm for 10 minutes, the supernatant was poured off, and the cell pellets were resuspended in 5 mL 100% TSB (30 g per L) growth media. Sterile packed bead columns, saturated with autoclaved 10% TSB (3 g per mL), were then injected with 1 mL of the concentrated cell inoculum. A 24 hour period was allowed for cell attachment, at which point flow of sterile, oxygenated growth media was induced to the columns at one of the assigned flow rates.

A total of three initial Reynolds numbers were targeted for investigation. The Reynolds numbers (Re) are 0.1, 1 and 10, where the Re values are determined from:

\[ Re = \frac{\rho V D}{\mu (1-n)} \]  
**Equation 21**

where \( \rho \) is the fluid density (g per mL), \( V \) is the superficial velocity, or Darcy velocity (cm per hr) defined as

\[ V = \frac{Q}{A} \]  
**Equation 22**

where \( Q \) is the fluid flow rate (mL per hr), and \( A \) is the cross-sectional area without the porous medium (cm²), \( D \) is the characteristic length for the porous medium (cm), taken to be the average grain size within the growth reactors, \( \mu \) is the dynamic viscosity for the fluid phase (g per cm per hr), and \( n \) is the aggregate porosity. For the column setups used in this experiment, these Reynolds numbers correspond to flow rates of 4.5, 45, and 450 mL per hr. Continuous flow was provided to each column growth reactor using continuous cycle syringe pumps along with a network of check valves. A graphical depiction of the experimental setup is provided in Figure 5-1. Growth media reservoirs were replaced in approximately 36 hr intervals, and all influent
lines were replaced every two days to minimizing biofilm growth upstream of the experimental columns. All growth media reservoirs were aerated by continuous sparging using filtered (0.22µm) air. Biofilm growth was allowed to continue under constant flow conditions for 11 days, at which point the columns were disconnected and prepared for contrast agent (barium sulfate suspension) injection and imaging.

5.3.4 Differential Pressure measurement
Differential pressure transducers (Honeywell Sensing and Control, 24PC Series) were affixed to each experimental column at the column entrance and exit in order to allow for measurement of differential pressure across each column length. Continuous differential pressure measurements were collected by a data acquisition system (Personal DAQ 50, Measurement Computing Corp., Norton, MA) connected to a computer.

5.3.5 DO measurement
Periodic measurements of dissolved oxygen (DO) were collected on the influent and effluent sides of each column for the duration of the biofilm growth phase of the experiment. Effluent DO measurements were made using flow through oxygen microprobes (Model 16-730, Microelectrodes Inc., Beadford NH). Influent DO measures were made using a separate DO probe (Symphony, VWR International) from samples extracted from the sterile media reservoirs. The equipment for measuring influent and effluent dissolved oxygen were kept separate for the duration of the experiment in order to prevent contamination. Dissolved oxygen probes were cleaned using a Tergazyme (Alconox.Inc, White Plains NY) protein removal solution after every set of measurements.
5.3.6 CT imaging

CT imaging was conducted at Argonne National Laboratory’s Advanced Photon Source (APS) using beamline BM13D GSECARS. Immediately prior to imaging, sample columns were disconnected from influent and effluent lines, followed by injection of the contrast agent solution. The contrast agent consisted of a medical grade barium sulfate suspension (Micropaque, Guerbet) mixed with sterile growth media. The stock concentration of Micropaque is 1.0 g barium sulfate per mL, and this was diluted to a usable concentration of 0.33 g barium sulfate per mL. The contrast agent was injected into the sample columns using a syringe pump at a rate of 1.2 mL per hr for the Re=0.1 and 1.0 columns and 5 mL per hr for the Re=10 columns. A total of 2 mL of contrast agent solution was injected to each column. The average pore volume prior to column inoculation is approximately 0.5 cm$^3$, so approximately 4 pore volumes of contrast agent was added to each column. The selection of a 0.33 g per mL contrast agent concentration was made to balance x-ray absorption through the column specimens with the saturation limit of the x-ray detector (camera). A thorough discussion of barium sulfate concentration ranges for synchrotron-based x-ray CT of biofilms is presented in Iltis et al. (2013b), who reported that the useful range of barium concentration for columns 5 to 7 mm in diameter is 33% (0.33 g per mL) to 50% (0.5 g per mL) barium sulfate. The K-shell absorption edge for barium is 37.4 keV and each column section was scanned at two energies, one above (37.54 keV) and one below (37.34 keV) this edge. The entire length of each column was scanned by moving the sample state in the vertical direction (generating overlapping “tiles” of volumetric results) to evaluate and compare the axial spatial distribution of biofilm at each of the flow rates.
5.3.7 Image Processing and Quantitative Analysis

All volume segmentation and quantitative analysis was accomplished using Avizo:Fire (www.vsg3d.com). After initial tomographic reconstruction a non-local means filter was applied to each grayscale data set. The non-local means filter evaluates each volume in total and identifies all regions with similar Gaussian neighborhoods. The mean of all of the equivalent regions is then used as a denoising parameter for the filtering operation. Additional details can be found in Buades et al. (2005) and a comparison of non-local means filtering to other filtering methods for evaluation of biofilm volumes can be found in Iltis et al. (2013b). Volume thresholding and segmentation was accomplished following the methodology presented in Iltis et al. (2013b), using factorization thresholding as the main method of phase isolation. A total of eight sets of CT scans were collected for each experimental column. After segmentation, the scans were recombined into a single volume for quantification.

After the complete column data sets were recombined, visual comparison of the segmented data sets to the grayscale source data showed good agreement, in general as can be seen in Figure 5-2. However, portions of the pore space in several column data sets were found to contain anomalous intensity variations. These areas of the column pore space, characteristically, are partially filled by easily identifiable barium sulfate; however, lower voxel intensity regions fill the remainder of the pore space. Within these areas voxel intensity gradients can be seen visually, and the voxel intensity values end up being higher than other areas attributed to biofilm and lower than areas containing barium sulfate. Examples of this phenomena are highlighted in green in Figure 5-2. There are several potential explanations for these areas including; (1) that the areas are biofilm that has grown or become trapped in the pore space, and, potentially,
biofilm that has developed a porous or channeled structure capable of letting some barium particles into the biofilm matrix. This explanation would help to account for regions where the clearly distinguishable barium exists on all sides of one of these regions, including the top. (2) The anomalous regions could be showing settling of the barium sulfate contrast agent. Unfortunately, neither of these explanations can be certified based on the evidence at hand.

In order to address this potential mislabeling of either biofilm or barium sulfate an alternate segmentation was implemented that focused on isolating biofilm containing regions by selecting a threshold value in the above edge volume (collected above the barium k-shell absorption edge), as opposed to relying on digital image subtraction. This method isolates a threshold value for the above edge data set which corresponds to the average value between the first two histogram peaks. Reasonable results were acquired for the Re=10 and Re=1.0 volumes, however, attempts to apply the alternate segmentation to the Re=0.1 data sets severely underestimated the amount of biofilm present within the compiled data sets. Representative cross-sectional images of the final segmented results for the A-series columns at each flow rate are also provided in Figure 5-2 for comparison to the factorization segmentation method as well as the original grayscale data. For simplicity, the factorization segmentation method will be abbreviated to FSM when discussing results generated from volumes segmented using that method. Results from volumes segmented using the alternate peak subdivision method will be identified using the abbreviation ASM.
5.4  Results

Quantitative results from this study fall into two categories; laboratory bulk parameter results, and quantitative image based results. Bulk parameter results will be discussed first, followed by discussion of the imaging results.

5.4.1  Laboratory investigation

The evaluation and monitoring of differential pressure and dissolved oxygen was the main focus of the laboratory investigation prior to column imaging. The temporal differential pressure (DP) record is shown in Figure 5-3. From this figure we see distinct trends that correlate directly to the applied flow rate. The error bars represent one standard deviation in the 12 hour average values presented. The columns subjected to the highest flow rate (450 mL per hr, Re=10) present the largest overall increase in DP, as well as a continuous steady increase in DP for the duration of the growth phase of the experiment. The Re=1.0 columns show very little change in DP for the first 8 days of the experiment, after which, DP steadily increases. The wide variation in the DP range associated with the Re=10 and Re=1.0 columns prior to the end of the experiment are notable. The fluctuations in DP may be the result of repetitive clogging and sloughing; a phenomenon that would result in both spikes in pressure, as well as the general increase in pressure as a whole. Contrary to these results, we see a slow but steady increase in DP in the Re=0.1 columns for the duration of the experiment, up to maximum values ranging from 3.3 to 3.6 cm H₂O with some cyclic variation starting on day 6.

A plot of the converted initial and final hydraulic conductivity (K) values, is presented in Figure 5-4. In general, we see decreases in hydraulic conductivity of 1 to 2 orders of magnitude for all experimental columns, values that are well within the range of 10⁻² to 10⁻⁴ reported in
other investigations (e.g. Cunningham et al., 1991; Seifert and Engesgaard, 2007; Thullner, 2010). The largest, measured, decrease in K is observed for the lowest flow rate columns (Re=0.1) and the highest flow rate columns have the highest final K values. At first glance the values appear to show a monomial correlation between initial Re value and final K value, however, other factors such as

(a) nutrient limitations (e.g. dissolved oxygen concentration),

(b) the shape and size of the porous medium, or

(c) the fact that the systems were run under constant flux conditions,

can all act as variables in biofilm growth, and as a result the correlation depicted in Figure 5-4 requires additional investigation and comparison to similar studies in order to determine if the apparent correlation appeared by circumstance or indicates a real relationship. Based on Figure 5-3, the fact that the Re=0.1 columns exhibit the smallest final K values seems counter intuitive since the Re=0.1 columns also presented the smallest change in measured differential pressure. However, given the fact that the applied flow rates for the Re=0.1 and Re=10 columns vary by 2 orders of magnitude, and that the final differential pressures (between the Re=0.1 and Re=10) only vary by approximately 1 order of magnitude, the finding that the Re=0.1 columns have final K values (1E-2 cm per sec for both columns) that are an order of magnitude smaller than those reported for the Re=10 columns (1.4E-1 and 7.8E-2 cm per sec) makes sense.

As mentioned previously, dissolved oxygen concentration was evaluated for the duration of the growth phase of the experiment in order to assess nutrient utilization across the length of the columns. Examination of the temporal dissolved oxygen (DO) record (Figure 5-5) helps to
explain the general trends and variability present in the DP record. Influent DO concentrations remain constant at, or about, the saturation limit for oxygen in the growth media for the duration of the experiment (average concentration of 8.05±0.29 mg per L). From Figure 5-5 we see that the high flow rate columns maintain an effluent concentration ranging from 1.5 to 6 mg per liter for the duration of the experiment, which indicates that at a Re value of 10 a threshold has been crossed at which point oxygen is no longer a limiting factor in biofilm growth, and instead, biofilm is able to grow continuously within the porous medium, until such a point as shear stresses induced by the increasingly restricted flow cause sloughing to occur. Evidence of this type of effect is demonstrated by the steady increase in both DP and DO concentration in the Re=1.0 column set (Figure 5-3 and Figure 5-5b) starting 6 days into the experiment. This increase in DP and DO readings suggests increased channeling within the columns allowing oxygenated media to fully pass through the column reactors. The low flow rate (Re=0.1) columns, however, maintain effluent concentrations of approximately zero for the duration of the experiment indicating that microbial growth is continuous, but nutrient limited, for the duration of the experiment (based on the continuous zero reading), but indicating that oxygen depletion should be considered when evaluating results from the CT analysis.

5.4.2 Quantitative Image Analysis

X-ray computed microtomography (CT) was used to image each of the experimental columns after addition of a barium sulfate contrast agent. Each of the volumes was segmented so that every voxel (the 3D equivalent of a 2D pixel) was assigned to one of 4 materials:

(a) the glass bead solid matrix

(b) barium sulfate, or the pore space available to flow
Quantitative analysis of the segmented data sets enables the direct calculation of bulk parameters such as the volume, surface area, and interfacial area of each material identified in the segmented volume. We also have the ability to quantify and evaluate the size and spatial distribution of discrete regions of each material.

One of the main benefits associated with incorporating x-ray CT into biofilm growth studies is the fact that changes in the porosity of a porous medium can be evaluated without requiring destructive sampling. Given the quantified biofilm, barium sulfate, and glass bead volumes, both the initial and final column porosities can be calculated. In addition, it is possible to measure both volume and surface area on a slice-by-slice basis, thus enabling the evaluation of the spatial distribution of parameters like biofilm volume, or change in porosity axially throughout the entire scanned region, which in our case is 30 mm of each column growth reactor. As a baseline evaluation of the quality of results generated using the two segmentation routines, the physically measured bulk initial porosity measurements were compared to the bulk initial porosity values determined using the segmented FSM and ASM volumes. The physically measured porosity values were calculated as each column was originally being constructed. Direct comparison of the physically measured initial porosity values to the initial porosity values calculated from the segmented CT data sets shows good agreement regardless of whether the FSM or ASM segmentation routine is used for segmentation. The largest discrepancy is associated with column Re=0.1, A where the physically measured initial porosity is 0.4 and the FSM and ASM volumes have initial porosities of 0.35 and 0.36, respectively. Summary plots of the initial and final
porosity distributions for each column, calculated from the FSM and ASM data sets, are provided in Figure 5-6. Direct comparison of the initial porosity distributions associated with the FSM volumes (Figure 5-6a) and ASM volumes (Figure 5-6c) are almost identical. The summary plots of the final porosity distributions (Figure 5-6b and d) primarily demonstrate that the main difference between results calculated using the FSM data sets and those calculated using the ASM data sets is a shift of 0.1 (or 10%) porosity. This conclusion is reinforced by the bulk final porosity values reported in Error! Reference source not found.. Trends between flow regimes are a bit more pronounced in the FSM data sets. As Figure 5-6b illustrates, the recorded final porosity values are very similar for the Re=10 and Re=1.0 columns, and that all four data sets fall into the same region of the plot, with measured porosity values gradually decreasing with elevation through the 30 mm column section. The Re=0.1 data sets, on the other hand, maintain final porosity values ranging from 0.2 to 0.33 for a majority of the column length. Finally, at elevations ranging from 21.5 to 27.5 mm final porosity values decrease significantly to final porosity values of 0.08 and 0.19 at the top of the Re=0.1 columns. This sudden and steep decline in column porosity suggests that biofilm is clogging or obstructing the effluent side of the column. Similar behavior, though much less pronounced, can be seen in the Re=1.0, A data set. In order to aid in investigating this further, summary plots depicting the measured change in porosity associated with each column and segmentation method are provided as Figure 5-7. By plotting the change in porosity for each column, we are able to examine the impact that biofilm growth is having on pore space internal to each column in the absence of the glass bead solid phase. The most prominent characteristic of the plots presented in Figure 5-7 is the near universal increase in porosity change (biofilm volume) near the top of the columns. One possible
reason for the consistent increase in biofilm volume near the top of each column is the column design. Each cylindrical column screws directly into a top-cap that has a small, ~1/8 inch opening through which fluid and sloughed biofilm are able to leave the column. It is entirely feasible that some sloughed biofilm became trapped either in the glass bead matrix near the top of the columns, or by the top-cap itself.

In an effort to verify the measured changes in porosity that have been quantified using the segmented CT data we have implemented the Kozeny – Carmen relationship in order to estimate the corresponding changes in hydraulic conductivity for each of the 6 experimental columns. Using the Kozeny-Carmen equation, hydraulic conductivity is calculated as (Bear, 1972):

\[ K = \frac{\rho g n^3}{\mu (1-n)^2} \left( \frac{d_m}{180} \right) \]

Equation 23

where \( \rho \) is the fluid density (g per mL), \( g \) is the gravitational constant (cm per sec\(^2\)), \( \mu \) is the dynamic viscosity of the fluid (N*sec per m\(^2\)), \( n \) is the column porosity, and \( d_m \) is the characteristic length for the porous medium (cm). Bear (1972) indicates that the characteristic length for the porous medium can be calculated as

\[ d_m = \frac{6}{M_s} \]

Equation 24

where \( M_s \) is the specific surface of the material, defined as the ratio of the total surface area of the solid phase to the volume of the solid phase (cm\(^2\) per cm\(^3\)). \( M_s \) values for each column were calculated using the bulk CT measurements for the glass beads within each respective column. The characteristic diameter, as calculated using \( M_s \), ranges from 1.6 mm to 1.7 mm. Incorporating this value into the Kozeny-Carman equation generates initial K values ranging from 1.46 to 2.2 cm per sec when using the total, CT calculated, initial porosity values; these
estimates are 3.5 to 4.5 times greater than the experimentally established initial hydraulic conductivities for the packed bead columns. Estimation of the final K values for each column using the total final porosity values succeeds in matching the physical data in only one case (Re=10 using the FSM data sets), as can be seen in Figure 5-8. In an effort to characterize why the K value estimates predicted using the Kozeny-Carman relationship fail to match the K values calculated using differential pressure measurements, a sensitivity analysis was carried out which evaluated the spatial distribution of changes in column porosity, and the impact that the variability discussed previously, and illustrated in Figure 5-6 and Figure 5-7, has on K estimates made using the Kozeny-Carman equation.

Instead of using the bulk average porosity values for each column, changes in reported porosity with column length were averaged at intervals ranging from 10.5 μm (1 voxel thickness) to 3.15 mm (300 voxels). The region(s) in each column that have the smallest porosity available for fluid transport are expected to have the greatest impact on the overall measured differential pressure for each column, and by association, these regions are expected to be the major contributing factor to reductions in column hydraulic conductivity.

Results from the sensitivity analysis are presented in Figure 5-9. As we expected, evaluating each column for the minimum porosity regions and calculating K values using the measured porosity values in the constriction points improves the comparison between physical and calculated results. The K value estimates made using the results from the ASM volumes are consistently higher than the physical values until the sensitivity analysis interval is reduced to 0.05 mm. at which point the estimated initial K values converge on the physical measurements and the reported final K values for the Re=10 and Re=1.0 columns are lower than the physical
measured values. The K value estimations for the low flow rate columns remain approximately one order of magnitude higher than the hydraulic conductivity determined from physical measurements throughout the sensitivity analysis. This is thought to be caused by the poor ability of the ASM routine to identify and isolate biofilm in the Re=0.1 data sets. As Figure 5-7c demonstrates, the change in porosity associated with isolated biofilm identified using the ASM routine was less than 2% consistently through the entire column length and as a result, the small constriction points that have been identified in other columns are not present in the ASM Re=0.1 data set. In the FSM data sets, adjusting the analysis interval impacted the estimated hydraulic conductivity values significantly. While various averaging intervals produced hydraulic conductivity estimates that match, or come close to the physically measured values, the general trend that is seen in the physical data, namely that bioclogging at low flow rates impacts hydraulic conductivity more severely than bioclogging at higher flow rates, never develops when applying the Kozeny-Carman relationship to the bioclogging results. There are several potential reasons that we couldn’t match the relationship. The first is that biofilm within the segmented results is treated as an impermeable material when applying the Kozeny-Carman relationship. This assumption has the potential to significantly over estimate decreases in K due to the possibility that the biofilm within each of the column reactors actually create a dual porosity system. For this reason, the over estimation of reductions in K at each of the different flow rates may be appropriate. In addition, visual comparison of the grayscale images of the Re=10 and Re=0.1 columns, previously presented in Figure 5-2, shows distinctly different biofilm morphology, some of which was not identified through segmentation. In the Re=0.1 columns the biofilm forms a visibly filamentous or porous structure, letting small amounts of barium sulfate
in between thin sections of biofilm. Many of these thin regions were not able to be completely isolated through segmentation using either segmentation method. The biofilms present in the Re=10 image, on the other hand, take up larger regions in the image, but appear to be denser as well. The unsegmented, fine structures present in the Re=0.1 data set could also potentially increase resistance to flow, further skewing the comparison between K values estimated using the Kozeny-Carman equation and quantified changes in column porosity associated with biofilm growth.

There are several limitations to using the Kozeny-Carman relationship for estimating changes in hydraulic conductivity associated with porosity variation due to biofilm growth. Firstly, biofilms can form largely as surface associated colonies, capable of changing the pore morphology of both pore throats and bodies, and significantly impacting bulk parameters such as K, while only restricting a small portion of the bulk pore space or porosity. Evidence of this kind of result has been presented by Bielefeldt et al. (2002) who measured decreases in hydraulic conductivity of up to 3 orders of magnitude, however reductions in porosity associated with biofilm growth were estimated to be between 3 and 8 percent. Secondly, the potential for biofilm to create a dual porosity environment is neglected in the application of the Kozeny-Carman model. Finally, as we have shown through this study, the reliance of the Kozeny-Carman equation on measured changes in porosity has the potential to generate faulty K value predictions when using bulk estimates of porosity in scenarios where the change in porosity throughout the system is not necessarily uniform. As we have demonstrated, biofilm growth within the column growth reactors varied spatially, and the measured bulk change in porosity for each column
averaged all of the various regions together resulting in average K value estimates that were not representative of actual conditions.

5.5 Conclusions:
The ability to visualize and quantify biofilm spatially in experimental growth reactors provides a new suite of capabilities for evaluating and validating biofilm growth studies in opaque porous media. Experimental measurements included in this study focused on differential pressure and dissolved oxygen, which were recorded temporally.

Direct comparison of measured bulk changes in hydraulic conductivity with changes predicted using the Kozeny-Carman equation in conjunction with the bulk average porosity measurements generated using segmented CT results showed poor agreement. Estimates of initial hydraulic conductivity generated using the Kozeny-Carman equation were 4 to 5 times higher than physically measured values, and estimates of the final hydraulic conductivity for biofilm containing columns were up to two orders of magnitude higher than physical measurements. The CT data sets enabled examination of changes in porosity throughout the experimental column and enabled the identification of the regions in each column that had the lowest measurable porosity. Recalculation of the hydraulic conductivity values based on the most restricted areas of each column resulted in estimates that were up to one to two orders of magnitude lower than the physically measured K values. Two segmentation routines were evaluated in this study, the factorization segmentation method (FSM) and the above-edge peak subdivision method (ASM). The main difference in the results generated by the two methods was that the final porosity measurements determined using the ASM were consistently 0.1 (10%)
higher than final porosity measurements determined using FSM, however, general trends were consistent regardless of which method was used for segmentation.

Differential pressure measurements for the lowest flow rate columns (Re=0.1) resulted in some of the lowest hydraulic conductivities measured during this study. The most likely cause for this decrease in K values is notable biofilm accumulation, and reduction in column porosity on the effluent side of the columns. However, biofilm grown under the lowest hydraulic loading rate did express a very different structure from biofilm subjected to higher loading rates. In particular, the low Re biofilm appeared to be more porous and filamentous in nature, allowing barium sulfate to surround and fill channels in the biofilm, thereby preventing complete isolation during thresholding and segmentation. This change in structure is thought to be influenced by oxygen limitations across the column axial length.

Given that techniques are now available for imaging biofilm in porous media using x-ray CT. Incorporation of CT imaging into future experiments enables the investigation and quantification of porous medium/biofilm interactions which previously have been difficult of obtain, such as change in porosity and spatial distribution of biofilm, a consideration which is just as important as bulk parameter measurement, since local changes in biofilm volume, even small changes, can effect differential pressure measurement, while being averaged out in a bulk porosity measurement.
5.6 Bibliography


Bibliography (Continued)


Figure 5-1: Continuous flow experimental column setup schematic.
Figure 5-2: Comparison of grayscale cross-sections to final segmented results generated using the factorization segmentation method (FSM) vs. the above edge peak subdivision method (ASM). Highlighted areas demonstrate regions where voxel intensity variation impacts the determination of biofilm from barium sulfate. The demonstration images correspond to: (a) high flow rate column; (b) middle flow rate column; (c) low flow rate column; (d) cross-sectional depictions of the 33% barium control column, above edge volume; (e) below edge column; (f) volume subtraction result; (f) result of segmentation using FSM.
Figure 5-3: Summary of the average temporal differential pressure record according to initial column Reynolds number (and associated flow rate). Differential pressure across column was measured continuously for the duration of the growth phase of the experiment. Each data point corresponds to the average of a 12 hr block of measurements for duplicate columns. Error bars correspond to one ± standard deviation of the averaged values.
Figure 5-4: Summary comparison of initial and final hydraulic conductivity for each experimental column.
Figure 5-5: Temporal experimental dissolved oxygen record for (a) Re=10 columns with an applied flow rate of 450 mL per hr, (b) Re=1.0 columns with an applied flow rate of 45 mL per hr, (c) Re=0.1 columns with an applied flow rate of 4.5 mL per hr, and (d) compiled influent dissolved oxygen for the duration of the experiment.
Figure 5-6: Summary comparison of spatial variation in column porosity in the axial direction as calculated using results from both segmentation methods. The quantified (a) initial and (b) final porosity distributions generated using the factorization segmentation routine are presented for direct comparison to the (c) initial and (d) final porosity measurements generated using the alternate, peak subdivision, segmentation method.
Figure 5-7: Direct comparison of calculated spatial changes in porosity calculated using the factorization segmentation routine as opposed to the alternate, peak subdivision, segmentation routine. The measured change in porosity data has been separated based on applied flow rate with (a) focusing on the Re=10 data sets; (b) contains the Re=1.0 data; (c) presents the Re=0.1 results; (d) shows the average change in porosity values for each flow rate. Note: The alternate segmentation method was unable to isolate biofilm in the Re=0.1, B data set. For this reason, (c) only contains one data set segmented using the alternate method.
Figure 5-8: Hydraulic conductivity (K) values estimated using the Kozeny-Carman equation, and direct comparison to K to experimental K values calculated using the terminal differential pressure measurements from the biofilm growth experiment. (a) Initial hydraulic conductivity values for the 6 experimental columns evaluated in this study, compared to the initial K values calculated by setting the characteristic length dm to equal 6/Ms. (b) Estimates of K values determined using three separate values for dm, in reference to the experimentally determined K values.
Figure 5-9: Hydraulic conductivity sensitivity analysis comparing Kozeny-Carman estimates calculated using various integration heights. (a) K estimates using the total porosity measurements for each column; (b) K estimates based on the minimum porosity averaged on a 3.15 mm interval; (c) K estimates using the minimum porosity average on a 1.58 mm interval; (d) K estimates using the minimum porosity averaged on a 0.53 mm interval; (e) K estimates based on the minimum porosity averaged on a 0.05 mm interval; (f) K estimates based on the minimum porosity measured in each column (10.5 μm interval).
6 GENERAL CONCLUSIONS
The incorporation of x-ray computed microtomography into biofilm growth and bioclogging experimental investigations has the potential to allow direct, visual, evaluation of biofilm growth internal to opaque porous media. A large number of advances in our understanding of biofilms have resulted from direct observation. The ability to visualize and image transparent 2D and pseudo 3D micromodels is one of the reasons that these tools have played a prominent role in porous media-associated biofilm growth research over the last 20 years. However, most porous media applications for biofilms are three-dimensional, and the applicability of results and models derived from 2D experimental systems is limited due to the significant difference in complexity between 2D and 3D systems. In order to more fully understand biofilm growth and bioclogging effects in porous media, as well as to validate computational models for 3D biofilm growth, new advances and new techniques are required which enable the visualization and quantification of biofilm growth in porous media applications. This has been the main focus and objective of the body of work contained in this dissertation. Two methods for imaging biofilm in porous media using x-ray computed microtomography have now been presented.

The first method focuses on the addition of silver-coated, hollow glass microspheres to systems containing biofilm. The silver microspheres affix to the biofilm aqueous interface enabling the delineation of that surface, internal to the pore space of a porous medium. Reconstruction of the interface is facilitated using a computational pointwrap algorithm which joins the discrete, biofilm-attached microspheres together into a triangulated mesh for quantitative analysis. This method was the focus of Chapter 2.
An alternate method that was developed concurrently focuses on the addition of a barium sulfate suspension to the aqueous phase in a porous medium. Instead of relying on surface attachment or coating of the biofilm, this method fills the entire hydraulically available pore space enabling delineation of biofilm from the aqueous phase based on a (a) density differences between barium sulfate and biofilm if a polychromatic x-ray source is used, or (b) identification of elemental barium if monochromatic, synchrotron-based x-ray CT is used. The initial work that our group conducted on this method focused on presenting the method and demonstrating, qualitatively, that barium sulfate can be used for identifying biofilm inside a porous matrix. However, additional work was required in order to quantitatively evaluate biofilm growth using this method. Chapter 3 focused on the development of a technique for systematically processing and quantifying the image data acquired using this method with polychromatic x-ray scanners. One of the major obstacles to the quantitative analysis of data acquired using this method with polychromatic x-ray sources is beam hardening, which occurs when low energy (soft) x-rays are preferentially absorbed during tomographic imaging. While beam hardening artifacts can be mitigated and/or addressed either during scanning or through the image processing techniques discussed in Chapter 2, monochromatic x-ray light sources can reduce the impact of beam hardening much more effectively. In addition, the use of synchrotron based x-ray sources can greatly shorten the time required for scanning to be accomplished, while still allowing for natural porous materials to be imaged. With this in mind, the barium sulfate method for imaging biofilm in porous media was modified and tested. This method development as well as
the development of a reliable segmentation routine for analyzing the resulting data stream was the topic of Chapter 4.

Finally, an investigation looking to evaluate biofilm growth subjected to different flow loading rates was the topic of Chapter 5. This experiment serves as one of the first applications of the barium sulfate method in the investigation of biofilm growth in porous media, and bioclogging in general. Results from this experiment are mixed. The experimental portion of the experiment demonstrated differences in differential pressure, measured hydraulic conductivity, and measured dissolved oxygen concentrations based on the flow rate applied to the experimental columns. Evaluation of the quantified CT results incorporated the analysis of two segmentation routines. In the end, the general trends shown in the quantified CT data sets were similar regardless of which segmentation routine was used. The main difference between the two sets of quantified results being a shift in evaluated porosity of approximately 10%. Direct comparison of measured changes in hydraulic conductivity to estimated values calculated using the Kozeny-Carman equation and changes in column porosity measured from the CT data was initially unsuccessful. However, CT imaging facilitates not only the quantification of parameters such as material volumes, surface areas and interfacial areas, but it allows for evaluation of the spatial distribution of these quantitative parameters as well. Evaluation of the spatial distribution of quantified changes in porosity axially through the columns revealed that the quantified bulk average porosity measurements were averaging out a number of smaller areas where porosity variations were reducing the area available for fluid flux far beyond what the average value indicated. Recalulation
of the Kozeny-Carman hydraulic conductivity measurements produced more reasonable results. This study also helped highlight several areas important for future research. Both of the methods developed herein have advantages and disadvantages. The advantages associated with the silver microsphere method of biofilm imaging include the fact that the silver microspheres adhere to the biofilm surface, that the microspheres can be added directly to the influent stream of growth media during normal flow conditions, as well as that identifying the microsphere using monochromatic CT is relatively straight forward so long as dual edge scanning is conducted. The three main critiques of the method both revolve around construction of the surface mesh that is used for quantitative analysis. First, the ability to create a surface mesh is directly dependent on the quality of coverage of the biofilm surface(s). If large regions of a growth reactor are not hydraulically available, or if portions of biofilm end up constricting flow, then the silver microspheres can be kept from accessing large regions of an experimental reactor, and any biofilm area that doesn’t have silver on it when imaging commences ends up not being included in quantified biofilm. Second, reconstruction of the triangulated mesh currently requires the use of a pointwrap algorithm. Being restricted to this algorithm results in the requirement that all of the biofilm be connected in some way throughout a given data set this also then requires that a uniform coverage occur throughout the volumetric data set, because the reconstruction algorithm is currently designed to mesh together the first contiguous network of points that it encounters, thus if you have two distinct regions of biofilm within a data set, only one will be meshed into a surface if there is not a material or a bridge to connect the two regions. Third, and
perhaps most important, is the fact that the silver microspheres outline the interface between the biofilm and the aqueous phase. The microspheres do nothing, though, to help in determining which side of the interface is biofilm and which is made up of the aqueous phase. Taking these considerations into account, there are two distinct scenarios where I see the silver microsphere method being incredibly useful in future investigations. The first scenario focuses on studies where particle or colloidal tracking is of interest, and the second scenario focuses on biofilm imaging studies where the developed biofilm is not as prolific as have been all of the biofilm studies contained in this work. Due to the ability of the silver microspheres to adhere to biofilm surfaces the method could be used in the detection of small amounts of biofilm which would go unnoticed, or unsegmentable using the barium sulfate method.

In terms of the barium sulfate method for imaging biofilm in porous media, this method is fairly easy to implement and administer. The barium sulfate does a very good job of filling the available pore space, so long a suitable concentration is used. As mentioned in Chapter 4, the advisable concentration of barium sulfate is a 50% concentration. The differences in fluid properties between the barium sulfate contrast agent and any growth media being administered does need to be considered in order to avoid flushing biofilm out of a system prior to imaging., however, we have consistently had good results by slowing the injection rate to $1/5^{th}$ to $1/10^{th}$ of the growth media application rate. The
most prominent issues with the method are settling can occur during imaging, and that fine details, particularly like the biofilm shown in Figure 5-2c can end up having a higher voxel intensity than other biofilm voxels because it is a small volume surrounded by a dense, x-ray absorbent material.

6.1 Future research
The main area that I see being vital to the continued advancement of techniques for imaging biofilm in porous media is the need for additional contrast agent development.

The most important way for this new area of biofilm research to continue to develop, I feel, is in the identification and development of new contrast agents. The area of confocal laser scanning microscopy is a perfect example where the actual tool, the microscope, merely facilitates experimental work while the broad and continuously growing range of stains used in CLSM imaging make the technique an invaluable asset to both biofilm research and microbiology in general. Now that we have a surface coating contrast agent as well as an aqueous phase-filling contrast agent, the logical next step is to develop a contrast agent that actually diffuses into cells and EPS, binds there, and is able to be flushed from the aqueous phase. I think that it is only through the development of a stain such as this that a lot of the uncertainties remaining about both the silver and barium contrast agents will be resolved. A number of options for accomplishing something like this are available, whether it be through antibody complexation, or through a successful variant of the gram stain. The development of a biofilm-specific stain will go a long way toward bringing the three-dimensional imaging of biofilm in opaque porous media into the mainstream.
7 BIBLIOGRAPHY


BIBLIOGRAPHY (Continued)


BIBLIOGRAPHY (Continued)


**BIBLIOGRAPHY** (Continued)


**BIBLIOGRAPHY** (Continued)


APPENDIX 1
IMAGING BIOFILM IN POROUS MEDIA USING X-RAY COMPUTED MICROTMOMOGRAPHY
IMAGING BIOFILM IN POROUS MEDIA USING X-RAY COMPUTED MICROTMOTOGRAPHY

Yohan Davit, Gabriel C. Ilitis, Gerald Debenest, Stephanie Veran-Tissoires, Dorthe Wildenschild, Magali Gerino, Michel Quintard

Journal of Microscopy

John Wiley & Sons Inc

350 Main Street

Malden MA 02148, USA

Volume 242

Issue 1
Imaging biofilm in porous media using X-ray computed microtomography

Authors: Y. DAVIT*,‡, G. ILTIS||, G. DEBENEST*,†, S. VERAN-TISSOIRES*,
D. WILDENSCHILD||, M. GERINO‡§ & M. QUINTARD*,†

* Université de Toulouse; INPT, UPS; IMFT(Institut de Mécanique des Fluides de
Toulouse), Allée Camille Soula Toulouse, France

†CNRS; IMFT F-31400 Toulouse, France

‡Université de Toulouse; INPT, UPS; ECOLAB Rue Jeanne Marvig F-31055 Toulouse,
France

§CNRS; ECOLAB F-31055 Toulouse, France

||School of Chemical, Biological, and Environmental Engineering, Oregon State University
Corvallis, Oregon, U.S.A.

Key words: Biofilm, imaging, porous media, X-ray tomography.
8.1 Summary

In this study, a new technique for three-dimensional imaging of biofilm within porous media using X-ray computed microtomography is presented. Due to the similarity in X-ray absorption coefficients for the porous media (plastic), biofilm and aqueous phase, an X-ray contrast agent is required to image biofilm within the experimental matrix using X-ray computed tomography. The presented technique utilizes a medical suspension of barium sulphate to differentiate between the aqueous phase and the biofilm. Potassium iodide is added to the suspension to aid in delineation between the biofilm and the experimental porous medium. The iodide readily diffuses into the biofilm while the barium sulphate suspension remains in the aqueous phase. This allows for effective differentiation of the three phases within the experimental systems utilized in this study. The behaviour of the two contrast agents, in particular of the barium sulphate, is addressed by comparing two-dimensional images of biofilm within a pore network obtained by (1) optical visualization and (2) X-ray absorption radiography. We show that the contrast mixture provides contrast between the biofilm, the aqueous-phase and the solid-phase (beads). The imaging method is then applied to two three-dimensional packed-bead columns within which biofilm was grown. Examples of reconstructed images are provided to illustrate the effectiveness of the method. Limitations and applications of the technique are discussed. A key benefit, associated with the presented method, is that it captures a substantial amount of information regarding the topology of the pore-scale transport processes. For example, the
quantification of changes in porous media effective parameters, such as dispersion or permeability, induced by biofilm growth, is possible using specific upscaling techniques and numerical analysis. We emphasize that the results presented here serve as a first test of this novel approach; issues with accurate segmentation of the images, optimal concentrations of contrast agents and the potential need for use of synchrotron radiation sources need to be addressed before the method can be used for precise quantitative analysis of biofilm geometry in porous media.

8.2 Introduction

Microorganisms (primarily bacteria, fungi and algae), in wet or aqueous environments, tend to aggregate and grow on surfaces, embedded within extracellular polymeric substances (EPS) (Costerton et al., 1995; Sutherland 2001). These sessile communities, termed biofilms, are ubiquitous in industry (Ganesh Kumar & Anand 1998), in medicine and natural environments (Hall-Stoodley et al., 2004). Biofilm cells, when compared with planktonic cells, have been documented to be more resistant to antibiotics and biocides (Costerton et al., 1999; Stewart, 2001; Davies, 2003; Hall-Stoodley et al., 2004). Hence, the development of biofilms can have undesirable and potentially harmful consequences in medical applications (Diosi et al., 2003; Lee & Kim, 2003), but can also be useful in natural or engineered systems such as wastewater treatment processes (Lazarova & Manem, 2000), bioremediation (Rittmann et al., 2000) or CO2 storage (Mitchell et al., 2009). In medical, natural, as well as engineered systems, biofilm control strategies, based on a better understanding of biofilm growth characteristics
as well as stress response behaviour, have become an important challenge (Stewart et al., 2000; Thormann et al., 2005; Xavier et al., 2005; Rittmann, 2007; Kim et al., 2009).

Within porous media (e.g. subsurface soil or rocks, or the riverine hyporheic zone), biofilm growth within the pore space can induce substantial modifications to mass and momentum transport dynamics (Taylor & Jaffé, 1990; Cunningham et al., 1991; Vandevivere & Baveye, 1992; Wu et al., 1997; Stoodley et al., 2005; Shafahi & Vafai, 2009). Evidence of this type of modification has been developed by observing variation, over time, of macroscopic parameters such as hydraulic conductivity and permeability as well as changes in porosity and dispersion, in conjunction with sampling indicating the presence of biofilm. A large amount of models, based on different conceptual schemes, processes or scales have been developed to describe the growth of biofilm and the associated consequences on porous media transport properties within the last decades. Historically, biofilms have been assumed, for modelling purposes, to form continuous layers (Williamson & McCarty, 1976; Taylor & Jaffé, 1990; Cunningham et al., 1991). Other propositions suggest that biofilms arrange in patchy aggregates within pore throats (Vandevivere & Baveye, 1992). Rittmann (1993) emphasized that both representations can be correct, that is, the spatial distribution of attached microorganisms strongly depends on the physical, chemical and biological properties of the medium and even on its history (Vieira et al., 1993; Telgmann et al., 2004). For example, hydrodynamics, nutrient conditions, microorganism species,
predation and bioturbation are found to have a strong impact on the growth dynamics of biofilms. Limitations of these empirical models have been widely discussed, for example interesting analysis concerning the ‘microcolony model’ (Molz et al., 1986), and the ‘biofilm model’ (Rittmann & McCarty, 1980) can be found in (Cunningham & Mendoza-Sanchez, 2006) as well as in (Baveye & Valocchi, 1989). Other models, based on a theoretical and numerical multiscale analysis of the processes, have emerged. For example, cellular automata (Picioreanu et al., 1998; Xavier et al., 2005) have been generally successful and provide interesting perspectives to investigate and understand microorganisms response to various environmental stresses (Molloy, 2006). Individual-based models have also been adapted to the problem of biofilm growth in three-dimensional (3-D) porous structures and can be used to study various phenomena, such as bioclogging (Graf von der Schulenburg et al., 2008). Other mathematical analyses focus on the development of upscaled biofilm-scale and Darcy-scale continuum descriptions of the transport processes. Such methods allow for the establishment of a direct connection between the microscopic topology of the porous medium and the macroscopic continuum behaviour. Various upscaling techniques have been adapted to the problem of biofilms in porous media such as the moment matching
method (Dykaar & Kitanidis, 1996) and the volume averaging theory (Wood & Whitaker, 1998; Wood & Whitaker, 1999; Wood et al., 2002; Golfier et al., 2009; Davit et al., 2010). In these theories, effective parameters, for example permeability and dispersion, are numerically calculated on a Representative Elementary Volume.

The fundamental issue with all these models, either empirical or theoretical, continuum or individual based, is that, in the context of porous media, they are often validated only against macroscale experiments, lacking crucial microscale direct observations. Without this microscale information, the multiscale development of pertinent macroscopic models as well as the determination of the fundamental parameters required to characterize the spatio-temporal distribution of biofilm within porous media is difficult. Yet, various methods have been developed for imaging biofilms, including confocal laser scanning microscopy (CLSM) (Lawrence et al., 1991; Kuehn et al., 1998), light microscopy (Bakke & Olsson, 1986; Bakke et al., 2001), electron microscopy (Priester et al., 2007), atomic force microscopy (Beech et al., 1996), nuclear magnetic resonance imaging (Lewandowski et al., 1992; Potter et al., 1996), infrared spectroscopy (Nivens et al., 1993), optical coherence tomography (Xi et al., 2006) and high-frequency ultrasound (Shemesh et al., 2007). Unfortunately, there
are constraints associated with each of these imaging techniques. Many of the
aforementioned techniques are not applicable to generic porous media structures,
due to their inherent opacity. The methods are also not well suited for imaging
regions larger than several porous media grains.

To circumvent the opacity problem, most of the work on pore-scale/biofilm-scale
observations in porous media has focused on 1-D or 2-D networks (Kuehn et al.,
1998; Thullner, 2010a). There has been some discussion of the differences induced by
experimental dimensionality (Baveye, 2010; Thullner, 2010a,b). Baveye (2010)
suggests that future work should focus on 3-D observations rather than on adapting
pseudo 1-D or 2-D results to 3-D configurations. The ability to image biofilm in
three dimensions within porous media would considerably aid in providing the
experimental data that has been lacking to validate the models that have been
presented so far. As a noticeable exception, Seymour et al. (2004a,b, 2007) used
noninvasive magnetic resonance microscopy to directly observe the 3-D velocity field
at the pore-scale and show that biofilm growth can induce anomalous transport.
The issue with this technique is that it does not allow spatial resolution of the
pore-scale geometry of the different phases within the porous matrix. Recent work
presented by (Iltis et al., 2010) focuses on the imaging of biofilm within porous media
using monochromatic synchrotron based X-ray computed microtomography. Results
from this work illustrate the ability of computed microtomography to provide experimental data for the validation of mathematical models of porous media associated with biofilm growth. However, the method is based so far on a cumbersome physical straining or on attachment of a contrast agent to the biofilm surface.

In this study, we present a method for imaging non a priori labelled microbial biofilms in porous media using a benchtop X-ray computed tomography setup. The presented method allows for the 3-D reconstruction of the solid, aqueous and biofilm phases within a porous matrix with a voxel size of 9 µm. A significant challenge inherent to imaging biofilm within porous media using X-ray absorption tomography lies in selecting proper contrast agents to aid in differentiating between materials with similar absorption coefficients, such as biofilm and water. Most conventional X-ray contrast agents diffuse readily into both the aqueous phase and biofilm (Iltis et al., 2010). The proposed method focuses on the use of a mixture of two different contrast agents that allow for differentiation of the solid, aqueous phase and biofilm regions within the experimental systems evaluated in this study.

The remainder of this paper is organized as follows. First, we present the different protocols that are used in this experimental study. Then, we validate the use of the contrast agents by comparison of 2-D images obtained by (1) optical shadowscopy
and (2) X-ray absorption radiography. Finally, the technique is applied to two different model porous media experimental systems containing polyamide or expanded polystyrene beads. Various reconstructed images are shown to illustrate the effectiveness of the method. The limitations of the techniques are discussed as well as suggestions for future work.

8.3 Material and methods

8.3.1 The porous models

Three types of porous media models were used for experimentation. Two-dimensional biofilm growth experiments were conducted using a porous media network consisting of expanded polystyrene beads (500–1500 µm) compressed between two PMMA (Plexiglas®), 3-mm-thick, transparent plates. Initial 3-D imaging was conducted using a polystyrene column (3.5 mm inner diameter) packed with 3 mm diameter polyamide beads. Additional 3-D biofilm imaging experiments were conducted using a polystyrene column (3.5 mm ID) packed with polystyrene beads (500–1500 µm). Expanded polystyrene has a lower X-ray absorption coefficient than polyamide, allowing an initial contrast between the biofilm and the beads. Schematics of the experimental devices can be found in Figs 1 and 2 for both the 2-D pore network and the 3-D column experiments.
8.3.2 Growing biofilms

Raw water from the river Garonne (France) was collected, filtered using a 500 µm screen and clarified via sedimentation for approximately 24 h. The river water was further amended with CH3COONa; 3H2O (carbon source) and KNO3 (electron acceptor) as indicated in Table 1. The prepared water was then placed in a plastic feed tank used as the reservoir for experimentation and constantly aerated using an air pump. The feed tank was refilled every 24–48 h with prepared river water for the duration of the experiments, to maintain the total water volume between 200 and 400 mL (depending mainly on evaporation). The microbial flora naturally present in the prepared river water was experimentally determined to form sufficient biofilm with the porous media for the purposes of this study. Flow within the experimental systems was induced using either a peristaltic or diaphragm pump (as detailed in Table 1). All experiments were conducted at 20°C ± 1°C in the absence of light to control the growth of phototrophic organisms. Additional details are provided in Table 1.

8.3.3 Contrast agent

As previously mentioned, both the biofilm and the aqueous phase have similar X-ray absorption properties. In addition, all the experimental systems evaluated in this study were designed using plastic materials to minimize the total X-ray exposure time of the microorganisms as well as to optimize the grey-level scaling. Unfortunately, the plastic beads used as the experimental porous medium also have
similar X-ray absorption properties to the biofilm and aqueous phase. Hence, obtaining contrast between the different phases requires the utilization of multiple contrast agents. Conventional contrast agents (e.g. potassium iodide) diffuse readily into biofilm when present in the aqueous phase. In this study, we use a medical suspension of micrometre-sized barium sulphate (Micropaque® , Guerbet) to enhance the absorption of the water-phase. Such medical suspensions are conceived to have specific physical properties (Cumberland, 1977; Plouraboué et al., 2004), that is, a high density to provide highly contrasted radiographs, low viscosity to readily penetrate within small sized areas and extremely reduced settling and flocculation of the particles to provide homogeneous absorption of the X-rays. Although barium is usually highly toxic, it is commonly safely used as a medical radiocontrast agent for X-ray imaging of the gastrointestinal tract or angiography because of its insolubility in water. It is also a low-cost product, known not to diffuse within the tissues. The idea behind the utilization of such a suspension is that particles are size excluded from the EPS matrix. If not totally immobilized, micrometre-sized cells within biofilms are known to be greatly constrained in their motion. Hence, diffusion of similar sized barium sulphate particles through the polymeric matrix itself is likely to be negligible. To what extent the contrast agent can penetrate into the biofilm following the flow within nutrients channels and how this depends on the matrix architecture as well as on the contrast agent viscosity and density remains to be fully characterized. It is interesting to emphasize that most
studies concerned with convective flows within biofilms involve submicrometre-sized particles (Stoodley et al., 1994) and that micrometre-sized latex beads grown with microbes seem to be immobilized (Drury et al., 1993). In addition, potassium iodide was added to the barium sulphate suspension to provide the required contrast between the polystyrene beads and the biofilm. Various ionic or nonionic iodinated radiocontrast agents are used for medical purposes (Aronson, 2006). In our case, we only require that it readily diffuses within the polymeric matrix. In this context, iodide (whether NaI or KI) has proved to be adapted to X-ray microtomography for noninvasively imaging biological specimens (Chen et al., 2004).

For the polyamide beads, experiments focused on obtaining an important contrast between the biofilm and the water phases, using only barium sulphate at higher concentrations. The details of the contrast agent mixtures used during experimentation are provided in Table 1. Preliminary scanning of the concentrations ratios were performed; herein, only the concentrations that proved to be the most successful are presented.

8.3.4 Imaging protocols

Two-dimensional imaging. The continuous flow of amended river water through the 2-D flow cells was induced and biofilm was allowed to develop for 10 days at which point optical imaging commenced using the system presented in Fig. 2. A white LED backlight (PHLOX®) applied a uniform illumination of the pore network from beneath the stage and images were captured from above using a 12 bit (SensiCam) camera linked to a computer by a fibre optic cable [as illustrated in Fig. (2)].
Following optical imaging, 10 mL of the contrast agent solution consisting of 0.33 g mL$^{-1}$ barium sulphate and 0.1 g mL$^{-1}$ potassium iodide was injected into the flow cell. The system was then set to rest for approximately 1.5 h to simulate the 3-D X-ray tomography image acquisition time frame (see next section). After this delay, a 2-D X-ray absorption radiograph was captured using a Skyscan 1174 tomograph with a pixel size of 12 µm.

Three-dimensional imaging. After 10 days of continuous flow, the experimental flow cells was removed from the water flow circuit. Ten millilitres of the contrast mixture, containing a suspension of barium sulphate, potassium iodide and water, was slowly injected through the porous model using a syringe. The concentrations of the contrast agent additives for the various experiments evaluated in this study are detailed in Table 1. The experimental flow cells then sat stagnant for approximately 15 min to allow for diffusion of the iodide into the biofilm. During these 15 min, a Skyscan 1174 tomograph was set to a tension voltage of approximately 50 kV and a current of 800 µA. All computed tomography imaging for this study was conducted at a resolution of 9 µm per pixel on a 360° rotation with a rotation step ranging from 0.5° to 0.7°. In each case, the total duration for tomographic imaging is approximately 1.5 h. The major technical limitation we encountered during tomographic imaging was ring artefacts, regardless of the use of the ring artefact reduction option in the commercial software.
NRecon (SkyScan). Meanwhile, there is no limitation in the method itself which prevents the utilization of synchrotron based tomography (monochromatic) or new generations of scanners capable of producing higher quality images.

8.3.5 Data analysis

Two-dimensional image analysis. 2-D (radiographic) X-ray absorption images (12 bit TIFF images) and 2-D optical images (12 bit TIFF images) were postprocessed using the open source software package ImageJ. For the X-ray images, we applied a fast-Fourier transform bandpass filter to reduce extreme frequency noise. Then, the two data sets are compared using pseudocoloration based on a LookUp Table. This coloration was choosen on the basis of visualization purposes, as guides for the eyes. Quantitative measures, such as correlation ratios, strongly depend on the segmentation procedure. This is beyond the scope of this work to propose such methods; rather, we provide a qualitative analysis of the results. Representative images used for comparison of the two data sets are provided in Fig. 3.

Three-dimensional tomography. The absorption projection images (12 bit TIFF images) were reconstructed using NRecon to obtain a set of cross-sectional slices (16 bit TIFF images) of the columns, using ring artefact and beam hardening correction. The various greyscale images presented in this paper are encoded as 8 bit images for visualization purposes. For the polyamide beads, images were slightly smoothed and undergo global binarization using ImageJ Otsu’s method. The surfaces are built in p3g, surface format, using the commercial software CTAn (Skyscan) and the 3-D geometry is observed using the software CTVol (Skyscan). The goal of this
work is to provide an operational technique for imaging biofilm that is to demonstrate that the use of a barium sulphate suspension as a contrast agent is feasible for imaging biofilm within a porous medium matrix.

8.4 Results

8.4.1 Two-dimensional experiments

The purpose of the 2-D investigation was to evaluate the behaviour of the contrast agent mixtures and to ensure that sufficient contrast between the various phases was achieved.

Potential issues identified include

*Potential issue A.* Exclusion of the barium sulphate suspension from the biofilm EPS needs to be verified.

*Potential issue B.* The contrast agents need to be investigated to see whether interactions between the microorganisms and the contrast agents modify the EPS geometry, thereby preventing the acquisition of representative images.

*Potential issue C.* The injection of the contrast agent mixture needs to be examined to determine whether the induced shear stress associated with injection results in biofilm detachment from the porous media matrix or in modifications of the EPS geometry.

*Potential issue D.* It is necessary to determine whether prolonged (1.5 h) X-ray exposure induces changes in the EPS geometry.
The presented X-ray computed tomography imaging method for biofilm investigations is non-invasive, in that the biofilm growth can be imaged \textit{in situ}. However, one caveat that must be taken into account is that X-ray exposure is expected to either severely retard microbial growth or kill the microorganisms all together. Thus, the technique can be considered nondestructive to the porous media–biofilm matrix, but the imaging technique is still terminal.

To investigate possible temporal changes to the biofilm matrix during imaging, a series of experiments were conducted to assess whether the potential issues previously identified as A, B, C and D negatively impact image accuracy and quality on the time-scale of a 3-D tomography acquisition (approximately 1.5 h of exposure time using the Skyscan 1174 tomograph). Thus, images of a 2-D pore network colonized by biofilm obtained using both optical shadowscopy and X-ray computed tomography were compared. Results of the 2-D investigation are presented in Fig. 3. Three zones, corresponding to different biofilm geometries, have been processed using a pseudocoloration to allow for comparison. Within Zone A, three biofilm filaments are clearly visible on both the optical image as well as the X-ray image. In Zones B and C, a clear correlation between the two geometries is apparent although discrepancies between the optical image and X-ray tomography image exist within these zones as well. Based upon the qualitative image comparison within these zones there appears to be good agreement between the two image capturing methods. Because the optical imaging method focuses, primarily, on a top-side view of the biofilm, the increased
distribution of barium sulphate within the radiograph can be attributed to an increased flow channel volume within the biofilm that is not visible within the depth of field captured using optical microscopy. Thus the qualitative results presented in Fig. 3 illustrate the utility of using X-rays (and the chosen contrast agent) to image biofilm, particularly when 3-D tomographs are captured as opposed to 2-D radiographs since the tomographs are capable of providing direct visualization of the channelling suspected to be present within the biofilm present in Zones A, B and C. The barium sulphate suspension used for imaging does not appear to enter the EPS layer readily within these zones. Rather the contrast agent seems to follow the aqueous phase flow channels. These conclusions are supported by the results, provided in the next section, concerning the successive use of barium sulphate and iodide. Hence, the issues previously detailed as A and B do not appear to significantly affect our imaging results. However, further investigations are required to elucidate the microscale behaviour of the particles, especially in relation to the density of the EPS matrix and the physical properties of the contrast agent suspension. Nevertheless, the use of barium sulphate as a contrast agent for imaging biofilm within porous media is promising since the delineation of the topology of the flow channels and the associated impact on the transport processes at the pore-scale is definable within relatively large volumes.

With respect to issue C, special care needs to be taken to ensure that the contrast agent or contrast agent mixture is injected at the same flow rate that was applied during the biofilm growth phase or at a lower flow rate to compensate for the slightly
larger viscosity of the contrast agents mixture. The introduction of air bubbles during injection should be avoided as well, as this introduces a fourth phase to the imaged system. Although special care was taken during the introduction of the contrast agent mixture used in the collection of the images detailed in Fig. 3, some detachment of biofilm, as well as the introduction of small bubbles into the porous medium, was noted (results are not presented). Within Fig. 3 we have identified, and circled in red, white regions within the radiograph that do not appear within the optical image. In these zones, the X-ray absorption coefficient is relatively small, meaning that the concentration of barium sulphate is lower than in the rest of the fluid phase. These are thought to correspond to air bubbles or detached biofilm, in which the contrast agent is volume excluded. However, these effects concern a relatively small proportion of the porous medium and biofilm volume, and this is a problem that can be addressed in future applications by careful experimentation.

Although issue D cannot be fully addressed using this 2-D experiment, we observed no substantial modifications to the EPS geometry after approximately 30 min of X-ray exposure. Although biofilm associated microorganisms are expected to be severely inhibited or killed by exposure to X-rays, the biofilm matrix appears to be stable after exposure times of up to 1.5 h from the benchtop tomography (Skyscan 1174) X-ray source used in this investigation. 3-D results concerning this aspect of the problem are discussed in the next section.
8.4.2 Results of the 3-D tomography and discussion

Single polyamide bead. The first set of 3-D experiments focuses on imaging of biofilm on 3-mm-diameter polyamide beads. For this case, only the barium sulphate suspension was introduced as a contrast agent. Examples of projection data are presented in Fig. 4 at time $t = 0$ without biofilm and at $t = 10$ days following the biofilm growth phase. Differences between these two raw images take the form of patchy white spots meaning, locally, lower X-ray absorption. These zones appear because biofilm has developed, constraining the local volume available for barium sulphate. This set of absorption data is used to reconstruct a set of cross-sectional slices on a single bead within the experimental column. Greyscale images as well as representative binary images are provided in Figs 5(a) and (b) at $t = 0$; Fig. 5(c) and (d) for $t = 10$ days. At $t = 0$, a cross-sectional circular shape, corresponding to the polyamide bead, is observed. After 10 days of biofilm growth, the boundary of the object that we imaged is tortuous and covers more surface. On the basis of the 2-D study presented in the preceding section, we interpret this additional area as biofilm. It is important to note that within Fig. 5(c) there is no contrast between the plastic bead and the biofilm grown on the bead, further reinforcing the proposition that the barium sulphate suspension is excluded from the EPS layer of the biofilm. A solution of potassium iodide was then flushed through the system. A depiction of the polyamide bead after potassium iodide addition is provided as Figs 5(e) and (f). Iodide, when present in the aqueous phase, diffuses readily into biofilm
present within the pore space. As a result, the contour of the polyamide bead is all that is clearly visible in Figs 5(e) and (f), thereby confirming that the tortuous zone surrounding the bead in Figs 5(c) and (d) is in fact biofilm. Some bright spots along the edge of the bead on Fig. 5(e) are also visible, corresponding to barium sulphate absorbed on the biofilm.

Surface reconstructions of the polyamide bead are provided in Fig. 6. The surface reconstructions correspond to $t=0$, prior to biofilm growth, and $t = 10$ days, after the biofilm growth phase. Contrast for both images is provided using the barium sulphate suspension. Within the imaged section, the biofilm appears to be highly heterogeneous and represents about 6% of the volume of the naked polyamide bead. Additional study is required to draw further conclusions on biofilm growth and development within our experimental system, however, the ability to image biofilm within porous media using the proposed technique has been established, which is the purpose of this study.

*Results for the polydisperse expanded polystyrene beads.* For more complex porous structures, such as polydisperse polystyrene beads, the alignment of tomography data captured both prior to, as well as following biofilm growth is not necessarily possible due to the potential for bead displacement due to fluid transport or biofilm growth. Thus, image processing techniques such as image subtraction are not applicable. A mixture of the barium sulphate and potassium iodide contrast agents at two different concentrations was utilized to differentiate between the
three materials present within the experimental system. Using this contrast mixture, tomographic imaging was performed. Preliminary imaging was carried out at time $t = 0$ after introducing the contrast agents mixture. Imaging was also conducted at time $t = 10$ days, approximately 15 min after injecting the mixture of both contrast agents. Comparative results are provided in Fig. 7 for the two data sets. Results for the $t = 0$ data set indicate that the contrast agent solution delineates, clearly, the beads contained within the column. At $t = 10$ days, the presence of three distinct phases is observed. The brightest phase corresponds to the barium sulphate (highest absorption coefficient). The dark regions correspond to beads and the intermediate greyscale values are interpreted as biofilm which the iodide has diffused into. Figure 8 illustrates the results of a comparative experiment examining biofilm growth within packed bead columns through which two different flow rates were applied. For this experiment, two columns containing polystyrene beads and connected to the same water supply were exposed to flow rates of 0.07 and 0.5 mL s$^{-1}$. Within the two columns biofilm growth appears to decrease with increasing flow rate. Although additional experiments are required to draw conclusions about biofilm growth within porous media, the presented results demonstrate that pore-scale information on biofilm growth within a porous medium is readily achievable using the proposed imaging method. Using the results generated using the presented method calculations of column or regional permeability can be performed numerically by solving Navier–Stokes equations. Darcy-scale dispersion tensors can also potentially be calculated using upscaling.
Successive imaging of a single column was conducted in an effort to further evaluate the effect of X-ray exposure on biofilm structure (issue D). The total exposure time was 3 h and consisted of a sequence of two imaging cycles. At the conclusion of this experiment, no change within the biofilm geometry were observable. This suggests that, at least for an acquisition time of 3 h or less, X-rays at the energy emitted by the Skyscan 1174 tomograph (50 kV and 800 µA) do not modify the geometry of the biofilm EPS matrix.

8.5 Conclusions

In this study, we present first results for a new method for imaging biofilm in porous media using X-ray computed tomography. We successfully use a mixture of two different contrast agents to obtain a three-phase contrasted 3-D representation of a model porous medium containing solids, water and biofilm. This method, because of its simplicity, accessibility and applicability to complex porous structures, provides an interesting and versatile framework for studying biofilm within porous media systems. The method can potentially be used in the calculation of porous media effective parameters. In particular, the presented method opens possibilities for systematic studies of biofilm response, within porous media, to changes in physical, chemical and biological parameters. For example, modifications of local Reynolds and Péclet numbers, nutrient availability, temperature and pH stresses, and the impact of biofilm biodiversity on biofilm geometry within the 3-D porous media matrix can potentially be
investigated. Although the use of synchrotron X-ray sources hold the potential to provide higher quality imaging data and the imaging of biofilm in porous media has been investigated using synchrotron light and silver microspheres as a contrast agent (Iltis et al., 2010), the method presented in this study is functional using both benchtop tomographs, such as the Skyscan 1174 as well as synchrotron X-ray sources, even though more sophisticated image processing procedures need to be developed. Thus the presented method is broadly applicable since imaging is not necessarily restricted by synchrotron accessibility and beam time constraints. On the other hand, one significant limitation associated with the use of benchtop tomographs is that the required imaging time for porous media materials such as glass beads, soil or rock materials is significantly greater than the 1.5 hour image acquisition time reported in this study. As a result, investigations using these types of porous media are anticipated to require synchrotron light sources. Future work will focus on (1) optimization of the image acquisition techniques, to obtain images that can be easily (and impartially) segmented into their respective phases (whether it is using a different polychromatic or a monochromatic imaging system, optimizing the concentrations of the contrast agents, using separate imaging of the solid phase, or similar), (2) a comparison of this work with other 3-D planar imaging techniques such as confocal laser scanning microscopy, for instance to provide further understanding of the interaction between the 1 µm BaSO4 suspension and the architecture of the biofilm, (3) application to real porous samples with heterogeneities of absorption
coefficients in the porous structures, and (4) an investigation of microbial retardation or mortality induced by X-ray exposure.
Table 1. Biofilm growth experimental design details.

<table>
<thead>
<tr>
<th></th>
<th>2-D pore network</th>
<th>3-D polyamide beads</th>
<th>3-D polystyrene beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beads diameters</td>
<td>500–1500 µm</td>
<td>3 mm</td>
<td>500–1500 µm diameters</td>
</tr>
<tr>
<td>Pump type</td>
<td>Prominent gamma/L diaphram</td>
<td>Ismatec Mini-S 820 peristaltic</td>
<td>Watson Marlow 505 Du type peristaltic</td>
</tr>
<tr>
<td>Nutrients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Introduced at days</td>
<td>0, 3, 6 and 9</td>
<td>0, 4 and 7</td>
<td>0, 3, 6 and 9</td>
</tr>
<tr>
<td>CH₃COONa; 3H₂O</td>
<td>0.66 g</td>
<td>0.16 g</td>
<td>0.66 g</td>
</tr>
<tr>
<td>KNO₃</td>
<td>0.33 g</td>
<td>0.06 g</td>
<td>0.33 g</td>
</tr>
<tr>
<td>Contrast agents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BaSO₄</td>
<td>0.33 g mL</td>
<td>0.66 g mL⁻¹</td>
<td>0.33 g mL</td>
</tr>
<tr>
<td>KI</td>
<td>0.1 g mL⁻¹</td>
<td>0 g mL⁻¹</td>
<td>0.1 g mL⁻¹</td>
</tr>
<tr>
<td>Flow rates</td>
<td>3.5 mL min⁻¹</td>
<td>6 mL min⁻¹</td>
<td>0.07 and 0.5 mL s⁻¹</td>
</tr>
</tbody>
</table>
Fig. 1. Flow system used for growing biofilm within (1) the two-dimensional pore network model and (2) the three-dimensional packed bead columns.
Fig. 2. Optical visualization system for the two-dimensional pore network.
Fig. 3. Comparison of two-dimensional images after 10 days of growth obtained using (1) the visualization device detailed in Figs 2 and (2) Skyscan 1174 X-ray absorption radiograph captured approximately 1.5 h after injection of the contrast agent mixture. On the radiograph, bright corresponds to low X-ray absorption and dark to high X-ray absorption. Three zones A, B and C, assessing various pore-scale geometries, underwent pseudocoloration using ImageJ on the basis of a LookUp Table (LUT). The dark-blue parts correspond to the beads, the blue-green-brown parts to the biofilm and the white parts to the aqueous phase. The parts circled in red on the radiograph correspond to either detached pieces of biofilm or gas bubbles that are not present on the optical shadowscopy.
Fig. 4. Examples of projection images obtained with the SkyScan 1174 using BaSO4 as the contrast agent at time $t = 0$ days on the left and $t = 10$ days on the right. Both images have undertaken a pseudocoloration in ImageJ on the basis of the unionjack LUT (only for visualization purposes). Blue corresponds to the highest X-ray absorption, red to intermediate absorption and white to lowest absorption.
Fig. 5. Cross-sectional reconstructed X-ray computed tomography data for a polyamide bead. Bright corresponds to high X-ray absorption and dark to low X-ray absorption. At $t = 0$ days, with BaSO$_4$ as the contrast agent, (a) is the reconstructed greyscale image and (b) is the binarized image obtained using ImageJ. At $t = 10$ days, after biofilm growth, with BaSO$_4$ as the contrast agent, (c) is the reconstructed greyscale image and (d) is the binarized image obtained using ImageJ. At $t = 10$ days, using potassium iodide as the contrast agent, (e) is the reconstructed greyscale image and (f) is the binarized image obtained using ImageJ. White spots along the edge of the bead on (e) correspond to barium sulphate absorbed on the biofilm.
Fig. 6. Three-dimensional surface reconstructions of the polyamide bead at time $t = 0$ using BaSO4 as the contrast agent and the biofilm (soft blue-green) and the polyamide bead (dark) at time $t = 10$ days.
Fig. 7. Greyscale cross-sectional X-ray computed tomography for the experimental columns packed with polystyrene beads at $t = 0$ with the mixture of contrast agents and at time $t = 10$ days. Bright corresponds to high X-ray absorption and dark to low X-ray absorption.
Fig. 8. Examples of reconstructed (X-ray Skyscan 1174 data) sectional slices for the entire length of the column obtained after 10 days at a flow rate of approximately (a) $Q = 0.07 \text{ mL s}^{-1}$ and (b) $Q = 0.5 \text{ mL s}^{-1}$ (a pseudocoloration has been applied to the images using ImageJ on the basis of the ceretec LUT and only for visualization purposes). The white-red parts correspond to the beads, the red parts to the biofilm and the blue-dark parts to the aqueous phase.
8.6 Bibliography


Bibliography (Continued)


Bibliography (Continued)


Bibliography (Continued)


Bibliography (Continued)


Bibliography (Continued)