pH Effects on Ceramic Membranes Fouled by Extracellular Polymeric Substances

Undergraduate Honors Research Thesis

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Abstract

Biofouling has proven to be a primary challenge for membrane filtration. Biofouling occurs when microbes secrete extracellular polymeric substances (EPS) which adhere to membrane surfaces. EPS impede the flow of treated water through membrane pores, resulting in increased pressure demands and operation costs. Importantly, the protein to polysaccharide ratio of EPS affects its propensity to adhere to surfaces. However, the effects of pH on EPS adherence have not been investigated over a broad pH range. This study investigates how pH affects the composition of EPS fouling layers that have been cleaned with ultrasound. This is significant because ceramic membrane technology allows for filtration at previously unfeasible pH levels, and it is unknown how operation under these pH conditions will affect fouling layer composition. In this study, 0.2 micron Whatman Anodisc ceramic membranes were fouled with EPS solution. During each filtration, flux measurements were taken. Membranes then underwent either timed sonication or no sonication. During filtration and sonication, pH was kept constant at a value of 3.5, 6.9, or 9.5. Confocal laser scanning microscopy, in conjunction with MATLAB image analysis, compared protein and polysaccharide intensities of each fouling layer. It was found that fouling layers initially exhibit a wide variety of protein to polysaccharide ratios, and that preferential removal of regions high in proteins occurs at pH 3.5. Additionally, EPS solutions exhibited faster filtration rates at pH 3.5 than at other pH values. Results were attributed to pH-mediated interactions between EPS components and the membrane surface and structural conformations of EPS.

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Field of Study

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Chapter 1: Introduction

To resist environmental stresses and facilitate adherence to a wide variety of surfaces [1], microorganisms secrete a protective matrix of extracellular polymeric substances (EPS). More than 99% of microorganisms secrete a network of EPS [2]. Membrane technology plays an increasingly prevalent role in water treatment, however, membranes are prone to fouling by EPS that adhere to their surfaces and block pores [3]. Membranes are made from polymers or ceramics, and operate by size exclusion. The effective diameter of a membrane's pores determines the size of molecule that it can exclude, giving rise to the classification system for membranes. Microfiltration and ultrafiltration membranes, pore size 10 to 0.1 µm and 100 to 2 nm respectively, are used for pretreatment of wastewater streams. Nanofiltration and reverse osmosis membranes have much smaller effective pore sizes, between 2 – 1 nm and less than 1 nm respectively, and are capable of excluding individual ions, primarily for drinking water treatment. Polymer membranes are more widely employed than ceramic membranes; however, ceramic membranes are becoming more popular due to their ability to withstand harsher cleaning conditions, such as high pH and temperature, which would normally destroy polymer membranes.

Membranes are susceptible to the phenomenon of fouling, and biofouling remains a major obstacle facing membrane technologies. EPS is the most significant contributor to biofouling [4]. EPS initially foul a membrane through the mechanisms of pore clogging and foulant adsorption [2]. Pore clogging depends on surface interactions

between EPS and membrane pores as well as closeness in size between the foulant and pores [2]. Surface adhesion depends on parameters such as surface charge and distance between the foulant and the membrane. Acidic conditions, calcium ion content, and high ionic strength can reduce energy barriers between EPS and a membrane surface, facilitating attachment [2].

EPS are a heterogeneous mixture of high and low weight polymers, most prominent among which are proteins and polysaccharides. The exact chemical composition of EPS is far from clear [1]. Instead, characterization of EPS usually focuses of the quantification of its primary components. An important parameter of EPS is the protein to polysaccharide ratio, which is abbreviated as PN/PS. The PN/PS of EPS determines several important traits. EPS with a low PN/PS exhibit higher adherence to membranes [4] and higher floc cohesiveness when subjected to shearing [5]. PN/PS also correlates negatively with the rate of membrane fouling [6]. PN/PS has also shown to be important in determining the surface charge of EPS. Polysaccharide content negatively correlates with hydrophobicity and surface charge. Overall, the polysaccharide portion of EPS is hydrophilic, and protein content has a positive correlation with surface charge and has been found to be hydrophobic [8]. These properties are due to the variety of functional groups displayed by EPS proteins and polysaccharides. Wang et al. (2011) found that positively-charged amino groups located in EPS proteins can neutralize portions of negative charge of acidic EPS functional groups, such as carboxylic and phosphoric groups [8].

Other substances present in EPS include humic substances, lipids, nucleic acids, and uronic acids [9]. EPS has to ability to complex with heavy metals due to its wide

variety of functional groups. Much like a cation exchange resin, when heavy metals are added to EPS solution, EPS will release Mg^{2+} and Ca^{2+} [9]. Ions such as calcium and iron associate with EPS proteins and assist in flocculating EPS through divalent cation bridging and double layer compression, both processes further contributing to surface adherence [9]. Further contributing to EPS flocculation is entanglement of long EPS polymers [9]. Molecular weights of EPS polymers can range from a few hundred Daltons to over 300,000 Daltons [1]. Diversity in functional groups lends EPS the ability to bind to both hydrophobic and hydrophilic surfaces, and to cohere to other EPS, via a variety of mechanisms [2]. One relevant mechanism is mediated by charge interactions between ionizable EPS functional groups, such as carboxyl, phosphoric, sulfhydryl, phenolic, and hydroxyl groups [9], and the surface potential of a membrane via electrostatic attraction [10]. EPS surface charge is neutral when it is at its isoelectric point but otherwise exhibits an inverse relationship with solution pH. Solution pH also affects the spatial distribution of EPS on a nanometer scale. For example, at pH 11, EPS was reported to be homogeneously distributed and swollen as functional groups had aquired repulsive charges. Conversely, decreasing the pH from 8.8 to 0.7 was shown to cause EPS aggregation into non-homogenous networks of dense domains, characterized by decreased distance between polymer chains [11]. The size of aggregates of EPS molecules also increases under elevated pH conditions [10]. Thus, solution pH is an important contributing factor to EPS adherence to ceramic membranes, especially given that ceramic membranes can tolerate a wider pH range than their polymer counterparts.

Extraction methodology is a critical part of any EPS study. EPS extraction starts with the collection of a biofilm. Unfortunately, there is no widely agreed upon method of

thereafter extracting EPS. Prevalent, high-yield extractions involving addition of formaldehyde and NaOH are shown to alter the chemical makeup of the extracted EPS [12]. Other issues include extraction chemicals remaining in final EPS solutions, such as in the case of the EDTA method [13], and disproportionate protein yield, such as in cation exchange resin extractions [12]. However, methodologies that do not alter EPS composition do exist. Ultrasound and centrifugation methods do not alter EPS functional groups, and additionally, do not rely on the addition or removal of outside compounds to the biofilm undergoing extraction [12].

Complementing the study of PN/PS, confocal laser scanning microscopy (CLSM) is a technique that can be used to directly visualize EPS on by tagging protein and polysaccharide portions with fluorescent markers [14]. CLSM can be used to create a 3D image of tagged polymers on a membrane surface, allowing for investigation of their relative prevalence and spatial distribution.

Fouling of ceramic membranes has not been studied as widely as polymeric membranes, and given the large differences in operating conditions between the two, it is important to expand the study of foulants across the range of operating conditions. This work explores the effects of solution pH on fouling and the removal of EPS proteins and polysaccharides from fouled ceramic membranes. EPS extracted from biofilms was filtered through Whatman Anodisc ceramic microfiltration membranes, pore size 0.2 μm, under pH 3.5, 6.9, and 9.5 conditions, in a dead-end filtration setup, resulting in the formation of fouling layers. An ultrasound bath applied shearing forces to the EPS to induce partial removal of the fouling layer. CLSM was used to image the fouling layers, followed by MATLAB image analysis to determine the ratio of protein probe intensity to

polysaccharide probe intensity. The objective of the analysis was to determine at which pH conditions a ceramic membrane system may operate in order to enhance filtration rates and ultrasonic cleaning for EPS-fouled membranes.

The following section, Methodology, details experimental methods. It includes all chemical analytical methods employed, methods for taking filtrations measurements, and microscopy procedures. The Results section lays out the results of assays and filtration measurements for EPS solution pHs of 3.5, 6.9, and 9.5. Additionally, this section details the analysis of confocal imaging data and presents results. The Discussion section draws upon data presented in Results to interpret data, compare it with findings in the literature, expresses limitations in the study's findings, and draws conclusions. The Conclusion section summarizes experimental results and outlines their implications to future research. Attached are also all references to all cited works, as well as Appendix A, image analysis histograms, and Appendix B, image analysis MATLAB scripts.

Chapter 2: Materials and Methods

EPS sampling and extraction

Biofilms used in EPS extractions were obtained from Jackson Pike Wastewater Treatment plant on 2015/07/08 and 2016/01/27. Using a plastic sampling cup, biofilms adhering to the walls of the final clarifier were collected. Furthermore, a sample of final clarifier water was also collected into a 100 mL Erlenmeyer flask. The biofilm samples were then transferred to 1 L glass beakers, which were sealed with Parafilm and refrigerated at 4 C for no more than 5 days. The final clarifier water sample was immediately tested for dissolved oxygen content, conductivity, and pH, before storage at 4 C.

The biofilms were then subjected to the ultrasound EPS extraction procedure similar to that reported by D'Abzac et al. Since the ultrasound reactor is not recommended for use with solids, biofilms were first diluted using DI water until they were fluid. The 2015 EPS was diluted to a final volume of 25% DI and 75% biofilm, while the 2016 EPS was diluted 50% with DI water. A Sorvall Legend RT centrifuge was turned on and allowed to come to 4 C. An ELAC Nautik USW 51 ultrasound transducer was attached to an ELAV Cesar RF Power Generator. The transducer and amplifier were set to generate ultrasound with a frequency of 20 kHz and an intensity of 300 W/L. The homogenized biofilm was transferred into the ultrasound reactor, where it was sonicated for one minute. Sonicated biofilm was transferred into six 15 mL centrifuge tubes, and immediately placed in the centrifuge. Centrifugation lasted for 20 minutes at 8000 G. The

supernatant containing the extracted EPS was then removed into a flask, and the pellet was discarded. To further clarify the EPS solution, the supernatant was centrifuged once more for 20 minutes at 8000 G. The extracted EPS solution was then stored in a 500 mL stock bottle at -3 C.

EPS Characterization

Extracted EPS were characterized by gravimetric analysis, protein content, and polysaccharide content. EPS and final clarifier water also underwent ICP analysis for calcium content.

Gravimetric analysis

The mass of an aluminum weigh boat was recorded, and EPS solution was pipetted into it. The dish was then reweighed. An oven was set to 105 C and the weigh dish containing the EPS was placed in the oven. After 24 hours had passed, the dish was placed in a desiccator and final weight was recorded.

Protein assay

The Pierce BCA Protein Assay Kit (Thermo Scientific, lot PA199207) was used to determine the protein content of the EPS solution. A stock solution of 200 mg/L bovine serum albumin was prepared from the provided ampules. An Isotemp 1013S incubator bath was set to 37 C and allowed to come to temperature. Standards covering the range of 0 mg/L to 200 mg/L were prepared by diluting stock solution with DI water to a final volume of 100 µL. Replicates of 100 µL EPS solution were measured into test tubes. The working reagent mixture was then added to the test tubes which were then vortexed. The

vortexed tubes were incubated for 30 minutes. Once 30 minutes were over, the test tubes were allowed to cool. Test tube contents were placed in cuvettes, and spectrophotometer was blanked with DI water and a Thermo Scientific NanoDrop 2000c spectrophotometer measured the absorbance of each cuvette at 562 nm.

Polysaccharide assay

The polysaccharide content of the EPS solution was measured using the anthrone method as follows. A 500 mg/L stock solution of D-glucose was first prepared. Glass test tubes were cleaned with soap and water and triple rinsed with DI water. Standards were prepared covering the range of 0 mg/L to 200 mg/L by diluting stock solution with DI water to a final volume of 600 µL. Replicates of 600 µL of EPS solution were also placed in glass test tubes. All test tubes were then placed on ice. 100 mg of anthrone was mixed with 2.5 mL of ethanol in a 100 mL Erlenmeyer flask. A stir bar was placed in the flask, and 47.5 mL of 75% sulfuric acid was added to the flask. The stir bar mixed the solution until all anthrone dissolved.

Working in a fume hood, a 1 L water bath was brought to a boil using a hot plate. 3 mL of anthrone solution was pipetted into each glass test tube. Test tubes were capped with Parafilm and pierced with a pin to prevent pressure buildup. Test tubes were swirled and placed in the boiling water bath. After 10 minutes of boiling, the tubes were removed and placed back into the ice bath until cool. The spectrophotometer was blanked with DI water and set to measure absorbance at 625 nm. The contents of each glass test tube were then pipetted into a cuvette and absorbance was measured by the spectrophotometer.

ICP-AES analysis

A Vista AX CCD Simultaneous ICP-AES was used to quantify elemental calcium concentrations of extracted EPS and clarifier samples. 12 mL falcon tubes were filled to the 10 mL mark with either calcium standards, diluted EPS, or diluted clarifier water. The argon tube furnace was fed with 2% nitric acid into the nebulizer, and emission for calcium was measured at wavelengths of 317.933, 318.127, and 396.847 nm. Blanks were run between measurement of standards and samples. Emission values were fitted to a standard curve with standards ranging from 0 to 25 mg/L, and calcium content was determined.

Membrane Fouling and Ultrasonic Cleaning

Two dead-end filtration apparatuses were first constructed. To construct an apparatus, a 60 mL syringe was fitted with a rubber stopper instead of a plunger, which was then glued in place. A 18 gauge syringe needle was then inserted through the rubber stopper. A 25 mm filter holder containing a membrane could then be attached to the Luer Lock end of the filtration apparatus, while a gas line for pressurization could be attached to the Luer Lock end of the needle.

To conduct the foulings and collect flux measurements, an Orion 5 Star (Thermo Scientific) conductivity meter and pH probe was calibrated using pH 4, 7 and 10 standards. The dead-end fouling apparatus was attached to a ring stand. A gas line connected the apparatus to a tank of nitrogen gas. 1 L of $CaCl_2$ stock solution was prepared and its conductivity adjusted to 950 µS/cm using KCl. For each pH, 3.5, 6.9, and 9.5, 100 mL of stock solution was dispensed into a 500 mL Erlenmeyer flask. If the filtration trial was to test clean water flux, no EPS was added. However, if the filtration trial was to test EPS flux, at this point 7.2 mL of the EPS solution which was extracted

during summer 2015 was added to the Erlenmeyer flask. To each Erlenmeyer flask was added a stir bar and the pH probe. Each flask was sparged with nitrogen, and dilute NaOH or HCl was used to adjust the pH of the solution. Simultaneously, forceps placed a 0.2 µm Whatman Anodisc membrane filter into a 25 mm filter holder. A gas line was connected to the filtration apparatus and turned on. For 2 minutes, nitrogen gas was allowed to flow through the apparatus, and then the gas was shut off and disconnected.

Once the pH was stable and at least 10 minutes of sparging had occurred, 30 mL of the pH-adjusted solution was syringed into the filtration apparatus, and the membrane-containing filter holder was attached to the outlet of the apparatus. A depressurized gas line was then connected to the gas inlet of the apparatus. Two graduated cylinders, one for each filtration apparatus, were filled to the 2.5 mL mark with DI water and positioned below the apparatus outlet. The gas pressure was recorded. The gas lines to the apparatus were then pressurized, and flux measurements were taken by recording the volume of the graduated cylinder. Once the graduated cylinder volume reached 16 mL the gas was shut off, and the gas lines were depressurized. The filter holder was disconnected, and a 50 mL beaker collected unfiltered solution remaining in the apparatus. The pH and conductivity of the unfiltered solution was then measured by the probes.

EPS-fouled membranes were then sonicated under the same pH conditions that they were fouled. 500 mL of 10^{-3} M KCl was prepared in a beaker and adjusted to the correct pH with dilute NaOH or HCl. This solution was then loaded into the reactor chamber of a 620 kHz ELAC Nautik USW 51 sonicator. The frequency generator and amplifier were set to 60 W and 620 kHz, respectively. The fouled membrane was then removed from its filter holder and placed into a sonicator basket face-down. The

sonicator basket was then lowered into the sonicator until it was 10.5 cm away from the transducer membrane. The sonicator was then turned on for a duration of 0, 2, or 4 seconds. After sonication was finished, the membrane was removed from the bath and placed face-up on a microscope slide which was labeled with its pH and sonication duration. The microscope slide was then placed into a petri dish and covered. Membranes were then refrigerated at 4 C.

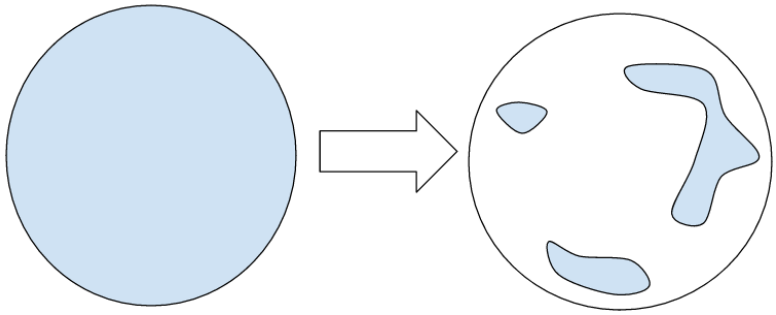

Figure 1. Cartoon depicting appearance of membrane before and after sonication with foulant represented in blue.

Staining and microscopy

Membranes were then stained with fluorescent probes according to the procedure outlined in Chen et al. (2006). In a dim room, 20 µM SYTO 63 was added dropwise to each membrane until each membrane surface was covered. The membranes, in their petri dishes, were then stacked, covered with tinfoil, and placed on a shaker table for 30 minutes at 20 rpm. The membranes were then unstacked and dipped in fresh solutions of

phosphate-buffered saline. This procedure was repeated until each membrane has also been covered with 0.1 M bicarbonate buffer, 10 g/L FITC, 0.2 g/L concanavalin A, and calcofluor white. After the final rinsing with phosphate-buffered saline, the membranes were stored at -80 C.

Frozen membranes were packed into a cooler and transported to the Olympus FV 1000 confocal laser scanning microscope. For imaging of SYTO 63, the CLSM excited at 633 nm and emission was detected at 650 – 700 nm. FITC was excited at 488 nm and detected at 500 – 540 nm. Calcofluor white was excited at 400 nm and detected at 410 – 488 nm. Concanavalin A was excited at 543 nm and detected at 550 – 600 nm. Either three or four images measuring 1.27 mm were taken of each membrane. Each image contained between 4 and 11 z slices, which were spaced either 5 or 10 μm apart. Imaged regions were selected for imaging based on the presence of foulant on the surface. Image spacing and image number were chosen such that a full profile of the fouling layer was captured. Laser intensity was chosen for each image to produce the clearest visualization of the fouling layer without introducing noise or bleaching. Images were stored on a flash drive as .oib files.

Chapter 3: Results

Analysis of EPS

Two solutions of EPS were extracted from biofilms, one collected during summer 2015 and the other during winter 2016. The solution containing the extracted EPS was analyzed for total solids concentration, protein concentration, polysaccharide concentration, and calcium concentration. Additionally, the calcium concentration of the final clarifier was determined. A comparison of these parameters for both EPS extraction dates can be seen in Table 1 below. The protein to polysaccharide ratio of the EPS solution was determined by dividing the protein content by the polysaccharide content. Propagated error was factored into calculations for total solids and protein to polysaccharide ratio. ICP results for clarifier calcium concentration were used to adjust the Ca^{2+} content of fouling stock solutions to levels with which the EPS were in equilibrium before extraction.

EPS Flux

Both EPS extracts were individually used to foul membranes. As fouling progressed, the volume of foulant solution filtered was recorded. Plots of volume filtered over time for each solution pH are shown in Figure 2. Volume measurements were not always taken at the same time. For example, measurements may have been taken at 1.5 and 2.5 minutes, but not at 2 minutes. In these cases, bracketing volume measurements and times were

used to linearly interpolate a volume measurement. To produce each plot, volume measurements were averaged and the standard deviation was calculated.

For both EPS solutions, the pH 3.5 solution filtered the most foulant solution in a 10 minute span. In the case of the 2015 EPS, the pH 3.5 fouling solutions filtered a mean volume of 12.6 mL in ten minutes whereas the pH 6.9 and pH 9.5 solutions filtered a mean volumes of 8.9 and 9.4 mL, respectively. In contrast, the 2016 EPS fouling solutions filtered faster overall. Variability in final volume after 10 minutes was much lower in the 2016 EPS. The pH 3.5 fouling solutions filtered 12.7 mL in ten minutes and the pH 6.9 and 9.5 fouling solutions filtered 12.0 and 11.5 mL, respectively. To show that fouling solution filtration rates were dependent on pH dependent properties of EPS, filtrations were conducted using a fouling solution containing no EPS, adjusted to the same pH and conductivity as the other fouling trials. Table 2 shows volume of EPS-free fouling stock filtered using a feed pressure of 30 psi.

Table 1. Characteristics of extracted EPS as determined by gravimetric analysis, Pierce BCA Protein Assay, the anthrone method for polysaccharide determination, and ICP.

Date of EPS extraction	2015/07/08	2016/01/27
Total solids (g/L)	1.170 +/- 0.001	0.70 +/- 0.05
Protein content (mg/L)	320 +/- 30	103 +/- 1
Polysaccharide content (mg/L)	60 +/- 10	46.3 +/- 0.5
Protein to polysaccharide ratio	5.3 +/- 0.3	2.22 +/- 0.01
Clarifier calcium concentration (mg/L)	61.1 +/- 0.8	77.4 +/- 0.3
Extracted EPS solution calcium concentration (mg/L)	75 +/- 1	40.6 +/- 0.3

Table 2. Clean water flux of unfouled membranes for three solution pH values.

pH	Flux of 285.4 mg/L $CaCl_2$*2H20 stock solution (mL/s)
3.5	0.870 +/- 0.004
6.9	0.89 +/- 0.01
9.5	0.826 +/- 0.007

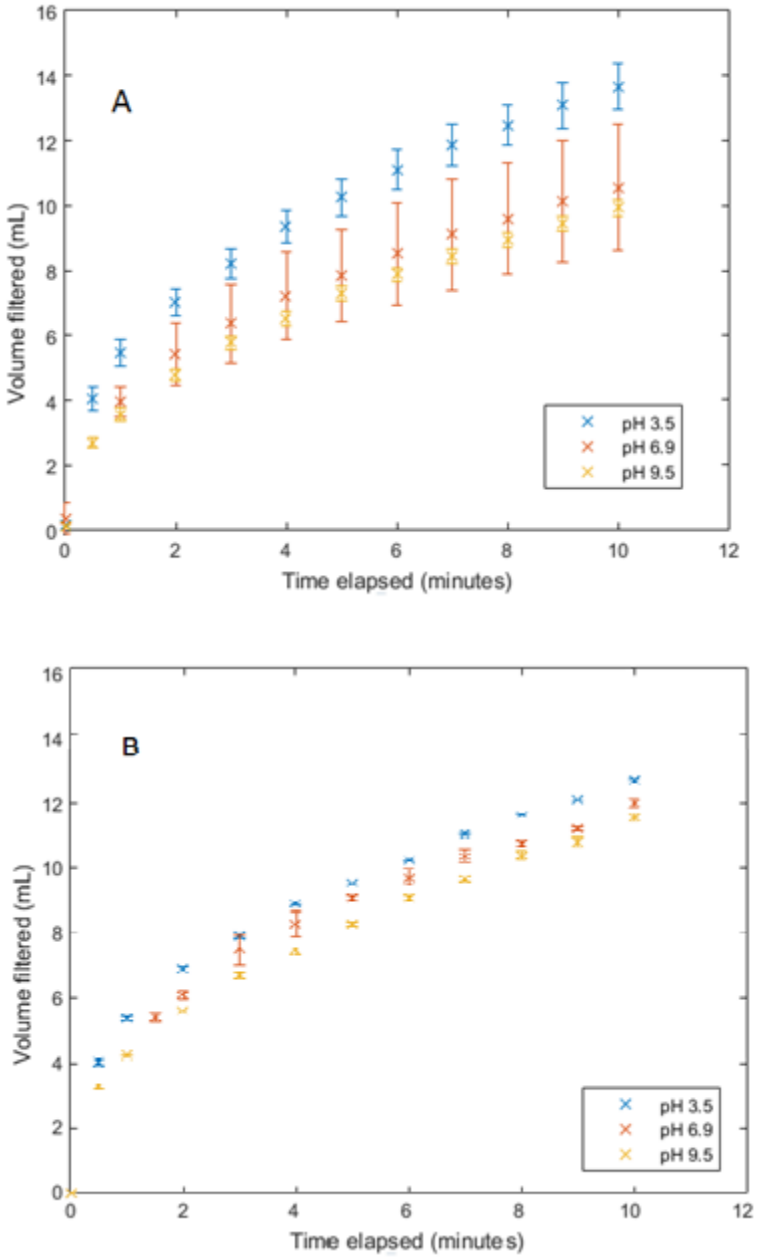

Figure 2. Volumes of EPS solution filtered over time for three pH values. Panel A shows the filtration of the 2015 EPS, the membranes used for which then went on to CLSM imaging. Panel B depicts the filtration of the 2016 EPS.

MATLAB Analysis of Confocal Microscopy

Confocal images were analyzed using MATLAB. Fiji was used to assign channel 1, calcofluor white, to the color red, and channel 2, FITC, to the color green. Channels 3 and 4 were empty for most images, and were not analyzed. The images were then exported in BMP format to MATLAB. All scripts used for image analysis used can be found in Appendix B. ImageFlattener.mat summed image intensity across the z-axis of each image stack, creating a 2D composite of each image. When called by imageAnalysis.mat and brokenImageAnalysis.mat, the function imageReader.mat then stored BMP image data as a .mat file. DataExpouser.mat loaded each .mat file and created a histogram for channel 1 and channel 2 by sorting pixel intensities for each channel into bins of width 10. From this data a histogram was created for each image, such as Figure 3 below. Histograms of all processed images can be found in Appendix A. Additionally, dataExposuer.mat calculated the 50^{th} percentile pixel intensity for each distribution.

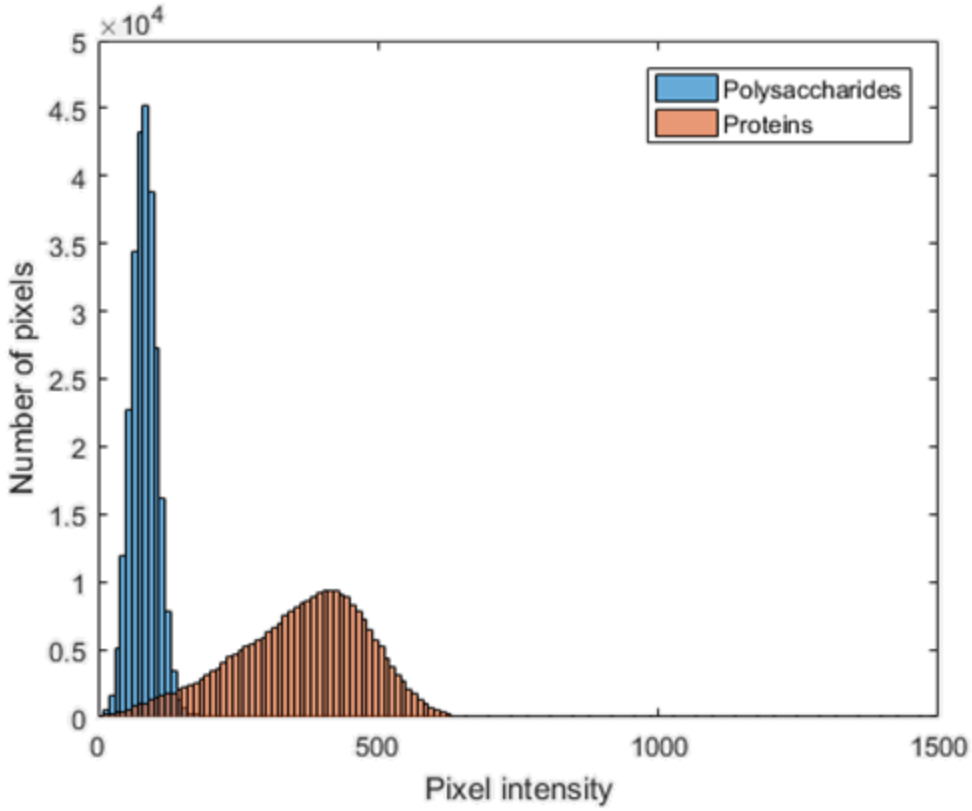

Figure 3. Typical distribution of proteins and polysaccharides. This image was taken of a membrane fouled and cleaned at pH 3.5, with sonication duration of 2 seconds. Its 50th percentile protein intensity is 380, its 50th percentile polysaccharide intensity is 90, and its 50th percentile PN/PS intensity ratio is therefore 4.2.

Image histograms revealed anomalies with a number of images, such as no distribution existing for one or both channels. For an image to be eligible for discarding, at least 10% or more of pixels must have exhibited zero intensity for proteins or polysaccharides, or the image must have exhibited a 90th percentile intensity of 10 or less

for proteins or polysaccharides. As a result, 14 images that were candidates for discarding were detected. Seven images displayed streaks instead of fouling layers and were discarded. Two images were blank upon inspection and were discarded. Four images showed only one type of stain and were discarded. In total, 14 images were discarded and 27 proceeded through the remainder of analysis. The function validImage.mat was used to determine if images met these criteria.

The final script to be called, statisticalAnalysis.mat, then divided the 50^{th} percentile intensity of channel 2 (proteins) by the 50^{th} percentile intensity of channel 1 (polysaccharides) to obtain a ratio that represents the relative, median prevalence of each channel on the image termed from hereon as the PN/PS intensity ratio. The data were then exported to Microsoft Excel, where, for each pH and sonication-duration pairing, PN/PS intensity ratios were calculated. To represent variance, a standard deviation was calculated for each combination of solution pH and sonication duration. The number of membranes and the number of images used in calculating each average is shown in Table 3. These averages and variances are shown in Figure 4. EPS filtered at pH 3.5 and 9.5 exhibited decreased averages in PN/PS intensity ratio with increased sonication duration, whereas the pH 6.9 trial exhibited mixed results with high variability. Variance within trials was high, visible by the large error bars associated with calculated PN/PS intensity ratios.

Table 3. Number of images of membranes used in developing each 50th percentile PN/PS intensity and corresponding standard deviations.

pH	3			6			9	
sonication duration (s)	0	2	4	0	2	4	0	2
Number of images analyzed	4	2	2	4	4	4	4	3
Number of membranes represented by analysis	2	1	1	2	2	1	1	1

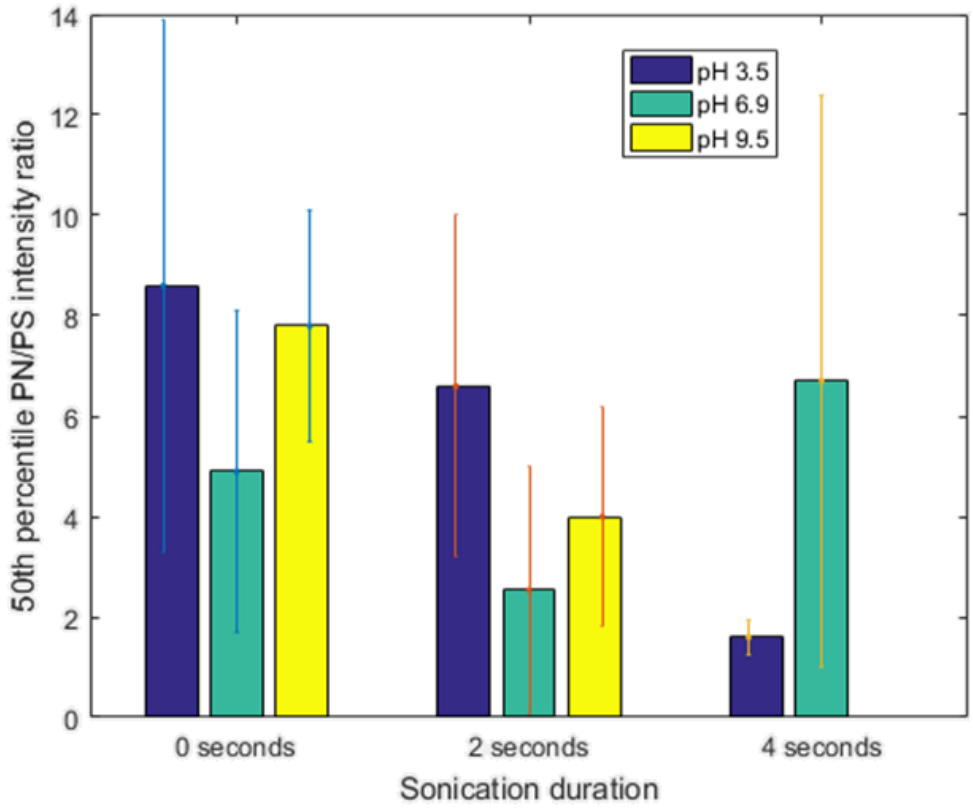

Figure 4. 50th percentile PN/PS intensity ratios for each combination of pH and sonication duration. No data exists for pH 9.5 with duration of 4 seconds because images met discard criteria.

Chapter 4: Discussion

Effects of pH and Sonication Duration on Fouling Layer PN/PS

All imaged membranes were fouled using the EPS extracted in 2015. Sonicated membranes exhibited large swaths of area in which neither proteins nor polysaccharides were present, as represented by Figure 1, and images were only taken in places where a fouling layer was still present.

For solution pH values of 3.5 and 9.5, images of the fouling layer after ultrasonication exhibited lower average PN/PS intensity than images taken of unsonicated membranes (Figure 4). Standard deviations are high for all image trials, with the exception of the pH 3.5, 4 second sonication. These results suggest two trends regarding EPS fouling layers. First, they suggest that the fouling layers do not have a homogenous PN/PS. Rather, fouling layers are highly heterogeneous with respect to PN/PS, which varies greatly from area to area. Second, the shifts from high PN/PS intensity to lower PN/PS with increased sonication suggests that regions of fouling layers with high PN/PS were more likely to be removed by sonication for those pH values. This implies that for solution pH values of 3.5 and 9.5, low PN/PS regions were less likely to be removed during cleaning than high PN/PS regions, resulting in the sonicated fouling layers exhibiting lower average PN/PS intensity ratios. Corroborating these results, a study using QCMD analysis observed that EPS adherence to PVDF ultrafiltration membranes increased with lower PN/PS [4]. Furthermore, the reduction in this variance displayed by

sonicated membranes at pH 3.5 suggests that a more narrow range of smaller PN/PS values were more likely to resist cleaning than the wide range of PN/PS values originally present on unsonicated membranes.

The preferential removal of high PN/PS regions of fouling layers may be explained by several factors. The protein portion of EPS has been shown to be hydrophobic [8], and therefore does not bind readily to hydrophilic alumina membranes. Surface charge interactions that mediate EPS electrostatic adhesion may also be a contributing factor to these patterns. Whatman Anodisc alumina membranes have been shown to have a point of zero charge around pH 8 [15]. Alumina membrane surfaces are therefore positively charged at solution pH values below 8 and negatively charged above pH 8. Under the conditions of this experiment, the alumina membrane had the most positive charge at pH 3.5. Since EPS with higher polysaccharide content have a more negative surface charge [8], EPS with low PN/PS will experience greater electrostatic attraction to alumina at pH 3.5 than EPS with high PN/PS. The protein portions of EPS are amino-dense, which accounts for higher hydrophobicity relative to the more polar, acidic groups which characterize the non-protein portion [8]. As a result, high PN/PS EPS will be more susceptible to removal through ultrasonic cleaning. The fact that PN/PS decreases with increased sonication at pH 3.5 conditions is reflective of the greater strength of attraction attributed to polysaccharides, especially since proteins have weak positive correlation with EPS surface charge [8] which would cause them to repel the membrane at pH values lower than 8.

The similar trend at pH 9.5 is harder to account for. At this pH, polysaccharide-associated acidic groups are deprotonated, and the membrane surface charge is negative.

However, while low PN/PS EPS may be electrostatically more repulsive at high pH than at low pH, this does not necessarily mean that they are more likely to be removed than high PN/PS EPS. To speculate, if protein hydrophobicity caused greater detachment from the membrane surface than the electrostatic repulsion of low PN/PS regions, then high PN/PS regions may be preferentially removed. However, more about the surface interactions of the specific EPS, such as the isoelectric point of the protein and polysaccharide portions of the EPS, would have to be known to attest for the presence of this trend with credence.

Overall, the pH 6.9 trials did not indicate a preferential removal of high or low PN/PS EPS. Inconsistent averages prevent the determination of anything but vague trends despite these trials being represented by greatest number of images and membranes (Table 3). A trend that generally exists for the pH 6.9 trials is high variance in PN/PS. High variance could mean that neither high PN/PS nor low PN/PS EPS are more likely to be removed, as the same high variance as seen in the unsonicated membranes remains present for all sonication durations. A possible explanation for this is that the EPS and the membrane surface are both closer to their isoelectric point, which would cause electrostatic attractions and repulsions to diminish. As a result, non-pH-dependent adherence mechanisms could take over. This is supported by reports that EPS isoelectric points are proximal to the EPS's environmental pH [11], pH 6.9 being the environmental pH level of EPS used in CLSM imaging.

The high variance of the imaging data, however, limits drawing solid conclusions. PN/PS has been shown to be highly variant on a membrane surface, and a large number of images are required to confidently state the means of data with such high variance.

Averages between membranes that underwent the same treatment had very high standard deviations, and so the imaging conducted should ideally be expanded until the number of samples can provide higher statistical confidence in reported average PN/PS. Currently, PN/PS outliers had a high propensity to affect averages due to small sample size.

To manage controllable sources of variability in imaging results, future procedures should vortex the fouling stock to ensure that EPS is fully homogenous before fouling takes place. Additionally, the staining procedure used by Chen et al. [14] requires dipping membranes repeatedly in phosphate buffered saline, which may remove foulant from membrane surfaces. Freezing membranes should not be practiced if spatial information about the fouling layer is to be conserved for imaging, since cold temperatures will warp the membrane, and the formation of ice crystals will dislocate the foulant. Finally, surface charge interactions could be commented on in more detail if points of zero charge of EPS proteins and polysaccharides were determined.

Nevertheless, it does appear that there is a trend toward lower PN/PS intensity regions of fouling layers remaining on alumina membranes when subject to cleaning at pH conditions of 3.5 and 9.5. This result implies that ceramic membranes that undergo repeated cycling of ultrasonic cleaning under acidic conditions may build up low PN/PS EPS during the initial stages of fouling.

pH Effects on Membrane Flux

The faster filtration 2015 EPS solution, as seen in Figure 2, may have been caused by a lack of pore blocking at a solution pH of 3.5. At low pHs, EPS are reported to de-swell from a network of homogenous density into a heterogeneous network of small, dense domains [11]. The result could be that large EPS aggregates present in low-pH solution

are not similar enough in size to the 0.2 μm pores to cause blockage. This is exemplified by the case of an EPS which fouled a membrane of 1.0 μm pore size faster than a 0.5 μm membrane because the size distribution of the EPS more closely matched the diameter of the pores [16]. EPS also have decreased surface at low pH conditions [7] resulting in reduced availability for interaction with membrane pore walls suggested by Sweity et al. [10].

Adsorption mediated by electrostatic attraction between the EPS and the membrane surface may also be responsible for the increased filtration at low pH. At a pH as low as 3.5, the EPS will likely be below its point of zero charge, rendering its surface charge to be net positive like the surface of the alumina membrane. If this were the case, increased electrostatic repulsion between the two would have detracted from EPS adsorption inside membrane pores, resulting in less pore blockage and higher flux. While there may be similar repulsion at a high pH, a corresponding increase in flux was not observed. This may be due the compensating effects of EPS networks swelling and become more inflexible under high pH conditions, causing more obstruction to the membrane surface [10].

The 2015 EPS was observed to filter faster at pH 3.5 when compared to pH 6.9 and 9.5. This trend was also observed for the 2016 EPS. However, in the case of the 2016 EPS, such a high contrast between filtration rates was not present between filtrations at different pH conditions. Without knowing more about the specific differences between the two EPSs, it is hard to say why this might be. It is known, however, that the 2016 EPS has a PN/PS of 2.22 whereas the 2015 EPS has a PN/PS of 5.3, calcium concentration also differs between the two EPSs, however, a better understanding of

these discrepancies in filterability necessitates gathering more detailed information about the two EPS solutions, such as point of zero of charge and molecular size distribution.

Chapter 5: Conclusion

It was found that unsonicated fouling layers exhibit a wide range of PN/PS. For a pH of 3.5, ultrasonicated fouling layers exhibited increased prevalence of areas with low PN/PS. It was proposed that these shifts were related to the hydrophobic character of the protein portion reducing the ability of high PN/PS regions to bind with the hydrophilic membrane surface. Additionally, relatively stronger electrostatic attraction of low PN/PS EPS to membrane surface resulted in cleaned membranes exhibiting lower PN/PS. As a result, repeated cleaning of ceramic membranes under acidic conditions could lead to buildup of low PN/PS EPS. Trends in EPS removal under pH conditions of 6.9 and 9.5 were less clear.

The comparison of two different EPS solutions showed that EPS of a low pH tends to filter faster, which was attributed to the tendency of EPS to form dense domains under low pH conditions. This may have been caused by the tendency of EPS to form low-surface area, dense aggregates under low pH condition, allowing EPS to pass through pores with reduced likelihood to obstruct pores. Surface charge effects may also be a contributing factor, since both EPS and the membrane surface were below the point of zero charge at pH 3.5.

Future imaging should incorporate a larger number of images to increase confidence in averages. Additionally, changes in the imaging procedure, such as avoiding repeated dipping in phosphate buffered saline and freezing steps, would improve the

quality of imaging results. Quantification of parameters related to adhesion, such as EPS surface charge and molecular size, should be incorporated in adherence related experiments.

References

[1] Kunacheva, C., & Stuckey, D. (2014). Analytical methods for soluble microbial products (SMP) and extracellular polymers (ECP) in wastewater treatment systems: A review. *Water Research*, 61, 1-18.

[2] Lin, H., Zhang, M., Wang, F., Meng, F., Liao, B., Hong, H., . . . Gao, W. (2014). A critical review of extracellular polymeric substances (EPSs) in membrane bioreactors: Characteristics, roles in membrane fouling and control strategies. *Journal of Membrane Science*, 460, 110-125.

[3] Meng, F., Chae, S., Drews, A., Kraume, M., Shin, H., & Yang, F. (2009). Recent advances in membrane bioreactors (MBRs): Membrane fouling and membrane material. *Water Research*, 43(6), 1489-1512.

[4] Sweity, A., Ying, W., Ali-Shtayeh, M., Yang, F., Bick, A., Oron, G., & Herzberg, M. (2011). Relation between EPS adherence, viscoelastic properties, and MBR operation: Biofouling study with QCM-D. *Water Research*, 45(19), 6430-6440.

[5] Sheng, G., Yu, H., & Li, X. (2006). Stability of sludge flocs under shear conditions: Roles of extracellular polymeric substances (EPS). *Biotechnology and Bioengineering*, 93(6), 1095-1102.

[6] Gao, W., Lin, H., Leung, K., & Liao, B. (2010). Influence of elevated pH shocks on the performance of a submerged anaerobic membrane bioreactor. *Process Biochemistry*, 45(8), 1279-1287.

[7] Wang, L., Wang, L., Ren, X., Ye, X., Li, W., Yuan, S., . . . Wang, X. (2011). PH Dependence of Structure and Surface Properties of Microbial EPS. *Environmental Science and Technology*, 737-744.

[8] Liao, B., Allen, D., Droppo, I., Leppard, G., & Liss, S. (2001). Surface properties of sludge and their role in bioflocculation and settleability. *Water Research, 35*(2), 339-350.

[9] Sheng, G., Yu, H., & Li, X. (2010). Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. *Biotechnology Advances, 28*(6), 882-894.

[10] Sweity, A., Ying, W., Belfer, S., Oron, G., & Herzberg, M. (2011a). PH effects on the adherence and fouling propensity of extracellular polymeric substances in a membrane bioreactor. *Journal of Membrane Science*, (378), 186-193

[11] Dogsa, I., Kriechbaum, M., Stopar, D., & Laggner, P. (2005) Structure of Bacterial Extracellular Polymeric Substances at Different pH Values as Determined by SAXS. *Biophysical Journal*, 89, 2711-2720.

[12] D'Abzac, P., Bordas, F., Hullebusch, E. V., Lens, P. N., & Guibaud, G. (2009). Extraction of extracellular polymeric substances (EPS) from anaerobic granular sludges: Comparison of chemical and physical extraction protocols. *Appl Microbiol Biotechnol Applied Microbiology and Biotechnology*, *85*(5), 1589-1599.

[13] Comte, S., Guibaud, G., & Baudu, M. (2006). Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties. *Enzyme and Microbial Technology*, *38*(1-2), 237-245.

[14] Chen, M., Lee, D., Yang, Z., Peng, X., & Lai, J. (2006). Fluorescent Staining for Study of Extracellular Polymeric Substances in Membrane Biofouling Layers. *Environmental Science & Technology.*, 40, 6642-6646.

[15] Kosmulski, M. (2006). PH-dependent surface charging and points of zero charge III. Update. *Journal of Colloid and Interface Science*, *298*(1), 730-741.

[16] Huang, C., Lin, J., Wu, C. L., & Chu, C. P. (2010). Recycling of spent filter backwash water using coagulation-assisted membrane filtration: Effects of submicrometre particles on membrane flux. *Water Science & Technology*, *61*(8), 1923.

Appendix A: Imaging Histograms

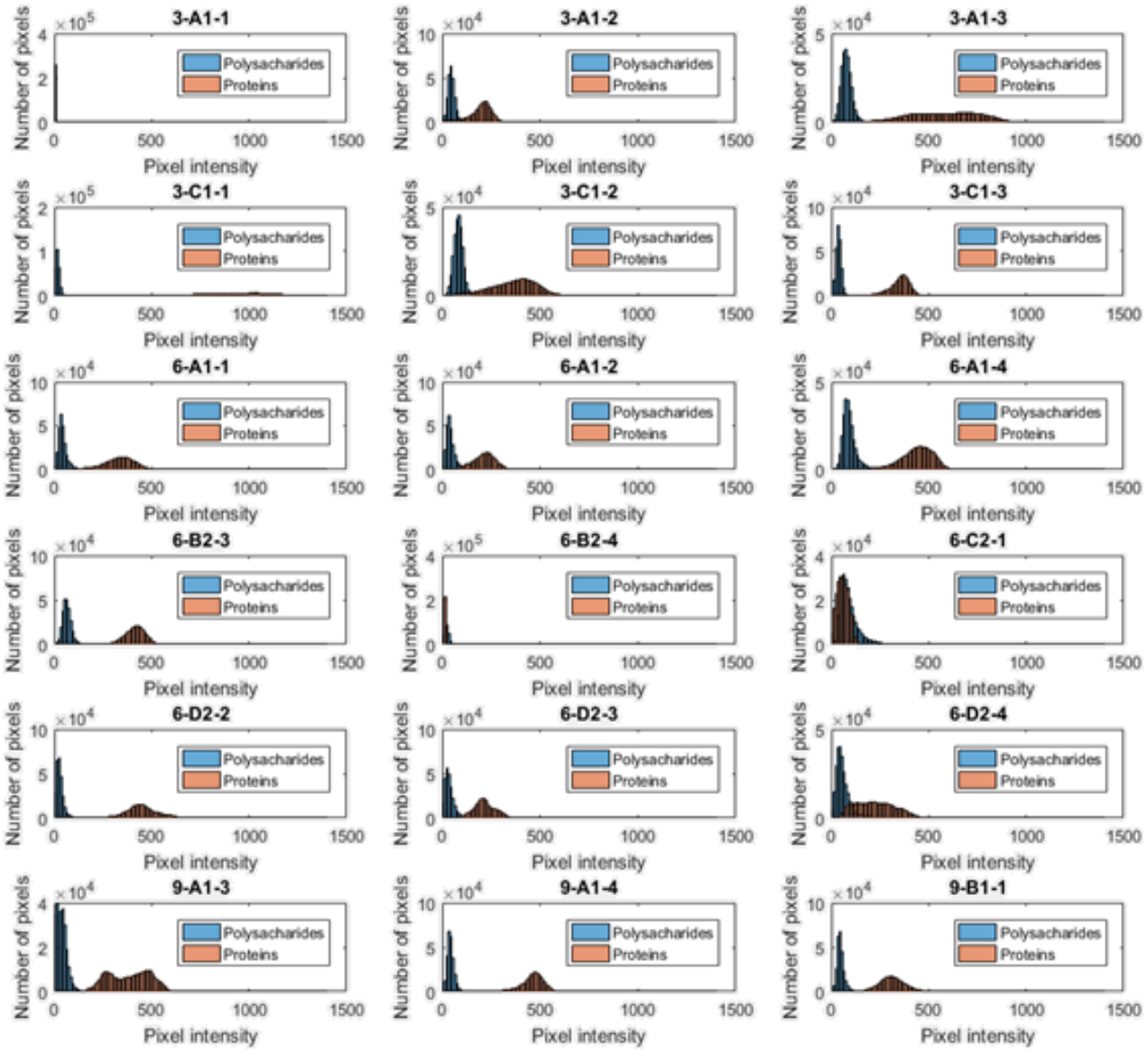

Figure A.1. Image histograms used in final processing of confocal microscopy data.

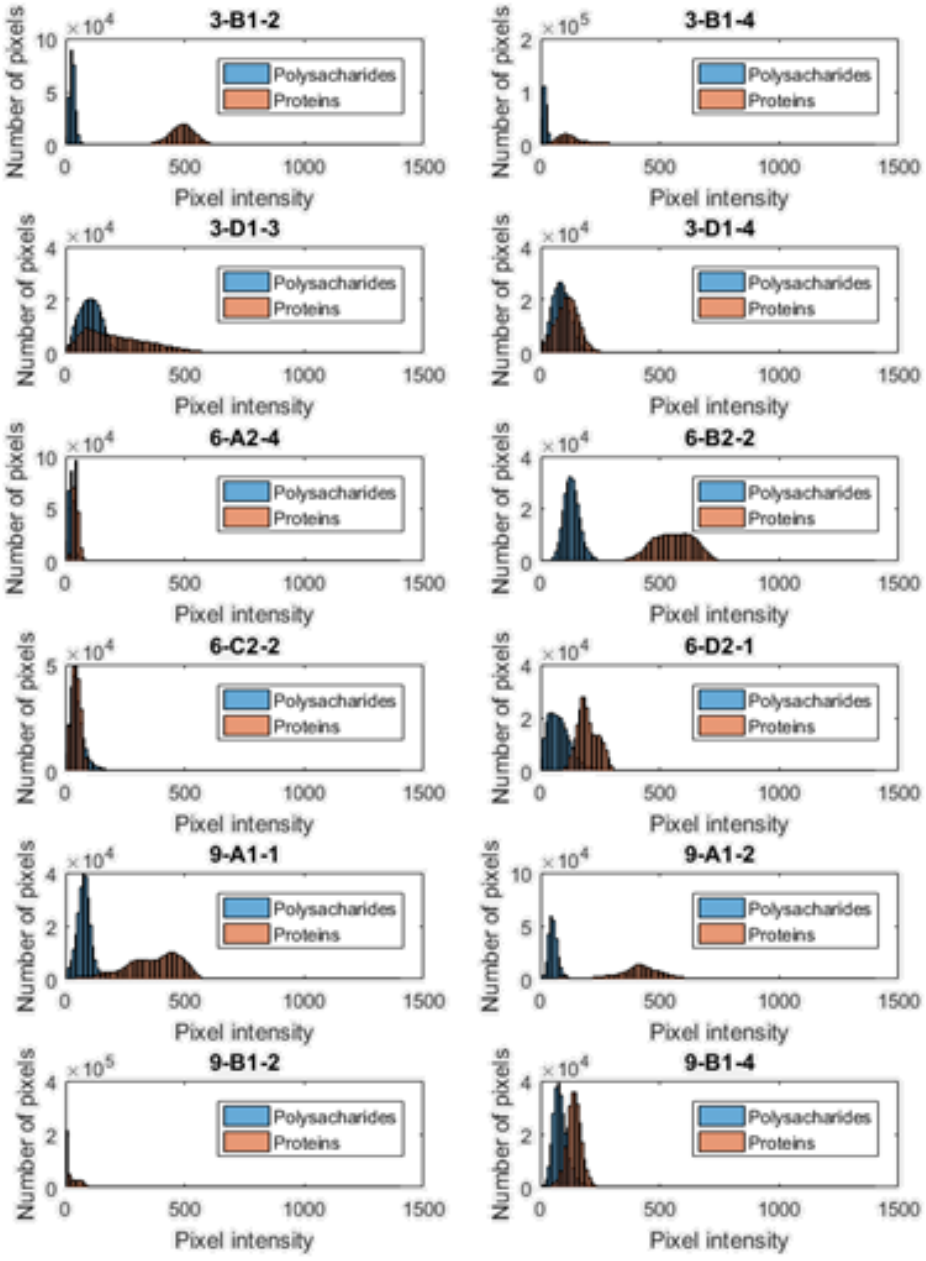

Figure A.2. Image histograms used in final processing of confocal microscopy data continued from previous page.

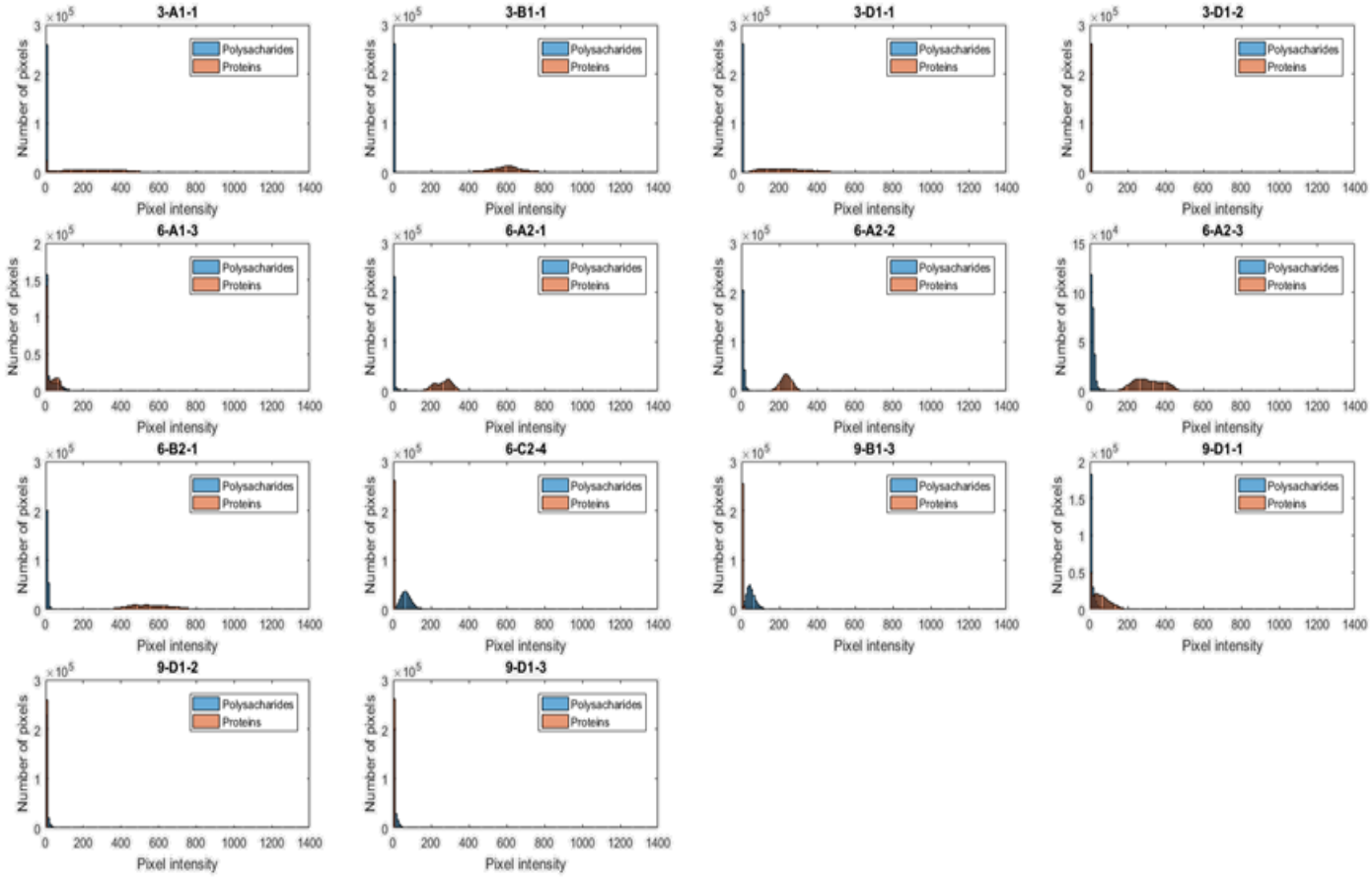

Figure A.3. Image histograms that met discard criteria and were not used incorporated into results.

Appendix B: Image Analysis Code

imageFlattener.mat

```
function[flatRGB] = imageFlattener(imName)
% ,z_step_microns,number_z_slices,micron_width_and_height,imageName
% create function that inputs image data files & outputs image
information
load(imName);

%Operate on the image data
%flatten RGB
```

imageReader.mat

```
function
imageReader(image_name_base,z_step_microns,number_z_slices,micron_width
_and_height)


%use imread function to load the images. Note: file to be read must be
in
%MATLAB's current folder, or added to path. (click and drag the folder
into
%the 'current folder' column, right click, select 'add to path ->
current folder'

%IMPORTANT: Polysaccharides must be colored RED, proteins must be
colored
%GREEN (referenced by variable z value. 1 == poly and 2 == protein),
for a BMP image file.

%Current naming convention is (pH series number)-(trial letter &
duplicate
%#)-(image number 0)(slice number [loops with %d] ).bmp

total_image_thickness= z_step_microns * (number_z_slices - 1);
%calculates the total thickness of the membrane image

%RGB = zeros(512,512,3,number_z_slices);

for z = 1:2 % z value determines color analysed during one cycle

for imgNumber = 0:(number_z_slices-1) %set to number of slices in stack

if     (number_z_slices >= 11) && (imgNumber <10) %takes care of
possibility of more than 10 slices --> extra zero needed in naming
convention.
```

```
imageName = sprintf('%s0%d.bmp',image_name_base,imgNumber) %constant
filename with number ammendment
else
imageName = sprintf('%s%d.bmp',image_name_base,imgNumber) %constant
filename with number ammendment
end

%RGB_matrix =imread(imageName);
RGB(:,:,:,imgNumber+1)=imread(imageName); %MATLAB reads image into the
variable space using the image's name... imageName

end %close z level loop

end %close color loop




save_name = strcat('2run',image_name_base,'.mat');
save(save_name)
```

imageAnalysis.mat

```
% new image analysis code using function imageReader.
clc
clearvars
close all

%imageReader function has fields imageReader(imagename,slice thickness,
%number of slices, surface area in sq mm)

% reads BMP images and calls function 'imageReader' to put them into
.mat
% files entitled with prefix '2run'. This function also stores the
number
% of slices, the slice thickness, and the xy dimensions of the image.


imageReader('3-A1-1',10,8,1272.3)
imageReader('3-A1-2',10,10,1272.3)
imageReader('3-A1-3',5,10,1272.3)


imageReader('3-A2-1',10,8,1272.3)
imageReader('3-A2-2',10,8,1272.3)
imageReader('3-A2-3',10,8,1272.3)
imageReader('3-A2-4',10,8,1272.3)
```

```
%imageReader('3-B1-1',???,???,1272.3) %check fiji to get value
%imageReader('3-B1-2',10,7,1272.3) %corrupted bmp image
%imageReader('3-B1-3',10,9,1272.3)
imageReader('3-B1-4',10,8,1272.3)


imageReader('3-B2-1',10,7,1272.3)
imageReader('3-B2-2',10,7,1272.3)
imageReader('3-B2-3',10,8,1272.3)
%imageReader('3-B2-4',???,???,1272.3)


%imageReader('3-C1-1',5,12,1272.3) %corrupted bmp image
%imageReader('3-C1-2',??,??,1272.3)%check fiji to get value
%imageReader('3-C1-3',?,??,1272.3)%check fiji to get value


imageReader('3-C2-1',10,8,1272.3)
imageReader('3-C2-2',10,9,1272.3)
imageReader('3-C2-3',10,8,1272.3)
imageReader('3-C2-4',10,7,1272.3)


imageReader('3-D1-1',5,9,1272.3)
%imageReader('3-D1-2',??,??,1272.3) %check fiji to get value
imageReader('3-D1-3',5,9,1272.3)
imageReader('3-D1-4',5,11,1272.3)


%imageReader('3-D2-1',??,??,1272.3)%check fiji to get value
%imageReader('3-D2-2',10,8,1272.3)%corrupted bmp image
imageReader('3-D2-3',10,7,1272.3)
%imageReader('3-D2-4',10,7,1272.3)%corrupted bmp image


imageReader('6-A1-1',10,8,1272.3)
imageReader('6-A1-2',10,8,1272.3)
imageReader('6-A1-3',10,8,1272.3)
imageReader('6-A1-4',10,8,1272.3)


imageReader('6-A2-1',10,6,1272.3)
imageReader('6-A2-2',10,5,1272.3)
imageReader('6-A2-3',10,5,1272.3)
imageReader('6-A2-4',10,6,1272.3)


imageReader('6-B2-1',10,6,1272.3)
imageReader('6-B2-2',10,8,1272.3)
imageReader('6-B2-3',10,6,1272.3)
%imageReader('6-B2-4',??,??,1272.3)%check fiji to get value
```

```
imageReader('6-C2-1',10,7,1273.3)
%imageReader('6-C2-2',??,??,1273.3)%check fiji to get value
%imageReader('6-C2-3',??,??,1273.3)%check fiji to get value
%imageReader('6-C2-4',??,??,1273.3)%check fiji to get value


%imageReader('6-D2-1',10,9,1273.3)%corrupted bmp image
%imageReader('6-D2-2',??,??,1273.3)%check fiji to get value
%imageReader('6-D2-3',10,8,1273.3)%corrupted bmp image
imageReader('6-D2-4',10,8,1273.3)


imageReader('9-A1-1',5,7,1273.3)
%imageReader('9-A1-2',5,8,1273.3)%corrupted bmp image
imageReader('9-A1-3',5,13,1273.3)
%imageReader('9-A1-4',??,??,1273.3)%check fiji to get value


%imageReader('9-B1-1',10,8,1273.3)%corrupted bmp image
imageReader('9-B1-2',5,9,1273.3)
%imageReader('9-B1-3',5,9,1273.3)%check fiji to get value
%imageReader('9-B1-4',5,8,1273.3)%corrupted bmp image


imageReader('9-D1-1',10,10,1273.3)
imageReader('9-D1-2',10,8,1273.3)
imageReader('9-D1-3',10,9,1273.3)
```

brokenImageAnalysis.mat

```
% CORRUPTED IMAGE READER
%read decorrupted image files

clc
clearvars
close all

imageReader('3-B1-1',10,9,1272.3) %check fiji to get value
imageReader('3-B1-2',10,7,1272.3) %corrupted bmp image
%imageReader('3-B1-3',10,9,1272.3)

imageReader('3-B2-4',10,7,1272.3)
```

```
imageReader('3-C1-1',5,12,1272.3) %corrupted bmp image
imageReader('3-C1-2',5,11,1272.3)%check fiji to get value
imageReader('3-C1-3',10,7,1272.3)%check fiji to get value

imageReader('3-D1-2',10,11,1272.3) %check fiji to get value



imageReader('3-D2-1',10,9,1272.3)%check fiji to get value
imageReader('3-D2-2',10,8,1272.3)%corrupted bmp image
imageReader('3-D2-4',10,7,1272.3)%corrupted bmp image

imageReader('6-B2-4',10,8,1272.3)%check fiji to get value


imageReader('6-C2-2',10,9,1273.3)%check fiji to get value
%imageReader('6-C2-3',??,??,1273.3)%check fiji to get value
imageReader('6-C2-4',10,8,1273.3)%check fiji to get value



imageReader('6-D2-1',10,9,1273.3)%corrupted bmp image
imageReader('6-D2-2',10,9,1273.3)%check fiji to get value
imageReader('6-D2-3',10,8,1273.3)%corrupted bmp image


imageReader('9-A1-2',5,8,1273.3)%corrupted bmp image
imageReader('9-A1-4',5,16,1273.3)%check fiji to get value


imageReader('9-B1-1',10,8,1273.3)%corrupted bmp image
imageReader('9-B1-3',5,7,1273.3)%check fiji to get value
disp('note: 9B13 acutally has 9 layers not 7')
imageReader('9-B1-4',5,8,1273.3)%corrupted bmp image

disp('finished. 3B13 not read.')
```

dataExpouser.mat

```
%run TrialFileProcessor in loop for all imName
% calls functions to do stat analysis
clc
```

```
clearvars
close all

%cycle through all possible combinations of image name mat files.
%check to see if the mat file is present in directory.
%if so, load the file's info.
%use the file info to get the imagenamebase.mat's info loaded into
local variablespace.
%use another loop to call functions to analyse the local data


load('valid_images.mat','valid*');

sub_i = 0;

savefilecounter   = 0;%used to count number of image files that are
successfully named by the loop
iteration_counter = 0; %counts number of times that the whole loop
iterates. used to index imagenamebases by single number

name_matrix=char(zeros(96,14));
discards = valid_image_vector;
%discards = [1,9,10,12,17,19,23,25,26,35,37,39,39,40,45,48,56];
%indexed by iteration_counter

%for making grouped subplots of trials by pH and sonciation duration
subplotIndex=0;

for pH = ['3','6','9']
    for membrane = ['A','B','C','D']
        for duplicate = ['1','2']
            for imageStack = ['1','2','3','4']

iteration_counter=iteration_counter+1;

discardTruth = iteration_counter == discards;
VALID = 0;
for discardCount = 1:1:length(discardTruth)
    if ((discardTruth(discardCount) == 1) && (1 ~= ((pH =='3' )&&
(duplicate =='2')))) || ( iteration_counter == 1)  %also discard 3.5-X2
due to sample mixup. Include reprehensible but must include 3A11
        VALID = 1;
    end
end

if (VALID == 1)

%strcat the name together along with '2run' (name ensured to be unique
due to existence of image
%files as well as .mat files named by X-L#-#

imagename = strcat('2run',pH,'-',membrane,duplicate,'-
',imageStack,'.mat');
```

```
isThere = exist(imagename); %returns 2 if named object is in matlab
path. returns zero if named object not in path

name_matrix(iteration_counter,:)= imagename;

%start of analysis

if  isThere == 2 %perform desired operations on imagename.mat's
contents

    savefilecounter=savefilecounter+1;% counts number of successfully
identified imagename.mat files

    %call analysis functions
    flatRGB(:,:,:,iteration_counter) = imageFlattener(imagename);

    %use Sam's code, decoder, to store information about membrane pH/
    %sonication duration

    [sonicationDuration(iteration_counter),trialpH(iteration_counter)]
= decoder(imagename);

    %categorize membrane. produce figure with all the histograms of
trial
    %included

binWidth = 10;
binMin=0;
binMax=1500;
binMax_DisplayOnly =1000;



    %pH 3.5 son 0
    if (sonicationDuration(iteration_counter) == 0) &&
(trialpH(iteration_counter) == 3.5)
        trial_ID_from_itCount(iteration_counter) = 1;

  %{
subplotIndex = subplotIndex+1
subplot(2,3,subplotIndex)

%catted(:,iteration_counter,1) =
reshape(flatRGB(:,:,1,imageInQuestion),[512^2,1]);% reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted= reshape(flatRGB(:,:,1,iteration_counter),[512^2,1]);% reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_polys(:,iteration_counter) =catted;
flatHists_polys=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);
hold on
```

```
%catted(:,iteration_counter,2) =
reshape(flatRGB(:,:,2,imageInQuestion),[512^2,1]); % reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted = reshape(flatRGB(:,:,2,iteration_counter),[512^2,1]); % reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_prots(:,iteration_counter) =catted;
flatHists_prots=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);

% label the graph
%title should include sonication duration, pH
titleLabel = strcat(pH,'-',membrane,duplicate,'-',imageStack);
title(titleLabel)
legend('Polysaccharides','Proteins')
ylabel('Number of pixels')
xlabel('Pixel intensity')

hold off

%}


    end
    %pH 3.5 son 2
    if (sonicationDuration(iteration_counter) == 2) &&
(trialpH(iteration_counter) == 3.5)
        trial_ID_from_itCount(iteration_counter) = 2;

   %{
subplotIndex = subplotIndex+1
subplot(2,3,subplotIndex)

%catted(:,iteration_counter,1) =
reshape(flatRGB(:,:,1,imageInQuestion),[512^2,1]);% reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted= reshape(flatRGB(:,:,1,iteration_counter),[512^2,1]);% reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_polys(:,iteration_counter) =catted;
flatHists_polys=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);
hold on
%catted(:,iteration_counter,2) =
reshape(flatRGB(:,:,2,imageInQuestion),[512^2,1]); % reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted = reshape(flatRGB(:,:,2,iteration_counter),[512^2,1]); % reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_prots(:,iteration_counter) =catted;
flatHists_prots=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);
```

```
% label the graph
%title should include sonication duration, pH
titleLabel = strcat(pH,'-',membrane,duplicate,'-',imageStack);
title(titleLabel)
legend('Polysaccharides','Proteins')
ylabel('Number of pixels')
xlabel('Pixel intensity')

hold off
       %}

    end
    %pH 3.5 son 4
    if (sonicationDuration(iteration_counter) == 4) &&
(trialpH(iteration_counter) == 3.5)
        trial_ID_from_itCount(iteration_counter) = 3;
%{
        subplotIndex = subplotIndex+1
subplot(2,3,subplotIndex)

%catted(:,iteration_counter,1) =
reshape(flatRGB(:,:,1,imageInQuestion),[512^2,1]);% reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted= reshape(flatRGB(:,:,1,iteration_counter),[512^2,1]);% reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_polys(:,iteration_counter) =catted;
flatHists_polys=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);
hold on
%catted(:,iteration_counter,2) =
reshape(flatRGB(:,:,2,imageInQuestion),[512^2,1]); % reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted = reshape(flatRGB(:,:,2,iteration_counter),[512^2,1]); % reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_prots(:,iteration_counter) =catted;
flatHists_prots=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);

% label the graph
%title should include sonication duration, pH
titleLabel = strcat(pH,'-',membrane,duplicate,'-',imageStack);
title(titleLabel)
legend('Polysaccharides','Proteins')
ylabel('Number of pixels')
xlabel('Pixel intensity')

hold off
%}
    end
```

```
        %pH 6.9 son 0
    if (sonicationDuration(iteration_counter) == 0) &&
(trialpH(iteration_counter) == 6.9)
        trial_ID_from_itCount(iteration_counter) = 4;
%{
        subplotIndex = subplotIndex+1
subplot(2,3,subplotIndex)

%catted(:,iteration_counter,1) =
reshape(flatRGB(:,:,1,imageInQuestion),[512^2,1]);% reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted= reshape(flatRGB(:,:,1,iteration_counter),[512^2,1]);% reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_polys(:,iteration_counter) =catted;
flatHists_polys=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);
hold on
%catted(:,iteration_counter,2) =
reshape(flatRGB(:,:,2,imageInQuestion),[512^2,1]); % reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted = reshape(flatRGB(:,:,2,iteration_counter),[512^2,1]); % reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_prots(:,iteration_counter) =catted;
flatHists_prots=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);

% label the graph
%title should include sonication duration, pH
titleLabel = strcat(pH,'-',membrane,duplicate,'-',imageStack);
title(titleLabel)
legend('Polysaccharides','Proteins')
ylabel('Number of pixels')
xlabel('Pixel intensity')

hold off
%}
    end
    %pH 6.9 son 2
    if (sonicationDuration(iteration_counter) == 2) &&
(trialpH(iteration_counter) == 6.9)
        trial_ID_from_itCount(iteration_counter) = 5;
%{
        subplotIndex = subplotIndex+1
subplot(2,3,subplotIndex)

%catted(:,iteration_counter,1) =
reshape(flatRGB(:,:,1,imageInQuestion),[512^2,1]);% reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted= reshape(flatRGB(:,:,1,iteration_counter),[512^2,1]);% reshape
command takes first input and reorders it columnwise into the shape of
second input.
```

```
catted_matrix_polys(:,iteration_counter) =catted;
flatHists_polys=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);
hold on
%catted(:,iteration_counter,2) =
reshape(flatRGB(:,:,2,imageInQuestion),[512^2,1]); % reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted = reshape(flatRGB(:,:,2,iteration_counter),[512^2,1]); % reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_prots(:,iteration_counter) =catted;
flatHists_prots=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);

% label the graph
%title should include sonication duration, pH
titleLabel = strcat(pH,'-',membrane,duplicate,'-',imageStack);
title(titleLabel)
legend('Polysaccharides','Proteins')
ylabel('Number of pixels')
xlabel('Pixel intensity')

hold off
%}
    end
    %pH 6.9 son 4
    if (sonicationDuration(iteration_counter) == 4) &&
(trialpH(iteration_counter) == 6.9)
        trial_ID_from_itCount(iteration_counter) = 6;
 %{
        subplotIndex = subplotIndex+1
subplot(2,3,subplotIndex)

%catted(:,iteration_counter,1) =
reshape(flatRGB(:,:,1,imageInQuestion),[512^2,1]);% reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted= reshape(flatRGB(:,:,1,iteration_counter),[512^2,1]);% reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_polys(:,iteration_counter) =catted;
flatHists_polys=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);
hold on
%catted(:,iteration_counter,2) =
reshape(flatRGB(:,:,2,imageInQuestion),[512^2,1]); % reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted = reshape(flatRGB(:,:,2,iteration_counter),[512^2,1]); % reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_prots(:,iteration_counter) =catted;
flatHists_prots=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);
```

```
% label the graph
%title should include sonication duration, pH
titleLabel = strcat(pH,'-',membrane,duplicate,'-',imageStack);
title(titleLabel)
legend('Polysaccharides','Proteins')
ylabel('Number of pixels')
xlabel('Pixel intensity')

hold off
%}
    end

    %pH 9.5 son 0
    if (sonicationDuration(iteration_counter) == 0) &&
(trialpH(iteration_counter) == 9.5)
        trial_ID_from_itCount(iteration_counter) = 7;
   %{
        subplotIndex = subplotIndex+1
subplot(2,3,subplotIndex)

%catted(:,iteration_counter,1) =
reshape(flatRGB(:,:,1,imageInQuestion),[512^2,1]);% reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted= reshape(flatRGB(:,:,1,iteration_counter),[512^2,1]);% reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_polys(:,iteration_counter) =catted;
flatHists_polys=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);
hold on
%catted(:,iteration_counter,2) =
reshape(flatRGB(:,:,2,imageInQuestion),[512^2,1]); % reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted = reshape(flatRGB(:,:,2,iteration_counter),[512^2,1]); % reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_prots(:,iteration_counter) =catted;
flatHists_prots=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);

% label the graph
%title should include sonication duration, pH
titleLabel = strcat(pH,'-',membrane,duplicate,'-',imageStack);
title(titleLabel)
legend('Polysaccharides','Proteins')
ylabel('Number of pixels')
xlabel('Pixel intensity')

hold off
%}
    end
    %pH 9.5 son 2
    if (sonicationDuration(iteration_counter) == 2) &&
(trialpH(iteration_counter) == 9.5)
```

```
        trial_ID_from_itCount(iteration_counter) = 8;
 %{
        subplotIndex = subplotIndex+1
subplot(2,3,subplotIndex)

%catted(:,iteration_counter,1) =
reshape(flatRGB(:,:,1,imageInQuestion),[512^2,1]);% reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted= reshape(flatRGB(:,:,1,iteration_counter),[512^2,1]);% reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_polys(:,iteration_counter) =catted;
flatHists_polys=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);
hold on
%catted(:,iteration_counter,2) =
reshape(flatRGB(:,:,2,imageInQuestion),[512^2,1]); % reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted = reshape(flatRGB(:,:,2,iteration_counter),[512^2,1]); % reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_prots(:,iteration_counter) =catted;
flatHists_prots=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);

% label the graph
%title should include sonication duration, pH
titleLabel = strcat(pH,'-',membrane,duplicate,'-',imageStack);
title(titleLabel)
legend('Polysaccharides','Proteins')
ylabel('Number of pixels')
xlabel('Pixel intensity')

hold off
%}
    end
    %pH 9.5 son 4
    if (sonicationDuration(iteration_counter) == 4) &&
(trialpH(iteration_counter) == 9.5)
        trial_ID_from_itCount(iteration_counter) = 9;
  %{
        subplotIndex = subplotIndex+1
subplot(2,3,subplotIndex)

%catted(:,iteration_counter,1) =
reshape(flatRGB(:,:,1,imageInQuestion),[512^2,1]);% reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted= reshape(flatRGB(:,:,1,iteration_counter),[512^2,1]);% reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_polys(:,iteration_counter) =catted;
flatHists_polys=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);
```

```
hold on
%catted(:,iteration_counter,2) =
reshape(flatRGB(:,:,2,imageInQuestion),[512^2,1]); % reshape command
takes first input and reorders it columnwise into the shape of second
input.
catted = reshape(flatRGB(:,:,2,iteration_counter),[512^2,1]); % reshape
command takes first input and reorders it columnwise into the shape of
second input.
catted_matrix_prots(:,iteration_counter) =catted;
flatHists_prots=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);

% label the graph
%title should include sonication duration, pH
titleLabel = strcat(pH,'-',membrane,duplicate,'-',imageStack);
title(titleLabel)
legend('Polysaccharides','Proteins')
ylabel('Number of pixels')
xlabel('Pixel intensity')

hold off
%}
    end

%select which images to analyse. flatRGB has 4th dimension which is
indexed by index variable "iteration_counter"
%imageInQuestion = iteration_counter; %analyses 3a12 3a13 3a21

%set histogram bin with, the mix and max bins
binWidth = 10;
binMin=0;
binMax=1400;
binMax_DisplayOnly =1000;

%figure
sub_i = sub_i+1;
subplot(6,5,sub_i)

catted= reshape(flatRGB(:,:,1,iteration_counter),[512^2,1]);% reshape
command

flatHists_polys=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);



hold on
catted = reshape(flatRGB(:,:,2,iteration_counter),[512^2,1]); % reshape
flatHists_prots=histogram(catted,'BinWidth',binWidth,'BinLimits',[binMi
n,binMax]);

% label the graph
%title should include sonication duration, pH

titleLabel = strcat(pH,'-',membrane,duplicate,'-',imageStack);
```

```
title(titleLabel)
legend('Polysacharides','Proteins')
ylabel('Number of pixels')
xlabel('Pixel intensity')

hold off



total_polys = nnz(flatHists_polys.Data); % ? check
total_prots = nnz(flatHists_prots.Data);

total_poly_check(savefilecounter) = total_polys;
total_prots_check(savefilecounter) = total_prots;

%find intensity of 50 and 90 percentiles of CDF

%for 50 percent:
cumulated_polys=0;
bin_number = 0;
while cumulated_polys < .5*total_polys

    bin_number = bin_number + 1;

    cumulated_polys = cumulated_polys +
flatHists_polys.Values(bin_number); %(bin intensity*no_in_bin)
    cumulative_50_polys(iteration_counter) = binWidth*bin_number;

end

cumulated_prots=0;
bin_number = 0;
while cumulated_prots < .5*total_prots

    bin_number = bin_number + 1;

    cumulated_prots = cumulated_prots + (
flatHists_prots.Values(bin_number)); %(bin intensity*no_in_bin)
    cumulative_50_prots(iteration_counter) = binWidth*bin_number;

end

% for 90%
cumulated_polys=0;
bin_number = 0;
while cumulated_polys < .9*total_polys

    bin_number = bin_number + 1;

    cumulated_polys = cumulated_polys + (
flatHists_polys.Values(bin_number)); %(bin intensity*no_in_bin)
    cumulative_90_polys(iteration_counter) = binWidth*bin_number;
```

```
end

cumulated_prots=0;
bin_number = 0;
while (cumulated_prots < .9*total_prots)

    bin_number = bin_number + 1;
   %
flatHist_Values_by_binNo(bin_number)=flatHists_prots.Values(bin_number)
;



    cumulated_prots = cumulated_prots +
(flatHists_prots.Values(bin_number)); %(bin intensity*no_in_bin)
    cumulative_90_prots(iteration_counter) = binWidth*bin_number;

end


end

end

%loop terminates and starts over.
            end
         end
    end
end



save('validImages_statisticalData_unweighted')
disp('dataExpouser_unweighted has finished.')
```

validImage.mat

```
% determine discards rigorously.
clearvars
close all
clc

load('discard_info_Data_unweighted.mat');

%if poly or prot less than ten or image coverage less than 95%

coverage_threshold = 512^2 * 0.90; % 90% coverage threshold
```

```
valid_image_no = 0;

for it_count = 1:1:91
        valid_image_no = valid_image_no + 1

if (total_prots_check(it_count) >= coverage_threshold) &&
(total_poly_check(it_count) >= coverage_threshold) &&
(cumulative_90_polys(it_count) >= 20) && (cumulative_90_prots(it_count)
>= 20)


    valid_image_vector(valid_image_no) = valid_image_no; end
end

save('valid_images')
```

statisticalAnalysis.mat

```
% analyse statistical data
clearvars
clc

load('statisticalData.mat'); %loads statistical data generated by
version dataExpouser_2016_03_15.mat

%sort the into matrices by pH and sonication duration

%analyze the 3.5/0 membranes using matrix trial_was_3__0

n = 0

A = 0;
B = 0;
C = 0;
D = 0;
E = 0;
F = 0;
G = 0;
H = 0;
I = 0;

for i = 1:1:length(cumulative_50_prots)
```

```
  %if discard(i) == 0 % stops processing of membranes indexed as
discards

    if (trial_was_3__0(i) ~= 0)
    %tabulate important stats, compute PN/PS
    A=A+1;
    PNPS_50(i) =  cumulative_50_prots(i)/cumulative_50_polys(i);
    PNPS_90(i) =  cumulative_90_prots(i)/cumulative_90_polys(i);

    stats_3_0(:,1,1,1,1,1,A) =
[cumulative_50_prots(i),cumulative_50_polys(i),cumulative_90_prots(i),c
umulative_90_polys(i),PNPS_50(i),PNPS_90(i)];

    end
    %{
    if trial_was_3__2(i) ~= 0
    %tabulate important stats, compute PN/PS
    n=n+1;
    PNPS_50(i) =  cumulative_50_prots(i)/cumulative_50_polys(i);
    PNPS_90(i) =  cumulative_90_prots(i)/cumulative_90_polys(i);
    end

    if trial_was_3__4(i) ~= 0
    %tabulate important stats, compute PN/PS
    n=n+1;
    PNPS_50(i) =  cumulative_50_prots(i)/cumulative_50_polys(i);
    PNPS_90(i) =  cumulative_90_prots(i)/cumulative_90_polys(i);

    end

        if trial_was_6__0(i) ~= 0
    %tabulate important stats, compute PN/PS
    n=n+1;
    PNPS_50(i) =  cumulative_50_prots(i)/cumulative_50_polys(i);
    PNPS_90(i) =  cumulative_90_prots(i)/cumulative_90_polys(i);

    end

    if trial_was_6__2(i) ~= 0
    %tabulate important stats, compute PN/PS
    n=n+1;
    PNPS_50(i) =  cumulative_50_prots(i)/cumulative_50_polys(i);
    PNPS_90(i) =  cumulative_90_prots(i)/cumulative_90_polys(i);
    end

    if trial_was_6__4(i) ~= 0
    %tabulate important stats, compute PN/PS
    n=n+1;
    PNPS_50(i) =  cumulative_50_prots(i)/cumulative_50_polys(i);
    PNPS_90(i) =  cumulative_90_prots(i)/cumulative_90_polys(i);

    end

        if trial_was_9__0(i) ~= 0
    %tabulate important stats, compute PN/PS
```

```
    n=n+1;
    PNPS_50(i) =  cumulative_50_prots(i)/cumulative_50_polys(i);
    PNPS_90(i) =  cumulative_90_prots(i)/cumulative_90_polys(i);

    end

    if trial_was_9__2(i) ~= 0
    %tabulate important stats, compute PN/PS
    n=n+1;
    PNPS_50(i) =  cumulative_50_prots(i)/cumulative_50_polys(i);
    PNPS_90(i) =  cumulative_90_prots(i)/cumulative_90_polys(i);
    end

    if trial_was_9__4(i) ~= 0
    %tabulate important stats, compute PN/PS
    n=n+1;
    PNPS_50(i) =  cumulative_50_prots(i)/cumulative_50_polys(i);
    PNPS_90(i) =  cumulative_90_prots(i)/cumulative_90_polys(i);

    end
    %}

  %end
end


disp('statisticalData has finished')
```