Efficacy of microscale/nanoscale aqueous ozone on the removal of *Bacillus* spp. biofilms from polyethersulfone membranes in the dairy industry

by

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Abstract

Biofilm formation on polyethersulfone (PES) membranes in the dairy industry is a concern to food safety and public health. Fouling of PES membranes leads to biofilm formation on membrane surfaces, resulting in reduction in membrane performance and premature replacement of membranes. The dairy industry must develop technology to discourage biofilm attachment, while also finding a more efficient, eco-friendly approach to biofilm removal from dairy processing equipment. Fouling of PES membrane surfaces creates an environment in which bacterial attachment and biofilm formation thrive. Biofilm removal from dairy processing membranes has become a point of interest as cost of producing quality products continues to rise. Experimentation was carried out in two phases.

Phase 1 evaluated the half-life of microscale/nanoscale aqueous ozone (MNAO) using a factorial design, with pH (2, 4, and 7) and temperature (5, 10, and 20°C) as independent factors with two replications. After statistical analysis of the half-life study using a completely randomized design, a pH of 4 at 10°C for MNAO was used in further studies. This study then evaluated the efficiency of MNAO on removal of biofilms from PES membranes.

Phase 2A first compared the efficacy of a deionized water wash and MNAO on *Bacillus cereus* and *Bacillus licheniformis* biofilms grown on PES membranes. MNAO was more effective ($p \leq 0.05$) than the water wash with an average reduction of 1.9 $\log_{10}$ cfu/cm^2 for *Bacillus cereus*, and 1.2 $\log_{10}$ cfu/cm^2 for *Bacillus. licheniformis*, respectively. The MNAO membrane wash reduced *B. licheniformis* counts on membranes by 2.3 log cycles, while the water wash reduced the population level by 1.9 log cycles ($p \leq 0.05$). Both the *Bacillus cereus* and *Bacillus licheniformis* data were compared to a non-treated control membrane.

Phase 2B analyzed the efficacy of a typical clean-in-place (CIP) cycle used in the dairy industry compared to a CIP cycle followed by MNAO wash. Treatment of *B. cereus* biofilms using a standard CIP protocol or the CIP+MNAO resulted in significant ($p \leq 0.05$) population reductions of 3.8 and 4.1 $\log_{10}$ cfu/cm^2 respectively. A significant difference was observed when comparing Side A and Side B data ($p \leq 0.05$). For *Bacillus licheniformis* containing biofilms, both treatments provided significant population reductions ($p \leq 0.05$) compared to the untreated control.

Phase 2C studied the efficacy of a typical CIP system alone in comparison to an MNAO wash followed by a typical CIP system. In the *Bacillus cereus* and *Bacillus licheniformis* study, CIP and MNAO+CIP treatments significantly reduced ($p \leq 0.05$) population levels of both organisms by 3.4 to 4.7 log cycles respectively; however, the reductions observed for the two treatments within bacteria type were similar ($p \leq 0.05$).

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Dedication

I dedicate this thesis to the love of my life, my parents, grandparents, mentors, the girls, and everyone in between who has come and gone throughout this adventure. To all who have put up with me during this crazy chapter of my life, I say thank you. I would not be here without you all. Always remember if you get tired, learn to rest, and never give up on your dream.

I love you all, God is good.

Chapter 1 - Introduction

1.1 Introduction

The buildup of proteins, fats, and minerals in a layer on the membrane surface is known as fouling. Fouling is a major challenge in the dairy industry, especially during membrane processing. Many factors influence membrane fouling, including surface charge, pore size, hydrophobicity, temperature, crossflow velocity, and pH (Hu and Dickson, 2015). As the membrane fouls over time, overall productivity of the membrane decreases in addition to shortening of the membrane life (Hu and Dickson, 2015). Many studies have shown that fouling is initiated mainly by proteins, minerals (Chamberland et al., 2017), sediments, and bacteria present in milk (Kumar et al., 2013).

Development of bacterial biofilms has been observed, contributing to decreased membrane performance and increased cost (Chamberland et al., 2017). Hinton et al. (2002) noted that adhesion of bacteria occurs more frequently on fouled surfaces compared to unfouled surfaces. According to Anand et al. (2014), biofilms survive membrane cleaning cycles, which can be detrimental for dairy processors. Biofilm growth on membranes, per Tang et al. (2010), results in a decreased membrane life due to microbial action and has been reported to be problematic. Milk is a good medium for microbial growth, which can cause spoilage of milk and its byproducts and can increase the risk for human illness. With public health at stake, biofilm-originating contamination has been linked to food poisoning, causing concern (Fink et al., 2017). Disinfection and cleaning of contaminated membranes is important, although elimination of biofilms on surfaces is an ongoing challenge. Spore-forming growth in the dairy industry negatively affects both product safety and quality. *Bacillus cereus*, which is a spore-former, causes two types of food poisoning syndromes: diarrheal and emetic type (Lücking et al., 2013).

Despite modern food technology and sterilization techniques, spoilage can lead to economic losses within food companies, creating a cause for concern.

Sanitation and disinfection of membranes are not only crucial for the quality and safety of products, but also for the prevention, removal, and killing of biofilms (Jang et al., 2006). Control of biofilms is achieved using strong oxidizing agents such as chlorine and peracetic acid. Effectiveness of clean-in-place (CIP) systems is determined by many factors and generally involves the use of caustic and acid wash steps, chemicals designed to remove organic and inorganic fouling layers, and sanitizers (Bremer et al., 2006). The dairy industry must now focus on environmentally sustainable cleaning protocols, which are found to be economical. There is an interest in nanobubble technology as its surface area-to-volume ratio theoretically increases the efficiency of antimicrobial components (Wilder et al., 2016). Generally recognized as safe, ozone is approved as an antimicrobial agent for direct applications in food. Ozone is a strong oxidant capable of inactivating *Listeria monocytogenes, Escherichia coli* O157:H7, and *Salmonella* spp. due to its potent microbicidal properties (Almeida and Gibson, 2016). Ozone has been used in many disinfectant applications and has the potential to be used more vigorously throughout the dairy industry alongside CIP systems. Using nanobubble technology with applied gaseous ozone could potentially revolutionize typical CIP systems.

This review focuses on the removal of generated *Bacillus* spp. biofilms on polyethersulfone (PES) membranes using microscale/nanoscale aqueous ozone (MNAO) as a sanitizer. Chapter 4 studies the efficacy of MNAO on the removal of *Bacillus cereus* and *Bacillus licheniformis* biofilms from PES membranes with a typical CIP system at different phases of the experiment.

1.2 References

Almeida, G. and K. E. Gibson. 2016. Evaluation of a recirculating dipper well combined with ozone sanitizer for control of foodborne pathogens in food service operations. J. Food Prot. 79:1537-1548.

Anand, S., D. Singh, M. Avadhanula, and S. Marka. 2014. Development and control of bacterial biofilms on dairy processing membranes. Comp. Rev. Food Sci. Food Saf**.** 13:18-33.

Bremer, P. J., S. Fillery, and A. J. McQuillan. 2006. Laboratory scale Clean-In-Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. Int. J. Food Microbiol. 106:254-262.

Chamberland, J., M. Lessard, A. Doyen, S. Labrie, and Y. Pouliot. 2017. Biofouling of ultrafiltration membrane by dairy fluids: Characterization of pioneer colonizer bacteria using a DNA metabarcoding approach. J. Dairy Sci. 100:981-990.

Fink, R., M. Oder, E. Stražar, and S. Filip. 2017. Efficacy of cleaning methods for the removal of *Bacillus cereus* biofilm from polyurethane conveyor belts in bakeries. Food Control 80:267-272.

Hinton, A. R., K. T. Trinh, J. D. Brooks and G. J. Manderson. 2002. Thermophile survival in milk fouling and on stainless steel during cleaning. Food Bioprod. Process. 80:299-304.

Hu, K., and J. M. Dickson. 2015. Membrane processing for dairy ingredient separation. IFT Press. Wiley-Blackwell, Hoboken, NJ.

Jang, A., J. Szabo, A. Hosni, M. Coughlin, and P. Bishop. 2006. Measurement of chlorine dioxide penetration in dairy process pipe biofilms during disinfection. Appl. Microbiol. Biotechnol 72:368-376.

Kumar, P., N. Sharma, R. Ranjan, S. Kumar, Z. F. Bhat, and D. K. Jeong. 2013. Perspective of membrane technology in dairy industry: A review. Asian-Australas J. Anim Sci. 26:1347-1358.

Lücking, G., M. Stoeckel, Z. Atamer, J. Hinrichs, and M. Ehling-Schulz. 2013. Characterization of aerobic spore-forming bacteria associated with industrial dairy processing environments and product spoilage. Int. J. Food Microbiol. 166:270-279.

Tang, X., S. H. Flint, R. J. Bennett, and J. D. Brooks. 2010. The efficacy of different cleaners and sanitizers in cleaning biofilms on UF membranes used in the dairy industry. J. Membrane Sci. 352:71-75.

Wilder, A. 2016. Evaluation of a novel commercial ground beef production system using a chlorinated nanobubble antimicrobial technology to control Shiga toxin-producing *Escherichia coli* and *Salmonella* spp. surrogates. MS Thesis. Food Science Institute. Kansas State Univ., Manhattan.

Chapter 2 - Research Objectives

The first part of this study assessed the half-life (k) value of ozone in aqueous solution under different temperature and pH constraints. The second part of this study focused on the evaluation of biofilm removal on polyethersulfone (PES) membranes in the dairy industry. Specific objectives are listed below:

1. The first objective was to study the half-life of microscale/nanoscale aqueous ozone (MNAO) using different pH (2, 4, and 7) and temperature (5, 10, and 20°C) combinations to determine optimal parameters for cleaning PES membranes.
2. The second objective was to evaluate the efficiency of MNAO in removing biofilms from PES membranes.

Chapter 3 - Literature Review

3.1 Dairy Technology

Dairy technology has been continually advancing since the early 1970s (Pouliot, 2008). This review focuses on the membrane processing, which is extensively used in the dairy industry as a unit operation to fractionate dairy proteins. Membranes in the food industry represent 20-30% of the current 250 million membranes used worldwide (Saxena et al., 2009). Over the years, there has been improvement in dairy membrane technology, which has enabled the manufacturing of high protein milk-based products such as high protein snacks, meal replacement shakes, and protein bars. Protein (2.3-4.4%), fats (2.5-5.5%), lactose (3.8-5.3%), water (85.3-88.7%), and minerals (0.57-0.83%) make up the composition of milk. Each differentiate in physical size, structure, and properties (Walstra et al., 2005).

Today, membrane separation technologies are being used to fractionate milk and its components by molecular weight and particle size. Reverse osmosis (RO), nanofiltration (NF), ultrafiltration (UF), and microfiltration (MF) are four of the most common methods used in the industry. Iorhemen et al. (2016) described problems associated with membrane processing, including a decrease in membrane performance, decrease in flux, fouling of the membrane, and biofouling of the membrane.

Cleaning and sanitation of separation membranes is crucial for continual membrane performance. If proper cleaning and sanitation protocols are not followed on a routine basis, it can lead to a premature replacement of membranes and can increase the cost of overall production. Microbial safety of products in the dairy and food industry is another factor that influences cleaning operations. The presence of bacterial biofilms has become a major concern in dairy manufacturing plants. Control of these biofilms is generally combatted by a process called

clean-in-place (CIP). Bremer et al. (2006) defines a typical CIP as cleaning of complete items of plant or pipeline circuits without dismantling or opening of the equipment and with little or no manual involvement on the part of the operator. Dairy industry CIP processes include a wide range of cleaning agents such as acids, bases, enzymes, and surfactants (Popović et al., 2010). Without these cleaning protocols, the microbiological safety of products being produced becomes compromised. Anand et al. (2014) described a previous study that found a multispecies biofilm on whey reverse-osmosis membranes despite regular CIP protocols. This leads to spoilage of product and potential illness if the cross-contaminated product is consumed. The types of spoilage and pathogenic microorganisms found in the dairy industry include but are not limited to *Lactobacillus, Staphylococcus, Klebsiella, Escherichia, Listeria, Enterococcus,* and *Bacillus* (Teh et al., 2015).

Detergents used in a typical CIP system include alkali or acid detergents. According to Thomas and Sathian (2014), typically used alkali detergents in the dairy industry include but are not limited to sodium hydroxide, potassium hydroxide, and sodium carbonate. Acid detergents include citric acid, phosphoric acid, and nitric acid. Other mineral acids that may be used include hydrochloric acid and sulfuric acid (Thomas and Sathian, 2014). The chemical cleaning process itself has brought about complications such as shortening of membrane life and issues related to disposing of harsh chemicals used during membrane cleaning. We are now looking for solutions to remove biofilms that a typical CIP may not remove from dairy membranes. Because of this, chemical cleaning needs to be minimized and other countermeasures need to be further researched to aid in cleaning. Ozone is an emerging cleaning and disinfection agent that is still in the experimental stages with the potential to be used alongside a typical CIP system.

This paper reviews common membranes used in the dairy industry, dairy ingredients produced during membrane filtration, the effects of fouling on dairy membranes, biofilm development on dairy processing membranes, cleaning protocols, and the effects of microscale/nanoscale aqueous ozone (MNAO) in the dairy industry.

3.2 Membrane Technology in the Dairy Industry

A barrier or interphase to the flow of molecular and ionic species present in liquids is known as a membrane (Saxena et al., 2009). Membranes are used in industry to remove components such as sediments, drugs, and microorganisms that impact product quality in a negative way (Kumar et al., 2013). Membranes are used in the dairy industry and serve many purposes. Some examples described by Kumari and Sarkar (2016) include the addition of non-thermal shelf life extensions of milk; standardization of milk; and concentration, fractionation, and purification of milk components. Uses of these membranes during processing involve filtration of solutions containing water, salts, amino acids, proteins, peptides, and other organic compounds such as sugars, organic acids, and vitamins (Saxena et al., 2009).

According to Daufin et al. (2001), membranes started gaining recognition after methods were established to use them in the processing of whey and other dairy applications. Henning et al. (2006) describes available membrane configurations including tubular, hollow fiber, spiral wound, and plate and frame configurations. Common membrane separation technologies used in the dairy industry and food industry include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO). Each of these filtration processes produces a permeate (feed which passes through the membrane) and a retentate stream (rejected feed materials). Table 3.1 compares the membrane types, pore size, molecular weight, compounds left in retentate, and application in the dairy industry.

Table 3.1 Commonly used membrane techniques in the dairy industry

Type	Pore Size	Molecular Weight	Compounds in Retentate	Application in Dairy Industry
Microfiltration (MF)	0.1-2 [micro] m	>200 kDa	Low retentate, separation of protein, bacteria and other particulates	Skim milk and cheese, dextrose clarification, bacterial removal
Ultrafiltration (UF)	1-500 [micro] m	1-200 kDa	Large retentate with casein micelles, fat globules, colloidal minerals, bacteria and somatic cells	Standardization of milk, reduction of calcium and lactose, protein, whey, milk concentration
Nanofiltration (NF)	0.5-2 nm	300-1,000 Da	Low productivity, separate monovalent salt and water	Desalting of whey, lactose-free milk, volume reduction
Reverse Osmosis (RO)	No pores	100 Da	Based on the principle of hyperfiltration of solubles, low productivity	Volume reduction, recovery of total solids and water

*Material compiled from Kumar, (2013); Pouliot, (2008); and Hu and Dickson (2015)

Each type of membrane is characterized by its own pore size and industry applications. MF typically runs at a low pressure and is used for larger particle size fractionation and separation (Hu and Dickson, 2015)**.** In the dairy industry, MF is widely used for bacteria reduction, fat removal from milk and whey, as well as for casein standardization (Van Hekken and Holsinger, 2000). In addition to reducing bacteria levels, it also increases microbiological safety of dairy products (Sahu, 2014). Reports have indicated that MF rejects microbes and fat while allowing other milk components to pass through the membrane (Kumar et al., 2013). According to Renhe and Corredig (2018), MF increases the casein-to-whey ratio in the retentate, which in turn provides an opportunity to create novel functional ingredients.

UF is a medium pressure-driven membrane filtration process. UF is a method for concentrating, purifying, and fractionating macromolecules (Henning et al., 2006). This filtration method is based on a membrane with a medium-open pore structure allowing most dissolved

components and some non-dissolved components to pass, while larger components are rejected by the membrane. UF is used for many applications such as powders, protein concentration, decalcification of permeates, protein standardization of cheese milk, and fresh cheese production. Another specialized type of UF is called diafiltration (DF). DF is a process in which the retentate is diluted with water and ultra-filtrated to reduce the concentration of soluble permeate components; DF increases the overall concentration of retained components (Kumari and Sarkar, 2016).

The highest operating pressures occur with nanofiltration (NF) and reverse osmosis (RO). These membrane pores are the smallest of the membranes discussed here. NF is a medium- to high-pressure-driven membrane filtration process. When applied pressures are higher than osmotic pressure, they allow permeation through the membrane (Pouliot, 2008). NF is a type of reverse osmosis where each membrane has a more open structure, which allows monovalent ions to pass through while rejecting the divalent ions (Kumar et al., 2013). In the dairy industry, NF is mainly used for special applications such as partial demineralization of whey, lactose-free milk, or volume reduction of whey.

RO is a high-pressure-driven membrane filtration process. This filtration method is commonly used for the concentration of liquids with removal of water from solutions. This can be used for concentration, condensate, and permeate (Forde, 2012). RO membranes have a small pore size that allow only small particles of a low molecular weight to pass through.

The engineering of membrane processing systems is dependent upon the needs of the manufacturer. Membrane material, surface texture, pore size, and hydrophilicity have an impact on fouling.

Membranes can be divided into ceramic membranes, polymeric membranes, and composite membranes (Iorhemen et al., 2016). Ceramic membranes have exhibited high chemical resistance, good filtration performance, pore size control, long lifespan, and low operational costs due to their inert nature and cleaning ease (Iorhemen et al., 2016). Polymeric membrane materials include but are not limited to polyethersulfone (PES), polyethylene (PE), polysulfone (PS), and polypropylene (PP). Approximately 75% of industrial membranes manufactured are polymeric (Hausmann et al., 2013). Composite membranes are used for water purification or water desalination systems. These membranes are semipermeable and can be modulated and optimized independently to obtain required thermal and chemical stability, permeability, and selectivity. Membrane material and surface texture has an impact on fouling (Choi and Ng, 2008).

There is a limit to PES membrane application due its hydrophobic properties and high cost (Jiang et al., 2015). Hu and Dickson (2015) stated that PES membranes also offer thermal stability, which enables high-temperature cleaning, thereby addressing the stronger cleaning requirements of the dairy industry.

3.3 Ingredients Derived from Milk

Of the unique components included in milk such as lactose, proteins, fats, and minerals, each of these elements differ in structure, size, and physical properties. Many dairy ingredients are used in the food industry and provide sensory and physical properties while also adding nutritional traits and benefits to products on the market today. Ingredients such as whey protein isolate and whey protein concentrate have become popular in many food products throughout the world. New dairy-based ingredients continue to expand the dairy foods market, creating value-added products with advanced functionality (Henning et al., 2006). In particular, casein and

whey proteins are valued as ingredients in dairy and non-dairy foods. Milk powder is being used as a functional ingredient in the food manufacturing industry to produce chocolates, beverages, bakery products, yogurt, and a variety of other items. Functional properties of milk powder include heat stability, aeration, emulsification, solubility, and viscosity building (Suthar et al., 2017). Milk protein concentrates (MPC) contain whey and casein proteins and are complete dairy protein complexes, providing a concentrated source of protein for nutritional value (Agarwal et al., 2015). MPCs can be used as a replacement for whole milk and skim milk powders. Equivalent protein and milk solids on a nonfat basis aid in formulating products with a lower lactose and a higher protein content (Agarwal et al., 2015). Another functional ingredient derived from milk is milk protein isolate (MPI), which contains 90-95% protein (Agarwal et al., 2015).

Agarwal et al. (2015) discuss the application of MPCs and MPIs as ingredients. MPCs and MPIs are used during the production of many products, including milk replacements, cheese milk, performance supplements, ice cream, cultured dairy products, and high-protein beverages. Also included are emulsions such as low-fat spreads, soups, and salad dressings. Milk proteins provide many functional properties, improve nutritional quality, and add value to the product. Separation technology helps provide a foundation for the increasing demand and value of the ingredients found in milk. UF plays an important role during separation of dairy ingredients. As a result, membrane fouling becomes an unavoidable occurrence during separation of these ingredients.

3.4 Fouling in Membranes

Fouling is described by Goode et al. (2013) as the unwanted buildup of material on a surface. Membrane fouling results from interactions of various components of the feed through

the membrane (Hu and Dickson, 2015). Fouling of dairy membranes is caused by buildup of proteins, fats, bacteria, and other minerals present in milk (Daufin and Merin, 1995; Garem and Jeantet, 1995; Turan et al., 2002; D'Souza and Mawson, 2005). The proteins found in the fouling layer after UF are whey proteins and caseins (Hu and Dickson, 2015). Ma et al. (2001) found that factors such as particle size, cross-flushing, and back-pulsing affect the fouling of membranes. Milk components smaller than the pore size of the membrane can pass through without any resistance. However, the components of the same size or larger than the pore size of the membrane can cause blockage of membrane pores and can be retained on the surface of the membrane. Whey proteins such as β-lactoglobulin and α-lactalbumin play a role in irreversible fouling and are adsorbed at the membrane surface (Chamberland et al., 2017). Consequently, the adsorbed protein particles cause a cake formation, which is also known as a fouling layer (Figure 3.1). Membrane fouling decreases flux rates and thereby increases energy consumption during membrane processing, leading to premature replacement of dairy processing membranes (Tang et al., 2010; Popović et al., 2010; Anand et al., 2012). This has a negative effect on plant efficiency (Fazel and Chesters, 2014; Yamamura et al., 2014).

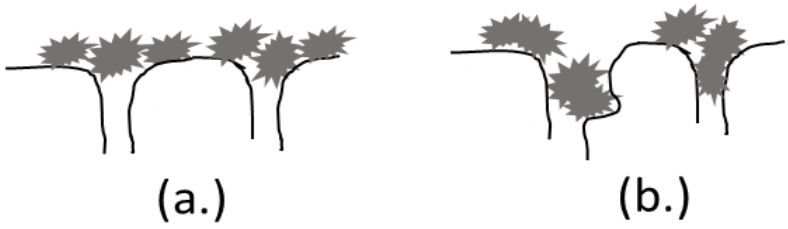

Figure 3.1 Mechanisms of membrane fouling: (a) external fouling and (b) internal fouling.

Fouling can be important because bacteria and spores must be retained as much as possible while other milk components are allowed to pass through the membrane during separation processes. Hu and Dickson (2015) state that fouling causes an increased membrane

resistance, leading to irreversible fouling. Particle fouling is expected to be removed during physical cleaning, but according to Psoch and Schiewer (2006), smaller particles contribute more to irreversible fouling. Accumulation of microorganisms growing and attached to an abiotic or biotic surface are known as biofilms. Biofilm contamination is known to be influenced by growth conditions and nutrient availability (Aswathanarayan and Vittal, 2014). Concentration gradient, or a phenomenon called concentration polarization, allows microorganisms involved in biofilm formation to grow on the surface of the fouled membrane (Hassan and Sanjeev, 2012).

3.5 Biofilms in the Food Industry

In 2010, the International Union of Pure and Applied Chemistry defined the term biofilm as an "Aggregate of micro-organisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substances adhere to each other and/or to a surface" (Teh et al. 2015, p. 2). Bacterial adhesion has been studied and the mechanism of attachment has been found to be dependent on thermodynamics and van der Waals attraction (Karunakaran and Biggs, 2011). Environmental biofilms and process biofilms are the two types of biofilms found in dairy manufacturing plants. Environmental biofilms form directly in the processing environment in places where complete sanitation is difficult to achieve. These biofilms can take weeks to fully develop (Teh and Bennett, 2015). Process biofilms develop on food contact surfaces that are directly in contact with flowing product. Biofilms also have rapid growth due to optimal environmental conditions and have been found to develop on stainless steel and separation membranes (Anand et al., 2014; Huttenlochner et al., 2017).

Biofilm formation on dairy membranes continually affects membrane performance, lowering trans-membrane flux (Anand et al. 2014; Stocia et al., 2018). The overall structure of the biofilm is dependent on environmental conditions, bacterial genome, and embedded

components in the matrix of the biofilm (Fysun et al., 2019). Biofilm generation occurs by adhesion of bacteria to proteins, forming quickly on membranes during the beginning stages of the filtration process (Chamberland et al., 2017). Temperature and nutrient availability are two favorable conditions that aid in the development of biofilms on surfaces.

The first step of biofilm formation is the attachment phase (Figure 3.2). This step requires the cell to form a semi-permanent association with the surface (Teh and Bennett, 2015). Biofilm formation and adherence is influenced by the presence of flagella, microbial growth phase, and cell surface charge attachment (Pagedar and Singh, 2011). In the dairy industry, there are many surfaces to which bacteria can attach, including stainless steel, plastic, rubber, polyurethane, epoxy surface coating, tiles, and membranes. The rate at which biofilms attach and are removed from surfaces is dependent upon the material being processed, age of surface, exposure to chemicals and cleaning agents, and surface damage due to mechanical operations (Teh and Bennett, 2015). Physical and chemical characteristics of the biofilm structure are dependent upon environmental conditions and bacterial species involved.

In the second step, biofilm must then form a semi-permanent association with the surface. Surface attachment and the rate at which bacteria attach will change depending upon the material in contact with the surface. Removal of attached bacteria will also vary depending upon exposure to cleaning agents, sanitizers, and age of the surface material (Teh and Bennett, 2015).

In the third step, biofilm formation involves the replication of cells producing extracellular polymeric substances (EPS). The fourth step then involves the formation of microcolonies on the surface. Teh and Bennett (2015) explain that the increased complexity and bulk of biofilms comes from the incorporation of EPS, food residues, and other microorganisms. The formation of microcolonies on the surface results from the replication and growth of the first

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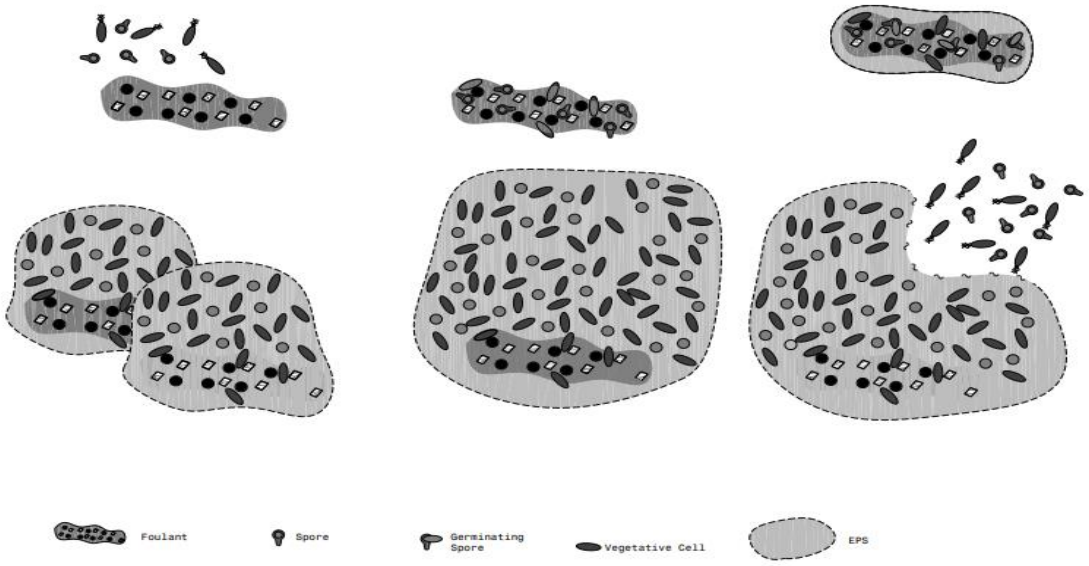

Figure reproduced from Teh and Bennett (2015).

Figure 3.2 Steps involved in biofilm formation on surfaces. Steps involved in biofilm formation over time (arrow) in a dairy processing plant under conditions of flow. (1) Initial reversible attachment of bacterium to substrate. (2) Irreversible surface attachment (3) Replication of cells, early development into biofilms. (4) Formation of micro-colonies on surface (5) Biofilm maturation (6) Biofilm dispersion

cells that attach to the surface. Independently, these microcolonies form a series of macro-colonies, establishing three-dimensional aggregates of cells and EPS upon the surface (Teh and Bennett, 2015). Herzberg et al. (2009) stated that as bacteria develop, they produce EPS, forming matrices otherwise called biofilms. Finally, in steps five and six, biofilm development involves maturation and dispersion. There are three distinct modes of biofilm dispersion including erosion, sloughing, and seeding. Dispersal from biofilms can be initiated or mediated by external forces such as abrasion, fluid shear, and cleaning (Teh and Bennett, 2015).

There are numerous bacteria known to attach to various food contact surfaces that can potentially develop into biofilms (Anand et al., 2012). *Bacillus cereus* ATCC10987 and *Bacillus cereus* ATCC6634 are known to have biofilm-forming capabilities (Karunakaran and Biggs, 2011). In upcoming sections, *Bacillus cereus* ATCC10987 and *Bacillus cereus* ATCC6634 are discussed in further detail.

3.6 *Bacillus cereus* and *Bacillus licheniformis*

Bacillus cereus is one of the many species of this genus found on food processing equipment and milk processing surfaces. It is a gram-positive, endospore-forming, rod-shaped bacterium (Pagedar and Singh, 2011) and can be found virtually everywhere in the environment. According to Sychantha (2014), food contaminated with *Bacillus cereus* can cause diarrhea, vomiting, and nausea when ingested. The optimum growth temperature for *Bacillus cereus* is between 30-40°C (Morris and Potter, 2013). *Bacillus cereus* produces two distinct types of toxins (emetic and diarrheal) (Fink et al., 2017) and is known to be responsible for non-gastrointestinal infections like keratitis and endocarditis (Kotiranta et al., 2000). In addition, *Bacillus cereus* is a spore-former that impacts the safety and shelf life of foods, making it a major threat to the dairy and food industry. *Bacillus cereus* exhibits different physiological cell stages such as vegetative, surface-adhered cells, and spores (Peng et al., 2001).

Food contamination by *Bacillus cereus* is common, especially in plant-based products (Morris and Potter, 2013). This organism is commonly isolated from meat products, spices, ready-to-eat vegetables, and dairy products. According to Saleh-Lakha et al. (2017), *Bacillus cereus* can even be reintroduced into milk products by poorly sanitized dairy processing pipelines, membranes, and other equipment. Saleh-Lakha et al. (2017) state that *Bacillus cereus* spp. can cause foodborne illness when present at 10^5 cfu/ml. Meanwhile, the growth of *Bacillus*

cereus can reach 10^7 cfu/ml before spoilage is evident in pasteurized milk. Food poisoning incidents involving *Bacillus* species are most associated with *Bacillus cereus* (Salkinoja-Salonen et al., 1999) and have brought attention to the *Bacillus* spp. group. Surveillance in the food industry has increased because of the psychrotrophic and spore-forming properties of this bacterium, which allow it to survive pasteurization and grow at refrigeration temperatures (Saleh-Lakha et al., 2017). Mesophilic strains of *Bacillus cereus* were found to be less heat-resistant than psychrotrophic strains (Christiansson et al., 1989; Morris and Potter, 2013).

Another species of *Bacillus* studied in this review is *Bacillus licheniformis*, also a rod-shaped, gram-positive bacterium. The optimal growth temperature for *Bacillus licheniformis* is 50°C, but this bacterium can survive at much higher temperatures. In harsh environments, *Bacillus licheniformis* will exist in the form of a spore. When conditions are good, it will resume its vegetative state. This bacterium is also a commonly found contaminant in milk and milk products (Dhakal et al., 2013). Global studies have identified *Bacillus licheniformis*, *Anoxybacillus* spp., and *Geobacillus* spp. as the top three spore formers found in dairy powders (Kent et al., 2016). Of the three species, Kent et al. (2016) stated that *Bacillus licheniformis* is the most prevalent spore former in dairy powders and raw materials. It has also been found to cause foodborne outbreaks associated with cooked meats, vegetables, raw milk, and baby food. This species of *Bacillus* produces spores, which can cause spoilage and compliance issues in dairy products (Dhakal et al., 2013), highlighting the importance for reduction and control of spore counts in the dairy industry. Proper cleaning using CIP systems is necessary to remove and control biofilm growth.

3.7 Cleaning Issues During Filtration

Biofouling on filtration membranes is a quality, safety, and performance issue in the dairy industry (Avadhanula, 2010; Chamberland et al., 2017). There are numerous bacteria known to attach to various food contact surfaces that can potentially develop into biofilms (Anand et al., 2012). Cleaning methods are often chosen based upon the unit configuration, fouling encountered, and physical and chemical resistance of the membrane (Bartlett et al., 1995). Formulated detergents, cleaning agents, and caustic solutions were found to have a positive effect on flux recovery (Malmrose et al., 2003). As a result of many studies, dairy processing membranes used in the industry are typically cleaned once per day to recover permeate flux (Popović et al., 2009). Whey proteins such as β-lactoglobulin and α-lactalbumin play a role in irreversible fouling and are adsorbed at the membrane surface (Chamberland et al., 2017).

There are many CIP cleaning protocols used in the industry, but generally a series of alkali, acid, and/or enzyme washes are required to break down fouling buildup on a membrane surface (Li and Chen, 2015). Commonly used CIP regimes in the dairy industry showed diverse effectiveness in removing *Bacillus cereus* biofilms. A typical CIP protocol as described by Ecolab (Saint Paul, MN) consists of the following: pre-flush water wash at 21°C, followed by an alkaline wash at pH 10.8-11 at 55°C, followed by another water flush at 21°C, followed by an acid wash at pH 1.8-2.0 at 55°C, and finishing with a final water flush at 21°C.

Tang et al. (2010) describes a typical CIP process consisting of an alkaline or acid wash pH 11–12 followed by a sodium hypochlorite wash (sanitization step). An alkaline treatment solubilizes proteins, carbohydrates, and fats; meanwhile, the acid wash dissolves minerals. As a result of the CIP, microbial levels in the membrane system decline, simultaneously removing the

foulant layer and microbial growth on the membrane surface. Three enzymatic cleaners were compared with sodium hypochlorite (pH 10.8–11) at 200 ppm free available chlorine (FAC) commonly used in the CIP of UF membranes in the dairy industry. Results showed that a standard CIP allowed a total aerobic plate count of 1.40–2.18 log cfu cm^{-2} to remain on the membrane surface (Tang et al., 2010). These residual bacteria counts are concerning when multiplied to reflect an area used in an industrial plant. Tang et al. (2010) notes that there is evidence that biofilms may protect against CIP chemical cleaning and culturable cells can remain after the CIP, causing rapid regeneration of biofilm cells on the surface.

The cleaning of membranes also involves decreasing microbial levels in the membrane system during sanitization (Hu and Dickson, 2015). Hu and Dickson (2015) also stated that there is an increasing concern over the environmental impacts and cost associated with current chemical sanitizers. Decreased membrane lifetime and disposal of the spent chemicals used during the membrane cleaning process are found to be problematic and an alternate approach may be needed (Malmrose et al., 2003). There is a need to investigate alternative environmentally, economically sustainable sanitation protocols in the dairy industry due to increased cost, chemical disposal, and environmental impact.

3.8 Microbubble and Nanobubble Technology

Microbubble and nanobubble technologies are newer concepts that have been used to support new developments in food safety interventions. This technology has been explored as a chemical-free cleaning agent for removal of biologically fouled membranes and other surfaces (Agarwal and Liu, 2017). Nanobubbles and microbubbles are small bubbles with a diameter of 10–50 μm and < 200 nm respectively. Tsuge (2014) explored two varieties of nanobubbles: oxygen-nanobubbles and ozonated-nanobubbles. Proposed surfactant abilities and the cleaning

effect of microbubbles have sparked an interest in the industry due to their applications in water treatment, biomedical engineering, and nanomaterials (Agarwal et al., 2011). Generation of free radicals, negative charge, self-pressurization, and interior gas are special properties associated with microbubbles (Agarwal et al., 2011). When in solution, the surface charge of the water-gas interphase is affected by the pH of water (Tsuge, 2014). According to Bauer (2016), nanobubbles provide increased surface area-to-volume ratio per mass as compared to standard water or other aqueous solutions.

Aqueous ozone has presented an assortment of microbubbles and nanobubbles with un-uniform sizes. Size of nanobubbles is often determined by a light scattering method and can be measured by zeta potential (Tsuge, 2014). Tsuge (2014) defines zeta potential as the surface charge of a suspended molecule in a fluid system; a high zeta potential will present stability within a solution. Agarwal et al. (2011) described a study using microbubbles for cleaning of hard surfaces and stated that destabilized and reduced biofilms were observed after the use of nanobubbles. When in solution, the surface charge of the water-gas interphase is affected by the pH of water (Tsuge, 2014). Using nanobubbles as a sanitation method was found to inactivate more than 99% of norovirus surrogates in oyster bodies after 12 hr (Tsuge, 2014).

Free radicals are generated when microbubbles and nanobubbles are used, catalyzing chemical reactions, and enhancing efficiency of detoxification (Agarwal et al., 2011). Laboratory-scale studies suggest that the cost of nanobubble production is higher than conventional chlorination techniques for use as a disinfectant (Agarwal et al., 2011). Agarwal and Liu (2017) explored this technology as a chemical-free cleaning agent for removal of biologically fouled membranes and other surfaces. Nanobubble technology is used most to aid in wastewater disinfection. Agarwal et al. (2011) reported that nanobubble pretreatment to

wastewater reduced overall biological, chemical, and physical loads, meanwhile reducing overall cost. There is limited knowledge and few reported applications of nanobubbles in the food industry. Because nanobubble technology is relatively underdeveloped, further studies should be done to determine real-world uses in food safety applications.

3.9 Ozone

With the growing concern for food safety, food processors are searching for an alternative antimicrobial agent. Ozone is known to be a strong oxidizing sanitizer and is widely used during sanitization (Demirci and Ngadi, 2012). With an oxidation potential of 2.07V, ozone is the strongest oxidant currently available for food applications (Demirci and Ngadi, 2012). Ozone is comprised of three oxygen atoms arranged at an obtuse angle and is naturally generated by reaction of free-radical oxygen with diatomic oxygen (Patil and Bourke, 2012). Ozone is holds a generally regarded as safe (GRAS) status as it leaves no residue (Dhillon et al., 2009). When used in the industry, ozone is generally produced using commercial ozone generators, and because of its reactiveness, it must be generated at the point of application due to its instability (Demirci and Ngadi, 2012). As temperature increases, ozone becomes less soluble and less stable, while becoming more reactive (Varga and Szigeti, 2016). Disinfection rates of ozone are reduced when organic compounds compete with microorganisms; thus, the absence of organic compounds in ozonated water is desirable (Varga and Szigeti, 2016). Due to its increased popularity, ozone is being studied as an alternative sanitation agent to chlorine, as ozone has no toxic residual effect after its decomposition to oxygen. This reduces industrial costs and environmental impacts (Fukuzaki, 2006; Patil and Bourke, 2012).

Multiple methods exist for ozone generation, including ultraviolet radiation, thermal, chemical, electric corona, electrolytic, and chemonuclear methods (Varga and Szigeti, 2016).

Ozone can be applied in gaseous form or as an aqueous solution (Dhillon et al., 2009). Ozone reacts in two ways when microbes and contaminates are present: by direct oxidation and through hydroxyl radicals generated during decomposition. These properties help ensure the microbiological safety of food products. Ozone has been shown to be active against viruses, bacteria, fungal spores, and fungi (Alexopoulos et al., 2013), and is a suitable disinfecting agent for many food industry-related applications including food surface hygiene and preservation, food plant sanitation, and wastewater reuse (Zhang et al., 2005).

The half-life of aqueous ozone can vary from seconds to hours depending on pH, temperature, and water quality (Demirci and Ngadi, 2012). Ozone solubility is affected by water purity. Due to the presence of minerals, ozone was found to dissolve faster in tap water when compared to deionized and distilled water (Kim, 1998). In a production environment, the use of ozone can potentially be harmful to human health. According to the United States Environmental Protection Agency, when inhaled, ozone reacts chemically with biological molecules in the respiratory tract, leading to several adverse health effects such as difficulty breathing, coughing, and airway inflammation. Ozone is unstable and comes out of solution easily; it is found to be more stable in gaseous form than in aqueous solution. Optimum dissolution of ozone in water occurs at a bubble size of 1-3 mm in diameter. Smaller ozone bubbles increase solubility and efficacy of ozonated water by increasing the surface area of contact (Katzenelson et al., 1974). Ozone as a disinfectant is used in a wide variety of applications. It has been found to be more powerful and potentially more versatile than chlorine (Neff, 1998); ozone is also more effective against a wider variety of microorganisms and has been found to kill foodborne pathogens at a faster rate than traditional sanitizers such as chlorine (Zhang et al., 2005; Patil and Bourke, 2012). Many studies have been done using ozone as a sanitizer on stainless steel coupons

(Greene et al., 1993; Marino et al., 2018). Greene et al. (1993) studied milk biofilms on stainless steel surfaces and found that both ozone and chlorine reduced bacteria populations by >99% with reductions of 1.24×10^5 to $8.56 \times l0^5$ cfu/cm^2 for *Pseudomonas fluorescens* and 1.53×10^4 to 8.56×10^5 cfu/cm^2 for *Alcaligenes faecalis.* Marino et al. (2018) studied *Pseudomonas fluorescens*, *Staphylococcus aureus*, and *Listeria monocytogenes* biofilms on stainless steel surfaces and reported the use of aqueous ozone at 0.5 ppm under static conditions resulted in a viability reduction of 1.61–2.14 log cfu/cm^2 after 20 min and 3.26–5.23 log cfu/cm^2 for biofilms treated in dynamic conditions .Another study explored disinfection of packages and equipment using ozone; Demirci and Ngadi (2012) found that ozone applied at 0.5-3.5 ppm in an aqueous or gaseous state caused significant microbial reductions.

Varga and Szigeti (2016) stated that adhered microorganisms on milk-contacting surfaces are tough to destroy and can cause depreciation in the quality of milk and other dairy foods. Due to limited studies involving MNAO on separation membranes in the dairy industry, the study described in this thesis sought to determine practical parameters and uses of MNAO on dairy processing membranes. Although the use of ozone has been studied in several applications, further investigation is needed. This thesis research evaluated the efficiency of MNAO on removal of *Bacillus cereus* and *Bacillus licheniformis* biofilms from PES membranes.

3.10 References

Agarwal, A., and Y. Liu. 2017. Enhanced microbubbles assisted cleaning of diesel contaminated sand. Marine Poll Bull. 124:331-335.

Agarwal, A., W. J. Ng, and Y. Liu. 2011. Principle and applications of microbubble and nanobubble technology for water treatment. Chemosphere. 84:1175-1180.

Agarwal, S., R. L. W. Beausire, S. Patel, and H. Patel. 2015. Innovative uses of milk protein concentrates in product development. J. Food Sci. 80:A23–A29.

Alexopoulos, A., S. Plessas, S. Ceciu, V. Lazar, I. Mantzourani, C. Voidarou, E. Stavropoulou, and E. Bezirtzoglou. 2013. Evaluation of ozone efficacy on the reduction of microbial population of fresh cut lettuce (*Lactuca sativa*) and green bell pepper (*Capsicum annuum*). Food Control. 30:491-496.

Anand, S., A. Hassan, and M. Avadhanula. 2012. The effects of biofilms formed on whey reverse osmosis membranes on the microbial quality of the concentrated product. Int. J. Dairy Technol. 65:451-455.

Anand, S., D. Singh, M. Avadhanula, and S. Marka. 2014. Development and control of bacterial biofilms on dairy processing membranes. Comp. Rev. Food Sci. Food Saf**.** 13:18-33.

Aswathanarayan, J. B., and R. R. Vittal. 2014. Attachment and biofilm formation of *Pseudomonas fluorescens* PSD4 isolated from a dairy processing line. Food Sci. Biotechnol. 23:1903–1910.

Avadhanula, M., A. Biswas, S. Anand, and A. N. Hassan. 2010. Formation of bacterial biofilms on spiral wound reverse osmosis whey concentration membranes and its influence on retentate quality. J. Dairy Sci. 93:77-78.

Bartlett, M., M. R. Bird, and J. A. Howell. 1995. An experimental study for the development of a qualitative membrane cleaning model. J. Membr. Sci. Technol. 105:147-157.

Bauer Nanobubbles. 2016. Retrieved September 28th, 2017 from http://www.nanobubbles.com/

Bremer, P. J., S. Fillery, and A. J. McQuillan. 2006. Laboratory scale Clean-In-Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. Int. J. Food Microbiol. 106:254-262.

Chamberland, J., M. H. Lessard, A. Doyen, S. Labrie, and Y. Pouliot. 2017. Biofouling of ultrafiltration membrane by dairy fluids: Characterization of pioneer colonizer bacteria using a DNA metabarcoding approach. J. Dairy Sci. 100:981-990.

Choi, J., and H. Y. Ng. 2008. Effect of membrane type and material on performance of a submerged membrane bioreactor. Chemosphere. 71:853–859.

Christiansson, A., A. S. Naidu, I. Nilsson, T. Wadström, and H. E. Pettersson. 1989. Toxin production by *Bacillus cereus* dairy isolates in milk at low temperatures. Appl. Environ. Microbiol. 55:2595-600.

D'Souza, N. M., and A. J. Mawson. 2005. Membrane cleaning in the dairy industry: a review. Crit. Rev. Food Sci. Nutr. 45:125–134.

Daufin, G., and U. Merin. 1995. Fouling of inorganic membranes in filtration processes of dairy products. Int. Dairy Fed. SI 9504 (Fouling and cleaning in pressure driven membrane Processes). 53-70.

Daufin, G., J. P. Escudier, H. Carrère, S. Bérot, L. Fillaudeau, and M. Decloux. 2001. Recent and emerging applications of membrane processes in the food and dairy industry. Food Bioprod Process. 79:89-102.

Demirci, A., and M. Ngadi, ed. 2012. Microbial decontamination in the food industry: Novel methods and applications. Woodhead Publishing Series in Food Science, Technology and Nutrition, Cambridge, UK.

Dhakal, R., K. Chauhan, R. B. Seale, H. C. Deeth, C. J. Pillidge, I. B. Powell, H. Craven, and M. S. Turner. 2013. Genotyping of dairy *Bacillus licheniformis* isolates by high resolution melt analysis of multiple variable number tandem repeat loci. J. Food Microbiol. 34:344-351.

Dhillon, B., D. Wiesenborn, C. Wolf-Hall, and F. Manthey. 2009. Development and evaluation of an ozonated water system for antimicrobial treatment of durum wheat. J. Food Sci. 74:E396-E403.

Fazel, M., and S. Chesters. 2014. RO membrane cleaning using microbubbles at 6,800 m^3/d wastewater RO plant in UAE. Desalination and Water Treat. 55:3358-3366.

Fink, R., M. Oder, E. Stražar, and S. Filip. 2017. Efficacy of cleaning methods for the removal of *Bacillus cereus* biofilm from polyurethane conveyor belts in bakeries. Food Control. 80:267-272.

Forde, A. 2012. Membrane technology: The whey to go for dairy. Dairy Ind. Int., 77:37.

Fukuzaki, S. 2006. The use of gaseous ozone as a cleaning agent on stainless steel surfaces fouled with bovine protein. Ozone Sci. Eng. 28:303-308.

Fysun, O. C., H. Kern, B. Wilke, and H. Langowski. 2019. Evaluation of factors influencing dairy biofilm formation in filling hoses of food-processing equipment. Food Bioprod Process. 113:39-48.

Garem, A., and R. Jeantet, 1995. Fouling occurring in nanofiltration of dairy products. Int. Dairy Fed. S.I. 9504:71–79.

Goode, K. R., K. Asteriadou, P. T. Robbins, and P. J. Fryer. 2013. Fouling and cleaning studies in the food and beverage industry classified by cleaning type. Comp. Rev. Food Sci. and Food Saf. 12:121–143.

Greene, A. K., B. K. Few, and J. C. Serafini. 1993. A comparison of ozonation and chlorination for the disinfection of stainless steel surfaces. J. Dairy Sci. 76:3617-3620.

Hassan, A., and Aand Sanjeev. 2012. Biofilm formation on dairy separation membranes as affected by the substrate and cheese starter. Midwest dairy association. 1-12.

Hausmann, A., P. Sanciolo, T. Vasiljevic, M. Weeks, K. Schroën, S. Gray, and M. Duke. 2013. Fouling mechanisms of dairy streams during membrane distillation. J. Membr. Sci. Technol. 441:102–111.

Henning, D. R., R. J. Baer, A. N. Hassan, and R. Dave. 2006. Major advances in concentrated and dry milk products, cheese, and milk fat-based spreads. J. Dairy Sci. 89:1179-1188.

Herzberg, M., S. Kang, and M. Elimelech. 2009. Role of extracellular polymeric substances (EPS) in biofouling of reverse osmosis membranes. Environ. Sci. Technol. 43:4393-4398.

Hu, K., and J. M. Dickson. 2015. Membrane processing for dairy ingredient separation. IFT Press. John Wiley & Sons, Incorporated, Hoboken, NY.

Huttenlochner, K., C. Müller-Renno, C. Ziegler, R. Merz, B. Merz, M. Kopnarski, J. Chodorski, C. Schlegel, and R. Ulber. 2017. Removing biofilms from stainless steel without changing surface properties relevant for bacterial attachment. Biointerphases. 12:02C404.

Iorhemen, O. T., R. A. Hamza, and J. H. Tay. sic. Membrane bioreactor (MBR) technology for wastewater treatment and reclamation: Membrane fouling. Membranes. 6:33.

Jiang, S., J. Wang, J. Wu, H. Zhou, and C. Jiang. 2015. Ultrafiltration performance and fouling resistance of PVB/SPES blend membranes with different degree of sulfonation. J. Mater. Res. 30:2688-2701.

Karunakaran, E., and C. Biggs. 2011. Mechanisms of *Bacillus cereus* biofilm formation: An investigation of the physicochemical characteristics of cell surfaces and extracellular proteins. Appl. Microbiol. Biotechnol. 89:1161-1175.

Katzenelson, E., B. Kletter and H. I. Shuval. 1974. Inactivation kinetics of viruses and bacteria in water by use of ozone. J. Am. Water Works Assoc. 725-729.

Kent, D. J., K. Chauhan, K. J. Boor, M. Wiedmann, and N. H. Martin. 2016. Spore test parameters matter: Mesophilic and thermophilic spore counts detected in raw milk and dairy powders differ significantly by test method. J. Dairy Sci. 99:5180-5191.

Kim, J. 1998. Ozone as an antimicrobial agent in minimally processed foods. PhD Dissertation. The Ohio State Univ., Columbus.

Kotiranta, A., K. Lounatmaa, and M. Haapasalo. 2000. Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes Infect. 2:189-198.

Kumar, P., N. Sharma, R. Ranjan, S. Kumar, Z. F. Bhat, and D. K. Jeong. 2013. Perspective of membrane technology in dairy industry: A review. Asian-Australas J. Anim Sci. 26:1347-1358.

Kumari, S., and P. K. Sarkar. 2016. *Bacillus cereus* hazard and control in industrial dairy processing environment. Food Control. 69:20-29.

Li, H., and V. Chen. 2015. Membrane fouling and cleaning in food and bioprocessing. Chapter 10. Membrane Technology. 213-254.

Thomas, A., and C. T. Sathian. 2014. Cleaning-In-Place (CIP) system in dairy plant – review. IOSR J. of Envir. Sci., Toxic. and Food Tech. 41-44.

Ma, H., L. F. Hakim, C. N. Bowman, and R. H. Davis. 2001. Factors affecting membrane fouling reduction by surface modification and backpulsing. J. of Membrane Sci. 189:255-70.

Malmrose, P., J. Lozier, J. Marie, M. Mickley, R. Reiss, J. Russell, J. Schaefer, S. Sethi, and J. Worley. 2003. Committee report: residuals management for low-pressure membranes. J. Am. Water Works Assoc. 95:68-82.

Marino, M., M. Maifreni, A. Baggio, and N. Innocente. 2018. Inactivation of foodborne bacteria biofilms by aqueous and gaseous ozone. Front. in Micro. 9:2024.

Morris., J. G., and M. Potter, ed. 2013. Foodborne infections and intoxications. 4th ed. Academic Press, London, UK.

Neff, J. 1998. New disinfectant from out of the blue: Ozone makes a pitch to displace chlorine as the disinfectant of choice in food processing. Food Processing, 59:135.

Pagedar, A., and J. Singh. 2011. Influence of physiological cell stages on biofilm formation by *Bacillus cereus* of dairy origin. Int. Dairy J. 23:30-35.

Patil, S., and P. Bourke. 2012. Ozone processing of fluid foods. 225–261. Novel Thermal and NonThermal Technologies for Fluid Foods. P. J. Cullen, B. K. Tiwari, and V. P. Valdramidis, ed. Academic Press, London, UK.

Peng, J., W. Tsai, and C. Chou. 2001. Surface characteristics of *Bacillus cereus* and its adhesion to stainless steel. Int. J. Food Microbiol. 65:105-111.

Popović, S., M. Djurić, S. Milanović, M. N. Tekić, and N. Lukić. 2010. Application of an ultrasound field in chemical cleaning of ceramic tubular membrane fouled with whey proteins. J. Food Eng. 101:296-302.

Pouliot, Y. 2008. Membrane processes in dairy technology-from a simple idea to worldwide panacea. Int. Dairy J. 18:735-740.

Psoch, C., and S. Schiewer. 2006. Resistance analysis for enhanced wastewater membrane filtration. J. Membr Sci. 280:284-297.

Renhe, I. R. T., and M., Corredig. 2018. Effect of partial whey protein depletion during membrane filtration on thermal stability of milk concentrates. J. Dairy Sci, 101:8757-8766.

Sahu, J., ed. 2014. Introduction to advanced food process engineering. 1st ed. CRC Press, Boca Raton, FL.

Saleh-Lakha, S., C. G. Leon-Velarde, S. Chen, S. Lee, K. Shannon, M. Fabri, G. Downing, and B. Keown. 2017. A study to assess the numbers and prevalence of *Bacillus cereus* and its toxins in pasteurized fluid milk. J. Food Prot. 80:1085-1089.

Salkinoja-Salonen, M. S., R. Vuorio, M. A. Andersson., P. Kampfer, M. C. Andersson, T. Honkanen-Buzalski, and A. C. Scoging. 1999. Toxigenic strains of *Bacillus licheniformis* related to food poisoning. Appl. Environ. Microbiol. 65:4637-45.

Saxena, A. P., B. K. Tripathi, M. Kumar, and V. Shahi. 2009. Membrane-based techniques for the separation and purification of proteins: An overview. Adv. Colloid Interface Sci. 145:1-22.

Stocia, I., E. Vitzilaiou, H. L., Roder, M. Burmolle, D. Thaysen, S. Knochel, and F. Van Den Berg. 2018. Biofouling on RO-membranes used for water recovery in the dairy industry. J. of Water Process Eng. 24:1-10.

Sychantha, D. 2014. Crystallization of Peptidoglycan *O*-acetyltransferase B from *Bacillus cereus* 10987. MS Thesis. Univ. of Guelph, Guelph, ON.

Tang, X., S. H. Flint, R. J. Bennett, and J. D. Brooks. 2010. The efficacy of different cleaners and sanitizers in cleaning biofilms on UF membranes used in the dairy industry. J. Membr Sci. 352:71-75.

Teh, K. H., S. Flint, J. Brooks, and G. Knight, ed. 2015. Biofilms in the dairy industry. Wiley-Blackwell, Chichester, UK.

Tsuge, H., ed. 2014. Micro- and nanobubbles: Fundamentals and applications. CRC Press, Boca Raton, FL.

Turan, M., A. Ates, and B. Inanc. 2002. Fouling of reverse osmosis and nanofiltration membranes by dairy industry effluents. Water Sci. Technol. 45:355–360.

Van Hekken, D., and V. Holsinger. 2000. Use of cold microfiltration to produce unique β-casein enriched milk gels. Lait. 80:69-76.

Varga, L., and J. Szigeti. 2016. Use of ozone in the dairy industry: a review. Int. J. Dairy Tech. 69:157-168.

Walstra, P., J. T. M. Wouters, and T. J. Geurts, ed. 2005. Dairy Science and Technology. 2nd ed. CRC Press, Boca Raton, FL.

Yamamura, H., K. Okimoto, K. Kimura, and Y. Watanabe. 2014. Hydrophilic fraction of natural organic matter causing irreversible fouling of microfiltration and ultrafiltration membranes. Water Research, 54:123-136.

Zhang, L., Z. Lu, Z. Yu, and Z. Gao. 2005. Preservation of fresh-cut celery by treatment of ozonated water. Food Control. 16:279-283.

Chapter 4 - Use of Microscale/Nanoscale Aqueous Ozone in the Removal of *Bacillus cereus* and *Bacillus licheniformis* Biofilms from Polyethersulfone Membranes in the Dairy Industry

4.1 Introduction

Fouling of membranes is a major operational problem for the dairy industry. Fouling occurs due to the accumulation of rejected solutes and particles on the membrane's surface (Tan et al., 2014) and can narrow the internal pores, causing a reduction in membrane performance. This leads to an early replacement of membranes (Anand et al., 2014). Membrane fouling can lead to an increased accumulation of undesirable bacteria and other fouling materials in pores of the membrane and consequently, can compromise the quality of milk and other dairy products (Tan et al., 2014). Dreszer et al. (2013) found that biofilms interfere with the performance of membranes by increasing hydraulic resistance, feed channel pressure, and transmembrane pressure by acting as a secondary membrane. Membrane cleaning was described by Hu and Dickson (2015) as the removal of fouling and biofilm material from the membranes prior to sanitization to reduce the microbial levels on the membrane to an acceptable level. *Corynebacterium*, *Micrococcus*, *Escherichia coli*, *Chryseobacterium*, *Streptococcus*, *Klebsiella*, *Aeromonas*, *Pseudomonas*, and *Bacillus* isolates were found to be the most resistant microflora against cleaning agents (Anand et al., 2014). Chemical and mechanical cleaning is often required to remove fouling and biofouling accumulated on the membrane during processing. Anand et al. (2014) reported that *Enterococcus*, *Staphylococcus*, *Klebsiella*, *Escherichia*, *Corynebacterium*, *Pseudomonas*, *Bacillus*, *Micrococcus*, *Streptococcus*, and *Aeromonas* were associated with biofilms found on reverse osmosis (RO) membranes used in the dairy industry. In addition to public health concerns and product spoilage, biofilms are responsible for the impedance of heat transfer processes, mechanical blockages, and increased corrosion rate of surfaces (Kumar and

Anand, 1998, lewski and Frank, 2003). In the dairy industry, ultrafiltration (UF) and RO systems are used during fractionation of milk and other liquids. The membrane filters allow microbial attachment on food contact areas blocking the pores, causing silting of the filter. Flow reduction is seen with decreased capacity and product loss. Effective cleaning mechanisms and procedures are necessary to prevent costly damages because of biofilms in the industry (Silagyi, 2007). The most found bacteria in dairy farms and processing plants come from the genus *Bacillus* (Ben-Ishay et al., 2017). *Bacillus licheniformis* and *Bacillus cereus* are common contaminants known to originate from raw milk but propagate as biofilm on manufacturing plant surfaces. These species can thrive on dairy equipment and in dairy products because of their ability to form robust biofilm during growth within milk (Ben-Ishay et al., 2017)

The efficiency of cleaning agents depends on many factors such as cleaning solution pH, pressure, optimum cleaning time, cleaning solution temperature, and crossflow velocity during cleaning (Anand et al., 2014). Tang et al. (2010) described a standard dairy clean-in-place (CIP) process, which consists of an acid wash followed by a sodium hypochlorite wash at pH 11-12 (200 ppm). This research by Tang et al. (2010) gave insight into the use of enzymes as a complement to cleaning agents when acid and alkaline washes were not sufficient for recovering membrane capacity and cleaning. RO plant operations are financially burdened by an increase in operation and maintenance costs due to fouling and biofouling (Herzberg and Elimelech, 2007). The overall cost of chemicals and equipment continues to rise with the destruction of membranes due to biofouling. With membrane fouling being a significant issue for dairy plants, successful biofilm removal is critically important. Ozone is a potent bactericidal agent used in diverse applications in the food industry.

Ozone is commonly used in aqueous solution with a bubble diameter measuring from millimeters down to nanometers (also called nanobubbles). Microbubbles and nanobubbles are not only small but have distinctive properties. Nanobubbles have longer suspension in solution due to their high gas solubility into liquid, increased internal pressure, and negatively charged surface area (Ebina et al., 2013). The half-life of Microscale/nanoscale aqueous ozone (MNAO) is important to consider for application. The definition of half-life is the amount of time it takes to reduce the amount of substance by half of its initial value. (McClurkin et al., 2013). Essentially, a longer half-life increases overall time high concentration levels of MNAO are available for use during microbial disinfection without recirculation. Different pH and temperature combinations effect the overall half-life of MNAO. At room temperature, ozone decomposes rapidly, and thus does not accumulate substantially without continual ozone generation and recirculation. At point of use there is a longer half-life of ozone and MNAO has an advantage over traditional aqueous ozone. MNAO bubbles are smaller in diameter and overall contact surface area is greater than traditional bubbles. Characteristics include small bubble size, decreased friction drag, and slow rising velocity (Tsuge, 2014). Combining gaseous ozone and nano-bubble technology could potentially be beneficial as both have shown to have antimicrobial effects (Coll Cardenas et al., 2011, Shah et al., 2009).

MNAO bubble technology is commonly used in food processing and wastewater disposal. The incorporation of MNAO into the dairy membrane cleaning process could aid in disinfection and removal of biofilms. There is limited research examining the removal of *Bacillus cereus* and *Bacillus licheniformis* biofilms on separation membranes in the dairy industry. Ozone has been found to be effective against a wide spectrum of microorganisms. In a study by Kim and Yousef (2000), a *L. mesenteroides* cell suspension 10^7 CFU/ml was exposed to

0.3, 1.1, and 2.1 ppm aqueous ozone. After 30 sec of exposure, the counts were 2.2, <1, and <1 CFU/ml, respectfully. Kim and Yousef (2000) also found that inactivation of this bacteria species is rapid and continues until all bacteria is eliminated or residual ozone becomes undetectable. Yesil et al., (2017) studied spinach leaves which were inoculated with *Escherichia coli* O157:H7 at approximate initial populations of 10^8 CFU/g, 10^7 CFU/g, and 10^5 CFU/g. Spinach leaves were treated with 1.5 g gaseous ozone per kg of gas mixture. Treated leaves decreased *E. coli* populations by 0.2, 2.1, and 2.8 $\log_{10}$ respectively, compared to the untreated control.

Incorporating MNAO into a typical CIP system may aid in the removal of these specific biofilms. The purpose of this study was to 1) determine the effect of pH (2, 4, and 7) and temperature (5, 10, and 20°C) of water on the half-life of MNAO and 2) evaluate MNAO efficacy on the removal of *Bacillus cereus* ATCC10987 and *Bacillus licheniformis* ATCC6634 from PES dairy membranes.

4.2 Materials and Methods

Experimental Design

A commercially available ozone generator system supplied by Tetra CleanCore Technologies LLC (Omaha, NE) was used to generate MNAO. This study was completed in 2 phases. In phase 1 bubble size distribution of MNAO was assessed. Optimum conditions of generated MNAO were evaluated to find the maximum half-life of MNAO using pH and temperature as the treatment structure. Polyethersulfone (PES) membranes were fouled during ultrafiltration (UF) of skim milk (SM) and were inoculated with *Bacillus licheniformis* ATCC6634 or *Bacillus cereus* ATCC10987 to promote biofilm formation.

The efficacy of MNAO was studied in 2 phases. Phase 1 evaluated MNAO system in terms of bubble size and half-life. Phase 1A studied ozone bubble size and Phase 1B evaluated the half-life of MNAO.

Findings from Phase 1 were applied to Phase 2. In Phase 2, the efficacy of MNAO was evaluated with a water CIP cycle as a comparison wash treatment. Phase 2A evaluated the efficacy of MNAO and water on bio-fouled PES membranes. Phase 2B evaluated the effectiveness of a CIP+MNAO wash treatment in comparison to a CIP system. Phase 2C evaluated the efficacy of the MNAO+CIP wash treatment in comparison to a CIP system. After each wash treatment further analysis of membranes was completed utilizing scanning electron microscopy (SEM) and confocal laser scanning microscopy. Refer to Table 4.1 for sequence of wash treatment, replications per treatment, and total sampling for Phase 2.

Statistical Analysis Overview

Statistical analysis for all experimentation was analyzed using SAS software version 9.4 (SAS Institute Inc., 2016). All statistics were completed and analyzed by the Department of Statistics at Kansas State University. *Bacillus cereus* and *Bacillus licheniformis* biofilms were evaluated after each cleaning treatment. Data were evaluated using a randomized complete block design (RCBD) with a split plot factor. If side found to be significant, data was analyzed using a RCBD with subsampling.

Table 4.1 Experimental design setup for Phase 2. This table lists the treatment, replications, and total membranes used in each subphase for *Bacillus cereus* ATCC10987 and *Bacillus licheniformis* ATCC6634 biofilm removal on PES membranes.

Bacteria	Phase	Wash Treatment	Membranes Per Treatment	Replications	Total Membranes
***Bacillus cereus* ATCC10987**	2A	MNAO	2	3	6
		Water	2	3	6
		Control*	2	3	6
	2B	CIP	2	3	6
		CIP+MNAO	2	3	6
		Control*	2	3	6
	2C	CIP	2	3	6
		MNAO+CIP	2	3	6
		Control*	2	3	6
***Bacillus licheniformis* ATCC6634**	2A	MNAO	2	3	6
		Water	2	3	6
		Control*	2	3	6
	2B	CIP	2	3	6
		CIP+MNAO	2	3	6
		Control*	2	3	6
	2C	CIP	2	3	6
		MNAO+CIP	2	3	6
		Control*	2	3	6

Control*: Non-treated membrane

4.3 Phase 1: Evaluation of MNAO system in terms of bubble size and half-life

A MNAO generator system (Figure 4.1) supplied by Tetra CleanCore Technologies Inc., (Omaha, NE) comprised of six ozone generators was used to generate the MNAO used in this experimentation in Phase 1A. The MNAO system consisted of a 19 L stainless steel reservoir to which approximately 9.5 L of distilled water was added. The distilled water was pumped using a diaphragm pump (NF1.300KT.18S, KNF; Trenton, NJ) through an attached liquid-gas mixing

device (injector) to uniformly disperse ozone gas into the water. The oxygen concentrator supplied concentrated oxygen to the ozone generators only when water was flowing through the system. Ozone and water mixture from the injector pumped through a specially designed reactor (U.S. Patent No. 8071526) to produce MNAO. This system can be operated in a continuous or recirculation mode and was set to recirculate for the current study. Ozone concentration and oxidation reduction potential (ORP) were monitored by an inline dissolved ozone monitor (Q45H/64; Analytical Technology Inc., Collegeville, PA) and an ORP monitor (BL-982411; Hanna Instruments, Leighton Buzzard, UK), respectively and calibrated as suggested by the manufacturers.

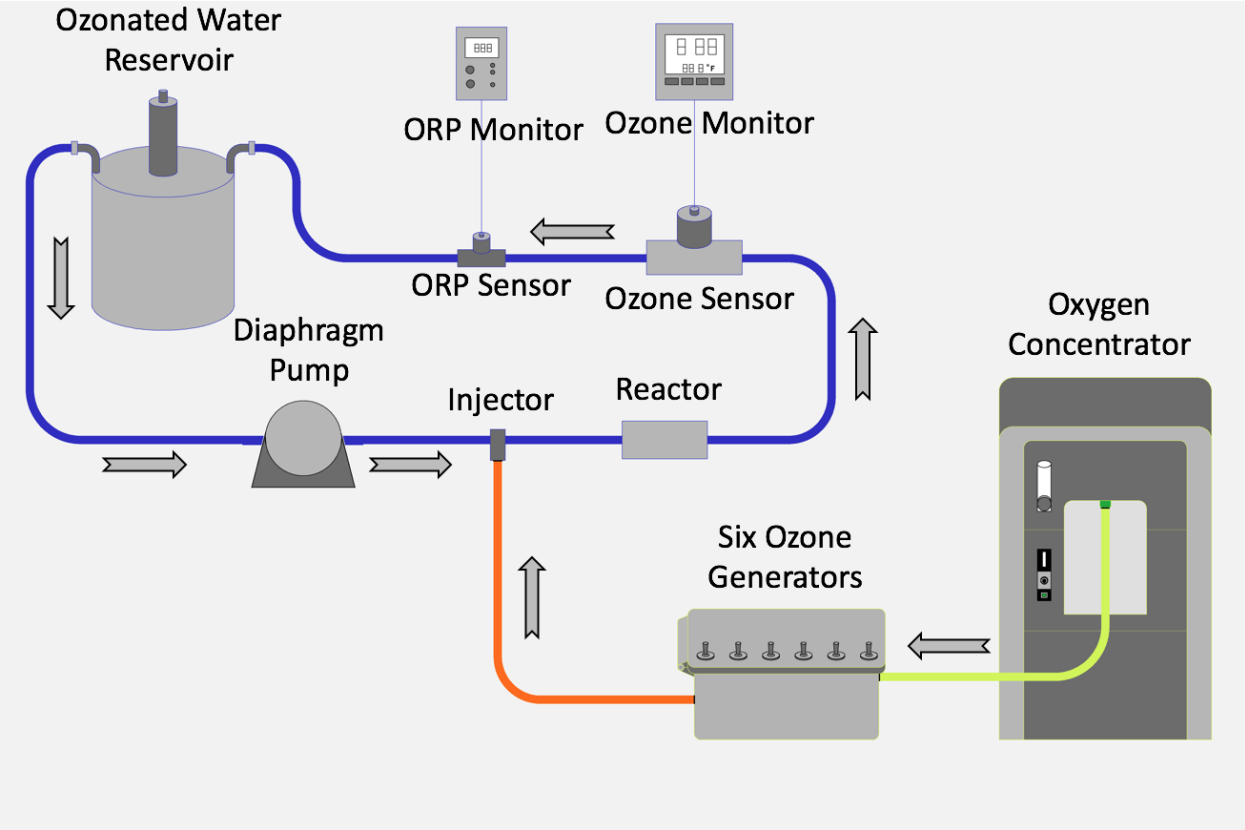

Figure 4.1 Aqueous ozone generator system provided by Tetra CleanCore Technologies Inc. (Omaha, NE) and set up for producing concentrated aqueous ozone using ozone generators. This flow chart displays the flow of the aqueous ozone through its closed system. (Green color represents oxygen flowing through the system. Orange color represents gaseous ozone flowing through the system. Blue color represents aqueous ozone flowing through the system.)

Phase 1A: Ozone Bubble Size

Phase 1A investigated ozone bubble size distribution in MNAO. This was evaluated using NanoSight (NS300, Malvern Instruments Ltd., Malvern, UK). Approximately 10 mL of representative sample of MNAO was collected from the MNAO reservoir using a syringe and directly injected into the flow cell of the NanoSight instrument. Measurement conditions such as viscosity of 0.92 cP, drift velocity of 1168 nm/s, and camera shutter speed of 32 ms with 24.99 given frames per second were used to acquire the bubble size distribution. When particles are exposed to laser light, scattered light is emitted from the particles. This spatial scattered light intensity distribution pattern changes in accordance with particle size.

Phase 1B: Half-Life

The half-life of MNAO was studied in Phase 1B of this experimentation, the ozonated water generation system described above was used. Combinations of time and pH required to deplete ozone concentration by 50% were evaluated using the parameters described by Dhillon et al. (2009). Approximately 9.5 L of distilled water was collected and cooled to the desired temperature (5, 10, or 20°C) using an ice water bath. The pH was then adjusted to 2, 4 or 7 using 12.5N sodium hydroxide (NaOH) or 2.7 N hydrochloric acid (HCl). Each combination of pH and temperature was recirculated in the MNAO system for approximately 15 min until an average ozone concentration of 14 ppm using the dissolved ozone meter was reached.

Next MNAO (14ppm) was pumped into a 500 mL glass beaker for half-life analysis. The ozone sensor from the ozone generator system, held tightly in place by screw top lid, was placed into the MNAO water. The container was sealed using an airtight gasket. The sample was then placed carefully into a static water bath (Lauda ecoline RE 307, Germany) to maintain a temperature of (5, 10, 20 °C) as per experimental design. Ozone concentration was recorded at

15 min intervals using a video monitoring device which captured images of the concentration levels over time until final MNAO concentration dropped below 1.0 ppm. First order rate constants (k) were calculated by a first order decay model using Equation 1 (Dhillon et al., 2009). The half-life of ozone ($t_{1/2}$) was then calculated using Equation 2 (Dhillon et al., 2009). A combination of pH and temperature, which produced the smallest k-value with the lowest pH and highest temperature, were chosen with recommendation from the MNAO system manufacturer. Noting a lower temperature and pH would decrease fluctuation in half-life.

$$\frac{d[O_3]}{dt} = -k[O_3] \quad \textbf{(Equation 1)}$$

$$t_{\frac{1}{2}} = \frac{ln(0.5)}{k} \quad \textbf{(Equation 2)}$$

The general experimental design was a completely randomized design. A 2-way factorial of pH (2, 4, and 7) x Temp (5, 10, and 20) served as the treatment structure. Replication of runs for combinations of pH and Temp were specified as the residual error term. Two response variables (k-value and ln half-life) were collected and analyzed using the GLIMMIX procedure within SAS software version 9.4 (SAS Institute Inc., 2016). F-tests were calculated for the two-way interaction of pH x Temp and individual main effects (pH and Temp). Simple (slice) effect comparisons investigated the cause of a present two-way interaction. Due to multiple comparisons, the chance of inflated family-wise error rate was controlled by performing a Tukey's adjustment. All tests used a specified significance level of $\alpha = 0.05$.

4.4 Methodology for Fouling and Biofouling of Polyethersulfone Membranes

Fouling of Polyethersulfone Membranes

PES ultrafiltration membranes (SAMP-SD-40B; Parker Hannifin Corp. Oxnard, CA) with a molecular cut off weight of 10 kDa in a flat sheet configuration were fouled during UF of SM. This was done to simulate real world fouling of PES membranes prior to biofilm development. UF of SM was carried out using a small-scale bench top plate-and-frame unit (SmartFlow Technologies Apex, NC). Unused (virgin) PES membranes were cut into flat sheet pieces with a surface area of 200 cm^2 and were stored in 1% sodium metabisulfite solution for up to 48 hr before use. SM from the Kansas State University Dairy Bar was used to foul each membrane using UF. Approximately 1.5 L of SM was tempered to 20 °C and was maintained at that temperature using a water bath during fouling. A transmembrane pressure of 207 kPa (30 psi) was held during processing. Diafiltration was carried out using deionized water at a rate of 60% of the original feed volume and concentrated to obtain 5x by volume. After concentrating to 5 x, the retentate was removed from the holding tank. Next, 4 L of distilled water at 20 °C was flushed through the closed system to remove loose proteins prior to biofilm formation. Following the water wash, fouled PES membranes were stored under refrigeration at 4 °C.

Biofilm Development on Fouled Polyethersulfone Membranes

The *Bacillus* spp. cultures used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Bacterial stock cultures were preserved on plastic beads in cryogenic vials at approximately -80^{o}C. Vegetative cells of the respective cultures were prepared by transferring the culture from the cryo-vials into Luria-Bertani (LB) Broth (Invitrogen, Waltham, MA) (15 mL) and storing at 37 ^{o}C for 24 hr to allow growth. The vegetative cells were then stored at 4^{o}C for a maximum of one week until further use. The

working culture was prepared by inoculating 500 mL of LB base with 10 μL of *Bacillus* growth and incubated for 48 hr at 37°C for further study of biofilm formation on PES membranes.

PES membranes with a pore size of 10kDa were fouled as previously described in *Fouling of Polyethersulfone Membranes* and inoculated with *Bacillus* spp. for biofilm development as described by Oliveria et al. (2010) with modifications. This method was proposed for stainless steel and it was modified for PES membranes under static conditions. Each membrane was placed into approximately 300 mL of LB inoculated with approximately 7 $\log_{10}$ cfu/mL *Bacillus cereus* or *Bacillus licheniformis*. Submerged membranes were incubated under static conditions for 48 hr at 37°C to promote biofilm formation. All inoculated broth was removed from each membrane dish after 24 hr incubation, and 300 mL of fresh inoculated broth was added to re-suspend the membranes for the remaining 24 hr. After 48 hr, inoculated membranes were removed from the incubator and were fully submerged and agitated for 30 sec three separate times in clean deionized water to remove any loose cells not associated with biofilms.

4.5 Evaluation of Fouling and Biofilm Formation on Polyethersulfone Membranes

Microbiological

During enumeration of PES membranes, each membrane was divided into two sections, (Side A and Side B). Each side was analyzed separately. The location of Side A and Side B were chosen based on the flow of the milk through the plate and frame system. SM entered the membrane system on the upper left corner of the membrane (Side A) and exited on the lower right-hand side of the membrane (Side B). The membrane sample was selected from approximately one-half inch from the top or bottom and edge of the membrane dependent on

sample side. Following cleaning, 6.45 cm^2 section from Side A and a 6.45 cm^2 section from Side B was scraped from top to bottom with one pass using a razor removing surface biofilms from the membrane (Figure 4.2). This method was repeated for each membrane after each wash treatment. Before each scraping, a sterilized template was placed on the membrane to ensure the correct area was sampled. The scraped layer was placed into 10 mL of 0.1% peptone water Difco, Becton Dickinson and Co., France) water and vortexed for 10 seconds. Serial 10-fold dilutions were prepared in sterile 0.1% peptone water and 0.1 mL was spread plated onto Mannitol Egg Yolk Polymyxin (MYP) Agar (Oxoid, Thermo Scientific, UK) (Downes and to, 2001). Plates were incubated at 37°C for 48 hr and colonies representative of *Bacillus* spp. were counted and reported as $\log_{10}$ cfu/cm^2.

Scanning Electron Microscopy

PES membranes were fouled under conditions described in *Fouling of Polyethersulfone Membranes and Biofouling of Polyethersulfone Membranes*. SM fouled membranes and inoculated membranes were analyzed using scanning electron microscopy (SEM). Side A and Side B sample membranes were cut into 1 cm x 1 cm pieces. Each membrane piece was placed on an aluminum stub coated with double-sided, conductive carbon, sticky tape and then sputtered with palladium using a Denton Vacuum Desk II sputter coater (Denton Vacuum, Moorestown, NJ). Membranes were then analyzed under the Hitachi S-3500N Scanning Electron Microscope Hitachi Science Systems Ltd., Tokyo, Japan) at an acceleration of 5-20 kV. Microscopy was performed and analyzed at the Nanotechnology Innovation Center at Kansas State University.

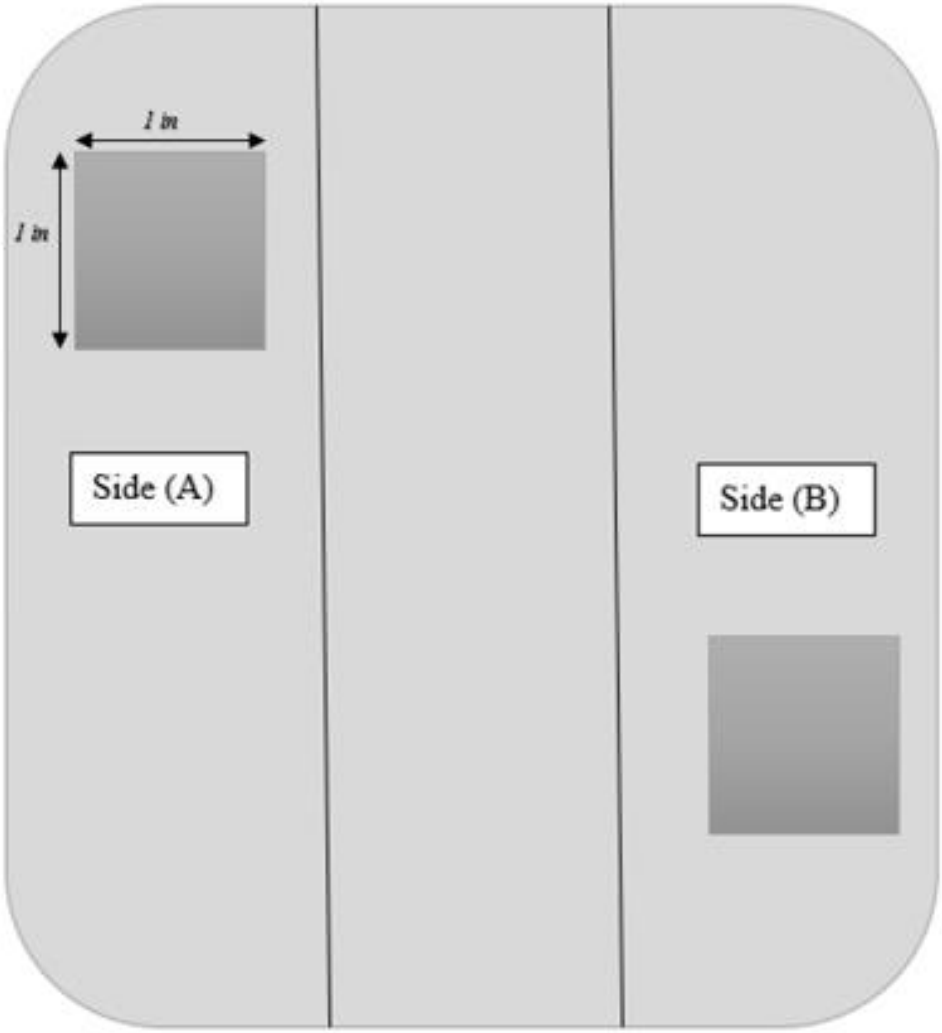

Figure 4.2 Example of membrane size showing Side A and Side B used during evaluation of membrane for biofilm. Above image Approximately 6.45 cm ^{2} (1 in x 1 in) of Side A and Side B were scraped and enumerated.

Confocal Laser Scanning Microscopy

A fluorescence-based optical microscope, Confocal Laser Scanning Electron Microscopy (CLSM), was used to obtain high-resolution optical images. Membrane samples were prepared by fouling PES membranes as described above in *Fouling of Polyethersulfone membranes and Biofouling of Polyethersulfone Membranes.* Lipids, proteins, and calcium were stained according

to Gandhi (2018) and each sample was analyzed in CLSM 5 Pascal (Zeiss, Arlington, VA). A Plain Neofluar 10x/0.3 Multitrack 1 objective was used and was set at 488 nm. All microscopy was performed and analyzed in the Microscopy Facility at Kansas State University.

4.6 Phase 2: Evaluation of efficacy of MNAO and comparisons with Water and CIP cycle

The experimental findings from Phase 1 were analyzed and applied to Phase 2. Experimental setup for Phase 2 was completed as described in Phase 1. SEM and CLSM were used to analyze and evaluate the efficacy of the wash treatments in Phase 2 as described in *Scanning Electron Microscopy and Confocal Laser Scanning Microscopy* sections.

Phase 2A: MNAO and Water Treatment Study

Phase 2A was divided into two treatments and a control. As previously described in *Generating Bacillus spp. Biofilms on PES Membranes*, PES membranes with attached biofilms were randomly separated into two cleaning treatments and a non-treated control (Water, MNAO, and Control. For the two treatments, membranes were installed in the plate and frame system independently. Each cleaning protocol was then performed for both *Bacillus* spp. independently. A bio-fouled membrane sample receiving no sanitizer treatment was also prepared as a non-treated control. After each treatment, all PES membranes were held under refrigerated conditions (4°C) to maintain bacterial populations prior to enumeration. As previously described in *Scanning Electron Microscopy* and *Confocal Laser Scanning Microscopy*, SEM and CLSM were used to analyze and evaluate the efficacy of the treatments in Phase 2.

Table 4.2 Outlines a standard Clean in place (CIP) cleaning protocol followed by MNAO and MNAO followed by a standard CIP cleaning protocol for dairy processing. Chemicals and protocol were provided by Ecolab (Saint Paul, MN). All wash steps were circulated at a flow rate of 3.78 L/min.

CIP-MNAO	Wash Step	Chemicals	Time (min)	Temperature (°C)	pH target
1	Deionized Water pre-flush		2	21	
2	Alkaline recirculation	Ultrasil 110, Ultrasil 101, XY12	6	55	10.8–11.0
3	Deionized water flush		2	21	
4	Acid recirculation	Ultrasil 76	5	55	1.8–2.0
5	Deionized water flush		2	21	
6	MNAO	Aqueous Ozone	6	10	10
7	Deionized water flush		2	21	

MNAO-CIP	Wash Step	Chemicals	Time (min)	Temperature (°C)	pH target
1	Deionized Water pre-flush		2	21	
2	MNAO	Aqueous Ozone	6	10	10
3	Deionized water flush		2	21	
4	Alkaline recirculation	Ultrasil 110, Ultrasil 101, XY12	6	55	10.8–11.0
5	Deionized water flush		2	21	
6	Acid recirculation	Ultrasil 76	5	55	1.8–2.0
7	Deionized water flush		2	21	

The MNAO system reservoir was filled with approximately 9.5 L of distilled water at pH 4 and 10°C as determined using the methods described above in *Phase 1B: Half-Life*. Distilled water was recirculated through the MNAO generator system until desired MNAO concentration of approximately 14 ppm was reached. The reservoir filled with concentrated MNAO was then connected to the plate and frame system and turned on (Figure 4.3). Concentrated MNAO was then recirculated through each membrane for 6 min. Treated membranes were removed and stored under refrigeration at 4°C for up to two hours until enumeration. This cleaning protocol was performed for both *Bacillus* spp. strains.

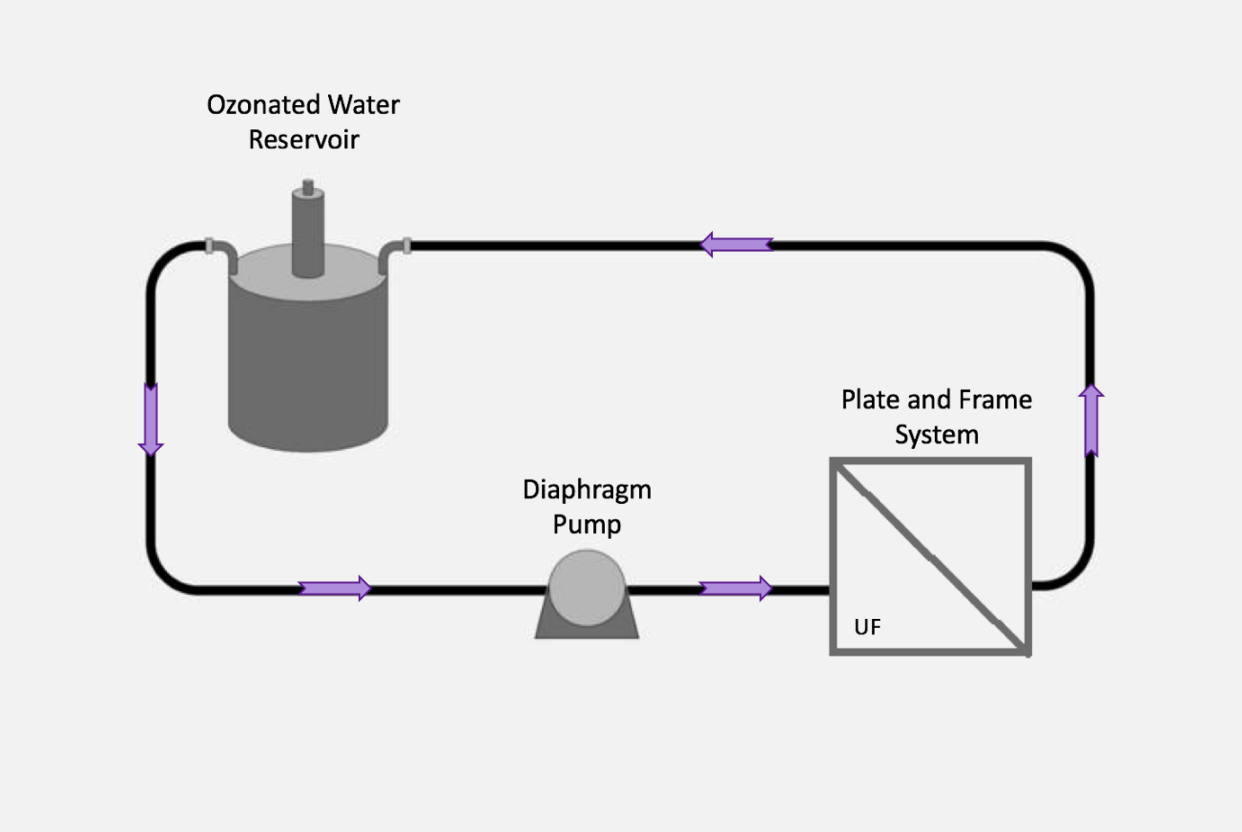

Figure 4.3 Ozone generator system with inclusion of plate and frame system. This system was used for cleaning of membranes with generated aqueous ozone. This figure shows the flow of ozonated water passing out of the water reservoir through the diaphragm pump into the plate and frame system unit. Aqueous ozone water is then pumped back into the water reservoir and the process is repeated until experimentation is complete.

The general experimental design was a randomized complete block design (RCBD) with subsampling. A 1-way factorial of Wash Treatment (Control, Water, and MNAO) served as the treatment structure. Period or recurrence served as blocking factor and Period * Wash Treatment were specified as the whole plot error term. This experimental setup was repeated for *Bacillus cereus* and *Bacillus licheniformis* and was analyzed independently using the MIXED procedure of SAS 9.4. F-tests were calculated for the individual main effect of Wash Treatment. Multiple comparisons utilizing Tukey's adjustments were used to isolate differences between wash treatments. Comparisons utilizing the control treatment served as the reference for calculating log reductions. All tests used a specified significance level of $\alpha = 0.05$

Phase 2B: Clean-In-Place (CIP) + MNAO Treatment Study

Phase 2B was divided into two treatments and a non-treated control (CIP cycle, CIP+MNAO, and Control). A typical Clean In Place (CIP) cycle and a CIP cycle followed by MNAO were used as treatments to evaluate efficacy of biofilm removal from PES membranes. A membrane sample receiving no sanitizer treatment was also prepared as a non-treated control. Time and temperature parameters for the treatment structure are described in Table 4.2. The pH of each solution was adjusted using 12.5 N sodium hydroxide (NaOH) or 2.7 N hydrochloric acid (HCl).

Chemicals provided by Ecolab (St. Paul, MN) were used for each CIP cycle. First bio-fouled membranes were installed into the plate and frame system. Deionized water at room temperature (21 °C) with a flow rate of 3.78 L/min was then pumped through the membrane as an initial rinse step. The alkaline wash was then pumped through the membrane system, followed by a deionized water wash, an acid wash, and concluded with a water wash. Membranes were

then removed from the plate and frame system and stored at 4℃ until enumeration. This treatment was completed for each *Bacillus* spp.

For the CIP+ MNAO treatment, bio-fouled membranes were installed in the plate and frame system. The CIP cycle was circulated through the plate and frame system as previously stated. Following the CIP cycle, MNAO was generated to 14 ppm in the ozone generator system and concentration was confirmed using the methods described above in *Phase 2A MNAO and Water Wash Treatment Study***.** MNAO was then circulated through the membrane for approximately 6 min. Membranes were then removed from the plate and frame system and were stored at 4°C until enumeration. This treatment was completed for each *Bacillus* spp.

In the case of *Bacillus cereus*, side was determined to be significant. This was the only case in the experimental design where side played a factor. The experimental design was a randomized complete block design (RCBD) subsampling. A 1-way factorial of Wash Treatment (Control, Water, and MNAO) served as the treatment structure. Period served as blocking factor and Period*Wash Treatment were specified as the whole plot error term. Subsample of Side nested within Period*Wash Treatment served as residual error term. Samples were collected and analyzed independently using the MIXED procedure. Smaller heterogeneous variance for the CIP+MNAO was accounted for in the model by a repeated statement for the group. F-tests were calculated for the individual main effect (Wash Treatment). Comparisons utilizing the control treatment served as the reference for calculating log reductions. All tests used a specified significance level of $\alpha = 0.05$.

For the *Bacillus licheniformis* study, side was not found to be significant. The general experimental design was a RCBD with split plot. A 2-way factorial of Wash Treatment (Control, Water, and MNAO)*Side (A and B) served as the treatment structure. Period served as blocking

factor and Period * Wash Treatment were specified as the whole plot error term. Side*Period*Wash Treatment served as residual error term. Samples were collected and analyzed independently using the MIXED procedure of SAS 9.4. Smaller heterogeneous variance for the CIP+MNAO was accounted for in the model by a repeated statement *Bacillus licheniformis.* F-tests were calculated for the individual main effects (Wash Treatment and Side) and interaction (Treatment*Side). Multiple comparisons utilizing Tukey's adjustment were used to isolate differences between main effects and interactions. All tests specified significance level of $\alpha = 0.05$.

Phase 2C: MNAO + Clean In Place (CIP) Treatment Study

Phase 2C was divided into two treatments and a non-treated control (CIP, MNAO+CIP, and Control*) A typical CIP cycle and MNAO followed by a CIP cycle were used as treatments to evaluate efficacy of biofilm removal from PES membranes. A membrane sample receiving no sanitizer treatment was prepared as a non-treated control. This cleaning protocol was performed and analyzed separately for both *Bacillus* spp. strains. After each treatment, all PES membranes were held under refrigerated conditions (4°C) prior to enumeration.

Bio-Fouled membranes were installed in the membrane system and a CIP cycle was run as described above in *Phase 2B: Clean-In-Place (CIP) + MNAO Treatment Study***.** Membranes were then removed from the plate and frame system and were stored at 4°C until enumeration. This treatment was completed for each *Bacillus* spp.

Next untreated bio-fouled membranes were installed in the membrane system. A MNAO treatment was circulated through membrane as described in *Phase 2B: Clean-In-Place (CIP)+MNAO Treatment Study***.** Following the MNAO treatment, a CIP cycle was applied to the

membrane. Membranes were then removed from the plate and frame system and stored at 4°C prior to enumeration.

The experimental design was a RCBD with a split plot. A 2-way factorial of Wash Treatment (Control, Water, and MNAO)*Side (A and B) served as the treatment structure. Period served as blocking factor and Period*Wash Treatment were specified as the whole plot error term. Side*Period*Wash Treatment served as residual error term. This experimental setup was repeated for *Bacillus cereus* and *Bacillus licheniformis* and was analyzed independently using the MIXED procedure of SAS 9.4. F-tests were calculated for the individual main effects (Wash Treatment and Side) and interaction (Wash treatment*Side). Multiple comparisons utilizing Tukey's adjustment were used to isolate differences between main effects and interactions. All tests used a specified significance level of $\alpha = 0.05$.

4.7 Experimental Results

Phase 1A: Ozone Bubble Size

Using distilled water (20°C), the maximum MNAO concentration generated was observed to be approximately 19 ppm and was achieved in 15 min with recirculation of MNAO in the closed system. Advantages of the ozone generator system included automated and continuous monitoring of dissolved MNAO concentration while running the system. The average bubble size in MNAO was observed to be 212 nm ± 87 nm at 23.5°C, with bubble size ranging from approximately 45-550 nm (Figure 4.4). The overall size of the bubble is dependent on supplied gas pressure and energy provided to the system (Meegoda et al., 2018). The bubble concentration was observed to be 1.59×10^8 particles/mL. Figure 4.4 shows distribution of bubbles plotted.

Phase 1B: Half-Life

The main effects of pH and temperature were significant ($p \leq 0.05$) and the two-way interaction between pH and temperature was also significant ($p \leq 0.05$), which indicates that the overall concentration of MNAO varied depending on pH and temperature factors. The highest half-life of MNAO was calculated to be 435 min at pH 7 and 10°C, with an average decay constant of 0.001 ppm/min. Further investigation, through aid of simple effect analysis, revealed the cause of the interaction in this case, the combination of pH 7 at 10°C, has a strong effect on half-life. Meanwhile, the lowest half-life of MNAO was observed for pH 7 at 20°C and was calculated to be 50 min with an average decay constant of 0.014 ppm/min. Table 4.3 shows average k-values and average half-life of MNAO at different temperature and pH combinations, which were observed in the present study.

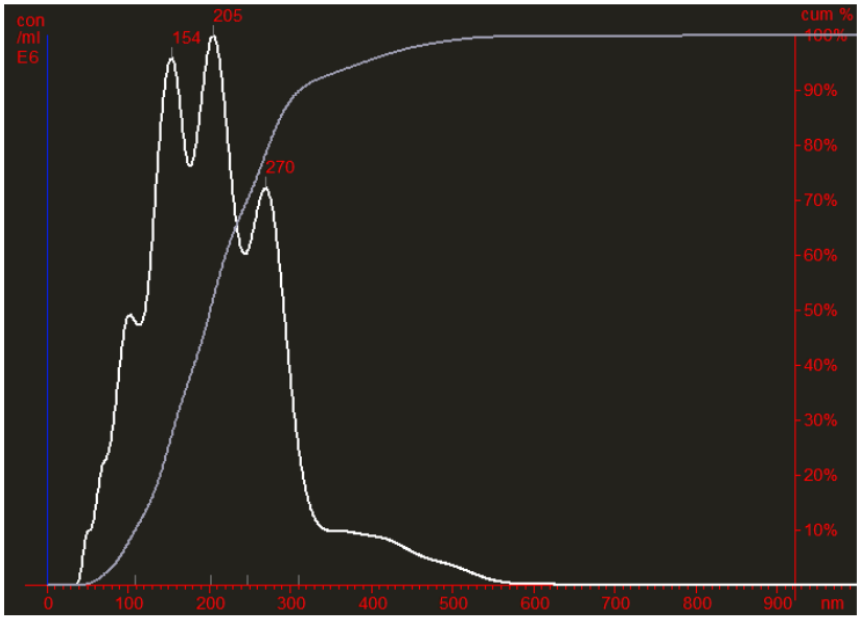

Figure 4.4 Particle size distribution of MNAO sample. Mean size = 212 nm. Mode size and concentration = 205 nm, 1.59×10^8 particles/mL, respectively.

Table 4.3 Half-life study results of microscale/nanoscale aqueous ozone (MNAO) generator system. Average half-life affected by temperature and pH was calculated using ozone decay constant (k) (Equation 1). Half-life of ozone ($t_{1/2}$) was calculated using Equation 2.

pH	Temperature (ºC)	Average k-Value (ppm/min) ± standard deviation	Average half-life (min) ± standard deviation
2	5	$0.0020 \pm 2.1x10^{-4\ c}$	358 ± 39^{a}
	10	$0.0027 \pm 1.4x10^{-4\ bc}$	257 ± 14^{ab}
	20	$0.0041 \pm 4.2\ x10^{-4\ b}$	170 ± 18^{bc}
4	5	$0.0027 \pm 4.2\ x10^{-4\ bc}$	260 ± 41^{ab}
	10	$0.0018 \pm 2.8\ x10^{-4\ c}$	390 ± 61^{a}
	20	$0.0026 \pm 7.7\ x10^{-4\ c}$	269 ± 65^{ab}
7	5	$0.0021 \pm 4.9\ x10^{-4\ c}$	348 ± 84^{ab}
	10	$0.0016 \pm 1.4\ x10^{-4\ c}$	435 ± 38^{a}
	20	0.0140 ± 0^{a}	50 ± 1^{c}

*Means (n=3) with different superscripts within each pH or temperature in each column vary statistically ($p \leq 0.05$)

Conformation of Fouling and Bio-Fouling of Polyethersulfone Membranes

To verify fouling, SM-fouled PES membranes were analyzed using confocal microscopy analysis (CSLM). Microstructure of the fouling layer was determined (Figure 4.5). A non-uniform distribution of proteins (stained green) was observed. Lipids (stained red) were more uniformly distributed in comparison to proteins. The blue calcium stain was not as prominent in color compared to the other two stains.

Using Scanning Electron Microscopy, images show non-uniform thickness throughout each fouling structure. SEM imaging of PES membrane fouling are illustrated in Figure 4.6, where image (A) shows a virgin unused membrane at 2.0k magnification with no fouling. The

membrane has a smooth surface and no detectable attached cells. Figure 4.6 (B) image displays a 45º tilt showing edges of fouled PES membrane. This image shows a cross section of the membrane enhancing the buildup of the fouled layer at 350 x magnification. Figure 4.6 (C) image and Figure 4.6 (D) image show a top view of fouled PES membranes. Figure 4.6 (C) image at 3.0k magnification and Figure 4.6 (D) image at 2.0 k magnification. This imaging confirmed the SM UF produced a fouling layer.

Biofilm growth of *Bacillus* spp. was observed from the topography and side view, which displayed a heterogeneous fouling structure throughout the used membrane and illustrated fouling (Figure 4.6). After the foulant layer was visualized, *Bacillus* spp. SEM images were captured at 24, 48, and 72 hr of incubation at 3.0 k and 5.0 k magnification to demonstrate biofilm formation. (Figure 4.7 and 4.8).

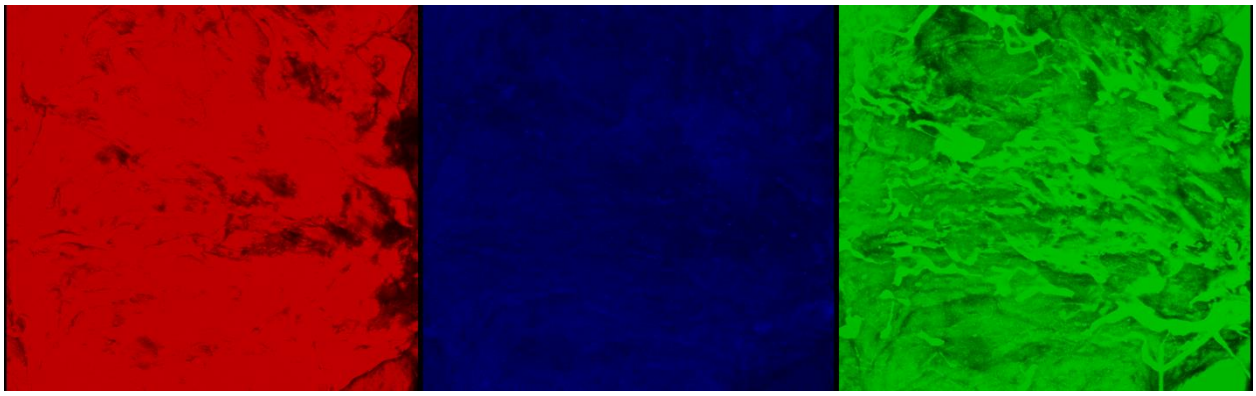

Figure 4.5 CLSM imaging of fouled PES membrane. Images show 3-D projection of Z-Series through microstructure of fouling film. Lipids are stained in red, calcium in blue, and proteins shown in green.

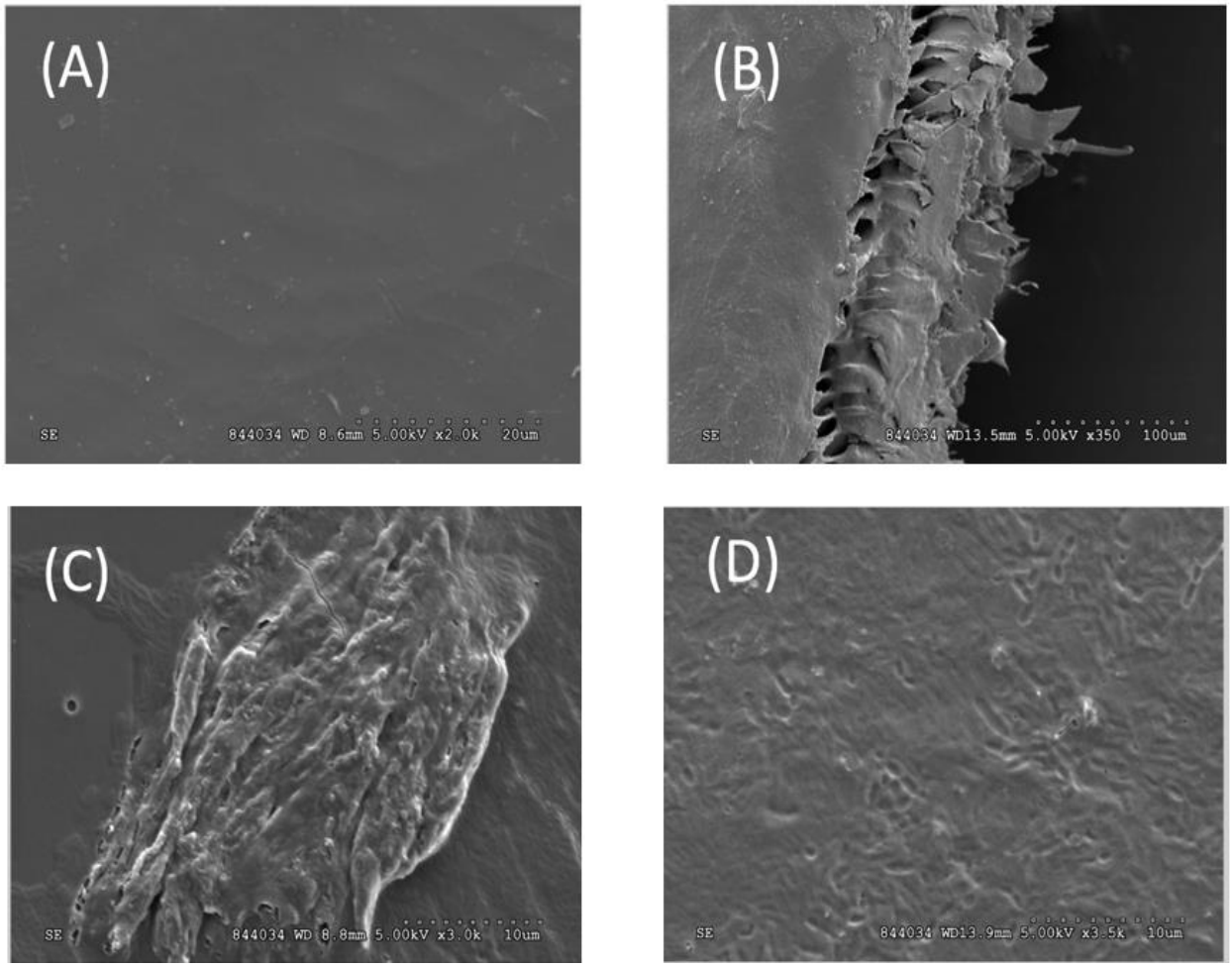

Figure 4.6 SEM imaging of PES membrane fouling. (A) Virgin unused membrane with no fouling at 2.0 k magnification (B) Image at 45º tilt showing edges of fouled PES membrane at 350 magnification. (C)(D) Images showing top view of fouled PES membrane (C) at 3.0 k magnification, (D) at 2.0 k magnification.

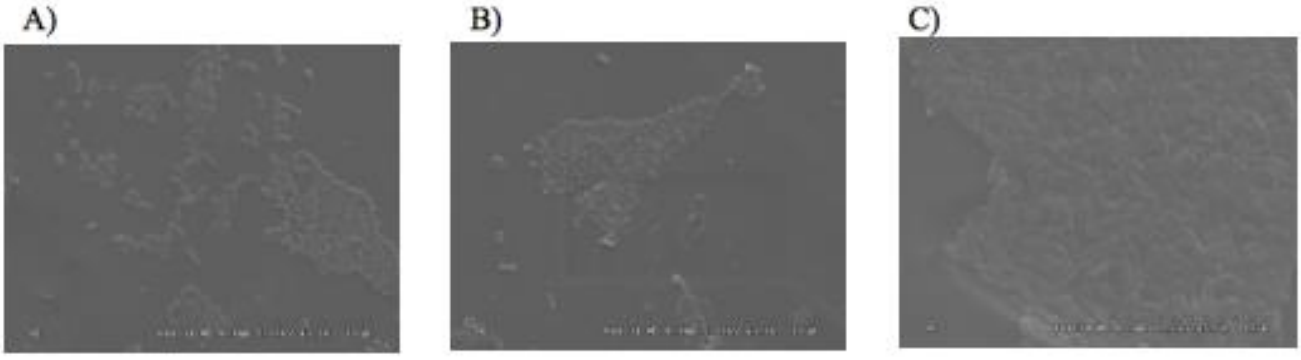

Figure 4.7 SEM micrographs with observed biofilm formation of *Bacillus cereus* ATCC10987 on PES membranes after 48 hr incubation at 37°C (A) and (B) illustrates *Bacillus cereus* biofilm formation at 3.0k magnification. Image (C) confirms *Bacillus cereus* biofilm formation at 5.0k magnification.

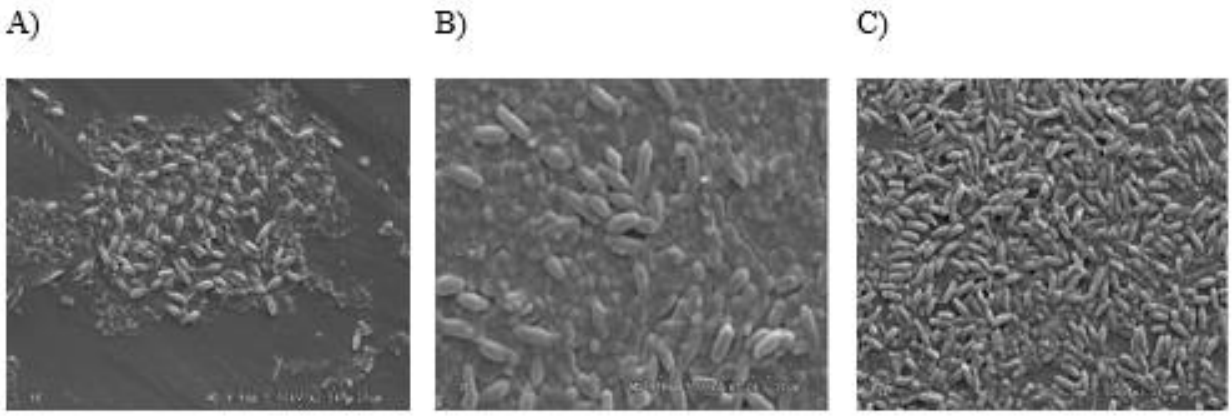

Figure 4.8 SEM micrographs illustrating biofilm formation of *Bacillus licheniformis* ATCC6634 on PES membranes at (A) 24 hr (2.0 k magnification), (B) 48 hr (5.0 k magnification), and (C) 72 hr (3.0 k magnification) incubation times

Phase 2A: MNAO and Water Treatment Study

All MNAO and water wash treatments were completed at an average ozone concentration of 14±0.69 ppm, and at a pH of 4, 10°C. *Bacillus cereus* and *Bacillus licheniformis* populations recovered from membranes after treatment were compared to the inoculated, but untreated, control membranes to quantify average $\log_{10}$ cfu/cm^2 reductions resulting from each treatment (Table 4.4).

Table 4.4 Effectiveness of MNAO (pH 4 at 10°C) versus water treatment for reducing *Bacillus* spp. populations on bio-fouled PES membranes.

Bacteria Strain	Treatment	Average $\log_{10}$ cfu/cm^2 ± standard error recovered from membrane surface	Average $\log_{10}$ cfu/cm^2 reduction
Bacillus cereus ATCC10987	Control	6.4±0.3[a]	~
	Water	5.2±0.3[b]	1.2
	MNAO	4.5±0.3[c]	1.9
Bacillus licheniformis ATCC6634	Control	6.9±0.4[a]	~
	Water	5.0±0.4[b]	1.9
	MNAO	4.6±0.4[c]	2.3

* Means (n=3) with different superscripts within a bacteria strain are different ($p \leq 0.05$).
*Control: Non-treated membrane. Average $\log_{10}$ cfu/cm^2 on non-treated control membranes minus average $\log_{10}$ cfu/cm^2 count on treated membrane.

For *B. cereus* inoculated membranes, both treatments reduced the population compared to the untreated control ($p \leq 0.05$). The MNAO treatment was more effective ($p \leq 0.05$) than the water wash treatment, with reductions of 1.9 and 1.2 $\log_{10}$ cfu/cm^2, respectively, when compared to untreated control membranes that harbored 6.4 $\log_{10}$ cfu/cm^2 of attached *B. cereus.* In this study MNAO and water treatment were significantly different. MNAO and the water treatment were also significantly different from the control sample.

Findings for *B. licheniformis* were comparable to *B. cereus*, with the *B. licheniformis* untreated control harboring 6.9 $\log_{10}$ cfu/cm^2. The MNAO membrane wash reduced *B. licheniformis* counts on membranes by 2.3 log cycles, while the water wash reduced the

population level by 1.9 log cycles. In this study MNAO and water treatment were significantly different from each other and the control sample.

Phase 2B: Clean-In-Place (CIP)+MNAO Treatment Study

Table 4.5 summarizes the effectiveness of including a MNAO wash as a final step in addition to a standard CIP cycle cleaning protocol for reducing populations of *Bacillus* spp. contained in biofilms on PES membranes. Untreated control membranes harbored 5.6 and 5.7 $\log_{10}$ cfu/cm^2 of *B. cereus* and *B. licheniformis*, respectively, within the developed biofilm.

Treatment of *B. cereus* membrane biofilms using only the standard CIP cycle, or the CIP cycle followed by the MNAO wash (CIP+MNAO) resulted in significant ($p \leq 0.05$) population reductions of 3.8-4.1 $\log_{10}$ cfu/cm^2, in comparison to the control membrane. A significant difference was observed when comparing Side A and Side B ($p \leq 0.05$). This was the only instance in experimentation where side played a factor and was treated as a subsample. When comparing the CIP and CIP+MNAO treatments, the samples were not significantly different.

For *B. licheniformis* containing biofilms, both treatments provided significant population reductions ($p \leq 0.05$) compared to the untreated control count. However, the standard CIP cycle protocol achieved lower ($p \leq 0.05$) *B. licheniformis* reductions (2.8 $\log_{10}$ cfu/cm^2) in comparison to the CIP+MNAO protocol (5.4 $\log_{10}$ cfu/cm^2), and CIP+MNAO was significantly more effective than CIP. In this case side was not significantly different.

Table 4.5 Effectiveness of including MNAO wash (pH 4 at 10°C) after standard Clean-in-Place (CIP) cycle application for reducing *Bacillus* spp. populations on bio-fouled PES membranes.

Bacteria Strain	Treatment	Average log_{10} cfu/cm^2 ± standard error recovered from membrane surface	Average log_{10} reduction cfu/cm^2
Bacillus cereus ATCC10987	Control	5.6 ± 0.5^a	~
	CIP	1.5 ± 0.5^b	4.1
	CIP+MNAO	1.8 ± 0.5^b	3.8
Bacillus licheniformis ATCC6634	Control	5.7 ± 0.3^a	~
	CIP	2.9 ± 0.3^b	2.8
	CIP+MNAO	0.3 ± 0.3^c	5.4

* Means (n=3) with different superscripts within a bacteria strain are different ($p \leq 0.05$). Control: Non-treated membrane. Average log_{10} cfu/cm^2 on non-treated control membranes minus average log_{10} cfu/cm^2 count on treated membrane.

Phase 2C: MNAO+Clean In Place (CIP) Treatment Study

Table 4.6 summarizes the impact of applying a MNAO wash before a standard CIP cycle to reduce *Bacillus* spp. populations on PES membranes. Untreated control membranes harbored 5.4 and 5.9 log_{10} cfu/cm^2 of *B. cereus* and *B. licheniformis* in attached biofilms, respectively. For both organisms, CIP cycle and MNAO+CIP cycle treatments significantly reduced ($p \leq 0.05$) population levels by 3.7 - 4.6 log cycles; however, the reductions observed for the two treatments within bacteria type were similar ($p \leq 0.05$). Side was not found to be significant in this treatment structure.

Table 4.6 Effectiveness of including a MNAO wash (pH 4 at 10°C) before standard Clean-in-Place (CIP) cycle application for reducing *Bacillus* spp. populations on bio-fouled PES membranes.

Bacteria Strain	Treatment	Average $\log_{10}$ cfu/cm^2 ± standard error recovered from membrane surface	Average $\log_{10}$ reduction cfu/cm^2
Bacillus cereus ATCC10987	Control	5.4±0.4[a]	~
	CIP	1.7±0.4[b]	3.7
	MNAO+CIP	1.5±0.4[b]	3.9
Bacillus licheniformis ATCC6634	Control	5.9±0.4[a]	~
	CIP	1.3±0.4[b]	4.6
	MNAO+CIP	2.2±0.4[b]	3.7

* Means (n=3) with different superscripts within a bacteria strain are different ($p \leq 0.05$). Control: Non-treated membrane. Average $\log_{10}$ cfu/cm^2 on non-treated control membranes minus average $\log_{10}$ cfu/cm^2 count on treated membrane.

Scanning Electron Microscopy of Cleaned Polyethersulfone Membranes

SEM imaging of membranes exposed to a typical CIP cycle and MNAO wash are provided in Figure 4.9 and Figure 4.10 The destruction of cell walls is seen in Figure 4.9 (B) and Figure 4.9 (C). Very few intact cells remained on the surface, although not all cell structures were removed during cleaning. Guzel-Seydim et al. (2004) stated the destruction of target microorganisms by ozonation can be explained by the oxidation of the cellular components. Ozone destroys bacteria by attacking the glycoproteins and glycolipids in the cell membrane, which results in rupture of the cell (Megahed et al., 2018). Cell wall destruction is not as prominent in Figure 4.10 image (B) and Figure 4.10 image (C). There were very few remaining cells left on the surface of this membrane sample.

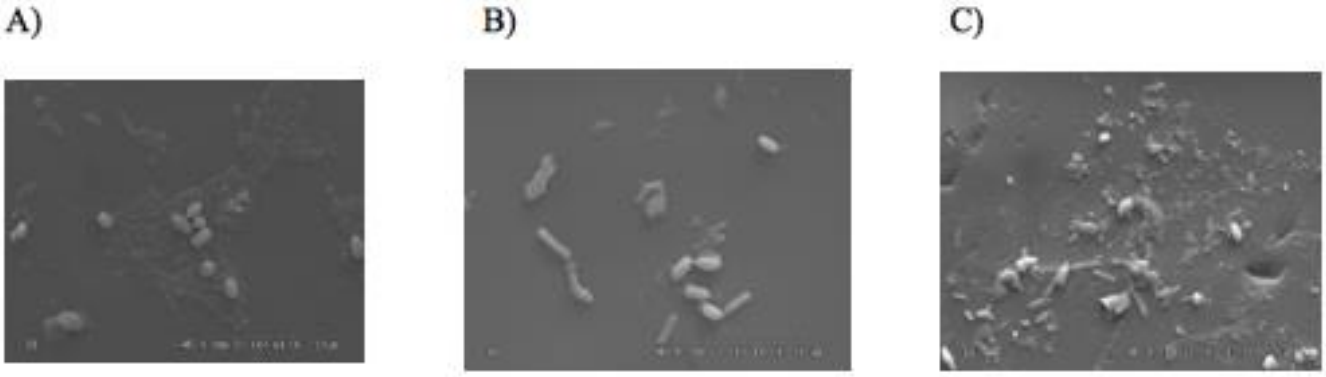

Figure 4.9 SEM micrographs of *Bacillus licheniformis* ATCC6634 biofilms on PES membranes after each cleaning cycle. (A) Control membrane with no sanitizer at 4.0 k magnification; (B) Membrane sanitized with CIP wash only at 4.0 k magnification; and (C) Membrane sanitized with MNAO then CIP wash at 3.0 k magnification.

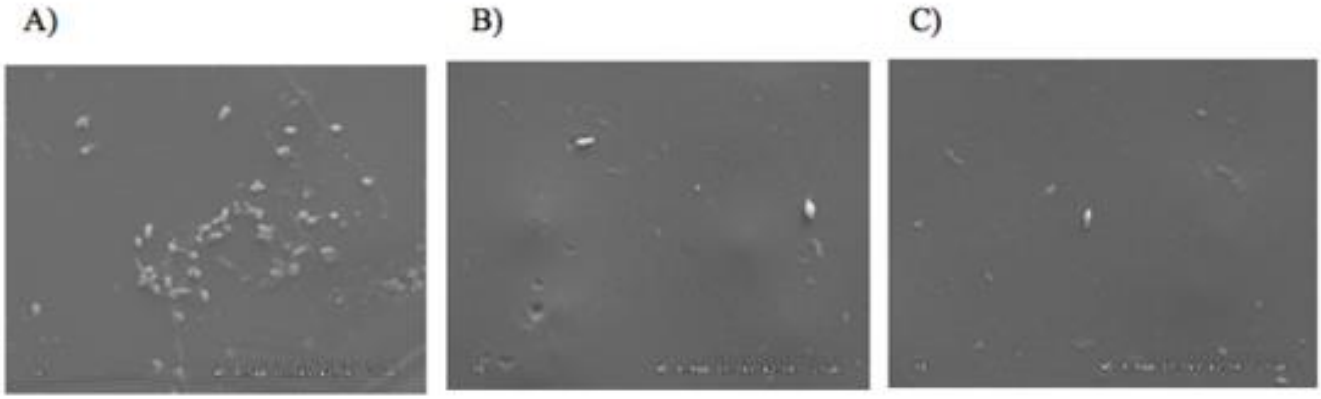

Figure 4.10 SEM micrographs of *Bacillus cereus* ATCC10987 biofilms on PES membranes after each cleaning cycle. (A) Control membrane with no sanitizer at 2.0k magnification; (B) Membrane sanitized with CIP wash only at 2.0k magnification; and (C) Membrane sanitized with MNAO then CIP wash at 2.0k magnification.

4.8 Experimental Discussion and Comparisons

Phase 1A: Ozone Bubble Size

Tsuge (2014) generated oxygen nanobubbles using equipment from Kyowa Corporation and estimated the concentration of oxygen nanobubbles to be 3.3 $x10^8$ particles/ml using a Zetasizer Nano (Malvern instruments Worcestershire, UK). The bubble size and concentration of the given mode peak was 72 nm and 4.6 $x10^8$ particles/ml.

When comparing the concentration of ozone nanobubbles in our study with the oxygen nanobubbles described by Tsuge (2014), the oxygen nanobubble concentration was observed to be higher than the ozone nanobubbles in this study. Differences observed could be related to gas solubility, and diffusion rates (Meegoda et al., 2018). Equipment may have also been a factor, as concentration of nanobubbles in the present study was measured using Nanoparticle Tracking Analysis (NTA).

Lee et al. (2018) conducted a study using a CIP cycle containing NaOCl at different concentrations using coarse bubbles and microbubbles to clean fouled flat sheet membranes. The aeration used in the CIP affected the foulant layer on the membrane using sheer force. The thickness of the foulant layer was observed to be thinner when treated using the microbubbles when compared to the coarse bubbles. Lee et al. (2018) improved cleaning efficiency using microbubbles with increased contact time. Although Lee et al. (2018) was not studying biofilm reduction on membranes, these data could aid in future research, as fouling plays an important role in biofilm attachment and removal.

Phase 1B: Half-Life of MNAO

Ozone decay constant (k) and half-life were evaluated using aqueous MNAO in this study. The goal of the MNAO half-life study was to determine the largest half-life of the

microbubbles and nanobubbles produced. A large half-life would increase the total time that the microbubbles (carrying gaseous ozone) would be in contact with the bio-fouled membrane. The half-life and ozone decay constant (k) reported by Dhillon et al. (2009) were compared to this research. At pH 6.5 and a temperature of 15°C, Dhillon et al. (2009) observed a minimum half-life of 7.6 min with a k value of 0.09 k. The maximum half-life was reported to be 9.19 min with a k-value of 0.07 k at pH 2 and 15°C (Dhillon et al., 2009). When compared to the study done by Dhillon et al. (2009), the largest half-life of MNAO observed in this study was approximately 43 times longer, with a maximum half-life of MNAO of 435 min at pH 7 and 10°C, with a maximum k-value of 0.0016 k. The half-life of MNAO detected in this research was approximately 187 times longer than the maximum k-value reported by Dhillon et al. (2009).

The pH and temperature combinations selected (pH 4 at 10°C) were important factors in the half-life of MNAO. Generating MNAO with a large half-life allows the solution to be stored and used later instead of at point of use. This is valuable, as you would not have to generate the solution on site and could instead potentially 1) source it from outside manufacturers, or 2) generate it in house and store it depending on concentration and calculated half-life.

Previously, it was discussed that an extended ozone half-life was needed when using ozone as a disinfection agent (Dhillon et al., 2009). It was reported that aqueous ozone in MNAO form sustained a longer half-life when compared to the study conducted by Dhillon et al. (2009) using aqueous ozone alone. MNAO increased the half-life of the microscale/nanoscale bubbles, potentially creating a more sustainable, eco-friendly bubble solution for cleaning and sanitation protocols. The data generated in the present study demonstrate that a pH of 7 at 10°C resulted in the longest half-life. The half-life of aqueous ozone solution at pH 4 and 10°C was not statistically different ($p \geq 0.05$) from pH 7 at 10°C; however, based upon recommendation from

the MNAO generation system manufacturer, a lower temperature and pH was suggested to avoid any fluctuation in half-life. Therefore, an aqueous ozone solution with a pH 4 at 10°C, and a half-life of 390 min, was selected to evaluate biofilm removal from dairy membranes in our subsequent study.

Phase 2A and Phase 2B: Clean In Place (CIP)+MNAO and MNAO+CIP Treatment

In a study reported by Marino et al. (2018), treated stainless steel coupons were inoculated with *Pseudomonas fluorescens, Staphylococcus aureus,* and *Listeria monocytogenes.* Treatment with aqueous ozone under static conditions resulted in an estimated viability reduction of 1.61–2.14 log cfu/cm^2 after 20 min, while reduction values were higher (3.26–5.23 log cfu/cm^2) for biofilms treated in dynamic conditions (Marino et al., 2018). In comparison, the contact time of the CIP-MNAO and MNAO-CIP cleaning treatments described in the present study were applied for a shorter 6 min and achieved larger reductions of 3.8-3.9 log cfu/cm^2 for *Bacillus cereus,* and 3.7-5.4 9 log cfu/cm^2 for *Bacillus licheniformis.* It is important to note, however, that *Bacillus cereus* and *Bacillus licheniformis* are spore forming bacteria grown on fouled PES, which likely contributed to the observed differences.

Previous research has reported that the spore form of *Bacillus* spp. attaches more readily than vegetative cells to stainless steel due to relatively high hydrophobicity and resistance to heat, chemicals, and surface-adhering capabilities. Increasing the contact time of MNAO with inclusion of CIP under dynamic conditions could potentially improve reductions. Although Ben-Ishay et al. (2017) stated that spore-formers can survive treatment with CIP protocols and cleaning reagents such as alkaline and acidic liquids even at high temperatures, our data demonstrate that *Bacillus* spp. reductions were achieved with the addition of MNAO.

Gandhi et al. (2018) reported a significant reduction of *Bacillus cereus* (ATCC10987) and *Bacillus licheniformis* (ATCC6634) biofilms at $\geq 2.88 \pm 0.22$ $\log_{10}$ cfu/cm^2 using the MNAO treatment on stainless steel coupons. Similar findings were observed in the present study using the CIP, MNAO+CIP, and CIP+MNAO treatments on bio-fouled PES membranes. Log reductions of 3.8-3.9 log cfu/cm^2 for *Bacillus cereus*, and 3.7-5.4 log cfu/cm^2 for *Bacillus licheniformis* were observed. Both studies were performed using similar experimental design and cleaning protocols.

Broadwater et al. (1973) reported a >2 $\log_{10}$ cfu/mL reduction of *Bacillus cereus* on stainless steel coupons following treatment with 0.12 ppm aqueous ozone for 5 min at 28 ºC. Similar results were reported in the present *Bacillus* spp. study using MNAO and CIP cleaning protocols on bio-fouled *Bacillus* spp. PES membranes.

There is little research comparing bacterial reductions on PES dairy processing membranes using aqueous ozone. Several studies investigated the use of microbubbles, gaseous ozone, and CIP cycle separately but not in combination. This study is the first of what could be potentially many research opportunities to discover improved biofilm removal from PES membranes in an affordable manner for the industry.

4.9 Conclusion

Investigation into microbial biofilm contaminating agents in the food industry is crucial although research proving the effectiveness of aqueous ozone as an antimicrobial agent against biofilms in membranes is scarce. These data sets were a first look at understanding the impact of MNAO, or a typical CIP cycle in combination with MNAO on bio-fouled PES membranes used in the dairy industry. Wash treatments were placed before and after the typical CIP cycle and

used to treat PES membranes fouled with *Bacillus* spp. Biofilms, comparing the effectiveness of the wash treatments against *Bacillus* spp. biofilms.

Using the NanoSight (NS300, Malvern Instruments Ltd., Malvern, UK) the average bubble size was found to be 212 nm ± 87 nm at 23.5 °C with a maximum MNAO water concentration of 19 ppm. Advantages of the ozone generator system included automated and continuous monitoring of dissolved MNAO concentration while running the system. Bubble size was an important factor contributing to the half-life of MNAO.

The half-life of MNAO used in this study was found to have a k-value of 0.0018 (390 min). A pH 4 at 10°C did not give the highest half-life but overall was not significantly different from pH 7 at 10°C. On a larger scale MNAO could potentially be used as a pre-generated solution rather than at point of use although stability of MNAO is cause for concern. Alongside the half-life, bubble size could have potentially played a role in the reduction of biofilm from bio-fouled PES membranes. Small bubble size in conjunction with a vetted sanitizer would allow for increased contact time on an affected surface with potential for decreased bacteria populations.

The MNAO wash treatment was compared to a deionized water treatment. The MNAO alone showed potential to be effective against *Bacillus* spp. biofilms as there was a significant difference between the water treatment and the MNAO treatment compared to the control. MNAO has potential to be used alone as a cleaning step in removal of *Bacillus* spp. biofilms.

In the dairy industry a CIP cycle is commonly used for cleaning of separation membranes. Based off the results of the MNAO and water treatment study we investigated MNAO in conjunction with a typical CIP cycle. MNAO was placed before and after the typical CIP cycle. Adding MNAO did not add a substantial benefit beyond what is currently used with a

CIP cycle. However, in the case of *Bacillus cereus* using a CIP cycle followed by MNAO there may be a benefit in adding MNAO. The CIP cycle alone compared to the CIP+MNAO treatment were significantly different.

Further investigation is needed to study effects of MNAO independently on PES membranes or in combination with other CIP cycle chemicals. Because biofilms that survive cleaning cycles become resistant over time, prevention strategies limiting adhesion of bacteria to membranes should be prioritized for sustainable control of biofouling. Increasing the contact time using MNAO with inclusion of a typical CIP cycle under dynamic conditions, potentially could also increase viability reduction in biofilm formation on the membrane surface. In addition, this research could further benefit the dairy industry by increasing cleaning efficacy, decreasing operational cost, and aiding in environmental sustainability.

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4.11 References

Anand, S., D. Singh, M. Avadhanula, and S. Marka. 2014. Development and control of bacterial biofilms on dairy processing membranes**.** Compr. Rev. Food Sci. Food Saf. 13(1):18-33.

Ben-Ishay, N., H. Oknin, D. Steinberg, Z. Berkovich, R. Reifen, and M. Shemesh. 2017. Enrichment of milk with magnesium provides healthier and safer dairy products. NPJ Biofilms and Microbiomes. *3*:24-7.

Broadwater, W. T., R. C. Hoehn, and P. H. King. 1973. Sensitivity of Three Selected Bacterial Species to Ozone. Appl Microbiol. 26(3):391-393.

Chmielewsky R. A. N., and J. F. Frank. 2003. Biofilm formation and control in food processing facilities. Compr. Rev. Food Sci. Food Saf. *2*:22-32

Coll Cárdenas, F., S. Andrés, L. Giannuzzi, and N. Zaritzky. 2011. Antimicrobial Action and Effects on Beef Quality Attributes of a Gaseous Ozone Treatment at Refrigeration Temperatures. Food Control 22(8):1442-447.

Dhillon, B., D. Wiesenborn, C. Wolf-Hall, and F. Manthey. 2009. Development and evaluation of an ozonated water system for antimicrobial treatment of durum wheat. J. Food Sci. 74:E396-E403.

Dreszer C., J. S. Vrouwenvelder, A. H. Paulitsch-Fuchs, A. Zwijnenburg, J. C. Kruithof, and H. C. Flemming. 2013. Hydraulic resistance of biofilms. J. Membr Sci. 429:436-447.

Ebina K., K. Shi, M. Hirao, J. Hashimoto, Y. Kawato, S. Kaneshiro, T. Morimoto, K. Koizumi, and H. Yoshikawa. 2013. Oxygen and Air Nanobubble Water Solution Promote the Growth of Plants, Fishes, and Mice. PLoS One. 8:E65339.

Gandhi, G. 2018. Study of high protein dairy powder (MPC80) susceptibility to fouling and efficacy of micro-nano-bubble aqueous ozone in removal of *Bacillus* spp. biofilms on stainless steel surfaces. MS Thesis. Food Science Institute. Kansas State Univ. Manhattan.

Gopal, N., C. Hill, P. R. Ross, T. P. Beresford, M. A. Fenelon, and P. D. Cotter. 2017. The Prevalence and Control of *Bacillus* and Related Spore-Forming Bacteria in the Dairy Industry. Front. Microbiol. 6:1418.

Guzel-Seydim, Z. B., A. K. Greene, and A. C. Seydim. 2004. Use of ozone in the food industry. Food Sci. Technol. *37*(4):453-460.

Herzberg, M., and M. Elimelech. 2007. Byesiofouling of reverse osmosis membranes: Role of biofilm-enhanced osmotic pressure. J. Membr. Sci. 295(1):11-20.

Hu, K., and J. M. Dickson. 2015. Membrane processing for dairy ingredient separation. IFT Press. John Wiley & Sons, Incorporated, Hoboken, NY.

Kim, J. G., and A. E. Yousef. 2000. Inactivation Kinetics of Foodborne Spoilage and Pathogenic Bacteria by Ozone. J. Food Sci. 65(3):521-528.

Kumar C. G., S. K. Anand. 1998. Significance of microbial biofilms in food industry: a review. Int. J. Food Microbiol. 42(1-2):9-27.

Lee, E. J., Y. H. Kim, M. J. Jeon, and H. S. Kim. 2018. The effect of aeration types on foulant removal in ex-situ chemical cleaning in place (CIP) with membranes fouled by secondary effluents. Chem. Eng. 333:730-738.

Lynn, 2011, Aqueous Ozone Solution for Ozone Cleaning System, Food Safety Technology LLC, U.S. Patent No. 8,071,526.

Marino, M., M. Maifreni, A. Baggio, and N. Innocente. 2018. Inactivation of foodborne bacteria biofilms by aqueous and gaseous ozone. Front. in Micro. 9:2024.

McClurkin, J. D., Maier, D.E. and Ileleji, K.E. (2013). Half-life time of ozone as a function of air movement and conditions in a sealed container. J. Stored Prod. Res. 55:41-47.

Meegoda, J. N., S. A. Hewage, and J. H. Batagoda. 2018. Stability of nanobubbles. Environ. Engr. Sci. 35(11):1216-1227.

Megahed, A., B. Aldridge, and J. Lowe. 2018. The microbial killing capacity of aqueous and gaseous ozone on different surfaces contaminated with dairy cattle manure. PloS One, 13(5):E0196555.

Oliveira, M. M. M., D. F. Brugnera, E. Alves, R. H. Piccoli. 2010. Biofilm formation by *Listeria monocytogenes* on stainless steel surface and biotransfer potential. Brazilian J. Microbiol. 41(1):97-106.

Shah, N. N. A. K., R. A. Rahman, L. T. Chuan, and D. M. Hashim. 2013. Application of gaseous ozone to inactivate *Bacillus cereus* in processed rice. 2009. J. of Food Process Eng. 34(6):2220-2232.

Silagyi K.S. 2007. Biofilm formation by E*scherichia coli* O157:H7. PhD Dissertation. University of Maryland, College Park.

Tan, T. J., D. Wang, and C. I. Moraru. 2014. A physicochemical investigation of membrane fouling in cold microfiltration of skim milk. J. Dairy Sci. 97(8):4759-4771.

Tang, X., S. H. Flint, R. J. Bennett, and J. D. Brooks. 2010. The efficacy of different cleaners and sanitizers in cleaning biofilms on UF membranes used in the dairy industry. J. Membr. Sci. 352(1-2):71-75.

Tsuge, H., 2014. Micro- and Nanobubbles. Singapore: Pan Stanford.

Yesil, M., D. R. Kasler, E. Huang, and A. E. Yousef. 2017. Efficacy of Gaseous Ozone Application during Vacuum Cooling against *Escherichia coli* O157:H7 on Spinach Leaves as Influenced by Bacterium Population Size. J. Food Prot. 80(7):1066-1071.

Appendix A - SAS Code

SAS CODE: MNAO-CIP

```
*********************************************************;
* Rachael Henderson Project: Ozone Wash_CIP             *;
* Coder: Nicholas Bloedow (Statistical Consulting Lab)  *;
* Date: 07/09/17                                        *;
*********************************************************;


/*
Notes:
*/



/* SAS Analysis */

ods rtf file = "C:\Users\bloedow\Documents\Consulting\Clients\Rachael
Henderson (Fall 2016)\
                    Statistical Analysis\SAS Output\Ozone Wash_CIP Analysis
(07_09_17).doc";

 title 'Ozone Wash_CIP Project (Rachael Henderson)';


/* Bacillus Cereus */
 title2 'Bacillus Cereus Dataset';

 *Imports the Bacillus Cereus Ozone Wash_CIP Dataset from Excel into SAS;
  proc import out=Ozone_CIP_BC
    datafile='C:\Users\bloedow\Documents\Consulting\Clients\Rachael Henderson
(Fall 2016)\Data\Stats Updated 6-19-17 Final.xlsx'
    dbms=xlsx
    replace;
    sheet="MNAO+CIP BC";
    getnames=yes;
  run;


*Creates formatted variables from the Bacillus Cereus Ozone Wash_CIP Dataset;
  data Ozone_CIP_BC;
    set Ozone_CIP_BC;
      log_CFU_cm2=round(log_CFU_cm2,0.0001);
      *CFU_cm2=round(CFU_cm2,0.0001);
      *if Dilution=0 then log_CFU_cm2=-0.66;
  run;

 *Prints out the Bacillus Cereus Ozone Wash_CIP Dataset;
  proc print data=Ozone_CIP_BC;
  run;


 /* Statistical Graphics */
```

```
  proc sgpanel data=Ozone_CIP_BC;
      panelby Treatment / Columns=3 rows=1;
    scatter x=Peroid y=log_CFU_cm2 / group=Side;
    colaxis label="Peroid" Min=0 Max=4 Values=(1 to 3 by 1);
      rowaxis label="Log(CFU/cm2)" Min=-1 Max=8 Values=(-1 to 8 by 1);
      refline 0 / axis=y ;
      title3 'Response Variable: Log(CFU/cm2)';
  run;


/* Statistical Analysis */

  data Ozone_CIP_BC;
    set Ozone_CIP_BC;
      if Treatment="CIP-O3" then BC_Grp=1; else BC_Grp=0;
  run;


  proc mixed data=Ozone_CIP_BC covtest plots=all;
    class     Peroid Treatment Sample Side BC_Grp;
      model     log_CFU_cm2 = Treatment|Side / residual outp=predresid;
      random    Peroid Peroid*Treatment;
      *repeated  / group=BC_Grp;
    lsmeans   Treatment / cl pdiff=all adjust=tukey;
    lsmeans   Side / cl pdiff=all adjust=tukey;
    lsmeans   Treatment*Side / cl pdiff=all slice=Treatment adjust=tukey;
      title3 'Response Variable: Log(CFU/cm2)';
      title4 'Mixed Model [ddfm=Containment]';
  run;

  proc sgplot data=predresid;
    scatter x=Peroid y=StudentResid;
  run;

  proc sgplot data=predresid;
    scatter x=Treatment y=StudentResid;
  run;

  proc sgplot data=predresid;
    scatter x=Side y=StudentResid;
  run;

  proc sort data=predresid;
    by StudentResid;
  run;



  proc mixed data=Ozone_CIP_BC covtest plots=all;
    class     Peroid Treatment Side BC_Grp;
      model     log_CFU_cm2 = Treatment / residual outp=predresid;
      random    Peroid Peroid*Treatment;
      repeated  / subject=Side(Peroid Treatment) group=BC_Grp;
    lsmeans   Treatment / cl pdiff=all adjust=tukey;
      title3 'Response Variable: Log(CFU/cm2)';
      title4 'Mixed Model [ddfm=Containment]';
  run;
```

```
  proc sgplot data=predresid;
    scatter x=Peroid y=StudentResid;
  run;

  proc sgplot data=predresid;
    scatter x=Treatment y=StudentResid;
  run;



/* Bacillus Licheniformis */
 title2 'Bacillus Licheniformis Dataset';

 *Imports the Bacillus Licheniformis Ozone Wash_CIP Dataset from Excel into
SAS;
  proc import out=Ozone_CIP_BL
    datafile='C:\Users\bloedow\Documents\Consulting\Clients\Rachael Henderson
(Fall 2016)\Data\Stats Updated 6-19-17 Final.xlsx'
    dbms=xlsx
    replace;
    sheet="MNAO+CIP BL";
    getnames=yes;
  run;


 *Creates formatted variables from the Bacillus Licheniformis Ozone Wash_CIP
Dataset;
  data Ozone_CIP_BL;
    set Ozone_CIP_BL;
      *CFU_cm2=round(CFU_cm2,0.0001);
      log_CFU_cm2=round(log_CFU_cm2,0.0001);
      *if Dilution=0 then log_CFU_cm2=-0.66;
      *if Treatment="CIP-O3" then Ozone_CIP_BL_Grp=1; *else
Ozone_CIP_BL_Grp=0;
  run;

 *Prints out the Bacillus Licheniformis Ozone Wash_CIP Dataset;
  proc print data=Ozone_CIP_BL;
  run;


 /* Statistical Graphics */
  proc sgpanel data=Ozone_CIP_BL;
      panelby Treatment / Columns=3 rows=1;
    scatter x=Peroid y=log_CFU_cm2 / group=Side;
    colaxis label="Peroid" Min=0 Max=4 Values=(1 to 3 by 1);
      rowaxis label="Log(CFU/cm2)" Min=-1 Max=8 Values=(-1 to 8 by 1);
      refline 0 / axis=y ;
      title3 'Response Variable: Log(CFU/cm2)';
  run;


/* Statistical Analysis */

  data Ozone_CIP_BL;
```

```
    set Ozone_CIP_BL;
      if Treatment="CIP-O3" then BL_Grp=1; else BL_Grp=0;
  run;


  proc mixed data=Ozone_CIP_BL covtest plots=all;
    class    Peroid Treatment Side BL_Grp;
      model    log_CFU_cm2 = Treatment|Side / residual outp=predresid;
      random   Peroid Peroid*Treatment;
      repeated / group=BL_Grp;
    lsmeans  Treatment / cl pdiff=all adjust=tukey;
    lsmeans  Side / cl pdiff=all adjust=tukey;
    lsmeans  Treatment*Side / cl pdiff=all slice=Treatment adjust=tukey;
      title3 'Response Variable: Log(CFU/cm2)';
      title4 'Mixed Model [ddfm=Containment]';
  run;


  proc sgplot data=predresid;
    scatter x=Peroid y=StudentResid;
  run;

  proc sgplot data=predresid;
    scatter x=Treatment y=StudentResid;
  run;

  proc sgplot data=predresid;
    scatter x=Side y=StudentResid;
  run;

  proc sort data=predresid;
    by StudentResid;
  run;

proc mixed data=Ozone_CIP_BL covtest plots=all;
    class    Peroid Treatment Side BL_Grp;
      model    log_CFU_cm2 = Treatment / residual outp=predresid;
      random   Peroid Peroid*Treatment;
      repeated  / subject=Side(Peroid Treatment) group=BL_Grp;
    lsmeans  Treatment / cl pdiff=all adjust=tukey;
      title3 'Response Variable: Log(CFU/cm2)';
      title4 'Mixed Model [ddfm=Containment]';
  run;

  proc sgplot data=predresid;
    scatter x=Peroid y=StudentResid;
  run;

  proc sgplot data=predresid;
    scatter x=Treatment y=StudentResid;
  run;

ods rtf close;
```

SAS CODE: CIP-MNAO

```
*******************************************************;
* Rachael Henderson Project: CIP_Ozone Wash             *;
* Coder: Nicholas Bloedow (Statistical Consulting Lab)  *;
* Date: 07/06/17                                        *;
*******************************************************;


/*
Notes:
*/



/* SAS Analysis */

ods rtf file = "C:\Users\bloedow\Documents\Consulting\Clients\Rachael
Henderson (Fall 2016)\
                  Statistical Analysis\SAS Output\CIP_Ozone Wash Analysis
(07_06_17).doc";

 title 'CIP_Ozone Wash Project (Rachael Henderson)';


/* Bacillus Cereus */
 title2 'Bacillus Cereus Dataset';

 *Imports the Bacillus Cereus CIP_Ozone Wash Dataset from Excel into SAS;
  proc import out=CIP_Ozone_BC
    datafile='C:\Users\bloedow\Documents\Consulting\Clients\Rachael Henderson
(Fall 2016)\Data\Stats Updated 6-19-17 Final.xlsx'
    dbms=xlsx
    replace;
    sheet="CIP+MNAO BC";
    getnames=yes;
  run;


 *Creates formatted variables from the Bacillus Cereus CIP_Ozone Wash
Dataset;
  data CIP_Ozone_BC;
    set CIP_Ozone_BC;
      log_CFU_cm2=round(log_CFU_cm2,0.0001);
      *CFU_cm2=round(CFU_cm2,0.0001);
  run;

 *Prints out the Bacillus Cereus CIP_Ozone Wash Dataset;
  proc print data=CIP_Ozone_BC;
  run;


 /* Statistical Graphics */
  proc sgpanel data=CIP_Ozone_BC;
      panelby Treatment / Columns=3 rows=1;
    scatter x=Peroid y=log_CFU_cm2 / group=Side;
```

```
    colaxis label="Peroid" Min=0 Max=4 Values=(1 to 3 by 1);
      rowaxis label="Log(CFU/cm2)" Min=-1 Max=8 Values=(-1 to 8 by 1);
      refline 0 / axis=y ;
      title3 'Response Variable: Log(CFU/cm2)';
  run;


/* Statistical Analysis */

  proc mixed data=CIP_Ozone_BC covtest plots=all;
    class    Peroid Treatment Side;
      model    log_CFU_cm2 = Treatment|Side / residual outp=predresid;
      random   Peroid Peroid*Treatment;
    lsmeans  Treatment / cl pdiff=all adjust=tukey;
    lsmeans  Side / cl pdiff=all adjust=tukey;
    lsmeans  Treatment*Side / cl pdiff=all slice=Treatment adjust=tukey;
      title3 'Response Variable: Log(CFU/cm2)';
      title4 'Mixed Model [ddfm=Containment]';
  run;


  proc sgplot data=predresid;
    scatter x=Peroid y=StudentResid;
  run;

  proc sgplot data=predresid;
    scatter x=Treatment y=StudentResid;
  run;

  proc sgplot data=predresid;
    scatter x=Side y=StudentResid;
  run;

proc sort data=predresid;
    by StudentResid;
  run;




  data CIP_Ozone_BC;
    set CIP_Ozone_BC;
      if Treatment="CIP-O3" then BC_Grp=1; else BC_Grp=0;
  run;

  proc mixed data=CIP_Ozone_BC covtest plots=all;
    class    Peroid Treatment Side BC_Grp;
      model    log_CFU_cm2 = Treatment / residual outp=predresid;
      random   Peroid Peroid*Treatment;
      repeated / subject=Side(Peroid Treatment) group=BC_Grp;
    lsmeans  Treatment / cl pdiff=all adjust=tukey;
      title3 'Response Variable: Log(CFU/cm2)';
      title4 'Mixed Model [ddfm=Containment]';
  run;

  proc sgplot data=predresid;
    scatter x=Peroid y=StudentResid;
```

```
  run;

  proc sgplot data=predresid;
    scatter x=Treatment y=StudentResid;
  run;


/* Bacillus Licheniformis */
 title2 'Bacillus Licheniformis Dataset';

 *Imports the Bacillus Licheniformis CIP_Ozone Wash Dataset from Excel into
SAS;
  proc import out=CIP_Ozone_BL
    datafile='C:\Users\bloedow\Documents\Consulting\Clients\Rachael Henderson
(Fall 2016)\Data\Stats Updated 6-19-17 Final.xlsx'
    dbms=xlsx
    replace;
    sheet="CIP+MNAO BL";
    getnames=yes;
  run;


 *Creates formatted variables from the Bacillus Licheniformis CIP_Ozone Wash
Dataset;
  data CIP_Ozone_BL;
    set CIP_Ozone_BL;
      log_CFU_cm2=round(log_CFU_cm2,0.0001);
      *CFU_cm2=round(CFU_cm2,0.0001);
      *if Dilution=0 then log_CFU_cm2=-0.66;
  run;

 *Prints out the Bacillus Licheniformis CIP_Ozone Wash Dataset;
  proc print data=CIP_Ozone_BL;
  run;


 /* Statistical Graphics */
  proc sgpanel data=CIP_Ozone_BL;
      panelby Treatment / Columns=3 rows=1;
    scatter x=Peroid y=log_CFU_cm2 / group=Side;
    colaxis label="Peroid" Min=0 Max=6 Values=(1 to 5 by 1);
      rowaxis label="Log(CFU/cm2)" Min=-1 Max=8 Values=(-1 to 8 by 1);
      refline 0 / axis=y ;
      title3 'Response Variable: Log(CFU/cm2)';
  run;
```

```
/* Statistical Analysis */

  data CIP_Ozone_BL;
    set CIP_Ozone_BL;
      if Treatment="CIP-O3" then BL_Grp=1; else BL_Grp=0;
  run;

  proc mixed data=CIP_Ozone_BL covtest plots=all;
    class     Peroid Treatment Side BL_Grp;
      model     log_CFU_cm2 = Treatment|Side / residual outp=predresid;
      random    Peroid Peroid*Treatment;
      repeated / group=BL_Grp;
    lsmeans   Treatment / cl pdiff=all adjust=tukey;
    lsmeans   Side / cl pdiff=all adjust=tukey;
    lsmeans   Treatment*Side / cl pdiff=all slice=Treatment adjust=tukey;
      title3 'Response Variable: Log(CFU/cm2)';
      title4 'Mixed Model [ddfm=Containment]';
  run;


  proc sgplot data=predresid;
    scatter x=Peroid y=StudentResid;
  run;

  proc sgplot data=predresid;
    scatter x=Treatment y=StudentResid;
  run;

  proc sgplot data=predresid;
    scatter x=Side y=StudentResid;
  run;

  proc sort data=predresid;
    by StudentResid;
  run;

  proc mixed data=CIP_Ozone_BL covtest plots=all;
    class     Peroid Treatment Side BL_Grp;
      model     log_CFU_cm2 = Treatment / residual outp=predresid;
      random    Peroid Peroid*Treatment;
      repeated / subject=Side(Peroid Treatment) group=BL_Grp;
    lsmeans   Treatment / cl pdiff=all adjust=tukey;
      title3 'Response Variable: Log(CFU/cm2)';
      title4 'Mixed Model [ddfm=Containment]';
  run;

  proc sgplot data=predresid;
    scatter x=Peroid y=StudentResid;
  run;

  proc sgplot data=predresid;
    scatter x=Treatment y=StudentResid;
  run;


ods rtf close;
```

SAS CODE: Water Wash – MNAO Wash

```
*******************************************************;
* Rachael Henderson Project: Biofilm_Ozone Wash        *;
* Coder: Nicholas Bloedow (Statistical Consulting Lab  *;
* Date: 02/06/17                                       *;
*******************************************************;



/* SAS Analysis */

ods rtf file = "C:\Users\bloedow\Documents\Consulting\Clients\Rachael
Henderson (Fall 2016)\
                    Statistical Analysis\SAS Output\Biofilm_Ozone Wash
Analysis (02_06_17).doc";

 title 'Biofilm_Ozone Wash Project (Rachael Henderson)';


/* Bacillus Cereus */
 title2 'Bacillus Cereus Dataset';

 *Imports the Bacillus Cereus Biofilm Ozone Wash Dataset from Excel into SAS;
  proc import out=Biofilm_OzoneWash_BC
    datafile='C:\Users\bloedow\Documents\Consulting\Clients\Rachael Henderson
(Fall 2016)\Data\
                  Experiment Aqueous Ozone (01_10_17 RH).xlsx'
    dbms=xlsx
    replace;
    sheet="BC Final";
    getnames=yes;
  run;

 *Creates formatted variables from the Biofilm Ozone Wash Dataset;
  data Biofilm_OzoneWash_BC;
    set Biofilm_OzoneWash_BC;
      CFU_cm2=round(CFU_cm2,0.0001);
      log_CFU_cm2=round(log_CFU_cm2,0.0001);
      Average_CFU_cm2=round(Average_CFU_cm2,0.0001);
      Average_log_CFU_cm2=round(Average_log_CFU_cm2,0.0001);
  run;


 /* Statistical Graphics */
  proc sgpanel data=Biofilm_OzoneWash_BC;
      panelby Treatment / Columns=3 rows=1;
    scatter x=Period y=log_CFU_cm2 / group=Side;
    colaxis label="Period" Min=0 Max=4 Values=(1 to 3 by 1);
      rowaxis label="Log(CFU/cm2)" Min=0 Max=8 Values=(0 to 8 by 1);
      refline 0 / axis=y ;
      title3 'Response Variable: Log(CFU/cm2)';
  run;


/* Statistical Analysis */
```

```
  proc mixed data=Biofilm_OzoneWash_BC covtest plots=all;
    class    Period Treatment Sample Side;
      model    log_CFU_cm2 = Treatment / ddf=4;
      random   Period Period*Treatment;
    lsmeans  Treatment / cl pdiff=all adjust=tukey;
      title3 'Response Variable: Log(CFU/cm2)';
      title4 'Mixed Model [ddf specified (4)]';
  run;

  proc mixed data=Biofilm_OzoneWash_BC covtest plots=all;
    class    Period Treatment Sample Side;
      model    log_CFU_cm2 = Treatment / ddfm=KR;
      random   Period Period*Treatment;
    lsmeans  Treatment / cl pdiff=all adjust=tukey;
      title3 'Response Variable: Log(CFU/cm2)';
      title4 'Mixed Model [ddfm=KR]';
  run;

/* Bacillus Licheniformis */
 title2 'Bacillus Licheniformis Dataset';

 *Imports the Bacillus Cereus Biofilm Ozone Wash Dataset from Excel into SAS;
  proc import out=Biofilm_OzoneWash_BL
    datafile='C:\Users\bloedow\Documents\Consulting\Clients\Rachael Henderson
(Fall 2016)\Data\
                    Experiment Aqueous Ozone (01_10_17 RH).xlsx'
    dbms=xlsx
    replace;
    sheet="BL Final";
    getnames=yes;
  run;

 *Creates formatted variables from the Biofilm Ozone Wash Dataset;
  data Biofilm_OzoneWash_BL;
    set Biofilm_OzoneWash_BL;
      CFU_cm2=round(CFU_cm2,0.0001);
      log_CFU_cm2=round(log_CFU_cm2,0.0001);
      Average_CFU_cm2=round(Average_CFU_cm2,0.0001);
      Average_log_CFU_cm2=round(Average_log_CFU_cm2,0.0001);
  run;


 /* Statistical Graphics */
  proc sgpanel data=Biofilm_OzoneWash_BL;
      panelby Treatment / Columns=3 rows=1;
    scatter x=Period y=log_CFU_cm2 / group=Side;
    colaxis label="Period" Min=0 Max=4 Values=(1 to 3 by 1);
      rowaxis label="Log(CFU/cm2)" Min=0 Max=8 Values=(0 to 8 by 1);
      refline 0 / axis=y ;
      title3 'Response Variable: Log(CFU/cm2)';
  run;


/* Statistical Analysis */
  proc mixed data=Biofilm_OzoneWash_BL covtest plots=all;
    class    Period Treatment Sample Side;
```

```
    model    log_CFU_cm2 = Treatment / ddf=4;
    random   Period Period*Treatment;
  lsmeans  Treatment / cl pdiff=all adjust=tukey;
    title3 'Response Variable: Log(CFU/cm2)';
    title4 'Mixed Model [ddf specified (4)]';
run;

proc mixed data=Biofilm_OzoneWash_BL covtest plots=all;
  class    Period Treatment Sample Side;
    model    log_CFU_cm2 = Treatment / ddfm=KR;
    random   Period Period*Treatment;
  lsmeans  Treatment / cl pdiff=all adjust=tukey;
    title3 'Response Variable: Log(CFU/cm2)';
    title4 'Mixed Model [ddfm=KR]';
run;


ods rtf close;
```

SAS CODE: Half - Life Study

```
ods rtf file = "C:\Users\bloedow\Documents\Consulting\Clients\Rachael
Henderson (Fall 2016)\
                    Statistical Analysis\SAS Output\Ozone_HalfLife_Final
Analysis (01_13_17).doc";

title 'Ozone_Half-Life Project (Rachael Henderson)';

 *Imports the Ozone Half-Life Dataset from Excel into SAS;
  proc import out=Ozone_HalfLife
    datafile='C:\Users\bloedow\Documents\Consulting\Clients\Rachael Henderson
(Fall 2016)\Data\
                  Half-Life Study Updated 11-7-16.xlsx'
    dbms=xlsx
    replace;
    sheet="Final";
    getnames=yes;
  run;

 *Creates formatted variables from the Ozone Half-Life Dataset;
  data Ozone_HalfLife;
    set Ozone_HalfLife;
      HalfLife=round(log(0.5)/K_Value,0.00001);
  run;

 *Prints out the Ozone Half-Life Dataset;
  proc print data=Ozone_HalfLife;
    title2 'Full Dataset';
  run;

/* Statistical Graphics */

 *Response Variable: K-Value;
  proc sgpanel data=Ozone_HalfLife;
      panelby Temperature_C / Columns=3 rows=1;
    scatter x=pH y=K_Value / group=pH;
    colaxis label="pH" Min=1 Max=8 Values=(1 to 8 by 1);
      rowaxis label="K-Value" Min=0 Max=0.015 Values=(0 to 0.015 by 0.0025);
      refline 0 / axis=y ;
      title2 'Response Variable: K-Value';
  run;


 *Response Variable: Log Half-Life;
  proc sgpanel data=Ozone_HalfLife;
      panelby Temperature_C / Columns=3 rows=1;
    scatter x=pH y=LOG_1_2_LIFE / group=pH;
    colaxis label="pH" Min=1 Max=8 Values=(1 to 8 by 1);
      rowaxis label="Log Half-Life" Min=0 Max=500 Values=(0 to 500 by 50);
      refline 0 / axis=y ;
      title2 'Response Variable: Log Half-Life';
  run;
```

```
/* Descriptive Statistics */

  proc tabulate data=Ozone_HalfLife format=8.5;
    class Temperature_C pH / mlf;
    var K_Value LOG_1_2_LIFE;
    table (K_Value*f=8.5 LOG_1_2_LIFE*f=8.3)*(n*f=3.0 mean stderr),
Temperature_C*pH;
  run;

/* Statistical Analysis */

 /* K-Value Analysis */
  proc glimmix data=Ozone_HalfLife plots=all;
    class    pH Temperature_C;
      model    K_Value = pH|Temperature_C / ;
    lsmeans  pH Temperature_C / cl pdiff adjust=tukey lines;
    lsmeans  pH*Temperature_C / cl pdiff lines slice=pH slicediff=pH
slice=Temperature_C slicediff=Temperature_C adjust=tukey;
      title2 'Response: K-Value';
  run;

 /* Log Half-Life Analysis */
  proc glimmix data=Ozone_HalfLife plots=all;
    class    pH Temperature_C;
      model    LOG_1_2_LIFE = pH|Temperature_C / ;
    lsmeans  pH Temperature_C / cl pdiff adjust=tukey lines;
    lsmeans  pH*Temperature_C / cl pdiff lines slice=pH slicediff=pH
slice=Temperature_C slicediff=Temperature_C adjust=tukey;
      title2 'Response: K-Value';
  run;


ods rtf close;
```