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Photochemical Disinfection of *Escherichia coli* in the Presence of Natural Aquatic Sensitizers: Influence of Solution Chemistry and Extracellular Polymeric Substances

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by

Amy Shin Hwei Gong

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Dissertation Committee:
Dr. Sharon L. Walker, Chairperson
Dr. David M. Cwiertny
Dr. Marylynn V. Yates

The Dissertation of Amy Shin Hwei Gong is approved:

_____

_____

_____
Committee Chairperson

University of California, Riverside

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Dedication

I would like to dedicate this dissertation to Christ, my husband, and loving family oversea who have provided a large amount of love, support, and encouragement throughout my path.

ABSTRACT OF THE DISSERTATION

Photochemical Disinfection of *Escherichia coli* in the Presence of Natural Aquatic Sensitizers: Influence of Solution Chemistry and Extracellular Polymeric Substances

by

Amy Shin Hwei Gong

Doctor of Philosophy, Graduate Program in
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Dr. Sharon L. Walker, Chairperson

The objective of this dissertation study was to elucidate how the level and composition of bacterial surface extracellular polymeric substances (EPS) contribute to indirect photochemical disinfection processes. This study was developed to generate mechanistic information on EPS function in bacterial die-off under natural occurring sensitizing environments to inform future process design. Specifically, this research focused on whether EPS promotes or prohibits disinfection, or be able to facilitate association with sensitizing surfaces.

To accomplish this, the influence of bacterial EPS has been studied in batch (solar simulator and reactor) and flowing (parallel plate) systems simulating groundwater and agriculturally impacted surface water. Model strains of *E. coli* and *Salmonella* were utilized. In the initial work, the model *Salmonella* was incubated in different solution chemistries for varying times. Three EPS extraction methods (lyophilization, ethanol, and sonication) were applied and compared over the range of conditions for the sensitivity and extent of cell lysis after the extraction processes. The sonication method

was deemed the best method for further use in subsequent disinfection kinetic studies in the solar simulator with *E. coli*. This work was followed by *E. coli* transport and deposition experiments in the flow chamber. Throughout the research, a series of physical-chemical-biological characterization techniques have been applied on the bacterial strains including EPS compositional analysis, cell lysis testing, and the measurement of bacterial hydrophobicity, zeta potential, surface charge density, and size.

The findings from this doctoral study provide the following fundamental insights. First, the composition and levels of EPS depends on the extraction protocol utilized, where no single method has the ability to separate all EPS from the cell surface without disturbing cell structure. Next, in the presence of nitrate photochemical disinfection was not sensitive to EPS level. Additionally, EPS did not hinder the disinfection by acting as a physical barrier or chemical quencher. Finally, this study has shown the ability of EPS to facilitate interactions between bacteria and sensitizers in photochemical disinfection processes.

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Chapter 1

Introduction

1.1 MOTIVATION AND BACKGROUND

Agricultural nonpoint source (NPS) pollution has been a major source of water quality impairments in rivers, lakes, wetlands, estuaries, and groundwater.[1] At least 1870 outbreaks from 1920 to 2002 have been reported related to contaminated drinking water, and accounts for 883,806 illnesses in United States.[2] More specifically, *Escherichia coli* O157:H7 causes an average of 73,000 cases of illness in United States each year.[3] Impacted surface waters – by agricultural run-off or municipal waste water – pose a significant health risk to humans getting in contact with recreational waters.[4] Therefore, a natural pathway for water treatment to disinfected or inactivate pathogens in large water bodies is needed.

Sunlight mediated photochemical disinfection is one of the economical natural attenuation processes that can be applied in large water bodies. However, direct photochemical disinfection relies solemnly on the UV spectrum, which only penetrates through a few inches of surface water. Indirect photochemical disinfection involves various chemical sensitizers in water that can absorb the energy in solar spectrum and form highly reactive, transient reactive oxygen species (ROS), responsible for bacterial disinfection. There are a few natural sensitizers regularly present in natural water bodies, including nitrate[5] from agricultural fertilizer, natural organic matter (NOM) from decayed plant material, and iron oxide from dissolved minerals.[6] Nitrate, when exposed to light of wavelengths from 300-340 nm,[5] produces hydroxyl radical (·OH) through the following reactions:[7, 8]

$$NO_3^- \xrightarrow{h\nu} NO_2 \cdot + O^- \cdot$$

$$O^- \cdot + H_2O \rightarrow OH \cdot + OH^-$$

NOM in water absorb both UV and visible solar energy, produce singlet oxygen ($^1O_2$) through the following reactions:[9]

$$^1HS + h\upsilon \rightarrow {}^1HS^* \rightarrow {}^3HS^*$$

$$^3HS^* + {}^3O_2 \rightarrow {}^1HS + {}^1O_2$$

Iron oxide present in natural waters in colloidal form, forms ·OH when it reacts with hydrogen peroxide (H_2O_2):[10]

$$H_2O_2 + Fe\ (II) \rightarrow Fe(III) + OH^- + {}^\bullet OH$$

Many of the previous studies on photochemical disinfection involve a metal photocatalyst such as TiO_2 in an engineered system.[11-13] Although the reaction efficiency is usually much greater with the presence of metal photocatalysts, the addition of the catalysts pose a considerable risk to humans through exposure to the photocatalysts in the water. Additionally, at the larger scale the cost of photocatalysts can be prohibitive. Therefore, natural oxidation mechanisms involving native constituents in water have a greater opportunity to be applied in low-tech water treatment processes. Photochemical processes involving the transformation of chemicals and pharmaceuticals have been extensively studied in literature.[14-17] Some studies have considered *direct* sunlight

disinfection processes as low-cost water treatment approaches.[18, 19] However, there has been little in the way of comprehensive research devoted to *indirect* photochemical disinfection with presence of sensitizers. Furthermore, few studies have clearly examined the contribution of biological factors to photochemical disinfection; including types of microorganisms, surface polymers (lipopolysaccharides (LPS) and extracellular polymeric substances (EPS)), other physical-chemical surface properties like zeta potential and charge density.[20-22]

Bacterial EPS are macromolecular polymers naturally excreted by bacteria during their normal metabolism process for protection.[23, 24] EPS contains majority protein[25, 26] and polysaccharides[27, 28] and trace amount of nucleic acids.[29] It has been well studied in bacterial transport and adhesion area that EPS can increase the level of interaction between bacterial surfaces and surrounding particles or other cells.[30, 31] When applied in photochemical disinfection process, the *increased association* between bacterial surfaces and particles may be able to result in *faster reactions* with more EPS presence in the system. Alternatively, EPS may provide *protective layer* that reduce the chance the ROS being able to access the bacterial surface. This protective layer may prevent ROS transport and access to the membrane surface, and may result in lower disinfection with more EPS presence around the cells.

One study using TiO_2 and ZrO_2 as visible light photocatalysts investigated the disinfection kinetics of heterotrophic biofilm bacteria with or without soluble EPS.[22] Their results suggested a faster reaction with less EPS presence, and competing physical

blocking or quenching effect on the photocatalytically produced ROS.[22] This is the only previous work done on the contribution of EPS, but it was a less mechanistic study utilizing complex biofilms and not from a single cell type.[22] In order to achieve a better understanding of the role EPS plays in photochemical disinfection process a well-controlled, systematic study is needed.

1.2 **OBJECTIVE AND SCOPE**

The overall objective of this dissertation study is to elucidate how the level and composition of bacterial surface EPS and solution chemistry (ex. sensitizer types and concentration) contribute to indirect photochemical disinfection processes. This study was developed to generate mechanistic explanations of EPS function in bacterial die-off under natural occurring sensitizing environments to inform future process design and modeling. *Specifically, this research focused on whether EPS promotes or prohibits disinfection, or be able to facilitate association with sensitizing surfaces.* This dissertation addresses this issue in three core chapters, investigating the composition of EPS (Chapter 2), the impact of EPS on the photochemical disinfection process (Chapter 3), and interactions between bacteria and surrounding sensitizing agents in surface water bodies (Chapter 4). Furthermore, the level of EPS was systematically modified and bacteria were disinfected in well-controlled photochemical batch system (Chapter 3). The bacteria with varied level of EPS were also applied in a parallel plate (PP) flow chamber system in order to understand the association between bacterial surface and natural chemical sensitizing agents (Chapter 4).

1.2.1 **Specific Objectives**

In order to accomplish the overall objective, a research plan and specific objectives and were developed. There are three specific objectives presented below and each specific objective is fully addressed in a separate chapter in following chapters.

1. *To evaluate the extent of EPS level and composition produced by model bacteria under environmentally relevant groundwater conditions.* Three EPS extraction methods were investigated and compared in varied solution chemistry that had been incubated for different lengths of time. Experiments were conducted in artificial ground water (AGW) utilizing *Salmonella* as model organism. To reveal the sensitivity of the three methods to catch subtle solution chemistry change, detailed statistical analysis was applied on the resulting EPS levels. (Chapter 2)
2. *To determine the role of bacterial EPS in direct and indirect photochemical disinfection processes. S*onication EPS extraction method was utilized to systematically modify the level of EPS remaining surrounds the bacterial surface prior to exposure in a batch solar simulator. These experiments seek to understand whether EPS contribute to the rate of photochemical disinfection in the presence of nitrate as a sensitizer. (Chapter 3)
3. *To understand contribution of EPS on the association between bacterial surfaces and sensitizing surfaces.* Experiments were conducted in a PP flow chamber, observing bacterial (with full or partial EPS) transport and adhesion behavior on three collector surfaces (quartz, SRHA, and α-Fe_2O_3) in real-time under

fluorescent microscopy. Experiments with SRHA and α-Fe_2O_3 surfaces represent two types of sensitizers, as compared to quartz as a control, to understand whether EPS facilitates association between bacteria and sensitizers. This study sought to evaluate the role of EPS, collector surfaces, and solution chemistry (IS) in the bacterial adhesion process (Chapter 4)

1.2.2 **Experimental Approach**

Representative model organisms have been selected to investigate the role of bacterial EPS in photochemical disinfection processes: *Escherichia coli* O157:H7[32-34], HU1[35], DC1[35], and SP4[36] as well as *Salmonella enterica* serovar Pullorum (SA1685)[32] for the initial EPS extraction study. *E. coli* O157:H7 strain p72 with pGFP tag was previously obtained from USDA, Dr. Pina Fratamico (USDA-ARS-ERRC, Wyndmoor, PA).[37] *E. coli* HU1 (HU stands for human, isolated from raw sewage samples) and DC1 (DC stands for dairy cattle, isolated from animal farms) were provided by Dr. Sloane Ritchey (Eastern Kentucky University). *E. coli* SP4, provided by Carl H. Bolster at the USDA (USDA-ARS, Bowling Green, Kentucky), was isolated from liquid swine effluent from a lagoon at Western Kentucky University. SA1685 was isolated from infected turkeys was obtained from the Salmonella Genetic Stock Centre, University of Calgary, Canada.

To accomplish the objectives mentioned above, the influence of bacterial EPS in various solution chemistries has been studied in batch (Chapter 2 and 3) and PP flowing (Chapter 4) systems simulating groundwater and agriculturally impacted surface water.

In Chapter 2, the model organism SA1685 was incubated in $10^{-2.5}$, 10^{-2}, and $10^{-1.5}$ M artificial groundwater (AGW) for 0, 6, 12, 18 and 24 h. Three EPS extraction methods (lyophilization, ethanol, and sonication) were applied and compared over the range of conditions for the sensitivity and extent of cell lysis after the extraction processes. The sonication method was applied further in the photochemical disinfection kinetic study in a solar simulator (Chapter 3) and the cell deposition study in the PP flow chamber (Chapter 4). The sonication method was selected due to its ability to remove EPS, cause relatively low levels of cell lysis, and to preserve whole living cells after the extraction process. Throughout the research, reported in all chapters, a series of cell characterization techniques have been applied on the *E. coli* strains including EPS compositional analysis, cell lysis testing, and the measurement of bacterial hydrophobicity, zeta potential, surface charge density, and size.

1.3 **ORGANIZATION OF THE DISSERTATION**

Following the Introduction, Chapter 2 in this dissertation entitled "Extraction and Analysis of Extracellular Polymeric Substances: Comparison of Methods and Extracellular Polymeric Substance Levels in *Salmonella pullorum* SA 1685" describes and compares three EPS extraction methods over a range of solution chemistries and exposure time. The three extraction methods (lyophilization, ethanol, and sonication) were compared base on the yield of extraction, EPS composition, and the extent of cell lysis caused by the extraction procedure. The ethanol method had the highest EPS yield but with largest variance. The sonication method being the only physical method had the

lowest yield, but with greater correlation between sugar and protein content. The result of this study was published in 2009 in *Environmental Engineering Science* (26 (10): 1523-1532).

Chapter 3, "Influence of Varying Levels of Extracellular Polymeric Substances (EPS) on Hydroxyl Radical Mediated Disinfection of *Escherichia coli*", has been recently submitted to the journal *Environmental Science and Technology*. The sonication extraction method for EPS was applied to remove different levels of EPS from the bacteria surfaces through gradually longer sonication times (20, 160, 300, 440, and 580 s). The bacteria with varied levels of EPS were then exposed to a lab-scale solar simulator and irradiated with presence of hydroxyl radical (·OH) produced by nitrate photolysis. A multi-target bacterial disinfection model was used to fit the experimental curves taking account of both the initial induction time (t_s) and the subsequent exponential decay. Results suggested greater steady-state hydroxyl concentration $[\cdot OH]_{ss}$ increased the efficiency of photochemical disinfection. The varied EPS levels and four *E. coli* strain types used in this study did not generate clear trend on both direct and indirect disinfection facilitated by nitrate, suggesting that EPS does not play a substantial role in the photochemical disinfection process.

Chapter 4 entitled "Influence of Surface Coating and Extracellular Polymeric Substances (EPS) on *Escherichia coli* O157:H7 Deposition in a Parallel Plate Flow Chamber", compares the contribution of full or partial EPS presence on bacterial adhesion to sensitizing surfaces in a PP flow chamber. Experiments were conducted

across a range of solution chemistry conditions (1-100 mM) with three different collector surfaces to identify contributing parameters involved in the interactions of the cells with the surfaces. The three kinds of solid collector surfaces selected included bare silica quartz (control), organic matter-coated quartz, and glass coated with hematite nanoparticles, the latter two chosen to simulate common sensitizing agents in photochemical disinfection. The bacterial surface macromolecules were partially removed through sonication using the approach developed in Chapter 2. Experimental results have been found sensitive to EPS levels, collector surfaces, and the IS tested. Greater EPS presence resulted in higher deposition, indicate that EPS may facilitate interactions between cells and sensitizer. Further discussion of the general trends and the findings from this doctoral research are summarized in Chapter 5, "Summary and Conclusions".

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Chapter 2

Extraction and Analysis of Extracellular Polymeric Substances: Comparison of Methods and Extracellular Polymeric Substance Levels in *Salmonella Pullorum* SA 1685

ABSTRACT

Extracellular polymeric substances (EPS) production and composition for *Salmonella pullorum* SA 1685 exposed to artificial groundwater (AGW) has been examined utilizing three EPS extraction methods: lyophilization, ethanol, and sonication. Experiments were carried out to evaluate the robustness of three EPS extraction methods and the sensitivity of each to subtle changes in solution ionic strength (IS) and duration of exposure. EPS extraction and analysis was conducted via sugar and protein analyses using the phenol sulfuric acid and Lowry methods, respectively, after 0-, 6-, 12-, 18-, and 24-h incubation times in AGW with $10^{-2.5}$, 10^{-2}, and $10^{-1.5}$ M IS. Lyophilization and ethanol methods resulted in a greater amount of EPS extracted than the sonication method (mass of EPS=cell), yet these methods fluctuated to a greater extent in the total amount—or level—of EPS extracted under the various test conditions. Systematic comparisons and extensive statistical analyses were conducted between the various experimental conditions. To our knowledge, this is the first study systematically comparing EPS extraction techniques utilizing Salmonella. As we investigated the relative EPS content in *Salmonella* SA1685 under conditions simulating groundwater, our results provide insight into the suitability of each method for detection of environmentally induced changes in bacteria suspended in simulated or real subsurface aquatic systems.

2.1 **INTRODUCTION**

Bacteria have unique physical, chemical, and biological characteristics, one of which is the production of extracellular polymeric substances (EPS)[1] which contributes to the degree to which the cells stick to surfaces[2-4] or each other[5-7]. EPS is a complex polymer matrix comprised of polysaccharides[8, 9], proteins[10, 11], and nucleic acids[12] on the outside of the cell membrane.[13] The EPS can be classified as either bound EPS or free EPS.[14] The bound EPS, also known as capsular EPS, is loosely bound around the cell surface, whereas the free or soluble EPS is exuded from the cell and released into the surrounding media.[14] Most cells produce EPS for protection,[15, 16] and to release waste as part of metabolic processes.[17]

EPS can serve an important role in cell adhesion and retention on mineral surfaces, impacting the fate and transport of microorganisms in groundwater environments.[2, 3, 18, 19] Recent literature demonstrates the ability of EPS to enhance cell retention on the surfaces through the formation of a conditioning film outside of the cells.[20, 21] The literature also suggests that the complexity and the gel forming properties of the EPS enhance the interaction of the cells with the other components around them.[2, 22-24] In the groundwater environment, this enhanced level of interaction of cells with mineral surfaces or other cells leads to greater removal from the pore water and reduced transport in the subsurface.[18]

There are numerous methods which have been developed for the extraction of EPS, each appropriate under different scenarios. The requirements of a good EPS

extraction method should include: i) causing minimal cell lysis; ii) not disrupting or altering the characteristics of EPS; and iii) the ability to release and collect all the EPS biopolymers.[14] In the literature, methods have been reported to achieve the task of separating EPS from the cell,[25-28] and they can be divided into three categories: physical methods, chemical methods, and a combination of the two for extracting the free and bound EPS from the cell.[14] Physical methods involve the techniques of separation with physical forces, for example centrifugation, dialysis, filtration, sonication[29], cation exchange[30], and heating[17, 31]. Chemical methods utilize chemical reagents, like ethylenediaminetetraacetic acid (EDTA), formaldehyde (H_2CO), sodium hydroxide (NaOH), and ethanol (C_2H_5OH) to fulfill the objective of separating EPS from the cells.[26] In the literature, the chemical methods have been reported to yield more than physical methods; however, with higher probability of contamination from the reagent or cell lysis.[14] Although physical methods usually yield less than chemical methods, the fact that they usually have minimal contamination and cell lysis, makes them also effective approaches for EPS extraction.[27] The combination of both chemical reagents and physical forces renders the method more reproducible and effective because a similar yield can be obtained without excessive contamination and cell lysis caused by the reagents.[14] To date, there have been limited studies on the extraction of EPS from *Salmonella*, or have comparisons of extraction methods with this important organism been reported.[32]

There are many techniques available for analyzing and quantifying the components of EPS, including colorimetric methods[33, 34], Fourier Transform Infrared

(FTIR) spectroscopy, X-ray Photoelectron Spectroscopy (XPS), Total Organic Carbon (TOC), High-Performance Size Exclusion Chromatography (HPSEC)[35], Gas Chromatography-Mass Spectrometry (GC-MS), Proton NMR (Hydrogen-1 NMR, or ^{1}HNMR)[36], High-Performance Liquid Chromatography (or High Pressure Liquid Chromatography, HPLC)[37], Atomic Force Microscope (AFM)[38, 39], DNA assays[40], Infrared Spectroscopy (IR spectroscopy), and Glucose-6-Phosphate Dehydrogenase (G6PDH)[14, 30, 41]. To quantify polysaccharides content in EPS, one traditional method is the phenol sulfuric acid (PSA) method.[33] Lowry[34], Bradford[42] , and Smith[43] methods are the typical approaches for bulk protein content quantification. These established colorimetric methods have been widely used, are easy to perform, and have been proven reliable. To ensure only extracellular content is evaluated, the extent of cell lysis also needs to be compared, even though extraction should be able to collect the EPS and keep the cells intact.[14] Typically, this is performed with an assay identifying presence of inner cell constituents.[14, 30, 41]

Salmonella is a foodborne and waterborne pathogenic bacteria which has caused a considerable number of outbreaks.[44-48] In the United States alone, approximately 30,000 to 40,000 culture-confirmed cases of nontyphoidal *Salmonella* are reported every year to the Centers for Disease Control and Prevention (CDC).[45, 48, 49] Common contaminated food includes meat related food like processed food with poultry, egg, and ground beef.[50, 51] *Salmonella*, a gram negative bacterium, can also be transmitted through water.[52] In order to study the fate of these organisms, the nature of the EPS must be characterized. To understand the extent to which the extracellular polymer (EPS) production and

composition responds to various environmental conditions, a combination of bound EPS extraction and analysis methods were conducted on cells exposed to relevant environmental conditions artificial ground water (AGW) at varying ionic strength. Systematic comparisons and statistical analyses were made between the three methods, three IS, and four different periods of time in which cells were exposed to the various experimental solution chemistry conditions, in regards to total sugar content, protein content, and the sugar to protein ratio in the extracted EPS. The objective of this work is to quantitatively compare three EPS extraction methods in their capacity to 1) extract EPS from *Salmonella* and 2) resolve the subtle changes in EPS composition in response to three different ionic strengths and five periods of time in which cells were exposed to the various solution chemistry conditions.

2.2 **MATERIALS AND METHODS**

2.2.1 **Cell Selection and Preparation.**

To perform this research, a model strain *Salmonella enterica* serovar pullorum (SA 1685) was used. This strain is an avian pathogen known to affect poultry.[53, 54] Related *Salmonella* species are notorious, having caused numerous outbreaks in both humans[46, 55] and animals[55, 56]. This particular strain was also selected as it is a non-flagellated, non-motile[57], extracellular polymeric substances (EPS) producing strain[32]. Prior to each experiment, a pre-culture was prepared by incubating a sample of SA1685 cells at 37 °C overnight in Luria-Bertani (LB) broth (Fisher Scientific, Fair Lawn, N. J.). The overnight pre-culture was utilized to inoculate a fresh culture (1:100 v/v) which was

incubated at 37 °C until reaching mid-exponential growth stage (3.5 hr). Subsequently, cells were harvested by centrifugation (5804R; Eppendorf, Hamburg, Germany) equipped with a fixed-angle rotor (F-34-6-38; Eppendorf) for 15 min at 4 °C and 3700 g. The sample was decanted, the pellet re-suspended in 10^{-2} M KCl, and the centrifugation repeated. This rinsing step was repeated two times, and the final pellet was re-suspended in 1 mL of 10^{-2} M KCl and utilized as a stock solution for the following experiments.

The stock solution concentration was measured for each harvested culture. This was done utilizing a cell counting chamber (Burker Turk Superior Marienfel) and light microscope (Fisher Scientific Micromaster). Cell concentrations were $3.44 \pm 1.64 \times 10^{10}$ cells/mL in the stock solutions.

2.2.2 **Exposure of Model Organism to Groundwater Conditions.**

Following the harvesting steps for SA 1685, the cells were exposed to a stress condition (varying AGW IS and time of exposure). Experiments were designed to simulate aquatic chemistry conditions existing in the subsurface environment. Cells were exposed to three representative IS of $10^{-2.5}$, 10^{-2}, and $10^{-1.5}$ M AGW solution (a liter of 10^{-2} M AGW contains 0.1071 g $CaCl_2 \cdot 2H_2O$, 0.0744 g $CaSO_4 \cdot 2H_2O$, 0.0595 g KNO_3, 0.1071 g $NaHCO_3$, 0.1042 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.1786 g $MgSO_4 \cdot 7H_2O$ [58] with slight modification) for durations of 6, 12, 18, and 24 h. Cell concentrations were maintained at 10^8 cells/mL in the AGW cell suspensions. During this exposure time cell suspensions were gently shaken at 3.5 rpm (VWR OS-500 Analog Orbital Shaker; West Chester, PA)

at room temperature (22-25 ^{o}C). All reagents utilized were ACS grade (Fisher Scientific).

2.2.3 **Extraction of EPS.**

To understand the extent to which the extracellular polymeric substances (EPS) production and composition responds to various environmental conditions, a combination of EPS extraction and analysis methods were conducted. For each exposure time as noted above, three different methods were utilized to separate EPS from the cell surfaces without lysing them. These methods were the lyophilization, ethanol[59], and sonication methods[18]. Subsequently, the composition and content of the EPS was analyzed and evaluated as a function of the imposed stress.

The first two extraction methods, lyophilization and ethanol, are similar in nature, as both were initiated with resuspending the freshly harvested cell pellet in 10 mL 0.22% formaldehyde (ACS grade, Fisher Scientific) in 8.5% sodium chloride for two hours in 4 °C incubator. Following the exposure to formaldehyde, the suspension was centrifuged (3700 g, 4 °C, 15 min) and the resulting pellet containing the EPS was resuspended in 10 mL deionized (DI) water (Millipore, Billerica, MA). Then the suspension was centrifuged again (3700 g, 4 °C, 15 min) to rinse away any remaining cellular material (non-EPS), the pellet was collected, its weight was measured, and 50 mL DI water per gram of pellet was added to resuspend the pellet. This solution was sonicated for 3 min (460/H Elma Transsonic Lab-Line Instruments, Melrose Park, IL) to purify the EPS and

final samples were centrifuged once more at 3700 g, 4 °C for 15 min (Eppendorf Centrifuge 5804R) to collect the purified EPS in the pellet.

The lyophilization and ethanol extraction methods deviated in the next and final stage of pellet handling. In the next stage of the lyophilization method the pellet in 50 mL centrifuge tube was then placed in the freezer (-80 °C) for 15 minutes. Finally, the sample was placed in a lyophilizer for a minimum of six hours at -60 °C and at a low pressure setting (~60 mtorr) (VirTis lyophilizer; Gardiner, NY). After six hours of lyophilization, the pelleted EPS was resuspened in 10 mL DI water (Millipore), mixed by vortexing and stored for later analysis.

The final step of the ethanol method was to resuspend the pellet in 5 mL 10^{-2} M KCl and 10 mL pure and cold ethanol and to incubate overnight at 4 °C resulting in precipitation of the EPS. After the incubation, the solution was then centrifuged (3700 g, 4 °C, 20 min), decanted, and 10 mL DI water (Millipore) added to the final pellet and resuspended for later analysis.

The third and final method for EPS extraction was the sonication method.[18] This method utilizes a cell disruptor machine (VirSonic Digital 600; VirTis, Gardiner, NY) to remove EPS from cell surfaces. This was done by taking the freshly harvested cell pellet, resuspending it in 10 mL of 10^{-2} M KCl, exposing it to the stress condition of choice, and then sonicating the suspension (10 mL of 10^{-2} M KCl) for total of 20 sec, with four runs of 5 sec on, and three runs of 5 sec pause in between the sonication in 3.5 Hz power level. The difference between this cell disruptor and the water bath sonication method utilized

in the other two methods was that the disruptor probe was placed directly into the bacteria suspension to have a higher degree of impact on the cell. For consistency, 1 cm of the metal probe was immersed while conducting sonication. Afterwards the suspension was once again centrifuged at 4 °C and 4000 g for 20 min. Following this step, the cells remained in the pellet and EPS in the supernatant. Finally, the solution was filtered through a 0.22 μm filter (Millipore, Fisher Scientific) to ensure a cell-free, EPS suspension.

2.2.4 **Evaluating Extent of EPS Production and Composition.**

Once the EPS was extracted, the resulting EPS suspension (resuspended in 10 mL DI water) was evaluated for the total amounts of sugar and protein using previously developed methods. Specifically, the sugar quantification was performed with Phenol-Sulfuric Acid (PSA) method,[33] and the protein was quantified using the Lowry method.[34] The PSA method involved adding 50 μL 80% (w/w) phenol solution (Fisher Scientific), followed by 5 mL of highly concentrated sulfuric acid 95.5% (Fisher Scientific) to 2 mL of resuspended EPS solution. The phenol, acid, and EPS suspension was incubated at room temperature (22-25 ^{o}C) for 10 min, followed by incubation in a water bath (Lab-Line Instruments) for 20 min at 25-30 ^{o}C. After the incubation steps, the dark yellow to brown color of the solution required an additional 4 hr for stabilization, and this was done by leaving the suspension at room temperature. Final measurement of the suspension was done spectroscopically (Biospec-mini Shimadzu Corp., Kyoto, Japan) at 480 nm with Xanthan gum as a standard.

Total protein amount quantification was performed with the Lowry method using bovine serum albumin (BSA, 1 mg/mL) (Fisher BioReagents, Fisher Scientific) as the standard and measured spectroscopically (BioSpec-mini) at a wavelength of 500 nm. Next, 0.3 mL of resuspended EPS solution (in 10 mL DI water) was put in a glass vial (20 mL in volume) and 1.5 ml alkaline copper reagent (made by combining 1 mL 2% $Na_2C_4H_4O_6$, 1 mL 1% $CuSO_4$, and 98 mL 2% $NaCO_3$ in 10^{-1} M NaOH) followed by the addition of 75 μL Folin reagent (Folin & Ciocalteu's Phenol Reagent, MP Biomedicals, LLC, Germany). The subsequent mixture was incubated at room temperature for 30 min and measured in the spectrometer at 500 nm. The absorbance reading was compared to the standard curve for BSA to determine the concentration of protein in each EPS sample.

2.2.5 **Further Cell Characterization**.

A cell lysis test was performed by measuring the amount of Glucose-6-Phosphate Dehydrogenase (G6PDH).[14, 30, 41] The kit consisted of two reagents, G6PD R1 reagent (NADP 1.5 mM, maleimide 12 mM, buffer, stabilizer, and lysing agent), and G6PD R2 reagent (Glucose-6-Phosphate 1.05 mM, buffer, magnesium salt, and sodium azide added as preservative) (Pointe Scientific Inc.; Canton MI). One mL of R1 reagent was added to 0.5 mL of resuspended EPS solution (in 10 mL DI water) and incubated at room temperature for 5-10 min. Then 2 mL of R2 reagent was added and the mixture was then incubated in water bath for 5 min. After the addition of R2 and water bath, an absorbance reading was immediately taken (340 nm) and again after exactly 5 min. The change in absorbance, normalized by the 5 min time increment, was calculated as the rate

of reaction occurring. This rate value was then compared to values on a standard curve comparing absorbance for known concentration of G6PDH, allowing for the severity of cell lysis under each cell condition and for all three extraction methods to be determined.

The cell lysis analysis was also performed in another test kit following Sigma protocol with chemicals from Sigma Aldrich (Sigma Chemical Co. St Louis, MO). The chemicals include Glycylglycine buffer, G 6-P, ?NADP, and $MgCl_2$. After mixing the buffer, G 6-P, ?NADP, $MgCl_2$, and the extracted EPS solution the absorbance was recorded immediately and after five min (time=0 and 5 min). G6PDH enzyme solution was applied as concentration standards.

Similar to the cell lysis analysis, viability tests were also performed under each experimental condition using the Live/Dead BacLight kit (L-7012; Molecular Probes, Eugene, OR) and an inverted fluorescent microscope (IX70, Olympus, Japan) with a red/green fluorescence filter set (Chroma Technology Corp., Brattleboro, VT). The Live/Dead BacLight kit could only be performed before the starting of the extraction process, as it requires whole cells. Therefore analysis of viability was conducted immediately after whole cells were exposed to AGW for the particular test condition. The G6PDH test could be performed after all of the EPS extraction steps, and indicated the cumulative cell membrane breakage through the measuring of inner cell enzyme concentration. Therefore, both methods were used.

2.2.6 **Statistical Analysis of Results.**

Analysis of variance (ANOVA) was performed to determine whether the three extraction methods tested (lyophilization, ethanol, and sonication) yielded significantly different values of EPS sugar and protein content under the various combinations of IS ($10^{-2.5}$, 10^{-2}, and $10^{-1.5}$ M AGW) and starvation period (6, 12, 18, 24 h) used to represent a range in environmental conditions. ANOVAs were performed using PROC MIXED in SAS (SAS Institute, 2003) and individual mean comparisons were based on Least Significant Differences (LSD) at $P \leq 0.05$. The experimental design was a split-plot design with the main unit having a 2-way factorial treatment structure (IS and exposure time) and subunit being extraction method. The Main unit had a randomized complete block design with 2 replicate blocks.

Pearson's correlation coefficients were calculated to determine if EPS protein concentrations were significantly correlated with EPS sugar concentrations for each of the three removal methods. In addition, multiple regression analysis was used to determine whether IS and starvation period had a significant effect on EPS sugar and protein concentrations for the three different extraction methods. All statistical analyses were performed using SAS version 9.1 (SAS Institute, 2003). Probability values less than 0.05 were considered statistically significant.

2.3 **RESULTS AND DISCUSSION**

2.3.1 **Experimental Results.**

The EPS composition of *Salmonella* SA1685 cells was determined using a combination of extraction and analysis methods. The response to various environmental conditions, notably IS of AGW and time of exposure to said conditions was evaluated through the quantification of EPS yield and sugar and protein levels. The results of these analyses are shown in Tables 2.1 through 2.3.

Table 2.1 Sugar Content of EPS Extracted by the Lyophilization, Ethanol and Sonication Methods.

IS (M)	Time (h)	Sugar content ($mg/10^{11}$ cells) Lyophilization	Ethanol	Sonication
10^{-5}	0[1]	3.46±0.75 a[2]	3.13±1.42 a	2.34±0.61 a
$10^{-2.5}$	6	2.64±0.07 a	3.02±0.20 a	1.00±0.09 a
	12	2.24±0.35 a	3.23±0.32 a	1.56±0.11 a
	18	3.86±1.28 a	2.95±0.78 a,b[2]	1.04±0.03 b
	24	3.48±0.27 a	3.55±0.16 a	2.24±0.16 a
10^{-2}	6	4.09±1.84 a	2.30±0.40 a,b	1.21±0.12 b
	12	3.57±0.83 a	2.94±0.89 a	2.36±0.71 a
	18	5.53±1.40 a	4.40±1.52 a,b	3.02±0.61 b
	24	2.44±0.44 a	3.40±0.96 a	1.57±0.37 a
$10^{-1.5}$	6	2.57±0.48 a	1.62±0.09 a	0.71±0.06 a
	12	3.10±0.48 a,b	3.66±0.63 a	0.71±0.02 b
	18	4.62±1.07 a	3.57±0.37 a	2.93±0.21 a
	24	7.88±2.47 a,b	8.42±3.99 a	5.75±0.18 b

[1] Solution of deionized water only, not supplemented with AGW.
[2] Letters in the same row (a and/or b) indicate a statistically significant difference based on LSD means separation.

Table 2.2. Protein Content of the EPS Extracted by the Lyophilization, Ethanol and Sonication Methods.

IS (M)	Time (h)	Protein content ($mg/10^{11}$ cells) Lyophilization	Ethanol	Sonication
10^{-5}	0[1]	128.29±17.59 a[2]	174.20±45.11 a	60.79±23.16 b
$10^{-2.5}$	6	89.13±5.97 a,b	112.46±29.62 a	23.48±2.02 b
	12	51.26±13.05 a	77.86±1.96 a	34.17±6.99 a
	18	99.13±43.27 a	76.22±15.79 a,b	18.96±0.32 b
	24	67.71±8.31 a	71.80±5.61 a	41.53±1.78 a
10^{-2}	6	111.31±25.86 a	75.86±8.65 a,b	24.13±1.95 b
	12	81.18±21.84 a	391.11±136.64 b	42.84±12.15 a
	18	172.85±26.41 a	137.86±50.04 a,b	76.33±24.78 b
	24	57.16±13.56 a	83.29±22.58 a	31.31±4.29 a
$10^{-1.5}$	6	75.90±17.20 a	51.32±6.43 a	5.02±1.23 a
	12	130.34±26.69 a	175.21±32.67 a	14.72±1.40 b
	18	105.44±11.29 a	89.99±5.18 a	64.97±5.36 a
	24	149.98±41.31 a	143.08±73.19 a	83.30±3.31 a

[1] Solution of deionized water only, not supplemented with AGW.
[2] Letters in the same row (a and/or b) indicate a statistically significant difference based on LSD means separation.

Table 2.3. Sugar to Protein Ratio of the EPS Extracted by the Lyophilization, Ethanol and Sonication Methods.

IS (M)	Time (h)	S/P Ratio Lyophilization	Ethanol	Sonication
10^{-5}	0[1]	0.027±0.003 a[2]	0.019±0.010 a	0.041±0.010 b
$10^{-2.5}$	6	0.030±0.002 a,b	0.028±0.005 a	0.043±0.003 b
	12	0.045±0.005 a	0.041±0.004 a	0.048±0.013 a
	18	0.041±0.005 a,b	0.038±0.002 a	0.055±0.001 b
	24	0.051±0.004 a	0.050±0.003 a	0.054±0.002 a
10^{-2}	6	0.035±0.009 a	0.030±0.003 a	0.050±0.003 b
	12	0.044±0.003 a	0.008±0.001 b	0.055±0.003 a
	18	0.032±0.004 a	0.032±0.002 a	0.041±0.007 a
	24	0.043±0.006 a	0.041±0.001 a	0.049±0.005 a
$10^{-1.5}$	6	0.034±0.002 a	0.032±0.003 a	0.153±0.057 b
	12	0.024±0.002 a	0.021±0.003 a	0.048±0.006 b
	18	0.043±0.006 a	0.040±0.003 a	0.045±0.004 a
	24	0.053±0.009 a	0.060±0.006 a,b	0.069±0.003 b

[1] Solution of deionized water only, not supplemented with AGW.
[2] Letters in the same row (a and/or b) indicate a statistically significant difference based on LSD means separation.

The relative performances of the three extraction methods in capturing the EPS trends under the range of environmental conditions tested have been examined by

statistical analysis. Letters in the same row (a and/or b) indicate a statistically significant difference between the three methods based on LSD means separation in Tables 2.1-2.3.

Table 2.4 ANOVA Summary Table For Sugar and Protein Content of EPS.

	Sugar		Protein	
Effect	**F value**	**P**	**F value**	**P**
Method	14.0	< 0.001	30.1	< 0.001
IS	5.4	0.0089	7.50	0.0019
Time	10.1	< 0.001	4.75	0.0068
Method*IS	0.24	0.92	2.56	0.055
Method*Time	0.55	0.77	6.16	< 0.001
IS*Time	7.83	< 0.001	5.65	< 0.001
Method*IS*Time	0.37	0.97	3.14	0.0038

Based on the ANOVA results, extraction method had a significant effect ($P<0.001$) on measured EPS sugar and protein content (Table 2.4). IS and exposure time also had a significant effect for all three extraction methods combined indicating that EPS protein and sugar content varied depending on the experimental conditions. For EPS sugar, all interaction terms with method of extraction were not statistically significant indicating the F test for extraction method (averaged over all IS and exposure time) is accurate. For the protein data the interaction terms were significant; however, because the F values were much lower for these interaction terms than the main effect for method, the F test for extraction method obtained for EPS protein is also accurate. Further discussion on the experimental trends and outcomes of our statistical analyses are discussed below.

2.3.2 **Comparison of the EPS Yield**

The three extraction methods, lyophilization, ethanol and sonication, were compared based upon the sugar and protein amount combined together as total EPS extracted. Between the lyophilization and ethanol methods, the total mass of the extracted EPS pellet – the mass of EPS measured before the final purification step by freeze drying or ethanol exposure – did not change significantly regardless of IS and exposure time (not significant with student's paired *t* test, P>0.1). Typically, the extracted pellet mass prior to the final purification step weighed approximately 0.5 g; however, this was a measured wet weight consisting not only of the EPS pellet but also residual DI water. Hence further analysis focused not on the EPS pellet mass, but rather emphasized and reported the sum of sugar and protein content as the total EPS extracted (EPS yield).

For a given IS and exposure time, we observed differences in the yield of the EPS pellet extracted by the three different methods. In particular, the lyophilization and ethanol methods always yielded higher values of sugar and protein content than the sonication method. For example, the lyophilization and ethanol methods, on average, yielded 2.4 and 2.2 times more sugar, respectively, than the sonication method. For protein content, the lyophilization and ethanol methods yielded, on average, values 4.2 and 4.6 times, respectively, more than the sonication method. For several combinations of IS and exposure time, the values obtained with sonication were significantly different

than those obtained with the lyophilization and/or ethanol extraction methods; of note, when EPS data were averaged over IS or time and were compared, the sonication method almost always yielded values significantly lower than the other two methods (data not shown). On the other hand, differences between the ethanol and lyophilization methods were not statistically significant under any of the environmental conditions tested (Tables 2.1 and 2.2). The lower observed EPS values obtained with the sonication method was attributed to this method being purely a physical extraction method; whereas, the lyophilization and ethanol methods were similar in their physical and chemical approach with only a difference in the last purifying step (see section 2.2). This finding is consistent with others who have reported lower yields with physical extraction methods [14, 26, 27]. For example, Comte et al. found that after comparing eight methods of extraction, including both chemical and physical methods, notably less EPS was extracted by physical methods than the chemical methods.

2.3.3 **Comparison of the Sugar and Protein Content as a Function of Extraction Method.**

When comparing data presented in Table 2.1 and displayed in Figure 2.1 (a), (b), and (c), it was observed that there was no notable difference between extraction methods in sugar content with changing solution chemistry (IS), as all three methods produced a fairly similar trend. There was an upward trend in sugar content as a function of exposure time observed in all three methods, particularly for cells exposed to the highest IS solution ($10^{-1.5}$ M). Over increasing periods of exposure, the effect of solution

chemistry (IS) became more evident, in that the overall increase in amount of sugar as a function of exposure time was more pronounced at the higher IS conditions tested.

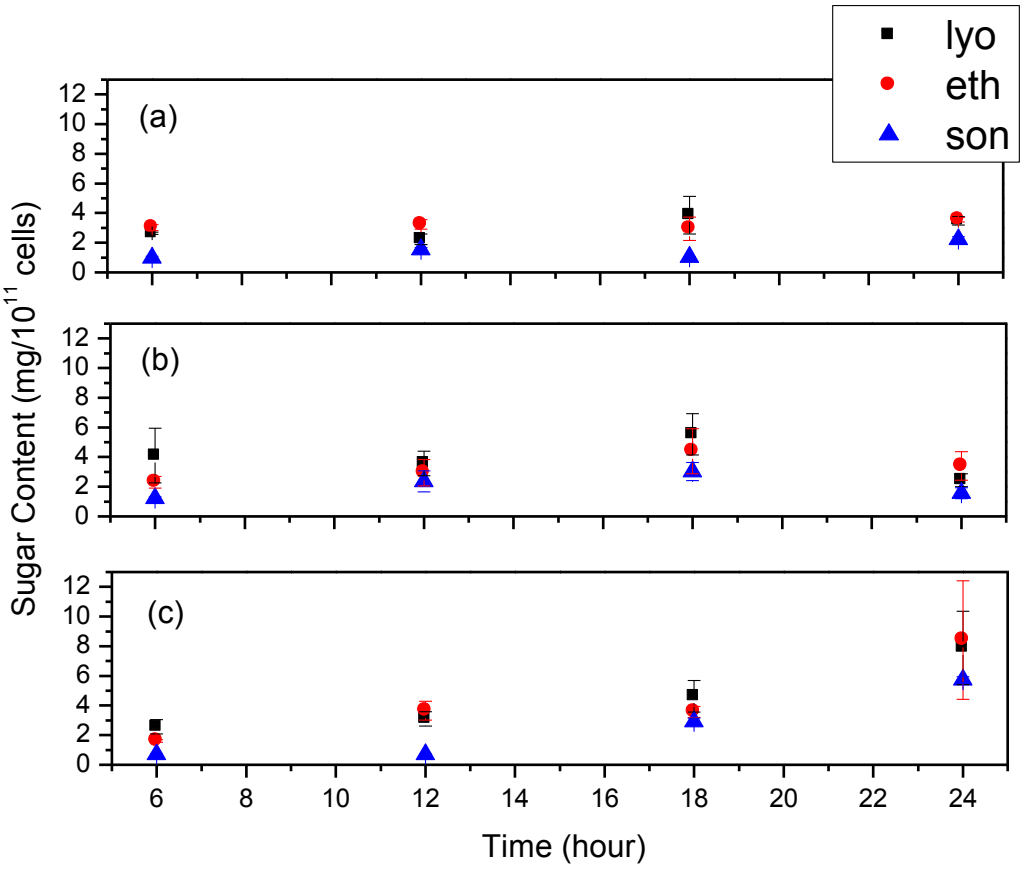


Figure. 2.1 Sugar content as a function of exposure time normalized by cell concentration (a) $10^{-2.5}$ M (b) 10^{-2} M (c) $10^{-1.5}$ M

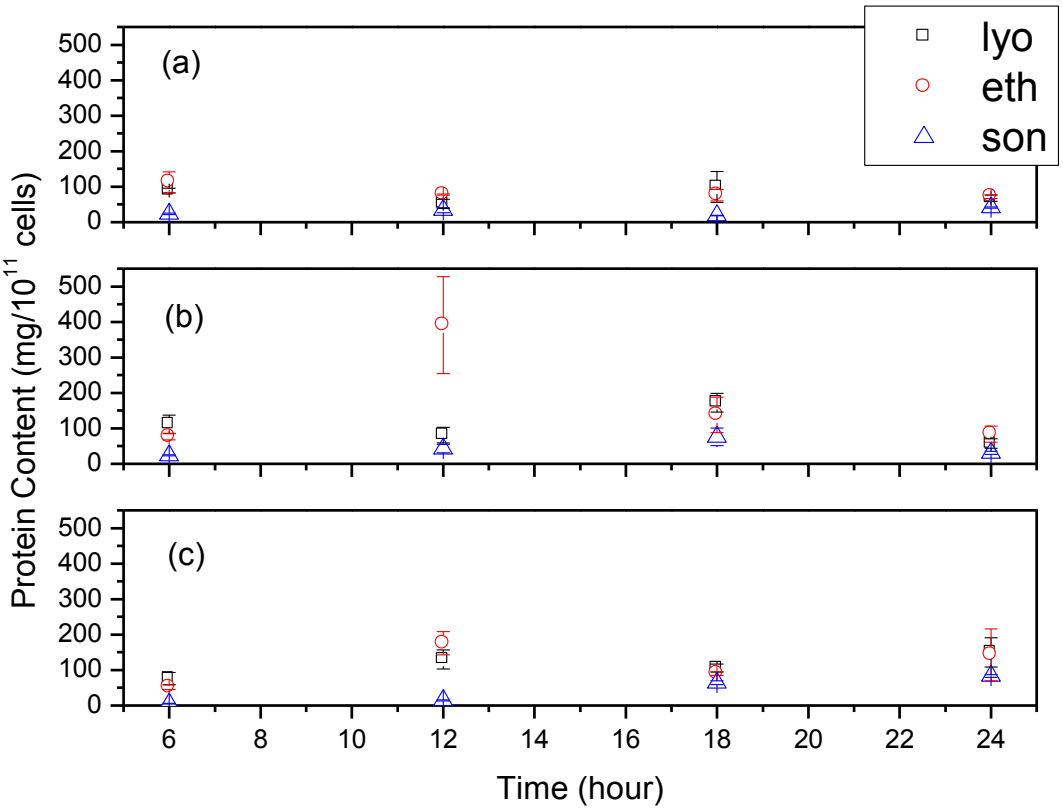


Figure 2.2 Protein content as a function of exposure time normalized by cell concentration (a) $10^{-2.5}$ M (b) 10^{-2} M (c) $10^{-1.5}$ M

The extracted protein content is presented in Figure 2.2, and Table 2.2, as a function of exposure time to AGW. There was no clear relationship between protein content, time, and IS (Figure 2.2). The changes in protein content as a function of exposure time for all extraction methods were minor. When comparing the three methods' sensitivity to time, as shown in Figure 2.2, all three followed a fairly similar trend for protein. The bulk of the comparisons were insignificant (24 of 39), however, the extraction methods were more sensitive to various environmental conditions (IS and

duration of exposure) when evaluating EPS protein versus sugar content. There was one outlying data point, for the ethanol sample after 12 h exposure to AGW (Table 2.2, Figure 2.2). This data point suggests an increase in protein after 12 h, but as the other two methods indicated, their protein levels were not changing significantly at the same condition (12 h), hence, this data point is considered an anomaly.

Further evaluation of the extraction methods was conducted to identify any correlation between extraction methods and corresponding protein and sugar levels. There were strong correlations between EPS sugar and protein content as measured by the sonication (r^2=0.92; $p<0.001$) and lyophilization (r^2=0.78; p=0.003) methods. For the ethanol extraction method, however, the correlation between EPS sugar and protein content was not significant (r^2=0.12, p=0.71).

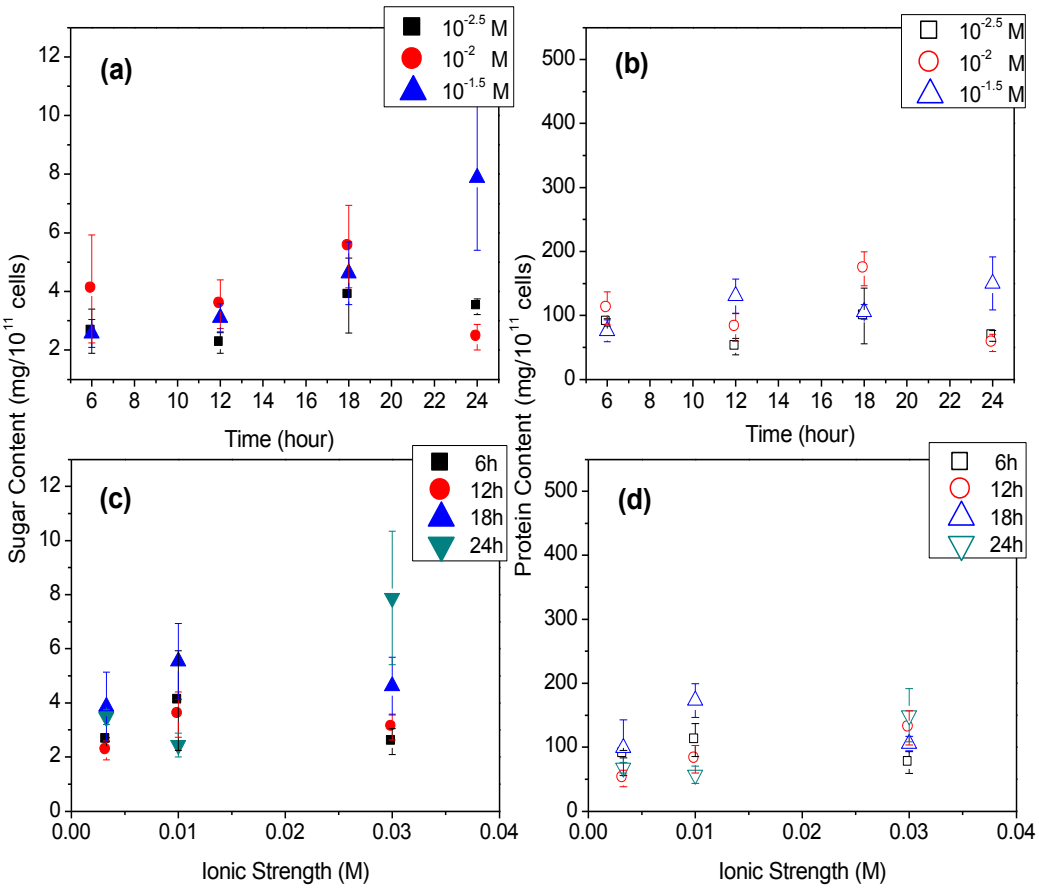


Figure 2.3 Sugar and protein content extracted by lyophilization method as a function of exposure time and IS: (a) sugar content to exposure time (b) protein content to exposure time (c) sugar content to IS (d) protein content to IS

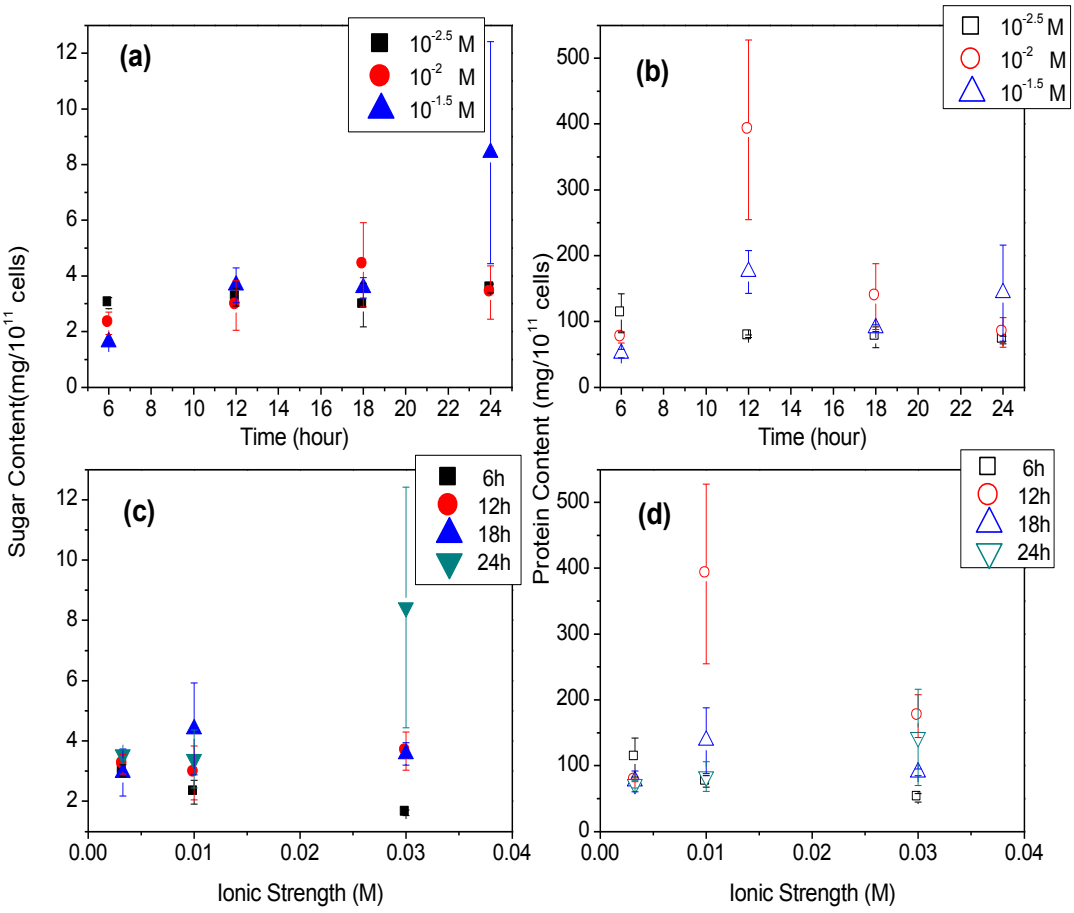


Figure 2.4 Sugar and protein content extracted by ethanol method as a function of exposure time and IS: (a) sugar content to exposure time (b) protein content to exposure time (c) sugar content to IS (d) protein content to IS

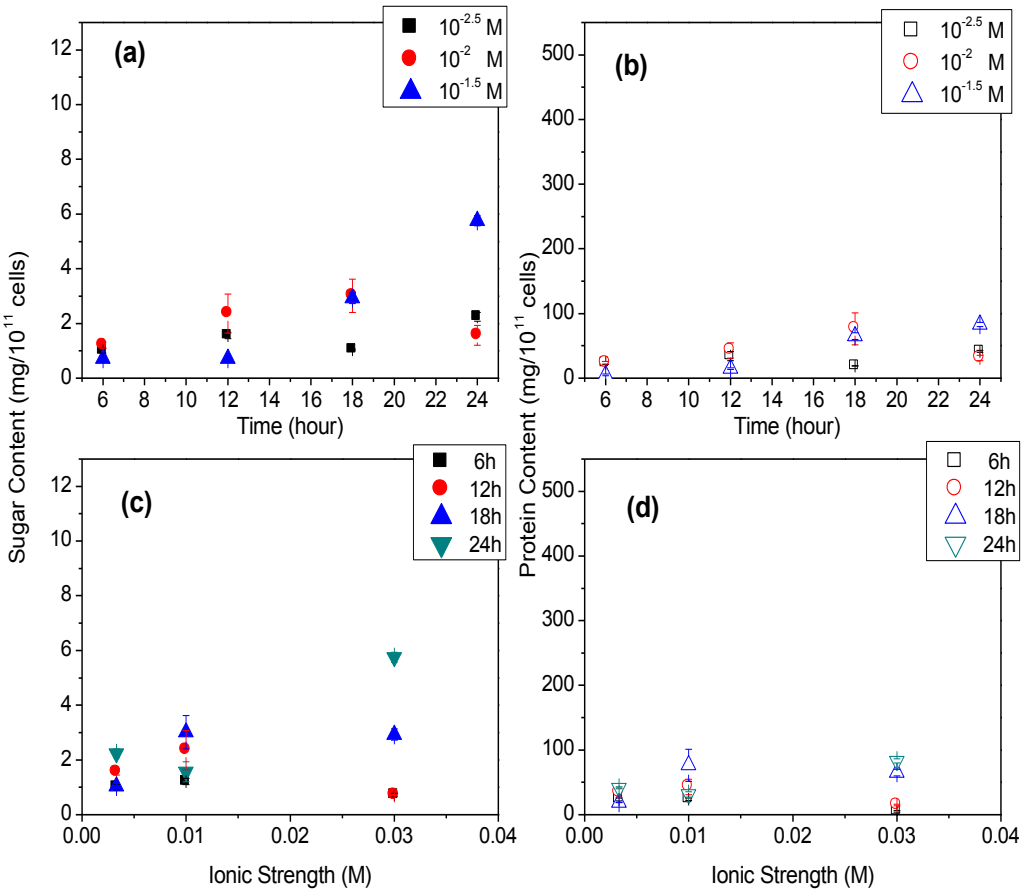
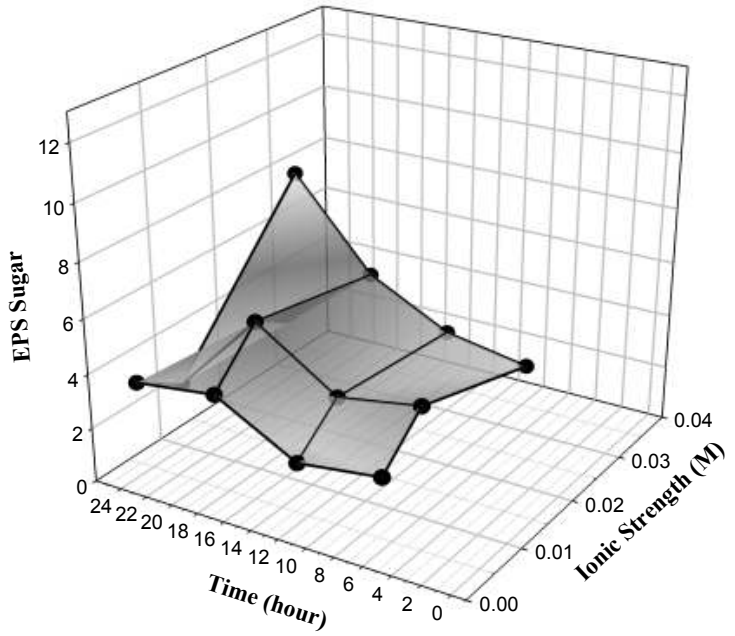


Figure 2.5 Sugar and protein content extracted by sonication method as a function of exposure time and IS: (a) sugar content to exposure time (b) protein content to exposure time (c) sugar content to IS (d) protein content to IS

An alternative presentation of the data is shown in Figures 2.3-2.8. In Figures 2.3-2.5, the EPS sugar and protein content extracted are presented as a function of exposure time and IS for each method. Multiple linear regressions performed on data from all 12 treatments showed significant increases in EPS content with increasing exposure time for sugar content as measured with the ethanol (slope = 0.15; P = 0.031)

and sonication (slope = 0.12; P = 0.035) extraction methods and for protein content as measured with the sonication extraction method (slope = 2.1; P = 0.041). No significant correlations, however, were observed with ionic strength. Figures 2.6-2.8 are more comprehensive, presenting the EPS sugar (part a) and protein (part b) content as a function of exposure time and ionic strength for each extraction method. These figures offer a different visual presentation of the data allowing individual trends with each method to be compared.

(a)



(b)

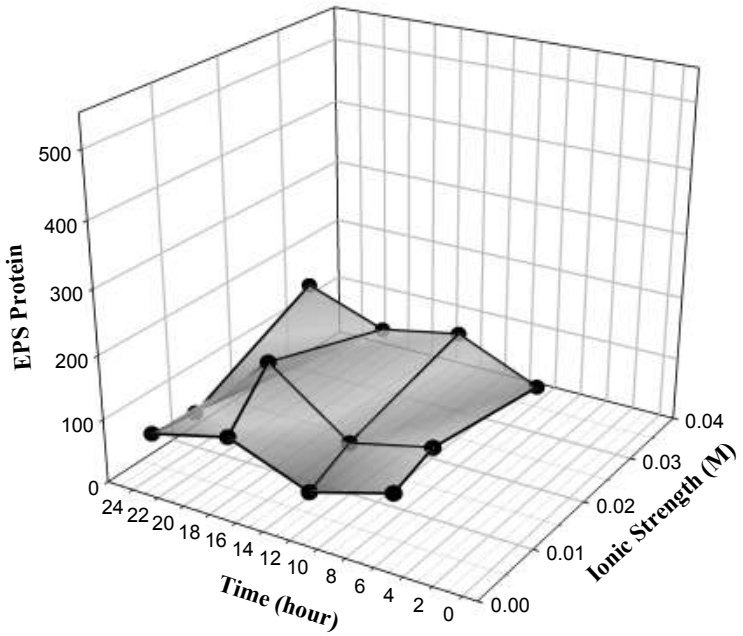
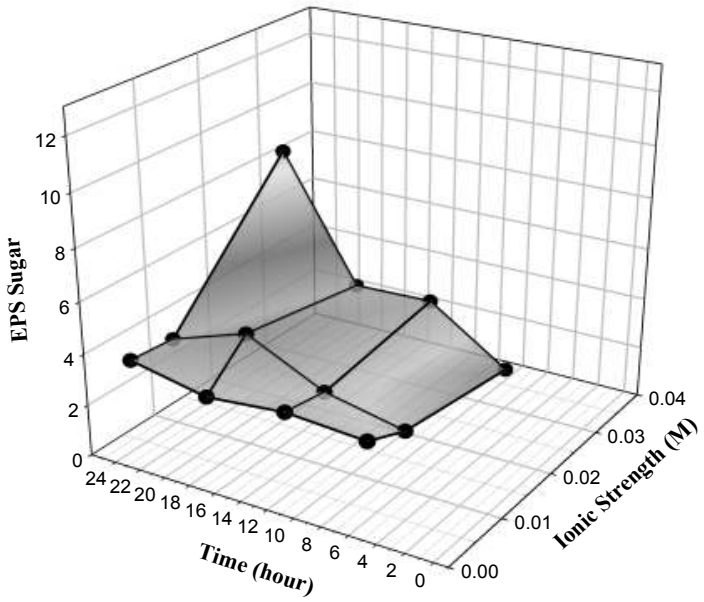


Figure 2.6 Lyophilization method extracted EPS three dimensional graphs: (a) EPS sugar content (b) EPS protein content.

(a)



(b)

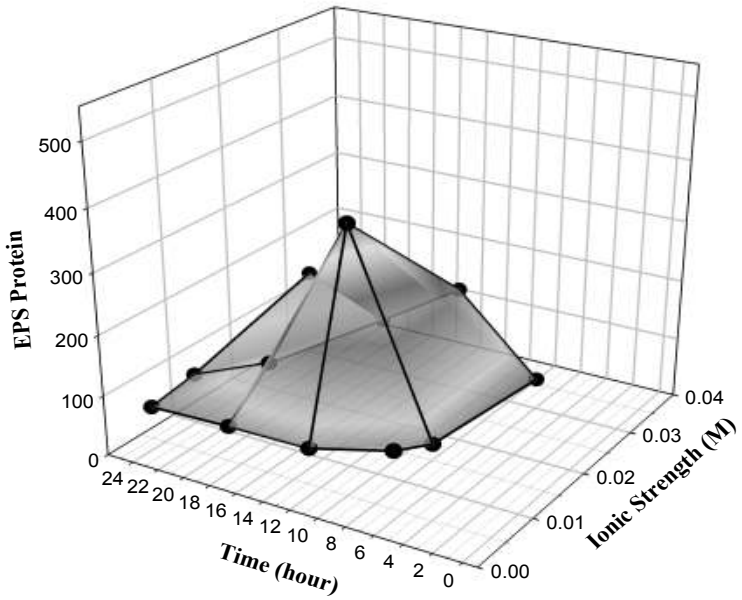
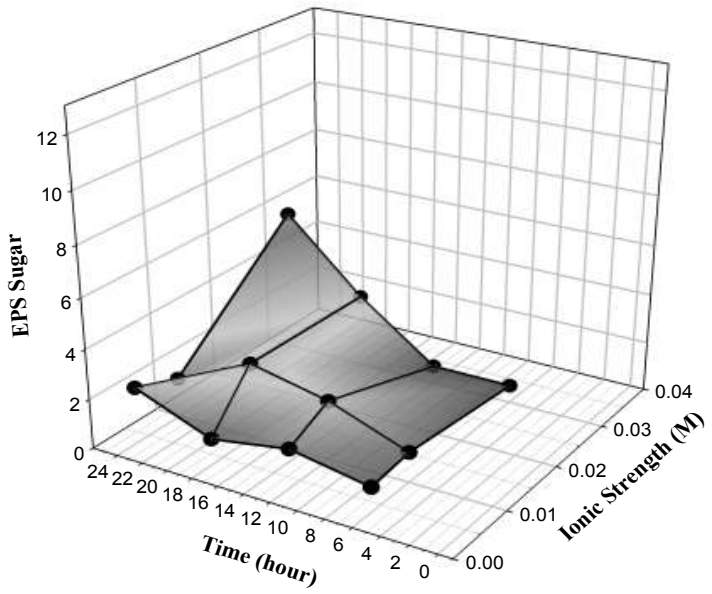


Figure 2.7 Ethanol method extracted EPS three dimensional graphs: (a) EPS sugar content (b) EPS protein content.

(a)



(b)

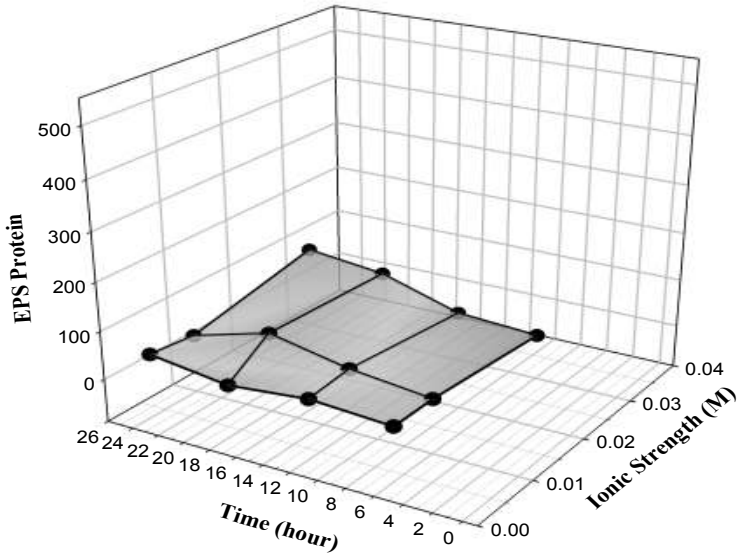


Figure 2.8 Sonication method extracted EPS three dimensional graphs: (a) EPS sugar content (b) EPS protein content.

2.3.4 **Comparisons Utilizing a Sugar/Protein Ratio.**

The ratio of sugar to protein (sugar/protein ratio) was another parameter that allowed for comparisons between the sensitivity of the three different extraction methods and has the potential to provide considerable insight into the cell response to subtle changes in environmental conditions (Table 3). Utilizing this approach, differences were observed between EPS extraction methods with regard to the resulting sugar/protein ratio. However, the letters, a and b, indicate two thirds (26 of 39) of the differences between methods were statistically insignificant. This suggests that the sugar/protein ratio can provide additional insight into changes in EPS. As mentioned before, the sugar and protein content extracted by the sonication method had a stronger linear correlation than lyophilization and ethanol methods. Hence we observed a more stable sugar/protein ratio from the sonication data than the other two methods.

2.3.5 **Comparisons Through Cell Lysis Analysis.**

The extent of cell lysis was shown to be relatively insignificant for all three extraction methods in various environmental conditions based upon both test kits and protocols as distributed by Sigma and Pointe Scientific (data not shown). The three methods had negligibly low levels of lysis, through the detection of G6PDH. Our results suggested the organisms' membranes remained intact up to the point of EPS extraction, as indicated by greater than 90% viability prior to extraction by BacLight test kit. During the following extraction processes by any of the three methods, only minimal lysis occurred as indicated by our detection protocols. Hence, it is assumed that quantifiable

levels of protein and sugar, as reported in Tables 3.1-3.3 are constituents of the EPS and not cytoplasmic or membrane based materials.

2.4 **CONCLUSIONS**

The ability to measure subtle changes in *S. pullorum* SA 1685 cells' EPS content was highly sensitive to the choice of extraction method. Notably, the ethanol method had the highest EPS yield of the three, slightly greater than lyophilization method and much more than sonication method in all conditions. The ethanol method results in the same general trends as the lyophilization method, but with higher variance. Sonication method may have lower yield, but was a much faster process to conduct needing no more than one hour versus the lengthy overnight steps involved in the other two methods.

The results of this study showed the sensitivity of three EPS extraction methods to a varied cell condition (IS of AGW and exposure time). This provided insight on these three methods and helped to determine the capability of each in detecting environmental changes in simulated or real subsurface environments. Based upon the conditions investigated in this particular study, ANOVA evaluation deemed the three extraction methods were all valuable and capable of differentiating EPS trends; however, choice of extraction method has a significant effect on the subsequent measured EPS sugar and protein content. Outcome of this study is intended to serve as guidance for environmental scientists in their selection of EPS extraction and analysis protocols, indicating the choice of methods can clearly influence the results and needs careful consideration.

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Chapter 3

Influence of Varying Levels of Extracellular Polymeric Substances (EPS) on Hydroxyl Radical Mediated Disinfection of *Escherichia coli*

ABSTRACT

Photolysis of nitrate, a prevalent constituent in agriculturally impacted waters, may influence pathogen attenuation in such systems through production of hydroxyl radical (·OH). This study focuses on the efficacy of ·OH generated during nitrate photolysis in promoting *E. coli* die-off as a function of extracellular polymeric substances (EPS) coverage. EPS levels of four *E. coli* isolates were systematically altered through a sonication extraction method and photochemical batch experiments with a solar simulator examined isolate viability loss as a function of time in nitrate solutions. *E. coli* viability loss over time exhibited two regimes; an initial induction time, t_s, with little decay was followed by rapid exponential decay characterized by a first-order disinfection rate constant, k. Increasing steady-state ·OH concentrations enhanced *E. coli* viability loss, increasing values of k and decreasing t_s values, both of which were quantified with a multi-target bacterial disinfection model. Notably, at a given steady-state ·OH concentration, values of t_s and k were independent of EPS levels, nor did they vary among the different *E. coli* strains considered. Results herein show that while ·OH generated via nitrate photolysis enhances rates of disinfection in surface water, the mechanism by which ·OH kills *E. coli* is relatively insensitive to common bacterial variables.

3.1 **INTRODUCTION**

Photochemically generated reactive oxygen species (ROS) including hydroxyl radical (·OH) and singlet oxygen ($^1O_2$) occur naturally in sunlit waters, where they play important roles in pollutant degradation[1, 2] and pathogen attenuation.[3-5] Among ROS, ·OH is often implicated as the primary entity responsible for bacterial disinfection in both natural[6, 7] and engineered[8-10] photochemical systems. While previous studies have often focused on the relative susceptibility of different pathogenic bacteria to ROS-mediated disinfection,[7, 11] the influence of several biological variables in these systems remains poorly understood. Notably, a variable likely to influence •OH activity but not yet extensively explored is extracellular polymeric substances (EPS), a high molecular weight polymer exuded by bacteria that consists primarily of polysaccharides[12, 13] and proteins,[14, 15] with some other components including nucleic acids.[16] To better predict the persistence of *Escherichia coli* (*E. coli*) in sunlit surface waters, a clearer understanding of the role of EPS in ROS-mediated disinfection is merited.

Bacterial disinfection in sunlit waters can occur by either direct or indirect photochemical processes. Direct photochemical disinfection is accomplished by UV light, especially in the UVB (280-320 nm) region of the solar spectrum, which is of sufficient energy to damage critical cellular components including DNA.[17, 18] In contrast, indirect photochemical disinfection involves the sensitized formation of extracellular ROS, whose bactericidal activity is typically attributed to reaction with cell outer membranes, causing decomposition of the membranes that leads to phospholipid

peroxidation or even cell death.[19] Therefore, it is crucial in indirect photochemical disinfection processes that the transient ROS produced in bulk solution can be promptly transported to the cell surfaces.[10, 11, 20-22]

While some EPS exuded by bacteria is dispersed as a soluble component in solution (i.e., soluble or free EPS), a portion stays in close proximity to the cells (i.e., bound EPS)[23] where it influences cell interactions with other particles and surfaces by bridging the cells to such substrates.[24-26] The physical presence of EPS around the cells also is known to protect cells from various environmental hazards.[27] Although not yet extensively investigated, it is anticipated that the bound EPS layer should provide some degree of protection from indirect photochemical disinfection by affecting the ability of ROS to react with and damage the cell outer membrane.[28] Specifically, highly reactive and non-specific ·OH is likely to oxidize the lipids, proteins, and nucleic acids comprising EPS,[29] in turn shielding more vital cell components from oxidative damage. Although soluble forms of EPS would likely function in a similar capacity, its influence on ROS activity is expected to be less than bound EPS that is more concentrated around the cell wall.

In a recent work with TiO_2 photocatalysts, Liu et al.[30] compared the bactericidal efficiency of ·OH toward biofilm heterotrophic bacteria, the same biofilm heterotrophic bacteria after removing soluble EPS, and a native EPS-deficient *E. coli* strain. They observed faster disinfection rates when the soluble EPS was stripped off the biofilm heterotrophic bacteria via centrifugation than for biofilm heterotrophic bacteria with

intact EPS. They proposed, therefore, that EPS may physically block or chemically quench the photocatalytically produced ·OH. However, the influence of native EPS level in non-biofilm *E. coli* has yet to be explored, leaving questions over whether native EPS levels associated with cell surfaces are sufficient to inhibit ROS activity.

The main objective of this study was to quantitatively test and identify the role of EPS in photochemical disinfection of *E. coli* in the presence of ·OH for four *E. coli* isolates with differing EPS levels, as well as systematically varied levels of bound EPS controlled by sonication. These model organisms were selected specifically to represent the type of species present in agricultural run-off waters in which photochemical disinfection processes may occur. Photochemical batch reactivity studies with each type of *E. coli* were conducted to determine the rate of viability loss over time using a standard viability assay.[31] Rates of viability loss were then compared to trends in EPS levels, as well as the concentration of steady-state ·OH present in each system. Trends in viability loss as a function of steady-state ·OH and EPS levels were then used to test our initial hypothesis that bound EPS functions as a scavenger for ·OH, thereby inhibiting its bactericidal activity.

As a model system for ·OH generation, we employ nitrate photolysis, which is recognized as a route for ·OH production in surface waters at levels with implications for pollutant fate.[1, 32] Average nitrate concentrations in surface waters are approximately 0-44 mg NO_3^-/L, and higher concentrations can occur in waters that are susceptible to agricultural runoff.[33] Thus, ·OH production arising from nitrate photolysis is likely to be

important in agriculturally impacted waters, although the implications of this process on bacterial pathogen fate are not yet established. A practical benefit of working with nitrate is that as a homogeneous system, it poses less complexity in bacterial studies relative to heterogeneity in ROS produced by other natural sensitizers such as dissolved organic matter (DOM).[34] For example, recent studies with natural organic matter (NOM) have suggested formation of microenvironments in close proximity to NOM where multiple forms of ROS exist at concentrations that can be orders of magnitude above those existing in bulk solution.[35, 36] In these alternative sensitizer systems, therefore, it is difficult to accurately identify the nature and level of ROS to which the bacteria are exposed, which complicates efforts to clearly establish the role of EPS in ROS-mediated disinfection.

3.2 **MATERIALS AND METHODS**

3.2.1 **Reagents.**

All solutions were made with reagent grade chemicals. Potassium phosphate monobasic (Fisher Scientific; 99.3%) was used to buffer pH. Potassium chloride (Fisher Scientific; 99.9%) was utilized to adjust ionic strength. Sodium nitrate (Fisher Scientific; 99.1%) was used to prepare nitrate solutions. Aqueous solutions of phenol (Sigma Aldrich; ≥ 99%) were used to measure steady-state ·OH concentrations. Sodium formate (Sigma Aldrich; ≥ 99%) was used as a radical quencher in selected experiments.

3.2.2 **Measurement of Steady-State ·OH Concentrations in Photochemical Batch Systems.**

A 450 W O_3-free xenon arc lamp (Newport Corporation, Irvine, CA) was used to produce a collimated beam. Light was first passed through a water filter to remove infrared radiation and then a 305 nm long-pass filter to produce light in the UV and visible range. The incident photon flux of the lamp was measured using a ferrioxalate chemical actinometer.[37] Briefly, equal volumes of ammonium ferric sulfate (Sigma Aldrich; 99%) and potassium oxalate monohydrate (Riedel-De Haën; ACS reagent) were irradiated for several minutes, and the incident photon flux was found to be 2.2×10^{-5} einstein min^{-1}. The visible light range (≥400nm) was found to have a photon flux of 1.2×10^{-5} einstein min^{-1} All experiments were conducted in a 50 mL (37 mm ID) jacketed beaker at 25 °C with 5 mM phosphate buffer at pH 7.0.

Steady-state concentrations of hydroxyl radical ($[\cdot OH]_{ss}$) were determined using phenol as a probe.[38] Batch reactors with varying concentrations of nitrate (50-500 mg/L as NO_3^-) and a known initial concentration of phenol (typically 100 μM) were exposed to light over several hours. Samples (1 mL) were taken hourly and transferred to a 2.5 mL crimp sealed amber vial for subsequent HPLC analysis. Samples were analyzed on an Agilent 1200 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector. An Eclipse XDB-C18 column (Agilent Technologies) was used with a mobile phase of 35% acetonitrile with 65% 1 mM sodium acetate and acetonitrile at a flow rate of 0.75 mL/min and a detection wavelength of 254 nm.

Phenol decay was modeled as a pseudo-first-order reaction with respect to phenol. A semi-log plot of normalized phenol concentration versus time provided the pseudo-first-order rate constant, k_{obs}, from which values of $[\cdot OH]_{ss}$ were determined via normalization with the rate constant for phenol reaction with ·OH ($k_{phenol} = 1.4 \times 10^{10}$ $M^{-1}s^{-1}$).[38]

3.2.3 **Cell Selection and Preparation.**

Four wild type *E. coli* isolates from dairy cattle (DC1),[39] human (HU1),[39] swine (SP4),[40] and a pathogenic strain (O157:H7)[41] were investigated in photochemical batch studies. The strains were also chosen as they are documented as EPS-producing strains with different amount of EPS coverage.

The day before the experiment, a fresh pre-culture was prepared from the stock culture on a Luria-Bertani (LB) (Fisher Scientific, Fair Lawn, N. J.) agar petri-dish. *E. coli* cells were selected from the plate and inoculated into 5 mL of LB broth in 37°C overnight (14-16 h). The overnight pre-culture was then used to inoculate (1:100 v/v) a 200 mL LB broth. This culture was incubated in 37°C for four hours (DC1 and HU1) and three and half hours (SP4 and O157:H7) to reach mid-exponential phase. Cells were harvested by centrifugation (5804R; Eppendorf, Hamburg, Germany) with a fixed-angle rotor (F-34-6-38; Eppendorf) for 15 min at 4 °C and 3700 g to separate cells from growth media. To further rinse the cells, after the centrifugation the media was decanted and 10 mL of 10 mM KCl was added and the suspension was vortexed before a second centrifugation for 15 min at 4 °C and 3700 g. The rinsing steps, including decanting,

adding electrolyte and vortexing were applied once more. After the third and final rinse cycle, 5 mL of 10 mM KCl was added to the pellet creating the stock solution to be used for further experimentation. The concentrations of each stock solution were quantified via a cell counting chamber (Buerker−Türk chamber, Marienfeld Laboratory Glassware, Lauda-Königshofen, Germany) and a light microscope (Fisher Scientific Micromaster). Typical cell stock solution concentrations in this study were between 2 to 10×10^{10} bacteria/mL. All cell media and electrolyte solutions were made by mixing deionized water (Millipore, Billerica, MA) and reagent grade salts (Fisher Scientific).

After the cell stock solution was prepared and the concentration was determined, the required volume of the cell stock solution was added either to 10 mL of 10 mM KCl for subsequent exposure to sonication, or directly added into 50 mL of nitrate solutions (50-500 mg/L as NO^{3-}) or 50 mL of phosphate buffer (5 mM at pH 7) to achieve a final 50 mL suspension of 5×10^{8} cells/mL in the batch reactor.

3.2.4 **Extraction and Evaluation of EPS.**

In certain experiments the level of EPS was experimentally controlled via partial removal of EPS achieved using probe sonication (Omni-Ruptor 250, Omni International, Kennesaw, GA) and centrifugation (Eppendorf). This method was previously reported on in detail.[42, 43] Briefly, sonication was applied via a cell disruptor machine with a 5/32" Micro-Tip sonicating probe at 30% intensity (150 Watt maximum). The probe was placed directly in the solution and approximately 10 cm was immersed in the solution. The bacterial solution was kept in ice during the entire sonication process. Sonication

was applied with fixed intensity (nominally 30%) and duration (20, 160, 300, 440, or 580 s total sonication time with a five second pause at every five second interval of sonication) to achieve the desired level of EPS removal. Afterwards, the sonicated solution was centrifuged at 4 °C and 4000 g for 20 min (Eppendorf) to separate the intact cells and the EPS that had been removed by sonication. After the centrifugation the supernatant was collected and passed through a 0.22 μm filter (Millipore, Fisher Scientific). The filtrate was collected as the EPS suspension for further compositional analysis. The pellet comprised of cells was re-suspended in 50 mL solutions composed of potassium nitrate and 5 mM phosphate buffer (pH 7) for later photochemical experiments.

The EPS composition, specifically the bulk sugar and protein amounts, were analyzed through established protocols. The sugar was quantified with Phenol-Sulfuric Acid (PSA) method,[44] and the protein was evaluated by the Lowry method.[45] The PSA method was performed by adding 50 μL of 80% (w/w) phenol solution, and 5 mL of 95.5% sulfuric acid to 2 mL of the filtered supernatant containing the EPS. The reaction between sulfuric acid, phenol, and sugar molecules creates heat and a color change from clear to brownish. The solution is allowed to cool in room temperature for 10 min and this is followed by further incubation in a water bath (Lab-Line Instruments, Inc., Melrose Park, IL) in 30°C for 20 min. Samples were put in a laminar flow hood for four hours at ambient temperature in order for the resulting color to stabilize. Subsequently, the absorbance was measured (Biospec-mini, Shimadzu Corp., Kyoto, Japan) at 480 nm with Xanthan gum (Sigma-Aldrich corporation, St. Louis, MO) as a standard. For

analysis of the EPS protein level, 1.5 mL of alkaline copper reagent (made by adding 1 mL of 2% $Na_2C_4H_4O_6$, 1 mL of 1% $CuSO_4$, and 98 mL of 2% $NaCO_3$ in 0.1 M NaOH) was added into 0.3 mL of the EPS suspension. The mixture was next incubated at room temperature for 10 min before adding 75 μL of Folin reagent (Folin and Ciocalteu's Phenol Reagent, MP Biomedicals, LLC, Germany). The mixture required another 30 min of room temperature incubation. Subsequently, the solution absorbance was measured at 500 nm with bovine serum albumin (BSA, 1mg/mL) (Fisher BioReagents, Fisher Scientific) as the standard. The absorbance values obtained from the sugar and protein analyses were interpolated using Xanthum gum and BSA calibration curves to determine the sugar or protein concentration, respectively, in the EPS removed from cells via sonication. All salts and reagents (unless otherwise noted) were ACS or reagent grade (Fisher Scientific).

3.2.5 **Exposure of Model Organisms to Light.**

All photochemical experiments were conducted with the aforementioned 450 W O_3-free Xe arc lamp and a custom-made jacketed photo-reactor, whose contents were well-mixed via a stir bar and maintained at 25 °C during all experiments via a circulating water bath (Thermo Scientific, Waltham, MA). Bacterial solutions containing a range of nitrate concentrations (0-500 mg/L as NO_3^-) were irradiated so as to vary the steady-state concentration of $^{\cdot}OH$ generated in each system. Experiments were conducted with *E. coli* DC1 cells with various levels of EPS obtained by varying the duration of sonication (from 20-580 s), as described previously. Additional *E. coli* strains (HU1, SP4, and

O157:H7) also were utilized to test the effect of cell strain at a fixed nitrate concentration (250 mg/L) and duration of sonication (300 s) for EPS removal.

During photochemical experiments, samples from the irradiated solution were withdrawn periodically over time and *E. coli* viability was measured. The percentage of viable *E. coli* was quantified using the Live/Dead BacLight kit (L-13152; Molecular Probes, Eugene, OR) and a fluorescent microscope (BX 51, Olympus, Japan) with a red/green fluorescence filter set (Chroma Technology Corp., Brattleboro, VT).

To identify the ROS type that is formed in irradiated nitrate solutions, a select number of photochemical experiments were conducted with bacteria in the presence of formate (50 mM) as a radical quencher. Control experiments were also conducted in which bacterial viability was measured in the presence of formate in both dark and irradiated batch systems without nitrate.

3.3 **RESULTS AND DISCUSSION**

3.3.1 **Analysis of *E. coli* Photochemical Decay Kinetics.**

During batch photochemical experiments (see Figure 3.1), changes in *E. coli* viability over time showed two distinct regimes of reactivity. Region 1 was an initial period of little to no viability change, which we hereafter refer to as the "induction time" (t_s), and region 2 was characterized by more rapid viability loss over time that followed exponential decay.

A multi-target bacterial disinfection model (equation 3.1) was applied to fit the experimental data taking account of the initial induction time (region 1) and the subsequent regime of exponential decay (region 2), which is typical of viability loss profiles observed in most pure culture bacterial disinfection processes.[46] The model takes the form of:

$$\mathbf{N} = \boldsymbol{N_0}[\mathbf{1} - (\mathbf{1} - \boldsymbol{exp}(-\boldsymbol{kt}))^{\boldsymbol{m}}] \quad (3.1)$$

where N and N_0 are the number of viable bacteria in the reactor and initial population, respectively, t is the illumination time (min), and the two parameters, k and m are constants obtained through the process of fitting this equation to the experimental data. For these model-fit parameters, k (min^{-1}) is the inactivation rate constant indicating the rate at which disinfection occurred in region 2, whereas m is simply a fitting parameter with no significant physical meaning.[46] Non-linear regression analyses were performed on $\ln(V/V_0)$ (V/V_0 means viability normalized by initial viability, both in percentage; when applying equation 3.1, $V/V_0=N/N_0$ in value) versus illumination time data using Origin 8.1 software (Origin Lab; Northampton, MA), providing best-fit parameters k and m. The initial induction time (region 1) at which population of cells begin to die-off is calculated using k and m, $t_s=\log(m)/[k\times\log(e)]$.[46] Values of k and t_s (region 1) were compared between different conditions. Fitted values for all experiments are reported in Table 3.1. To evaluate the significance of trends in model fit parameters for *E. coli* DC1 as a function of experimental variables (e.g., $[\cdot OH]_{ss}$, EPS level, presence or absence of

radical scavengers), k, m, and t_s values were evaluated using analysis of variance (ANOVA).

3.3.2 **Hydroxyl Radical Production in Illuminated Nitrate Suspensions.**

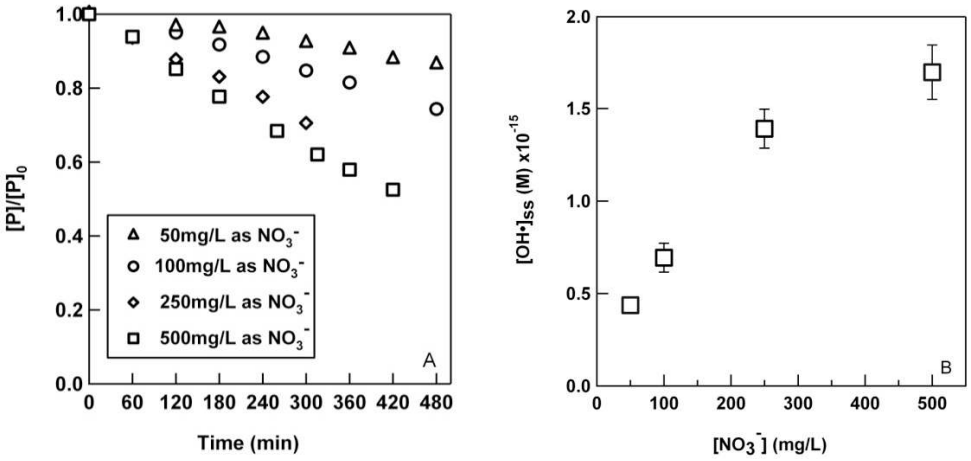

Figure 3.1 (a) Normalized phenol decay ([P] = phenol concentration; $[P]_0$ = initial phenol concentration) over time indicates the production of hydroxyl radical (·OH) by nitrate at wavelengths ≥ 305nm. (b) Calculated steady-state hydroxyl radical concentrations ($[\cdot OH]_{ss}$) as a function of nitrate concentration (mg/L). These ·OH concentrations (4.37×10^{-16}-1.70×10^{-15} M) generated over the range of aqueous nitrate concentrations (50-500 mg/L) are slightly lower than other studies of ·OH production from nitrate photolysis,[1] most likely due to differences in our reactor setup and lamp power output.

Values of $[\cdot OH]_{ss}$ (4.37×10^{-16}-1.70×10^{-15} M) determined from experiments with phenol are shown as a function of nitrate concentration (50-500 mg/L) in Figure 3.1. Values of $[\cdot OH]_{ss}$ initially increased linearly with nitrate concentration, however, ·OH concentrations began to plateau at higher concentrations (≥250 mg/L). This behavior is consistent with previous work,[1, 47] and attributable to the photolytic formation of nitrite, which can acts as a hydroxyl radical scavenger. For the majority of experiments,

therefore, concentrations of nitrate were typically kept below 250 mg/L to avoid competition with nitrite for ·OH. For experiments in which EPS levels were systematically varied via sonication, a nitrate concentration of 250 mg/L (corresponding to a value of $[\cdot OH]_{ss}=1.39 \times 10^{-15}$) was employed.

3.3.3 **Effect of Hydroxyl Radical Concentration on Bacterial Disinfection.**

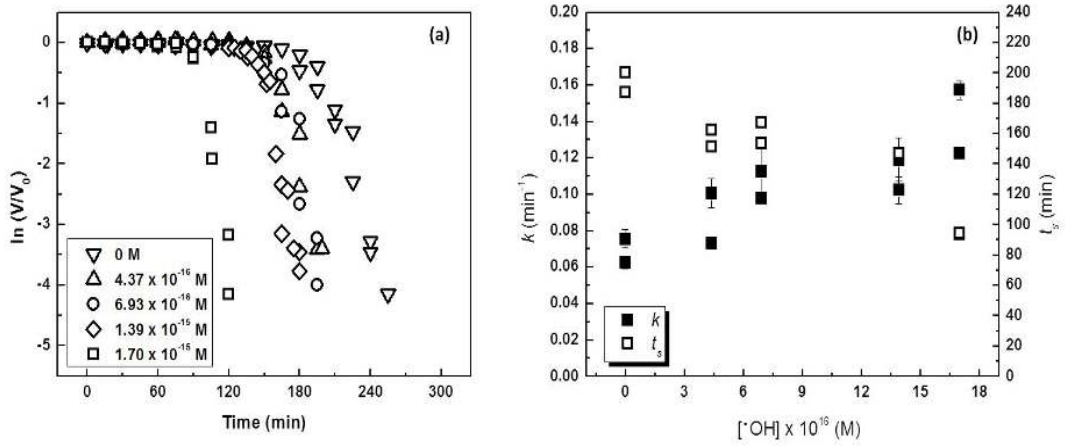

Figure 3.2 Results from photochemical disinfection of *E. coli* DC1 exposed to light with wavelength≥305 nm. a) Disinfection data presented as the natural log of cell viability normalized by initial viability versus the time of light exposure as a function of steady-state hydroxyl radical concentration ($[\cdot OH]_{ss}$). b) Results from non-linear regression analysis obtained from best-fit analyses of data presented in (a) with equation 1. The induction time (t_s) and viability loss rate constant (k) are shown as a function of steady-state hydroxyl radical concentration. Note that direct photochemical disinfection is the only process occurring when $[\cdot OH]_{ss}=0$.

Results from photochemical batch experiments conducted with DC1 containing a natural level of EPS and exposed to different concentrations of $[\cdot OH]_{ss}$ and light at wavelengths (λ)≥305 nm are shown in Figure 3.2. Direct photochemical disinfection

occurred when $[\cdot OH]_{ss}=0$, resulting in the onset of complete viability loss after approximately 4 h (Figure 3.2 (a)). Increasing values of $[\cdot OH]_{ss}$ shortens the time required to kill *E. coli* DC1 exposed to light at a wavelength≥305 nm. As the $[\cdot OH]_{ss}$ was increased incrementally, distinct patterns were observed. Faster rates of disinfection due to the presence of ·OH were inferred by both the shortening of the induction time (t_s), and the increase in the rate constant for viability loss (k) relative to values obtained via direct photochemical disinfection. Over the range of $[\cdot OH]_{ss}$ concentrations investigated (Figure 3.2 (b)), the rate constant for viability loss (k) doubled compared to the value measured for direct photochemical disinfection. Meanwhile, the induction time t_s also decreased by a factor of two over the range of $[\cdot OH]_{ss}$ investigated.

In our batch photochemical systems, indirect disinfection accomplished by $[\cdot OH]_{ss}$ works in complement with direct photochemical disinfection, stimulating viability loss. Indeed, concurrent direct and indirect disinfection facilitates bacterial disinfection more effectively than direct disinfection accomplished by the available UV light alone. One-way ANOVA tests on k and t_s identified significant changes over different $[\cdot OH]_{ss}$ values.

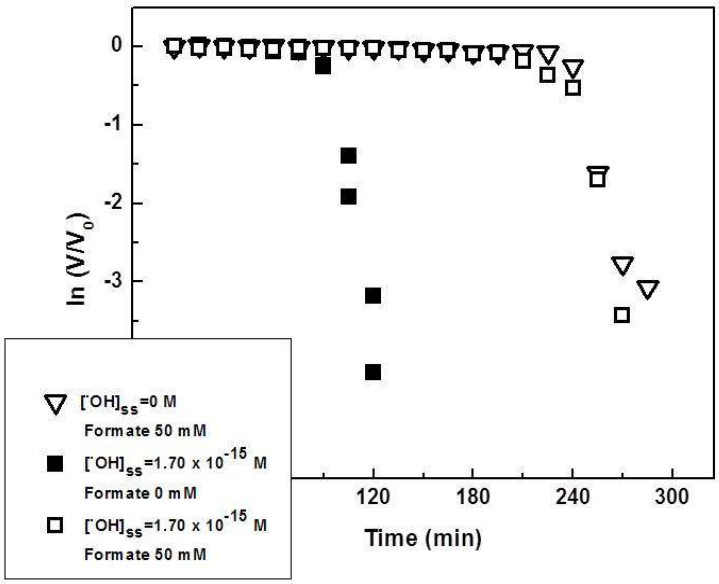

Figure 3.3 Photochemical disinfection experiments of *E. coli* DC1 exposed to light at wavelength≥305 nm conducted at 0 and 1.7×10^{-15} M $[\cdot OH]_{ss}$ with or without the presence of excess formate ($CHOO^-$, 50 mM), a known ·OH scavenger. Data presented as the natural log of cell viability normalized by initial viability versus the time of light exposure. Data is consistent ·OH as the primary agent responsible for bacteria kill in all systems. Photochemical experiments with *E. coli* DC1 that were conducted with $[\cdot OH]_{ss}$ in the presence of excess formate revealed viability losses consistent with a direct photochemical reaction only.

To validate ·OH as the primary entity responsible for the enhanced rates of die off in irradiated nitrate solutions, excess formate was added as a radical quencher (see Figure 3.3). Resulting viability losses observed in the presence of formate were consistent with viability losses measured in systems free of nitrate (i.e., equivalent to viability losses from direct photochemical reaction), consistent with ·OH as the active ROS responsible

for enhanced *E. coli* viability loss in our systems. The result of reactions with formate are also consistent with trends reported in the literature.[48]

3.3.4 **Effect of EPS Extraction on Rates of Photochemical Disinfection.**

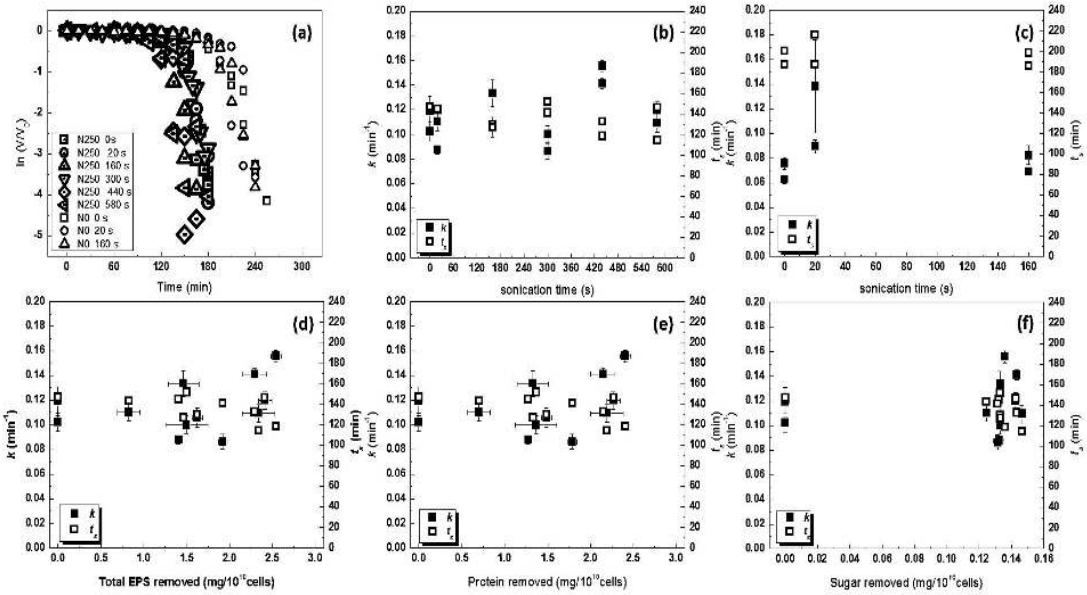

Figure 3.4 Results of photochemical disinfection of *E. coli* DC1 exposed to light≥305 nm as a function of EPS level. (a) Changing viability in cell population due to *direct* (open symbols) and *indirect* (symbols with dot in center). Indirect disinfection experiments were conducted in the presence of 250 mg/L nitrate which generated $[\cdot OH]_{ss}=1.39 \times 10^{-15}$ M. Data are presented as the natural log of cell viability normalized by initial viability versus the time of light exposure. The remaining figures provide the rate constant, k (min^{-1}) and induction time, t_s (min) with respect to sonication times for experiments conducted (b) in the presence ($[\cdot OH]_{ss}=1.39\times10^{-15}$ M) and (c) absence ($[\cdot OH]_{ss}=0$) of photochemically generated hydroxyl radical. In panels (b) and (c), longer sonication times imply a greater extent of EPS removal. For systems conducted with $[\cdot OH]_{ss}=1.39\times10^{-15}$ M, values of k and t_s are also shown with respect to (d) total EPS, (e) protein, and (f) sugar removed from cell surfaces

Figure 3.4 (a) displays semi-log viability curves from photochemical batch experiments conducted with DC1 exposed to a fixed concentration of $[\cdot OH]_{ss}$ (1.39×10^{-15} M) and light of $\lambda\geq305$ nm. Data are shown for DC1 with six different levels of EPS that were achieved by varying sonication time during extraction (data shown as dotted symbols in (a)). For comparison, results are also presented from control experiments conducted in the absence of $[\cdot OH]_{ss}$ (i.e., 0 mg/L nitrate) for DC1 with three different EPS levels. In these controls, direct photochemical disinfection is the only mechanism involved in cell death (data shown as open symbols in (a)).

Figure 3.4 (b) presents values of k (left y-axis) and t_s (right y-axis) obtained from non-linear regression analyses of data in Figure 3.4 (a) from experiments with DC1 with six different EPS levels in the presence of 1.39×10^{-15} M $[\cdot OH]_{ss}$. Values of k and t_s are plotted as a function of sonication time (x-axis), where longer sonication times correspond to greater extents of EPS removal. For comparison, Figure 3.4 (c) shows values of k (left y-axis) and t_s (right y-axis) as a function of sonication time (x-axis) from control experiments with DC1 conducted in the absence of $[\cdot OH]_{ss}$ (i.e., direct photochemical disinfection). Finally, for experiments conducted in the presence of 1.39×10^{-15} M $[\cdot OH]_{ss,}$ values of k (left y-axis) and t_s (right y-axis) are also presented with respect to total EPS (d), protein removed (e), and sugar removed (f) via the sonication process.

Similar patterns of disinfection were observed when EPS was removed from surfaces of bacteria, when applying sonication for varied times (20, 160, 300, 440, and

580 s). The greater sonication time removes an increasingly proteinaceous fraction of the EPS and retains lower total levels of EPS around the cells. However, all values of t_s observed were within the range of 110 to 150 min (see Figure 3.4 (b)) and no clear trend was exhibited with the application of longer sonication times (i.e., increasing amounts of EPS removal). Values of k also showed no clear trend with the extent of EPS removal (see Figure 3.4 (b)). This suggests no clear connection between the level of EPS on the *E. coli* surface and reactivity metrics for $^{\cdot}$OH-mediated disinfection (e.g., values of k or t_s) at the EPS level considered in this study.

Notably, our results also convincingly illustrate that extraction of EPS from *E. coli* DC1 does not affect the rate of direct photochemical disinfection when the cells are exposed to light with $\lambda \geq 305$ nm (Figure 3.4 (c)). Any differences in measured rates of direct photochemical disinfection over the range of variables considered are statistically insignificant.

The lack of an observable EPS effect for direct photochemical disinfection is justifiable; the organic functionalities present in EPS are not likely present in large enough quantities to effectively absorb higher energy UV-light so as to protect critical cellular components from sustaining direct photochemical damage. In contrast, the lack of an EPS effect on indirect disinfection is surprising and may seem counterintuitive. However, the trends may be attributed to the fact that not all EPS was removed by sonication. This is a limitation of the sonication method, as compared to others used for EPS extraction which can remove substantially more of the surface polymers.[42]

However, this is the only method that leaves cells intact for subsequent disinfection studies. Hence, following sonication there is potentially a substantial EPS fraction remaining on the cell surface. Since previous work has suggested that it is the extracellular protein which reacts with $^{\cdot}OH$ leading to disinfection,[29] the subtle changes in the remaining proteinaceous EPS content may result in minor differences in indirect disinfection patterns.

3.3.5 **Effect of EPS Extraction on Rates of Photochemical Disinfection in Four Wild Type *E. coli* Strains.**

To ensure that the observed lack of involvement of EPS in DC1 disinfection processes was not strain specific, EPS was extracted from four *E. coli* strain types (DC1, HU1, SP4, and O157:H7) (Figure 3.5). In these experiments a consistent length of sonication was applied (300 s), which resulted in different remaining levels of EPS bound to the cell surface.

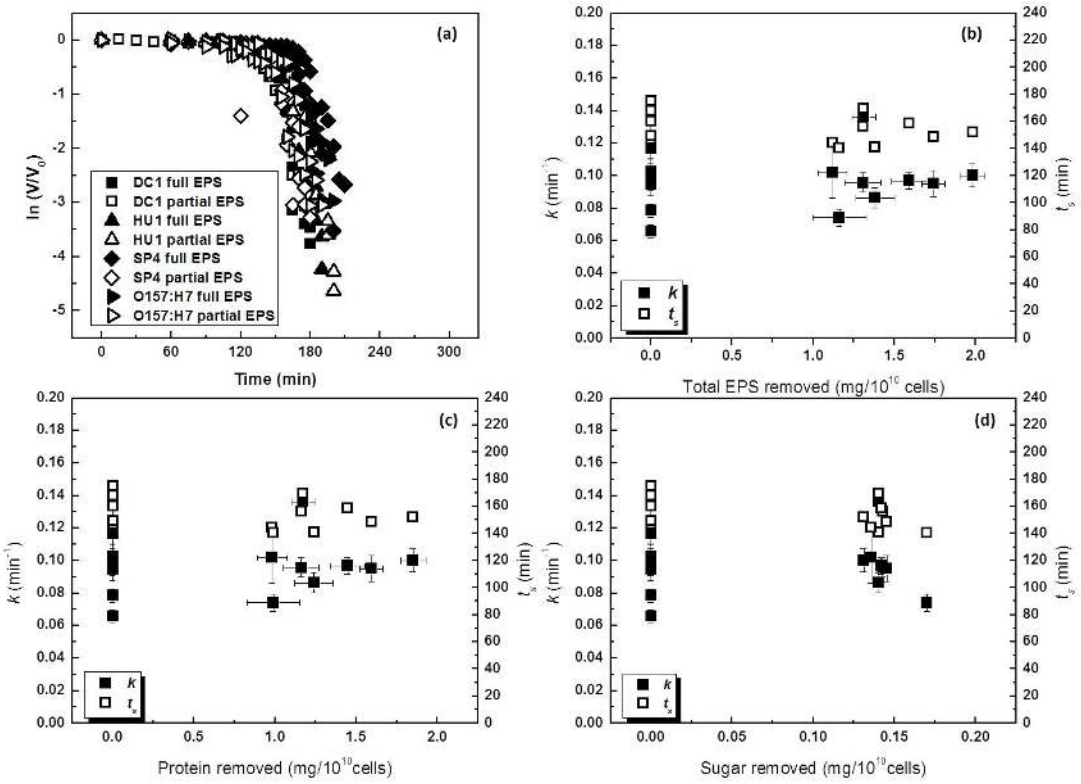

Figure 3.5 Results of photochemical disinfection of four different *E. coli* isolates exposed to light≥305 nm as a function of EPS level. (a) Photochemical disinfection of *E. coli* strains DC1, HU1, SP4, and O157:H7 in the presence of a constant hydroxyl radical concentration ($[\cdot OH]_{ss}=1.39\times10^{-15}$ M). The EPS was removed via 300 s sonication and compared to full EPS presence. Data presented as the natural log of cell viability normalized by initial viability versus the time of light exposure. The remaining figures provide the rate constant, k (min^{-1}) and induction time, t_s (min) with respect to (b) total EPS, (c) protein, and (d) sugar removed from cell surfaces.

For DC1, the removed amount of sugar and protein was averaged to be 0.136±0.001 and 1.545±0.432 mg/10^{10} cells, respectively. For HU1, the removed amount of sugar and protein was 0.141±0.002 and 1.167±0.006 mg/10^{10} cells, respectively. The levels of sugar and protein removed for SP4 was 0.140±0.007 and 1.289±0.433 mg/10^{10}

cells, respectively. Finally, the EPS extraction for O157:H7 revealed sugar and protein levels removed of 0.156±0.020 and 1.219±0.324, mg/10^{10} cells, respectively.

After the EPS extraction step, the cells were exposed to light (wavelengths≥305 nm) and an equal concentration of nitrate, producing an $[\cdot OH]_{ss}$ of 1.39×10^{-15} M and allowing for indirect disinfection processes to occur. All four strains were fully disinfected within an essentially identical 3-3.5 h time period (Figure 3.5). A two-way ANOVA test on the rate of disinfection with factors 1) EPS level (full or partial remaining after the 300 s of sonication), and 2) *E. coli* strain also indicate a statistically insignificant relationship between the four *E. coli* isolates tested. These results clearly show that the insensitivity of ·OH-mediated disinfection to bound EPS levels is a general phenomenon for *E. coli*, and not strain specific.

Table 3.1 Resulting Data From Photochemical Disinfection: Rate Constant (k), Fitting Parameter (m), Reduced Chi^2, Adjusted R^2, and Induction Time (t_s)

Strain[1]	Nitrate Conc. (mg/L)	Sonication[2] Time (s)	k[3] (min^{-1})	k error	m[4]	m error	Red. Chi^2	Adj. R^2	t_s[5] (min)
DC1	0	0	0.076	0.005	3.82E+06	4.43E+06	0.024	0.983	200
DC1	0	0	0.063	0.003	1.21E+05	8.58E+04	0.010	0.990	187
DC1	0	20	0.139	0.038	1.01E+13	8.98E+13	0.028	0.962	216
DC1	0	20	0.090	0.005	1.84E+07	2.04E+07	0.010	0.990	187
DC1	0	160	0.069	0.003	3.60E+05	2.02E+05	0.007	0.995	186
DC1	0	160	0.082	0.008	1.26E+07	2.19E+07	0.027	0.971	199
DC1	0	0	0.076	0.005	3.82E+06	4.43E+06	0.024	0.983	200
DC1	0	0	0.063	0.003	1.21E+05	8.58E+04	0.010	0.990	187
DC1	50	0	0.101	0.008	1.28E+07	1.90E+07	0.015	0.984	163
DC1	50	0	0.073	0.003	6.64E+04	4.00E+04	0.008	0.993	151
DC1	100	0	0.112	0.016	1.49E+08	4.38E+08	0.036	0.954	168
DC1	100	0	0.098	0.003	3.45E+06	1.59E+06	0.003	0.998	154
DC1	250	0	0.102	0.007	2.87E+06	3.52E+06	0.015	0.987	145
DC1	250	0	0.119	0.012	3.98E+07	7.81E+07	0.075	0.954	147
DC1	500	0	0.157	0.005	2.56E+06	1.46E+06	0.003	0.998	94
DC1	500	0	0.123	0.002	1.08E+05	2.61E+04	0.001	0.999	94
DC1	250	0	0.102	0.007	2.87E+06	3.52E+06	0.015	0.987	145
DC1	250	0	0.119	0.012	3.98E+07	7.81E+07	0.075	0.954	147
DC1	250	20	0.110	0.007	7.39E+06	9.02E+06	0.018	0.989	143
DC1	250	20	0.088	0.003	3.30E+05	1.78E+05	0.003	0.996	145
DC1	250	160	0.106	0.008	9.27E+05	1.09E+06	0.026	0.979	130
DC1	250	160	0.133	0.011	2.32E+07	3.61E+07	0.009	0.990	127
DC1	250	300	0.100	0.007	4.04E+06	4.91E+06	0.009	0.988	152
DC1	250	300	0.086	0.006	1.98E+05	1.96E+05	0.024	0.970	141
DC1	250	440	0.141	0.004	1.35E+08	8.73E+07	0.005	0.998	132
DC1	250	440	0.156	0.005	1.06E+08	6.87E+07	0.005	0.998	119
DC1	250	580	0.109	0.007	2.70E+05	2.73E+05	0.018	0.989	114
DC1	250	580	0.120	0.007	3.97E+07	4.75E+07	0.014	0.991	146
DC1	250	0	0.102	0.007	2.87E+06	3.52E+06	0.015	0.987	145
DC1	250	0	0.119	0.012	3.98E+07	7.81E+07	0.075	0.954	147
DC1	250	300	0.100	0.007	4.04E+06	4.91E+06	0.009	0.988	152
DC1	250	300	0.086	0.006	1.98E+05	1.96E+05	0.024	0.970	141
HU1	250	0	0.096	0.005	1.59E+06	1.30E+06	0.023	0.984	150
HU1	250	0	0.117	0.006	1.38E+08	1.40E+08	0.012	0.989	160
HU1	250	300	0.136	0.007	1.10E+10	1.45E+10	0.018	0.988	170
HU1	250	300	0.096	0.006	3.24E+06	3.51E+06	0.044	0.976	156
SP4	250	0	0.079	0.004	9.59E+05	7.62E+05	0.011	0.984	175
SP4	250	0	0.095	0.007	7.97E+06	1.10E+07	0.027	0.968	168
SP4	250	300	0.095	0.008	1.44E+06	1.96E+06	0.031	0.964	149
SP4	250	300	0.102	0.016	2.48E+06	6.47E+06	0.171	0.878	145
O157:H7	250	0	0.100	0.007	9.85E+06	1.31E+07	0.020	0.973	161
O157:H7	250	0	0.066	0.004	3.75E+04	2.73E+04	0.017	0.975	160
O157:H7	250	300	0.097	0.005	4.73E+06	4.39E+06	0.013	0.984	159
O157:H7	250	300	0.074	0.005	3.42E+04	2.91E+04	0.028	0.967	141
DC1[6]	0	0	0.069	0.006	1.22E+07	1.95E+07	0.025	0.972	236
DC1[6]	50	0	0.080	0.007	8.90E+07	1.58E+08	0.019	0.975	230
DC1[6]	250	0	0.080	0.003	1.95E+08	1.35E+08	0.004	0.996	239
DC1[6]	500	0	0.111	0.010	3.24E+11	8.04E+11	0.012	0.983	240

[1] Different strain types of *E. coli,* DC1 stands for source dairy cattle, HU1 stands for source human, SP4 stands for source swine, and O157:H7 is a pathogenic strain also from swine.

[2] Probe sonication were applied by immerse 10 cm of probe into 10 ml of bacterial solution with 2.5×10^9 cells/ml using 30% (45 watt) intensity for varied lengths of total sonication break down in 5 second on and 5 second off cycles.

[3] k is photochemical disinfection rate constant calculated by fitting experimental curves with equation 3.1, the larger the k indicate the faster the disinfection.

[4] m is also calculated through fitting the experimental curves with the equation mentioned above, however the value of m have no significant physical meaning.

[5] $t_s=\log(m)/[k*\log(e)]$ is the initial lag phase where no or very few viability loss have been observed due to bacterial self-repair during photochemical disinfection.

[6] Excess formate ($CHOO^-$, 50 mM) were added as hydroxyl radical quencher where the destruction ability of $^{\bullet}OH$ were quenched by reacting immediately with formate.

Table 3.2 Characterization of EPS Content Removed From Bacterial Surfaces by the Sonication Method.

Sample Name[1]	Total EPS Removed[2] (mg/per 10^{10} cells)	total EPS Stdev	Protein removed[3] (mg/per 10^{10} cells)	Protein Stdev	Sugar removed[4] (mg/per 10^{10} cells)	Sugar Stdev	SP ratio[5] (S/P)
DC1 30% 20s-1	0.825	0.132	0.701	0.132	0.124	0.001	0.178
DC1 30% 20s-2	1.405	0.021	1.273	0.022	0.132	0.003	0.104
DC1 30% 160s-1	1.622	0.061	1.489	0.061	0.133	0.000	0.089
DC1 30% 160s-2	1.463	0.182	1.330	0.182	0.133	0.000	0.100
DC1 30% 300s-1	1.500	0.243	1.367	0.243	0.133	0.000	0.097
DC1 30% 300s-2	1.920	0.052	1.788	0.051	0.131	0.001	0.074
DC1 30% 440s-1	2.291	0.143	2.148	0.143	0.143	0.000	0.067
DC1 30% 440s-2	2.543	0.062	2.407	0.063	0.136	0.001	0.057
DC1 30% 580s-1	2.340	0.192	2.193	0.192	0.147	0.001	0.067
DC1 30% 580s-2	2.413	0.078	2.271	0.078	0.143	0.001	0.063
DC1 30% 300s-1	1.981	0.077	1.850	0.078	0.131	0.001	0.071
DC1 30% 300s-2	1.379	0.120	1.239	0.119	0.140	0.002	0.114
HU1 30% 300s-1	1.312	0.072	1.172	0.070	0.140	0.002	0.120
HU1 30% 300s-2	1.306	0.112	1.163	0.110	0.143	0.003	0.123
SP4 30% 300s-1	1.741	0.070	1.595	0.071	0.145	0.001	0.091
SP4 30% 300s-2	1.118	0.091	0.982	0.091	0.136	0.001	0.139
O157:H7 30% 300s-1	1.590	0.109	1.448	0.108	0.142	0.000	0.098
O157:H7 30% 300s-2	1.160	0.164	0.990	0.162	0.170	0.001	0.175

[1] Sample name contain four part including 1)"DC1" *E. coli* strain type, 2)"30%" as sonication intensity, 3)"300s" as total sonication time, and 4)"-1" or "-2" means duplicates.
[2] Total EPS removed were calculated by adding sugar removed and protein removed together.
[3] Protein removed from bacterial surfaces by applying sonication in 30% intensity (45 watt) for varied lengths and analyzed with Lowry method.
[4] Sugar removed from bacterial surfaces by applying sonication in 30% intensity (45 watt) for varied lengths and analyzed with phenol-sulfuric acid method.
[5] SP ratios were calculated by dividing every sugar removed with according protein removed, the resulting value shows the relative characteristics of experimental condition.

3.4 **CONCLUSIONS**

·OH generated during nitrate photolysis can enhance rates of viability loss in *E. coli* through indirect photochemical disinfection mechanisms. Counter to our original hypotheses, the extracellular polymer substances (EPS) bound to the cell surface has no observable impact on the rates of direct photochemical disinfection as well as ·OH

mediated indirect disinfection of *E. coli*. Notably, this study has also demonstrated that different agriculturally relevant isolates of *E. coli*, with full and partial EPS content on the cell surface, behave similarly in the presence of a fixed ·OH concentration. Our initial intent was to demonstrate that EPS is an important biological variable that would impact photochemical disinfection rates; however, we found that not to be the case. Furthermore, there was remarkable consistency in rates of disinfection, regardless of native EPS levels or strain.

Although limit literature is available as to the influence of EPS on ROS mediated disinfection processes, the findings presented herein are contrary to previously reported trends. Liu et al.[30] previously reported that the presence of full EPS on cells in a biofilm decreased the disinfection efficiency than when soluble part of the biofilm EPS had been removed. The rate of photocatalysis disinfection also was greater using intact *E. coli* cells than full EPS containing heterotrophic biofilm bacteria. They concluded EPS did have an impact of reducing rate of disinfection, and suggested a mechanism where EPS competes with bacterial cell wall for ROS.[30] The disparity between this study and ours is likely due to the levels of EPS encountered on the bacterium. The EPS levels are much less in planktonic *E. coli* as investigated here than EPS surrounding bacteria found in heterotrophic biofilms which accounts up to 90% of organic carbon.[49]

These findings bode well for future efforts in modeling and prediction efforts for planktonic bacterial pathogen persistence in surface waters. Specifically, EPS levels appear to unimportant relative to other factors known to influence photochemical

disinfection efficiency (e.g, presence of different endogenous sensitizers,[50, 51] water chemistry,[52, 53] etc.) and future model development must incorporate these known influencing parameters, but does not need to consider EPS level.

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Chapter 4

Influence of Surface Coating and Extracellular Polymeric Substances (EPS) on *Escherichia coli* O157:H7 Deposition in a Parallel Plate Flow Chamber

ABSTRACT

Bacterial transport and adhesion behavior were investigated in parallel plate (PP) flow chamber with three kinds of solid collectors (bare quartz, natural organic matter, and hematite) as a function of extracellular polymeric substances (EPS) level. Suwannee River humic acid (SRHA) and hematite (α-Fe_2O_3) have the ability to facilitate photochemical disinfection through the production of reactive oxygen species (ROS) under UV and visible light. However, due to the extremely short lifetime of these radicals, the disinfection efficiency is limited by the successful transport of ROS to bacterial surfaces. This study was designed to quantitatively investigate the role of collector surfaces, EPS, and solution chemistry (ionic strength, IS) on bacterial interaction with ROS producing material. Higher IS increased the extent of bacterial adhesion, with few exceptions. Furthermore, interaction between collector surfaces and cells can be explained by electrostatic forces, where SRHA reduces and α-Fe_2O_3 enhances deposition significantly. More deposition was observed with greater EPS level, indicating the ability of EPS to facilitate greater interaction between cells and particles in aquatic environment. These results demonstrate more polymer-induced forces involved in the interactions. The findings from this well-controlled study on the mechanisms of cell-sensitizer interactions provide insight for the future design of photochemical disinfection processes.

4.1 **INTRODUCTION**

E. coli O157:H7 is a notorious strain that has been involved in numerous outbreak events causing foodborne and waterborne diseases, resulting in hemolytic-uremic syndrome.[1] In United States alone, O157:H7 causes an average of 73,000 infections and 61 deaths each year.[2] *E. coli* O157:H7 can be associated with various farm animals as sources for contamination of water and food,[3, 4] as well as causing impairment to surface water associated with agricultural run-off.

Indirect photochemical disinfection is a natural attenuation process that utilizes energy from the sun and is capable of treating large surface water bodies in a low tech, low cost way. Previous work has looked at indirect photochemical disinfection processes and quantified disinfection rates as a function of various solution parameters (cell type, cell polymer presence, sensitizer type, solution chemistry, etc.). One study revealed the insensitivity of disinfection rate to cellular extracellular polymeric substances (EPS) levels in a homogeneous (constituents fully dissolved) aquatic system with nitrate as the sensitizer.[5] However in heterogeneous systems such as those with un-dissolved natural organic matter present, studies have shown the existence of microenvironments near the interface with the sensitizer where the concentration of reactive oxygen species (ROS) formed is more than two orders of magnitude greater than the bulk, resulting in much faster disinfection[6] and inactivation[7] rates. Due to the short life span of the ROS, successful disinfections require prompt transport of ROS to cell surfaces.[8, 9] It has been shown that bacterial EPS have ability to initiate adhesion process to form aggregates or

biofilms.[10, 11] This can contribute to cell interaction with sensitizers and possibly influence ROS access to the cell surface. Therefore, there is a need for a study that can provide fundamental insight to the interfacial interactions between cells and the sensitizing materials and the contribution of EPS level in the disinfection process.

The interactions between cells and surfaces depend on many factors. Common controlling factors influencing cell-surface interactions or biofilm formation in water include classical Derjaguin–Landau–Verwey–Overbeek (DLVO) type forces (van der Waals and electrostatic interaction),[12, 13] gravity,[14, 15] steric repulsion,[16] electrosteric force,[17, 18] hydrophobic properties,[19] and hydration conditions of cells and solid surfaces[20]. The interactions between cells and surfaces in water also depend on solution chemistry including pH, ionic strength (IS), and valance.[21, 22] Other than these physicochemical factors, the interactions also depend on biological factors like quorum sensing,[23] and the presence of bacterial surface macromolecules including flagella, pili, fimbriae, proteins, lipopolysaccharides (LPS), and EPS.[1, 24] Previous studies on O157:H7 transport and adhesion in porous media revealed deviation from DLVO predictions due to electrosteric repulsion,[25] pH associated electrosteric stabilization,[26] and the presence of EPS.[25-27] However, the mechanisms involved in interaction between O157:H7 cells and surfaces that can act as sensitizing agents responsible for ROS production (such as $^{\bullet}OH$, $^{1}O_2$, and H_2O_2) has not been clearly determined.

Many studies have focused on the role of surface macromolecules in cell interaction and adhesion behavior in such systems, as batch adhesion tests,[28] packed-bed

columns,[29-33] radial stagnation point flow (RSPF) systems,[17, 18, 32, 34] parallel plate (PP) flow chamber,[35] and direct observation through atomic force microscopy (AFM)[36-38]. A number of these studies demonstrated surface macromolecules play a significant role in well controlled adhesion experimental systems. However, results deviate on the contribution of macromolecules where some suggest an increase in adhesion,[28, 30, 34] and others observed hindrance[17, 18, 28, 29] because of the presence of surface macromolecules. Due to the discrepancy in the literature, a straightforward experimental system with well-defined conditions focusing on a specific macromolecule is needed to investigate the disinfection scenario where cells interact with sensitizing surfaces.

This study was designed to investigate the role of EPS on the interaction between cells and aquatic sensitizing agents through observing the adhesion behavior of *E. coli* O157:H7 in PP flow chamber.[39-41] The PP flow chamber system simulates the condition where flow stream is parallel to collector surfaces in porous media, and the fundamental mechanism influencing bacteria adhesion to solid surfaces can be revealed in well-defined hydrodynamic flow field. This technique can provide fundamental insight though direct observation of the bacterial interactions with model sensitizing agents (NOM and iron oxide (hematite, α-Fe_2O_3) under a microscope with cells of varying EPS levels. The higher the level of interaction between the cell and solid collector surfaces (made of desired ROS-generating material), the greater the potential for successful disinfection under UV and visible light. The initial hypotheses developed were 1) deposition of bacterial cells with full or partial EPS would be sensitive to increasing IS due to increased electrostatic interactions; 2) EPS is sensitive to IS changes due to

conformational and possible compression of the macromolecules. This sensitivity will change the relative steric interactions with the surfaces; 3) With the NOM coated quartz, less attachment would be observed than to pure quartz surfaces; 4) With α-Fe_2O_3 coated surface, a significantly higher deposition would be observed than on pure quartz surface due to the electrostatic attraction. The validity of these hypotheses and the observed trend are described in detail in the following sections.

4.2 **MATERIALS AND METHODS**

4.2.1 **Cell Selection, Preparation, and Characterization**

A pathogenic strain *E. coli* O157:H7/pGFP strain 72 with ampicillin resistance was chosen as a model organism in this study.[42, 43] This strain was selected because it is a rod-shaped, Gram-negative bacteria with very good fluorescence expression.[43] Bacterial cells were pre-cultured by inoculating bacterial colonies streaked from a Luria-Bertani (LB) agar plate into 5 mL LB media (Fisher Scientific, Fair Lawn, NJ). Cells were grown in the presence of 0.1 g/L ampicillin the day before the experiment and incubated at 37 ˚C in an incubator overnight (16 h). The pre-culture step was followed by a culturing process in which 2 mL of the pre-culture was added to 200 mL of LB media (1:100 v/v) and incubated at 37 ˚C for 3.5 hours to reach mid-exponential growth phase. After reaching this growth stage, the culture was harvested by centrifugation (5804R; Eppendorf, Hamburg, Germany) with a fixed-angle rotor (F-34-6-38; Eppendorf) at 4 ˚C 3700 g for 15 min to separate the cells from the growth media. The centrifugation step was at 4 ˚C 3700 g for 15 min, followed by decanting the supernatant, and resuspending

in 10 mL of electrolyte (10 mM KCl, Fisher Scientific). This was repeated twice to remove all traces of growth media. Next, a stock suspension was prepared by resuspending the pellet in 5 mL of electrolyte, at the same ionic strength required for subsequent transport or cell characterization steps.

The concentrations of bacteria were determined by direct visualization under a light microscope (Fisher Scientific Micromaster) with a cell counting chamber (Buerker−Türk chamber, Marienfeld Laboratory Glassware, Lauda-Königshofen, Germany). The resulting cell concentrations in this particular study were in between 4×10^{10} and 6×10^{10} bacteria/mL. All solutions used were made from reagent grade chemicals (Fisher Scientific) and deionized (DI) water (Millipore, Billerica, MA).

4.2.1.1 **Extraction of EPS by Sonication Technique.**

Bacterial EPS was extracted through a previously established protocol applying probe sonication (Omni-Ruptor 250, Omni International, Kennesaw, GA) and centrifugation (Eppendorf).[44] Bacteria in stock solution were re-suspended in 10 mL of 1, 10 or 100 mM KCl. A 5/32" Micro-Tip sonicating probe (Omni International) was immersed 10 cm into the solution in a 15 mL centrifuge tube. The temperature was maintained below 4˚C by immersing the tube in an ice bath throughout the sonication process. Sonication was repeatedly applied to the bacterial cells by way of five sec duration pulses with five sec rest periods in between at a fixed intensity (30% of the 150 Watt = 45 Watt) for a total of 300 sec. After sonication, the bacterial solution was centrifuged at 4 °C and 4000 g for 20 min (Eppendorf), and the supernatant was collected

and passed through a 0.22 μm filter (Millipore, Fisher Scientific) for further EPS composition analysis. Finally, the pellet was resuspended in electrolyte for subsequent characterization and transport experiments.

The sugar and protein content of the EPS were evaluated through the Phenol-Sulfuric Acid (PSA) method[45] and the Lowry method[46] with Xanthan gum (Sigma-Aldrich corporation, St. Louis, MO) and bovine serum albumin (BSA, 1mg/mL) (Fisher BioReagents, Fisher Scientific) as the standards, respectively. Both are colorimetric methods where the chemical reaction induced color change can be measured by spectroscopy (BioSpec-mini, Shimadzu Corp. Kyoto, Japan) and compared with a standard curve for sugar or protein concentration. The sugar level was determined by adding 50 μL of 80% (v/v) phenol and then 5 mL of highly concentrate sulfuric acid (95.5% Fisher Scientific) into 2 mL of an extracted EPS sample. The mixing of these chemicals produces heat and a color change. Therefore, the solution required 10 min at room temperature to cool down, followed by immersion in a water bath (Lab-Line Instruments, Inc., Melrose Park, IL) at 25-30 °C for 20 min, and finally, room temperature incubation for 4 hr to stabilize the color before measuring the absorbance at 480 nm (Biospec-mini, Shimadzu Corp.). The protein level was evaluated by adding 1.5 mL of alkaline copper reagent (made by mixing 1 mL of 2% $Na_2C_4H_4O_6$, 1 mL of 1% $CuSO_4$, and 98 mL of 2% $NaCO_3$ in 0.1 M NaOH) to 0.3 mL of EPS sample. After adding the alkaline copper reagent, the sample required incubation at room temperature for 10 min. Next, 75 μL of Folin reagent (Folin and Ciocalteu's Phenol Reagent, MP Biomedicals, LLC, Germany) was added into the mixture, the solution was vortexed

(AutoTouch Mixer Model 231, Fisher Scientific), and incubated at room temperature for 30 min before measuring absorbance at 500 nm (Biospec-mini, Shimadzu Corp.). All chemicals used were ACS grade (Fisher Scientific).

4.2.1.2 **Cell Surface Characterization.**

Bacterial cell sizes, surface charge densities, hydrophobicity, and zeta potentials were determined according to previous established protocols.[47] The size and shape of bacteria were evaluated by taking images of 10^{10} bacteria/mL with a light microscope (Fisher Scientific Micromaster). Images with more than 50 cells were processed through MATLAB software (Matlab, the MathWorks, Inc., Natick, MA), and individual bacteria widths and lengths were determined to calculate the effective diameters.

Surface charge densities were evaluated by potentiometric titrations in an auto titrator (798 MPT Titrino, Metrohm, Switzerland). Briefly the amounts of base added into bacterial solution for the pH value to increase from 4 to 10 were measured. The bacterial concentration in the titrator was 1×10^8 bacteria/mL, and the base used to titrate the bacterial solution was 0.3 N NaOH.

The hydrophobicity of the bacterial cells was measured by the microbial adhesion to hydrocarbon test (MATH test).[48] The relative hydrophobicity was determined by the percentage of 4 mL bacterial cells partitioned into 1 mL of n-dodecane (laboratory grade, Fisher Scientific) after vortexing for 2 min (AutoTouch Mixer Model 231, Fisher Scientific) and a 15 min room temperature incubation. The optical density of the water

phase was determined by spectroscopy (Shimadzu Corp.), and the percentage of bacteria which passed to the oil phase was calculated.

The bacterial electrophoretic mobility (EPM) was measured by a ZetaPALS analyzer (Brookhaven Instruments Corporation, Holtsville, NY) in 1, 10, and 100 mM KCl with unadjusted pH (5.6-5.8). Bacterial solution was diluted to an optical density of OD_{600} = 0.2 to 0.225 before running through ZetaPALS. The zeta potential of the cells was then calculated by electrophoretic mobility values using the Smoluchowski equation.[49]

4.2.2 **Parallel Plate Experiments**

Deposition behavior of *E. coli* O157:H7 was observed in real time with a custom-made PP flow cell system (Figure 4.1), inserted on the stage of an upright fluorescence microscope BX-51 (Olympus, Tokyo, Japan). Images were taken by a 40× objective passing through a fluorescent filter set (Chroma Technology Corp., Brattleboro, VT). The flow cell is rectangular shaped with an inner chamber 6 cm × 1 cm × 0.08 cm in size, formed by attaching the deck, rubber gasket, and a microscope glass slide with a thin layer of vacuum grease. The flow deck was made with a pocket in the center, 9 mm in width, 20 mm in length (parallel to flow direction), and 1.5 mm in depth. This pocket allows for different collector surface samples (PP coupons) of this size to be inserted into the pocket and be fixed with vacuum grease. The inlet and outlet of the flow cell were attached to a 60 mL syringe pump where the flow rate of the bacterial solution was kept at 0.1 mL/min for all conditions. The concentration of bacterial solution was optimized

to be 10^8 cells/mL in the flow cell for all conditions tested. Images of bacterial deposition on desired surfaces were taken by QImaging Retiga EXi digital camera (QImaging, Surrey, BC, Canada) every 30 sec over the course of 30 min and then analyzed with the supplied software (SimplePCI, Precision Instruments, Inc., Minneapolis, MN). The number of cells attached to the surface for five or more consecutive images were counted and the particle flux was determined from the linear slope of the number of cells deposited verses time and normalized by the microscope viewing area (211 μm × 168 μm). Resulting bacterial transfer rate coefficients k (m/sec) were calculated from the deposition flux J (cells/sec×m^2), and injection concentration C_0 (cells/m^3).

$$k = \frac{J}{C_0} \tag{4.1}$$

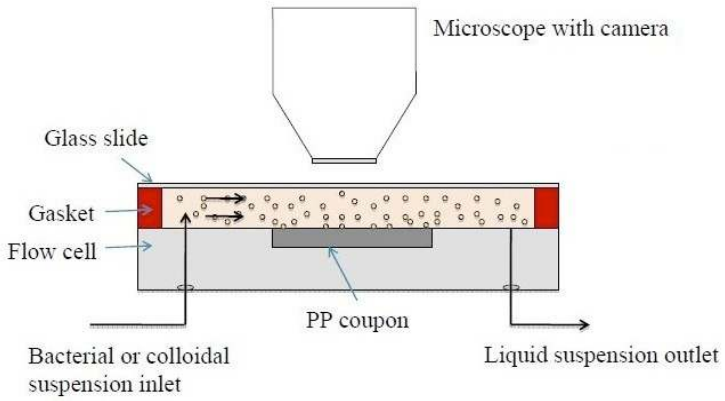

Figure 4.1 Schematic of the custom made flow cell with pocket for the controlled deposition studies with various collector surface. Modified figure with permission from supporting information for "The Effect of Packing Hydrophilization on Bacterial Attachment and the Relationship with the Performance of Biotrickling Filters"[50]

4.2.3 **Surface Preparation and Characterization**

4.2.3.1 **Cleaning Bare Quartz/Glass**

Bare quartz and glass slides were used as the sample or coupon in the PP system. They were cut to 9 mm × 20 mm in size and cleaned thoroughly with a standard cleaning procedure[51] before insertion into the PP flow cell. The surfaces were first cleaned with detergent, a 2 % (v/v) Extran and sonicated in a water bath for 15 min followed by further sonication in 2 % (v/v) RBS 35 (Fisher Scientific, Pittsburgh, PA) for 15 min at 50°C. After sonication, the surfaces were immersed overnight in a mixture composed of highly concentrated sulfuric acid and NOCHROMIX® solution (Godax Laboratories, Inc., Takoma Park, MD). Before the PP experiments, the surfaces were further rinsed 3 times with DI water and 3 times with ethanol (200 proof, KOPTEC, King of Prussia, PA). The assembled PP flow cell was rinsed by flowing DI water for 10 min and the desired electrolyte (KCl 1, 10, or 100 mM) for 10 min at a 1 mL/min flow rate.

4.2.3.2 **Coating Quartz Substrate with Natural Organic Matter.**

For select experiments with organic matter, the quartz surfaces were cleaned with the procedure mentioned above, and subsequently were coated with Suwannee River humic acids (SRHA) according to a previously established flow through method.[52] In between flowing DI water and KCl solutions, 2 mL of poly-L-lysine (PLL)-free solution, 2 mL PLL, 2 mL PLL-free, and 2 mL SRHA solution were flow through the system. PLL-free solution was comprised of 10 mM N-(2-Hydroxyethyl) piperazine-N'-(2-

ethanesulfonic acid) (HEPES) (Sigma-Aldrich, St. Louis, MO) and 100 mM KCl. PLL solution was made by adding 0.1 g/L PLL into PLL free solution. SRHA solution was made by adding 28.3 mg of dry SRHA powder (International Humic Substance Society) into 52 mL DI water and shaken for 2 hr. All solutions made were passed through 0.22 μm cellulose acetate membrane filters. When flowing through the PP flow cell, the PLL binds to the quartz. Subsequently, the SRHA solution was passed through the flow cell and bound to the PLL layer. Hence a layer of SRHA is formed on the quartz surface.

4.2.3.3 **Coating the Glass Substrate with Iron Oxide (α-Fe_2O_3).**

α-Fe_2O_3 nanoparticles were made by adding 24 mL of 1 M ferric nitrate solution into 200 mL of boiling DI water at a steady speed of about 1 mL every 30 sec (finish adding 24 mL in 12 min).[53] The solution turned from light brown to dark brown to very dark brown, and then the reaction took place to turn the solution to dark red in boiling condition. The solution was boiled on hot plate to reduce the volume from 200 mL to 100 mL in about 30 min. After forming 100 mL dense solution followed by a cooling step, dark red precipitate forms with very small particle sizes (5-10 nm). Next, a 10-min 9000 rpm centrifugation was applied on the solution. After centrifugation the supernatant was decanted and 100 mL DI water added and vortexed to re-dissolve the α-Fe_2O_3 in DI water. Glass surfaces were cleaned with the procedure mentioned before and pretreated with Piranha solution (the mixture of 30% H_2O_2 and 95.6% H_2SO_4 in 1:3 v/v ratio) to hydroxylate the glass surface, making the glass surface hydrophilic. The hydrophilic glass then was coated with α-Fe_2O_3 nanoparticles with spin coating method at room

temperature and 1500 rpm for 25 sec using a Laurell spin coater (WS-400A-6NPP/LITE) (Laurell Technologies Corp.; Johnstown, PA). The α-Fe_2O_3 solution was made by the method mentioned above and diluted (1:10 v/v) with DI water. Afterward, the coated glass samples were dried at room temperature. Before each PP experiment, the glass coated with α-Fe_2O_3 was installed in the PP flow cell and cleaned with DI water and ethanol. The cleaning process with DI water and ethanol did not alter the conformation of the α-Fe_2O_3 coating. To ensure the glass substrate was successfully coated with α-Fe_2O_3, the pretreated glass and the coated glass were measured via UV-visible spectroscopy (Figure 4.2) where the difference in absorbance was due to the presence of the α-Fe_2O_3 nanoparticle coating.

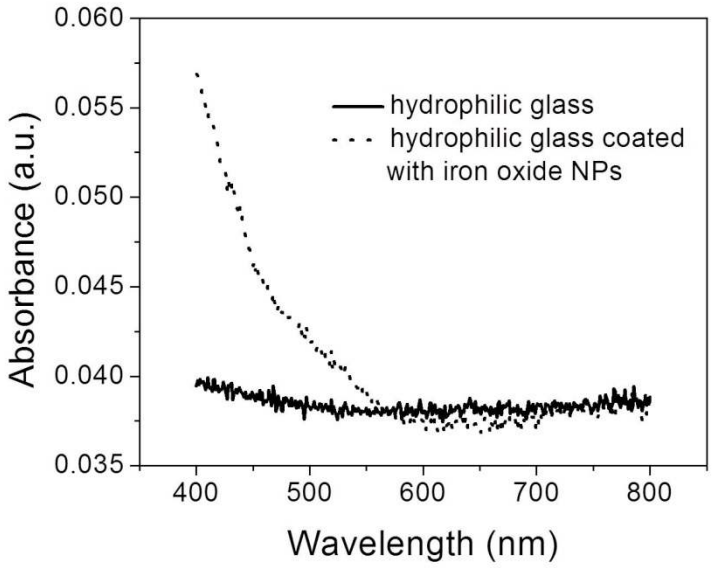

Figure 4.2 Absorbance values of substrates from UV-visible spectroscopy with and without α-Fe_2O_3 coating on glass surface. The trend of increasing absorbance at lower wavelengths is indicative of the α-Fe_2O_3 nanoparticles coating the glass substrate

4.2.3.4 **Streaming Potential Analysis**

The zeta potential of quartz, SRHA coated quartz, and α-Fe_2O_3 coated glass surfaces were measured with a streaming potential analyzer (EKA, Anton Paar, Graz, Austria) equipped with an asymmetric clamping cell according to a previously established protocol.[54] Before taking the measurements, the EKA machine was thoroughly rinsed with 2L of DI water and 1L of testing electrolyte (1, 10, or 100 mM KCl). After 3 rinsing cycles, the flow check was performed from left to right and from right to left to ensure the system was running properly. Subsequently, the EKA measurements in 1, 10 or 100 mM KCl were taken.

4.3 RESULTS AND DISCUSSION

4.3.1 Predicted Interactions Between O157:H7 Cells and Collector Surfaces.

Table 4.1 System Characterization Data: Collector Surface and Cell Zeta Potential, and Calculated Energy Barrier Height (*kT*) Between O157:H7 and Collector Surface as a Function of IS.

Collector surface zeta potential (mV)[1]				**O157:H7 zeta potential (mV)**	
IS(mM)	quartz[2]	SRHA	α-Fe_2O_3	full EPS[4]	partial EPS[5]
1	-24.7	ND[3]	ND	-2.71±0.32	-3.44±-0.62
10	-19.8	-23.40±1.03	0.14±1.85	-0.25±2.18	-0.15±1.21
100	-3.41	19.66±24.43	44.82±25.37	2.36±1.95	-3.35±3.70

Energy Barrier Height[6] (*kT*)						
	O157:H7 with full EPS			O157:H7 with partial EPS		
IS (mM)	quartz	SRHA	α-Fe_2O_3	quartz	SRHA	α-Fe_2O_3
1	5.6	ND	ND	9.77	ND	ND
10	NB[7]	NB	NB	NB	NB	NB
100	NB	NB	NB	NB	NB	NB

[1] Collector surface zeta potential measured using a streaming potential analyzer (EKA).
[2] Quartz surface zeta potentials from reference.[55]
[3] ND: not determined.
[4] O157:H7 with full EPS zeta potentials measured with freshly harvested cells diluted in test electrolytes.
[5] O157:H7 partial EPS zeta potentials measured with cells after applying probe sonication for EPS extraction.
[6] Energy barrier heights calculated using DLVO theory.[12, 13]
Hamaker constant used was 6.5×10^{-21} J.[56]
[7] NB: no energy barrier.

To understand the interactions between *E. coli* O157:H7 and the collector surfaces, zeta potential measurements were performed at the same solution chemistries used in transport experiments. These values were used to predict the interaction forces between cells and surfaces according to DLVO theory[12, 13] (Table 4.1). Generally speaking, the streaming potential of all three types of collector surfaces (bare quartz, SRHA, and α-Fe_2O_3) became less negative (or transitioned to positive) with greater IS tested. Zeta potentials of the bare quartz surface appeared to be most negative at 1 mM KCl and increased with IS towards neutral. For SRHA coated surfaces, the coating seemed to result in slightly more negative zeta potential in 10 mM KCl than bare quartz. However, in 100 mM KCl the charge of SRHA coated surface was reversed to a positive value, but with large variation. The increased error is likely due to the conditions approaching the upper limit of IS that the streaming potential analyzer can handle. The α-Fe_2O_3 coating on the glass surface resulted in positive zeta potentials in both 10 and 100 mM KCl and a more positive value was observed in 100 mM than in 10 mM KCl.

Bacterial surfaces appear to be near neutral according to ZetaPALS measurements, whereas most nonpathogenic *E. coli* are more negatively charged at similar pH and IS conditions. The zeta potential of O157:H7 also showed less sensitivity to the changing IS[25-27] as compared to other nonpathogenic *E. coli* strains.[47, 57] Similar values of O157:H7 zeta potentials were found with full and partial EPS coated cells in 1 and 10 mM KCl where no significant difference was detected between full EPS bacteria and their partial EPS counterpart ($p>0.1$). In 100 mM KCl, zeta potential of full EPS cells was observed to be significant higher than their partial EPS counterpart ($p<0.1$).

The large variation observed for O157:H7 with partial EPS in 100 mM KCl is likely due to the IS condition approaching the upper capacity of the ZetaPALS.

The DLVO calculation based on resulting zeta potential values (Table 4.1) indicated there were no energy barriers to cell interactions with all three surfaces, for cells with full and partial EPS at 10 and 100 mM KCl. The energy barrier in 1 mM KCl with both full and partial EPS was very low, below 10 kT in value, which may be easily overcome by other attractive forces like Brownian motion[58] and diffusion[59]. These predictions with classic DLVO theory suggest chemically favorable conditions for O157:H7 interaction with the surfaces under all conditions tested in the PP system. The implications of this are discussed below.

4.3.2 **Adhesion Behavior of O157:H7 in the Parallel Plate Flow Chamber.**

Experimental results from O157:H7 transport experiments with the three test collector surfaces, in 1, 10, or 100 mM KCl, and with full or partial EPS bacteria are reported in Table 4.2 and Figure 4.3.

Table 4.2 O157:H7 Bacterial Transfer Rate Coefficients (*k*) for O157:H7 with Full or Partial EPS on Quartz, SRHA-, and α-Fe_2O_3-coated Surfaces.

Collector Surface	Ionic Strength (mM KCl)	EPS	k (m/s)
Quartz	100	Full	$2.54\times10^{-8}\pm2.95\times10^{-9}$
Quartz	100	Partial	$1.66\times10^{-8}\pm2.52\times10^{-9}$
Quartz	10	Full	$1.20\times10^{-8}\pm4.48\times10^{-9}$
Quartz	10	Partial	$2.71\times10^{-9}\pm1.29\times10^{-10}$
Quartz	1	Full	$1.58\times10^{-8}\pm3.95\times10^{-9}$
Quartz	1	Partial	ND[1]
Quartz SRHA	100	Full	$1.24\times10^{-8}\pm4.93\times10^{-10}$
Quartz SRHA	100	Partial	$7.75\times10^{-9}\pm1.24\times10^{-9}$
Quartz SRHA	10	Full	$1.19\times10^{-8}\pm2.61\times10^{-9}$
Quartz SRHA	10	Partial	$1.08\times10^{-9}\pm5.87\times10^{-10}$
Glass iron oxide	100	Full	$3.71\times10^{-8}\pm1.85\times10^{-9}$
Glass iron oxide	100	Partial	$1.75\times10^{-8}\pm3.18\times10^{-9}$
Glass iron oxide	10	Full	$2.76\times10^{-8}\pm8.51\times10^{-10}$
Glass iron oxide	10	Partial	$2.36\times10^{-8}\pm1.86\times10^{-9}$

[1]ND: No deposition detected

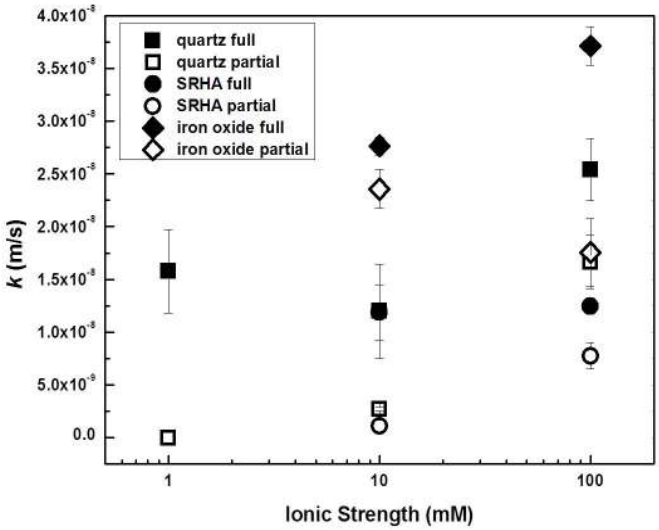

Figure 4.3 O157:H7 bacterial transfer rate coefficient (k) as a function of IS observed in the PP flow chamber with quartz, SRHA, and α-Fe_2O_3 as solid collectors. Full or partial refers to EPS levels. Error bars indicate one standard deviation.

4.3.2.1 **Effect of Collector Surface on Adhesion Behavior of O157:H7**

Surface coating was found to impact deposition trends across the IS conditions tested. For example, the bacterial transfer rate coefficient (k) on the bare quartz in 10 mM KCl were $1.20\times10^{-8}\pm4.48\times10^{-9}$ and $2.71\times10^{-9}\pm1.29\times10^{-10}$ for full and partial EPS, respectively. Bacteria with full EPS attached on SRHA surfaces in 10 mM KCl resulted in similar transfer rate coefficient (k=$1.19\times10^{-8}\pm2.61\times10^{-9}$) compared with same condition on quartz, but the partial EPS bacteria attached less on SRHA (k=$1.08\times10^{-9}\pm5.87\times10^{-10}$) than on quartz, where k for quartz is 2.5 times the SRHA value. Solid surfaces with α-Fe_2O_3 coating appeared to be more favorable for bacterial deposition than

bare quartz surfaces in 10mM KCl for both full and partial EPS-coated bacteria. Specifically, deposition on α-Fe_2O_3 resulted in 2.3 and 8.7 times greater k (k=$2.76\times10^{-8}\pm8.51\times10^{-10}$ for full EPS; k=$2.36\times10^{-8}\pm1.86\times10^{-9}$ for partial EPS) than on quartz.

The bacterial transfer rate coefficients (k) on the bare quartz in 100 mM KCl were $2.54\times10^{-8}\pm2.95\times10^{-9}$ and $1.66\times10^{-8}\pm2.52\times10^{-9}$ for full and partial EPS, respectively. The SRHA coating made the surface less favorable than bare quartz in 100 mM KCl for both full and partial EPS bacteria (k=$1.24\times10^{-8}\pm4.93\times10^{-10}$ for full EPS; k=$7.75\times10^{-9}\pm1.24\times10^{-9}$ for partial EPS). Comparing the bacterial transfer rate coefficients for full and partial EPS in 100 mM KCl, the transfer rate coefficients in quartz were about 2 times greater than on SRHA, for full and partial EPS. Similar to what was observed in 10 mM KCl, the α-Fe_2O_3 coating on surface brought in higher k (k=$3.71\times10^{-8}\pm1.85\times10^{-9}$ for full EPS; k=$1.75\times10^{-8}\pm3.18\times10^{-9}$ for partial EPS), which were 1.5 (full EPS) and 1.1 (partial EPS) times greater than for quartz.

Adding a surface coating, either natural organic matter or α-Fe_2O_3, generally resulted in significant differences in transfer rate coefficients, as determined with a student's t test. Insignificant differences were only found between 1) quartz and SRHA-coated quartz in 10 mM KCl with full EPS; 2) quartz and iron oxide-coated glass in 100 mM KCl with partial EPS. The reason the surface coatings impacted the extent of attachment is due to a combination of mechanisms. Electrostatic interaction is one critical mechanism. For example, the addition of SRHA coating on the quartz increased the negativity of quartz surface, thus reducing the attractive forces between bacterial

surfaces and the solid. The greater deposition on α-Fe_2O_3 surfaces than on bare quartz is likely due to the α-Fe_2O_3 coating being positively charged (as confirmed by zeta potential measurements in Table 4.1), leading to greater electrostatic attraction with the negatively charged bacteria (as compared to cells' interaction with the negatively charged bare quartz).

Similar to these trends, work in porous media has shown the increased attachment efficiency when bacteria attach on quartz coated with iron oxide, and further addition of natural organic matter on iron coated quartz lowered the efficiency and thus increased the transport of bacteria.[60, 61] Another study found increased bacterial deposition rates and maximum surface coverage on iron oxide.[62] Negatively charged colloids were also found to have reduced deposition in porous media when both the silica collector and colloids were coated with humic acid.[59] These studies from the literature are in agreement with the current study.

4.3.2.2 **EPS Effect on Adhesion Behavior of O157:H7**

Generally, lower bacterial transfer rate coefficients (k) were observed for cells with partial EPS than with full EPS across all conditions tested. For example at 100 mM KCl, the k values were $2.54\times10^{-8}\pm2.95\times10^{-9}$ and $1.66\times10^{-8}\pm2.52\times10^{-9}$ for full and partial EPS on quartz surfaces, respectively, where the rate coefficient k for cells with full EPS is 1.5 times greater than for cells with partial EPS in the same condition. At 10 mM KCl, significantly lower deposition was observed with partial EPS than full EPS, with $k=2.71\times10^{-9}\pm1.29\times10^{-10}$ (partial EPS), and $1.20\times10^{-8}\pm4.48\times10^{-9}$ (full EPS), where the rate

coefficient *k* for cells with full EPS is 4.3 times that for cells with partial EPS value in the same solution. With SRHA coated surfaces, *k* values for cells with full EPS in 10 and 100 mM accounts for 11 and 1.6 times of partial EPS, respectively. With α-Fe_2O_3 coating, full EPS *k* values in 10 and 100 mM accounts for 1.2 and 2.1 times of their partial EPS value, respectively. This indicates that EPS association with cell surfaces may facilitate the interaction between cells and collector surfaces. Previous studies reported adhesion of bacteria increase with more EPS produced and present around the cells,[10, 63, 64] which is in agreement with these reported trends (Table 4.2). Overall, the data suggest that EPS facilitates the association between bacteria and solid surfaces through a combination of mechanisms. These include polymer bridging[65-67] in lower IS and the increase of heterogeneity[68, 69] on bacterial surfaces.

4.3.2.3 **Adhesion Behavior of O157:H7 With Respect to Ionic Strength**

Increasing IS generally was found to enhance the amount of deposition. This was the case for cells with partial EPS on quartz and SRHA, as well as cells with full EPS on α-Fe_2O_3. Specifically, the *k* values for cells with partial EPS onto quartz and SRHA increased by 6.13 and 7.18 times, respectively when the IS was increased from 10 to 100 mM. The value of *k* for cells with the full EPS onto the α-Fe_2O_3 increased 1.34 times over that same change in solution chemistry.

The difference between bacterial cells deposited on quartz surfaces with full EPS at 1 and 10 mM KCl was insignificant ($p>0.1$). Bacterial cells with full EPS attach

similarly on SRHA coated quartz in 100 mM KCl and in 10 mM KCl, with no significant difference ($p>0.1$).

The only deviation from this trend of increasing IS leading to greater deposition was for cells with partial EPS depositing on α-Fe_2O_3 surfaces. Bacterial cells with partial EPS attached less on α-Fe_2O_3 in 100 mM KCl (k=1.75×10^{-8}±3.18×10^{-9}) than in 10 mM KCl (k=2.36×10^{-8}±1.86×10^{-9}). Possible mechanisms include the stabilization of the surface polymers by the increased IS,[55, 70] or the extraction of EPS from cells may have altered the local charge heterogeneity,[68, 71, 72] such that the electrostatic attractive force of the surface may be reduced in 100 mM KCl.

4.4 CONCLUSIONS AND IMPLICATIONS FOR PHOTODISINFECTION

The results from this study generally showed a good agreement with the initial hypotheses. The decrease of the bacterial EPS level lowered the deposition of cells as expected. This indicates the bacterial macromolecules enhance interactions with other surfaces in aquatic systems. The SRHA surface coating lowered the rate of deposition in both 10 and 100 mM KCl as compared to bare quartz. In contrast, the α-Fe_2O_3 surface coating resulted in greater attachment on the surface than on quartz. These observations clearly demonstrate the electrostatic forces play a significant role on the O157:H7 deposition as demonstrated in the PP system.

Deposition of bacterial with full and partial EPS increased with greater IS. The general trend increased attachment with IS agreed with DLVO calculations. The few exceptions suggest some behaviors are not captured by classic DLVO theory, specifically due to the presence of EPS[73], which causing surface heterogeneity[74, 75] and facilitate steric interactions[28, 36].

The increased association between bacterial and sensitizing surfaces may facilitate more successful ROS transport to cell surfaces, which result in greater disinfection rate in photochemical disinfection. Approaches to optimize the EPS production, and hence cell-sensitizer interactions, includes modifying carbon source, carbon to nitrate ratio, phosphate, and nutrient levels.[76, 77] The formation of bacterial surface macromolecules and their composition can be optimized for greater photochemical disinfection by means of modifying related solution chemistry conditions, e.g. IS[36], pH[78], and temperature[79, 80]. Through optimizing the production of EPS, the effect of EPS may be exploited to maximize interaction with surrounding agents. Additionally, the more favorable interactions between bacteria and α-Fe_2O_3 surfaces suggest the presence of iron oxide in the form of colloidal iron may also facilitate photochemical disinfection in natural water.

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Chapter 5

Summary and Conclusions

The overall goal of this doctoral research was to investigate the role of bacterial extracellular polymeric substances (EPS) and the influence of its composition in indirect photochemical disinfection processes with nitrate, natural organic matter, and iron oxide as natural sensitizers in surface water. The study was carried out in three distinct parts, systematically working towards the identification of the fundamental governing mechanisms facilitated by the surface bound EPS controlling the disinfection rates and the degree of association of bacteria and sensitizers naturally present in surface water. Specifically, the role of EPS was examined through 1) the comparison of EPS extraction methods; 2) disinfection kinetics relative to EPS levels in photochemical disinfection; and 3) bacteria cell-sensitizer interactions as a function of EPS levels. To accomplish the goal, a few wild type *Escherichia coli* and *Salmonella* strains were selected as model organisms. Batch and parallel plate (PP) flow chamber systems were also applied to the study. To reveal the role of EPS, the sonication EPS extraction method was applied to systematically remove partial surface bound EPS, leaving different EPS levels around the cells. A series of techniques characterizing cell surface properties (EPS composition, hydrophobicity, size, shape, charge density, electrophoretic mobility (EPM), and zeta potential) were also performed to complement the kinetic studies in batch and PP flow chamber.

In Chapter 2 presented three methods to separate EPS from the cell through *Salmonella* EPS extraction and analyses processes. It was found that protein component removed by the three extraction methods (lyophilization, ethanol, and sonication) were all more sensitive to various environmental conditions (IS and duration of exposure) than

sugar content. Lyophilization and ethanol methods combined chemical and physical approach, whereas sonication extracted EPS through physical separation. Comparing the three methods found that the sonication method resulted in sugar content that had a stronger correlation with protein content than lyophilization and ethanol methods. The ethanol and lyophilization methods were similar in nature, and resulted in similar yield and trends. However, there was greater variance in EPS extracted by ethanol method than by the lyophilization method. Although the sonication method extracted much less sugar and protein than the other two methods, the greater consistency and being shorter in process time make this method the more desirable approach than the others. The primary finding from this method development study was the establishment of a standard protocol for EPS extraction and analyses, and this method was applied in the later studies to elucidate the role of EPS in various applications.

Chapter 3 investigated the role of EPS and nitrate concentration in photochemical disinfection processes. The sonication method was applied to four *E. coli* strains to partially remove EPS from the bacterial surface to compare with cells with full EPS in disinfection experiments. Results suggested a linear relationship between hydroxyl radical concentration and resulting disinfection rate constant (k) and induction time (t_s). When comparing the results for *E. coli* cells with full versus partial EPS, there was no significant trend in direct and indirect photochemical disinfection. These tests were carried out on four different types of *E. coli*, demonstrating these results were not a strain specific phenomenon.

In Chapter 4, the interactions between bacteria and sensitizers were observed microscopically under flow in a PP flow chamber. Bacterial transport and adhesion in the PP flow chamber were found to be sensitive to the EPS level, type of sensitizer (collector surface chemistry), and the ionic strength (IS). Noticeably less attachment on the natural organic matter coated surface and more attachment on hematite coated surface was observed than on bare quartz. Increasing IS of the electrolyte solution resulted in greater cell deposition as predicted by Derjaguin-Landau-Verwey-Overbeek (DLVO) calculations. The partial removal of EPS from the bacterial surfaces resulted in lower deposition than for full EPS bacteria, indicating the presence of EPS around the cells increased the interaction between the solid collector and bacteria. Although EPS did not play a significant role directly in photochemical disinfection processes as Chapter 3 suggested, the finding in this study revealed that EPS may enhance the rate of photochemical disinfection through facilitating the interactions between bacteria and sensitizers in water.

The findings from this doctoral study provide the following fundamental insights for consideration of EPS in the design of photochemical disinfection processes. First, the composition and levels of EPS depends very much on the extraction protocol utilized, where no single method has the ability to separate all EPS from the cell surface without disturbing cell structure. This suggests that when extracting extracellular material from cells, careful consideration must be taken in the selection of the method most appropriate for the particular study. Second, in the presence of nitrate, photochemical disinfection was not sensitive to EPS level; neither did EPS hinder the reaction by acting as a physical

barrier or chemical quencher. Finally, this study has shown the ability of EPS to facilitate interactions between bacteria and collector surfaces that may serve as sensitizers in photochemical disinfection processes. This suggests that the bacterial growth conditions may be optimized to enhance EPS production and result in more effective sensitizer-cell interactions leading towards disinfection. However, further work is merited on the topic of EPS production and composition such that specific components of EPS may be found that can be exploited to further promote attachment and disinfection. Through the application of microbiology, cell biology, and proteomics tools, this approach may be further developed. This dissertation study has demonstrated the function of bacterial surface EPS in photochemical disinfection processes, and with this knowledge the design of future low-tech and low-cost disinfection and water treatment processes may be feasible.