Determination of Prevalence of *E. coli* and *Salmonella* in Dairy Lagoon Liquid Manure and Development of Ozone and Air Injection Treatment Methods for Pathogen Reduction

by

PRACHI PANDEY

THESIS

Submitted in partial satisfaction of the requirements for the degree of

Master of Science

in

Biological and Agricultural Engineering

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

_____

Ruihong Zhang, Chair

_____

Pramod Pandey, co-chair

_____

Xunde Li

Committee in Charge

2023

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENT

I would like to express my deep appreciation to my advisor, **Dr. Ruihong Zhang**, for the invaluable guidance and support she provided throughout my studies. Dr. Zhang's wisdom and unwavering support have been a source of inspiration not only to me but to all students under her mentorship. I wish to convey my appreciation to **Dr. Pramod Pandey** for his invaluable guidance, patience, generous allocation of time, and continuous support throughout my MS studies. Dr. Pandey's valuable counsel and expertise have played crucial role in enhancing the quality of my work and ultimately contributing to the achievement of my project goals. Both Drs. Zhang and Pandey provided me with numerous opportunities to gain new knowledge in waste management and proficiency in laboratory procedures to enrich my expertise in the field of Biological and Agricultural Engineering.

I extend my sincere gratitude to **Dr. Xunde Li**, who provided me opportunities to learn and explore more about the fundamental biological research and advance molecular technique. Additionally, I would like to express my appreciation to Dr. Li's students, including Katie, Megan, Yuyu, and Sharon, who offered valuable assistance in various experimental methods, data analysis, and interpretation. The accomplishment of my research objectives is intricately tied to the support and resources provided by Dr. Li's lab.

I want to express my sincere appreciation to **Dr. Vikrant Singh, Dr. Navneet Rai and Dr. Noha Amaly** for dedicating their valuable time to help me clearing my concepts on various topics in the field of chemistry and microbiology. Their suggestions, extensive knowledge and expertise helped me to achieve my project goals.

I am very thankful to my fellow lab mates: Jiang, Aditya, Julia, Peja, Yuxin, Justin and Harika for their priceless contributions in my research. Mr. Dennis Da Silva (owner of Da Silva Farm), and Mr. Joe Melo (Technical Field Officer from California Dairy Campaign (CDC)) provided enormous help in explaining the manure management in dairy farm, and assisted in sample collections from multiple dairy farms. I am truly appreciative of their support, dedicated time, and priceless assistance.

Last but not least, I would like to thanks my friends and most important my family for their love, encouragement and support throughout my life and during the MS study.

ABSTRACT

Dairy industry is important for milk production and has substantial contribution to the California's economy, however, dairy farms also produce manure, which impacts the environment. Dairy manure contains carbon, nitrogen and phosphorus, which are beneficial to soil. Manure also contains many bacteria including human pathogen, such as *Salmonella* and *Escherichia coli* (*E. coli*) which poses risk to public health. In this research, an intensive reconnaissance survey of manure stored in dairy lagoon was conducted to determine the prevalence of *E. coli* and *Salmonella.* In addition, an attempt was made to develop the ozone and air-based treatment method to reduce pathogen in liquid manure. First a comprehensive review on microbial pollution of manure, and various control method was performed (Chapter 1). Secondly, a field study was undertaken to collect dairy manure from 20 dairy farms to determine the prevalence of indicator *E. coli*, Shiga toxin producing *E. coli* (STEC), and *E. coli* O157:H7 (Chapter 2). Finally, a lab scale experiment was developed to determine the impact of ozone and air injection on *E. coli* inactivation in liquid manure (Chapter 3). The first chapter addresses the issues of microbial pathogen in manure and associated risk to human, animal and environment and impacts of various treatment methods on bacteria control. Previous studies showed that anaerobic digestion reduces *E. coli* and *Salmonella* under prolonged incubation and high temperature conditions, however, many anaerobic pathogens such as *Clostridium* survive anaerobic environment. In anaerobic incubation, *E. coli* level is reduced, but these bacteria are not eliminated completely. In the second chapter, several bacteria testing methods were used to determine the prevalence of *E. coli*, *Salmonella*, STEC and *E. coli* O157:H7 which are common pathogens in dairy manure. About 177 manure samples were collected from 20 dairy farms from primary and secondary lagoons to test the bacteria using agar culture-based methods, polymerase chain reaction (PCR) method for detection of *E. coli* and *Salmonella.* In addition, a real- time PCR based method was used to

determine the presence of *E coli* O157:H7. Results showed that the prevalence of *Salmonella* in manure sample is lower than *E. coli*. The bacteria detection approach presented here could reduce the number of testing required during downstream confirmation process. The presence of *Salmonella* was found in 2.26 % of the samples, and both the culture-based and PCR methods yielded comparable outcomes in detecting *Salmonella*. Moreover, approximately 11.30% of the total samples out of the 177 were identified as positive for STEC by qPCR. None of the lagoon samples were positive to *E coli* O157:H7 by qPCR. The outcomes of this study hold substantial importance to determine the microbial quality of lagoon manure, and aid in the selection of appropriate methodologies for determining the prevalence of pathogenic organisms in dairy manure. In the third chapter, lab scale experiments were conducted to determine the impacts of ozone injection (Ozonation) and air injection (Aeration) in liquid dairy manure on *E. coli*reduction. Liquid manure was exposed to ozone and air for multiple durations (0 min, 60 min, 120min, and 240 min) and *E. coli* levels were determined. Further, manure samples were analyzed forpH, salt content, potassium (K^+), sodium (Na^+), nitrate ($NO3^-$), calcium ($Ca2^+$), and electrical conductivity (EC, mS/cm). Results showed that Ozonation can be an effective treatment method to reduce *E. coli* levels in liquid manure. Further nitrate ions were reduced substantially by Ozonation. The analysis of variance (one way and two way) revealed significance difference in *E.coli* and nitrate reductions in liquid slurry among ozone and air treatments (p <0.05). The study's results carry significance importance in terms of developing a simple field-scale method for reducing microbial and nutrient pollutions from dairy lagoon manure.

CHAPTER 1: INTRODUCTION

1.1 Overview of microbial pollution in dairy manure

While dairy industry is valuable for milk production, it also generates a large amount of manure, and many pathogenic bacteria present in dairy manure poses risk to human health (Han et al. 2019, Hodgson et al. 2016). Many foodborne outbreaks of disease are linked with the livestock waste (Bicudo and Goyal 2003, Bintsis 2018), and substantial number of foodborne diseases are reported to be caused by contaminated fresh produce and animal products (Bintsis 2018, Davis et al. 2016). There are many pathogenic bacteria such as *E. coli* O157:H7, *Salmonella*, STEC, and *Campylobacter* in manure, and they poses health risks to human. The infective dose for each bacteria is different (low to high depending on pathogens) [Table 1.1], however, there has been increasing concern about the effects of livestock pathogens on human, and particular attention has been given to *E. coli, Salmonella,* and *Campylobacter* (Bicudo and Goyal 2003, Bintsis 2018). For example, raw milk can cause disease by *Campylobacter* (Davis et al. 2016), and many fresh produce outbreaks by Shiga toxin-producing *Escherichia coli* (STEC) are reported in the USA (Atwill et al. 2015, Baker et al. 2019). More than 265,000 illnesses are reported annually in the USA by STEC (Baker et al. 2019), and because of STEC high virulence (5-50 cells can cause infection), it is a major concern for dairy industry (Farrokh et al. 2013). In addition, *Salmonella* is responsible for salmonellosis, commonly linked with poultry litter and fecal contamination. Though infective dose is high [1,000-10,000 cells], *Salmonella* is responsible for over 2 million cases of infection annually and around 2,000 deaths/year (Bicudo and Goyal 2003, Lung et al. 2001). Outbreaks related with *E. coli* and *Salmonella* are often related with the consumption of unpasteurized products and raw vegetables, which were connected with the use of manure during growing and harvesting (Lung et al. 2001).

Table 1.1: Common sources and infection doses of major pathogens (STEC, *E. coli*, *Salmonella*, and *Campylobacter*).

Bacteria	Infective dose	Common sources	References
E. coli O157:H7	Low [<10-100 bacteria]	Livestock waste, uncooked food, unclean water	(Baker et al. 2019, Cho et al. 2006, Farrokh et al. 2013)
E. coli (STEC)	Low [< 10 cells]	Animal waste, dairy, raw produce, uncooked ground beef	(Bicudo and Goyal 2003, Radhika et al. 2014)
Salmonella	High [1000-10,000 cells]	Poultry, egg, unpasteurized milk, salad	
Campylobacter	Low [500 – 800 CFU]	Dairy manure, raw chicken, raw milk, water	(Bicudo and Goyal 2003, Chen et al. 2019, Davis et al. 2016)

1.2 Persistence of STEC, *E. coli* and *Salmonella* in dairy manure

While prolonged storage of manure degrades pathogens in manure, *E. coli* and *Salmonella* are shown to persist for extended period of time depending on the moisture and environmental conditions (Table 1.2). Pathogenic microorganisms, which are zoonotic in nature (i.e., *Salmonella*, *E. coli*, *Campylobacter*) are present in manure, and when animal manure is spread on cropland, these pathogens are exposed to various environmental conditions, and often many of these pathogens survive for extended period of time depending on the weather, manure handling, and treatment of manure prior to application (Bicudo and Goyal 2003, Biswas et al. 2018). For example, *Listeria* and *E. coli* can survive in solid and liquid manure for many months (Biswas et al. 2018, Biswas et al. 2016, Black et al. 2021). Viable pathogens present in soil amended with manure can be transported to environment by various processes such as leaching, and surface run-off (Black et al. 2021). In general, treatment of manure by composting has a potential to eliminate pathogens because of higher temperature during composting process (> 55 °C), and studies have shown that *Escherichia coli* O157:H7 and *Salmonella* survived in animal waste, which went

through composting process (Chen et al. 2018). Pathogens such as STEC, and *Escherichia coli* O157:H7 are also reported in organic dairy farms, and understanding the prevalence, survival, and dissemination of these pathogens is crucial in order to prevent the

Table 1.2: Prevalence of STEC, *E. coli* and *Salmonella* in dairy manure

Descriptions	**Material/source**	**References**
High prevalence of pathogens in animal feces was observed in stored manure. *E. coli* survival rate was observed relatively higher in stored manure as compared to milk, bedding, fecal and soil samples.	Solid dairy manure	(Toth et al. 2013)
A significantly higher prevalence of *E. coli* O157 was noted in herds that grazed in land, which was amended with manure, and farm level control of pathogens may provide benefits	Manure amended soil	(Hancock et al. 1998)
The prevalence of *E. coli* O157 and *Salmonella* spp. in dairy manure was 15.4% and 6.6% respectively.	Solid dairy manure	(Chen et al. 2019)
E. coli and *Listeria* survived in the solid dairy manure piles for over 29 weeks. In slurry samples, *E. coli* was not detected after 14 weeks, but *Listeria* survived more than 29 weeks.	Solid and liquid dairy manure	(Biswas et al. 2018)
Presence and survival of *Escherichia coli* in liquid fraction of dairy slurry on acidic environment.	Liquid slurry	(Soares et al. 2019)
A prevalence rate of 9% for stx1 and/or stx2and 19% for rfbE was observed from the 518 dairy manure samples	Dairy manure	(Baker et al. 2019)
Prevalence of Shiga Toxin-Producing *Escherichia coli* stx1, stx2, eaeA, and *E. coli* O157:H7 in Organic dairy farm manure	Dairy manure	(Franz et al. 2007)

spread of microbial pathogens to the environment and ambient water (Franz et al. 2007). Spreading of manure on cropland, and its subsequent transport to surface water by runoff can cause food

borne contamination. For example, studies were able to detect *E. coli* and antibiotic resistant bacteria in vegetables and produce crops grown in land receiving manure as fertilizers (Black et al. 2021, Nicholson et al. 2005, Tien et al. 2017).

1.3 Dairy manure treatment methods for pathogen removal

In a dairy farm, both liquid and solid manure waste is produced in a large volume and controlling pathogens in manure require on-farm treatment methods. In California dairy farms, liquid solid separation and manure storage in lagoons are most common practices. Solid manure separated from liquid after flushing the barn is often dried in outdoor by sunshine and stored in the form of piles (Biswas et al. 2018, Nicholson et al. 2005, Pandey et al. 2018, Spiehs and Goyal 2007). In addition, some dairy farms use composting process to treat manure. More recently, dairy digesters are built in dairy farms to treat manure and produce biogas. All these existing manure treatment methods have numerous benefits in terms of handling the manure, improving the farm conditions, and controlling greenhouse gases and odor (Horan et al. 2004, Pandey et al. 2015, Smith et al. 2005). However, the impacts of these manure treatment methods on pathogen removal is often uncertain because pathogen survival can depends on many factors such as moisture and temperature (Nicholson et al. 2005, Pandey and Soupir 2011). A range of studies have reported various outcomes in terms of pathogens survival in various manure treatment processes (Table 1.3). Studies have shown that anaerobic digestion process is able to reduce *E. coli* and *Salmonella* under certain conditions, however, many other pathogens such as clostridia are not removed, and temperature of anaerobic digestion seems to play a big role in pathogen removal (Biswas et al. 2016, Costa et al. 2017, Horan et al. 2004, Pandey et al. 2016).

Table 1.3: Manure treatment methods and pathogen removal.

Treatment methods	**Effect on pathogen removal**	**References**
Large-scale manure treatment (i.e., liquid solid separation, screening clarifier, anaerobic	Anaerobic digestion found to remove manure indicator bacterial, however, liquid solid separation has minimal impact in pathogen removal.	(Liu et al. 2016)
Anaerobic digestion and storage	Anaerobic digestion significantly reduced indicator bacteria like lactobacilli, coliforms, and streptococci, and it lowered coliforms in pig slurry, and streptococci in dairy manure. Clostridia was not reduced.	(Costa et al. 2017)
Mesophilic anaerobic digestion	Mesophilic digestion was found to reduce *E. coli*, *Listeria,* and *Salmonella*. However, the numbers of *Campylobacter* were unchanged.	(Horan et al. 2004)
Moderate, mesophilic and thermophilic anaerobic digestion	*E. coli* inactivation was greatly depends on temperatures and incubation period. At thermophilic temperature, *E. coli* inactivation was relatively faster than mesophilic and moderate temperature.	(Pandey and Soupir 2011)
Composting of dairy manure	Large populations of *E. coli* O157:H7 survived for 36 days during composting at an external temperature of 21 °C, however, *E. coli* was inactivated in 7-14 days when the external temperature of the bioreactor was 50 °C.	(Jiang et al. 2003)
Composting of pig manure	Composting process reduced *E. coli* and *Enterococcus* and eliminated *Salmonella. Bacillus licheniformis* and *Clostridium sporogenes* were recoverable in compost products.	(Mc Carthy et al. 2011)
On-farm dairy manure composting	Composting with turning can be an option to inactive *E. coli* O157:H7 on-farm dairy manure.	(Shepherd et al. 2007)
Pig litter composting	Windrow composting successfully removed Salmonella and decrease fecal coliform.	(Tiquia et al. 1998)

In general, temperature in composting as compared to anaerobic digestion is higher, and high temperature during composting process is found to be more suitable in removing pathogens. However, the removal of pathogens could vary depending on the temperature level, moisture and turning frequency during compost process (Jiang et al. 2003, Mc Carthy et al. 2011). In addition to composting, desiccation process found to play a role in elimination of coliform. The number of coliforms is reduced by $10^2 – 10^4$ in manure with the progression of composting process, and once the temperature of compost reaches to thermophilic conditions (Larney et al. 2003), *E. coli* levels in dairy manure is reduced substantially with periodic pile turning (Shepherd et al. 2007).

1.4 Dairy manure aeration

Dairy manure produces odors, and aeration of manure in lagoons is found to control odor and ammonia (Schroeder et al. 2011, Westerman and Zhang 1997, Zhang et al. 2004). While energy is a major factor which prohibit continuous aeration of lagoons, sporadic aeration of lagoon is common in dairy farms, which increases oxygen levels in manure (Zhang et al. 2004). Because of low oxygen environment in liquid manure stored in lagoons, the decomposition of organic matter under anaerobic condition occurs, which produces odorous gases such as ammonia, hydrogen sulfide, and volatile compounds (Westerman and Zhang 1997). Experiment showed that sporadic aeration in headspace of anaerobic digester reduces hydrogen sulfide substantially without affecting methane production rate (Mulbry et al. 2017). Aeration process assist in enhancing aerobic decompositions, reducing volatile fatty acids (VFA) and Biological Oxygen Demand (BOD), and pathogens (Zhang et al. 2004).

Aeration of dairy manure slurry stored in lagoons is done by various methods (Figure 1.1) such as injection of compressed air, mechanical surface aeration, mechanical subsurface aeration, and liquid pumping/recycling to lagoons (Westerman and Zhang 1997). In general, both air injection

and mixing is used in dairy lagoons to create aerobic environment. Majority of the aeration systems used in dairy lagoons are in two categories: 1) surface aeration; and 2) diffuse aeration (Figure 1.1). In surface aeration, motor-mounted propeller are used to circulate surrounding lagoon water, which facilitate air (Fig. 1.1(C)). Oxygen transferring into the liquid is done by either shearing the liquid surface or by injecting air through orifices (Rosso et al. 2008). Motor-

Figure 1.1: Aeration of dairy manure slurry: A) diffuse aeration; B) aeration by bank mounted aeration pump; C) mechanical aeration; and D) tractor PTO driven lagoon pump. Modified online images [A, B, C, D]. Available from: https://blog.wastewater.com/technical-bulletin-178-selecting-lagoon-aeration-systems; https://www.netsolwater.com/mechanical-wastewater-vs-diffused-air-aeration-systems.php?blog=2007; https://www.hydroinnovations.com.au/training/aeration-systems-are-important; http://nuhn.ca/products/manure-pumps/lagoon-pump/; https://www.youtube.com/watch?v=YctXi9jDK8U. [Accessed 3 December, 2023].

-mounted propeller-based aerator are portable and can be easily moved from one farm to another. However, these are considered to be less efficient (Mukhtar et al. 2010). Venturi aeration system

mix the liquid and aerate lagoons (Fig. 1.1 (B)), which requires pump to draw slurry from the lagoons and pumps liquid into the venturi system that creates mixing and oxidation zone effectively (Yadav et al. 2021). The venturi aerator are small in size, and facilitate mixing and equalization in lagoons (Fig. 1.1 (D)), and are considered to be useful for sewage treatment (Anastasi et al. 2012, Anastasi et al. 2013) and supplying oxygen steadily to liquid manure efficiently (Mukhtar et al. 2010, Yadav et al. 2021). In addition, diffuse aerator are also used in dairy lagoons (Fig. 1.1 (A)), which provides oxygen from the bottom to the top, which facilitate mixing more uniformly (Vagheei 2021). These systems are found to be suitable in avoiding overloading and toxic conditions, and also provide continuous aeration at low cost without creating the turbulence and turbidity in lagoons (Rosso et al. 2008, Vagheei 2021). In general, aerator are found to be capable of reducing ammonia and odor, however, studies have shown that aeration does not reduce nitrate and bacteria, and therefore, Ozonation can be a good option because it can affect both nitrate and bacteria (Alkoaik 2009, Huang et al. 2022, Lin and Wu 1996, Schroeder et al. 2011)

1.5 Ozonation of dairy manure

Ozone is effective in removing odor and disinfecting the waste water (Botondi et al. 2023). Ozone is a strong oxidizing agent and ozone induced oxidation process can reduce bacteria and pathogens. Results showed that over 60% odor was reduced when ozone was injected into liquid slurry (Alkoaik 2009). The use of ozone technology (Figure 1.2 (A-D)) is considered to be eco-friendly for sanitization of the products of dairy supply chain (Botondi et al. 2023). For example, ozone is used for sanitizing air, water and food (Rangel et al. 2021). Ozone can be easily produced in large quantity from atmospheric air with the help of ozone generator, and ozone enhances the characteristics of sludge (Ried et al. 2014). It can assist in controlling buildup of foam in waste

water during treatment process (Ried et al. 2014). In waste water treatment, liquid solid separation is essential, and it can be affected adversely by bulking and foaming (Figure 1.3 (A-B)). Even low-dose gaseous ozone is found to be effective in removal of pathogenic bacteria. In a study, chronically infected wounds were exposed to the low-dose ozone, and results showed that eight strains: *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* were completely inhibited by ozone (Fontes et al. 2012). Ozone inhibit the cell viability of *E. coli*, *P. aeruginosa* and *A. baumannii* (15%) considerably (Rangel et al. 2021).

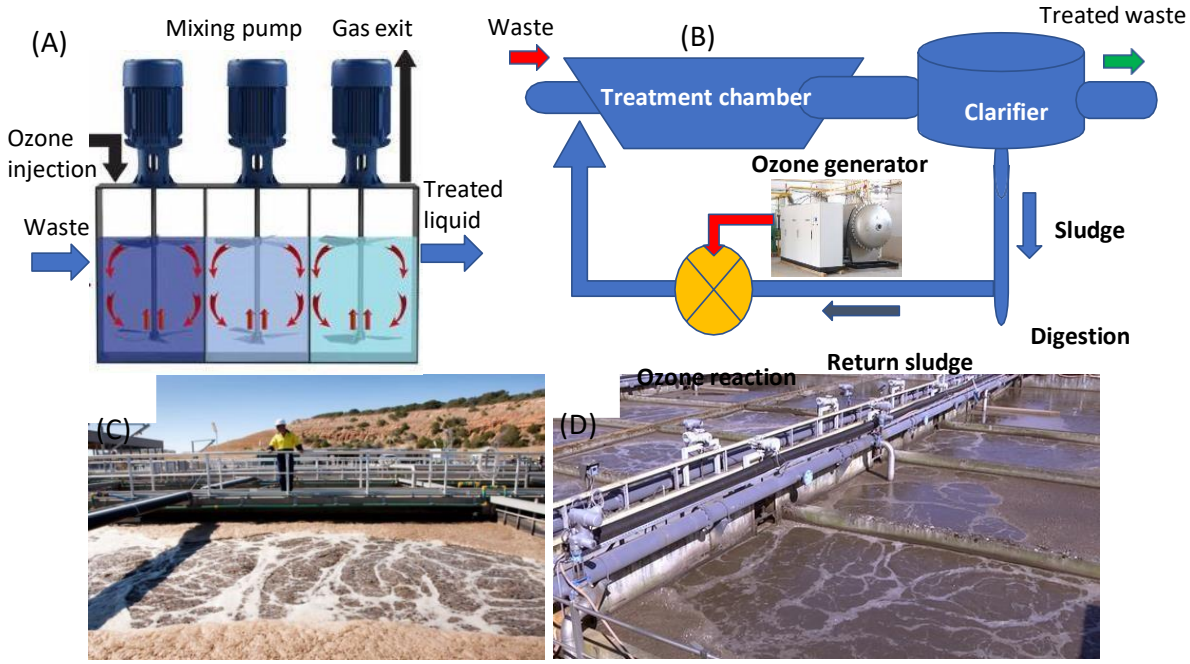


Figure 1.2: Ozone based treatment of wastewater: A) ozone injection and mixing; B) ozone reaction in clarified connected waste water system; C) ozone filtration; and D) ozone wastewater treatment. Modified online images [A, B, C, D]. Available from: https://chemtech-us.com/articles/effectiveness-and-benefits-of-ozone-filtration-for-wastewater-treatment/; https://www.ozcon.co.uk/applications/ozone-water-treatment/ozone-wastewater/; https://www.indiamart.com/proddetail/used-ozone-generators-20170002355.html; https://www.wateronline.com/doc/expansion-and-upgrade-of-wwtp-ozone-disinfection-system-reduces-cost-by-0001. [Accessed 3 December, 2023].

Because of ozone's unique properties such as high reactivity, and no leftover harmful residues, ozone is considered to be one of the potential options to disinfect dairy farm, and improve hygiene and biosecurity of dairy products (Megahed et al. 2018). Research showed that low level of ozone for shorter time can also be effective in removal of pathogens from various material surfaces, which are hard to disinfect (Megahed et al. 2018).

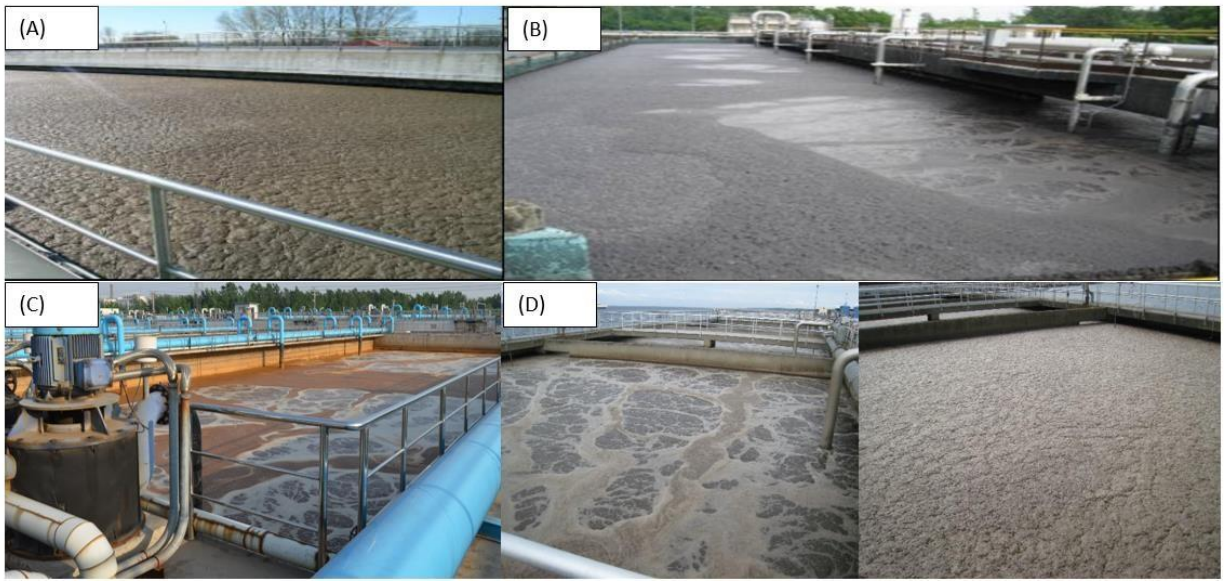


Figure 1.3: Impact of ozone on controlling foam formation: A) foam formation in waste water treatment; B) reduced foam formation by Ozonation (Ried et al. 2014); and C,D) Ozone treatment of wastewater. Modified online images [A, B, C, D]. Available from: https://www.semanticscholar.org/paper/Ozone-to-Control-Bulking-and-Foaming-in-Municipal-Ried-Wang/8023d02b3ef143cb5eb072cac029ce17b8945df4/figure/2 ; https://ozonesolutions.com/wastewater; https://www.semanticscholar.org/paper/Application-of-ozone-in-wastewater-treatment-%3A-For-Nilsson/9947e5257ee1a79f5381b7899446a6d58e9d7ff1#extracted. [Accessed 3 December, 2023].

Although pathogenic bacteria in dairy manure pose serious risks to public and animal health, there are limited options to control microbial pollution in dairy manure mainly because the existing methods such as drying and heating are cost prohibitive for enormous volume of manure produced in each dairy farm. While sporadic aeration is used in dairy farms for mixing the liquid slurry and

controlling the odor, there is limited information on the removal of pathogenic organisms from dairy lagoon manure by aeration. In wastewater treatment system, Ozonation is used as a tertiary treatment method to eliminate harmful bacteria and odor, however, existing knowledge on the use of ozone for treating dairy manure stored in lagoon is weak. In addition, there is a knowledge gap in the prevalence of pathogens such as STEC, *Salmonella*, and *E. coli* O157:H7 in manure stored in dairy lagoons.

The overall goal of this study is to develop an improved understanding of pathogen contamination in liquid manure stored in dairy lagoons, and evaluate the potential treatment methods to control pathogenic bacteria in liquid manure prior to its application as fertilizer. The specific objectives of this study are to: 1) determine the relative prevalence of *E. coli*, *Salmonella*, and STEC in dairy lagoon; 2) determine the impacts of Ozonation of bacteria removal in dairy lagoon slurry; and 3) evaluate the impacts of Aeration on *E. coli* removal, and compare with Ozonation process. In this thesis, there are three chapters. The first chapter is focused on understanding the issues of microbial pathogen in manure, and associated risks to human, animal, and environment. The second chapter is focused on determining the prevalence of pathogens in dairy lagoons in California. Subsequently, the third chapter is focused on developing the manure treatment methods for controlling microbial pathogens in dairy manure stored in dairy lagoons.

CHAPTER 2

Determining *Escherichia coli, Salmonella*, and Shiga toxin-producing *Escherichia coli* in dairy lagoons

2.1 Introduction

Dairy industry is one of the top agriculture industry in the United States, which contributes to U.S. economy substantially, and it supports approximately 0.2 million jobs, and $21 billion in economy (Naranjo et al. 2020, Valldecabres et al. 2022). In dairy farming, one of the major environmental and health concerns is the excessive amount of manure production, and potential spread of pathogens from manure to food, water, and environment, which poses potential health risks to humans and animals (Atwill et al. 2015, Biswas et al. 2018, Pandey et al. 2014, Semenov et al. 2007). In many agricultural and food product-associated, dairy manure was found to be a potential source of contamination (Biswas et al. 2018, Pell 1997, Sheng et al. 2019). The manure from cattle (raised in confined feeding operations) contains pathogens such as *E. coli*, *Salmonella,* and *Listeria*, which causes outbreaks of foodborne diseases that has received substantial attention in the USA (Bicudo and Goyal 2003, Biswas et al. 2018, Tabe et al. 2016, Varsaki et al. 2022).

E. coli and *Listeria,* are often found in dairy manure which are linked with microbial pollution of water (Some et al. 2021, Subirats et al. 2022, Varsaki et al. 2022). A survey in the dairy farm environment of 13 dairy operations for detecting five animal borne pathogens (*E. coli* O157:H7, *Campylobacter jejuni, Mycobacterium avium* ssp., *Salmonella enterica*, *Cryptosporidium parvum*) showed that *E. coli* O157:H7 was detected on 6 farms, *Mycobacterium avium* ssp. was detected on 10 farms, and *Cryptosporidium parvum* was detected in feces samples in majority of farms. The *Campylobacter jejuni* and *Salmonella enterica* were found less frequently (Toth et al. 2013).

Another study reported that the environment of cattle farms retains a relatively higher incidence of *Listeria monocytogenes* (Varsaki et al. 2022). While studying the occurrence of *Escherichia coli* O157 and *Salmonella* spp. in bovine manure, research found the prevalence of *E. coli* O157 and *Salmonella* spp., were 15.4% and 6.6% respectively (Chen et al. 2019). In addition to conventional dairy, the harmful STEC is also prevalent in manure from organic dairy farm (Franz et al. 2007).

On-farm practices such as waste treatment and herd management play an important role in controlling microbial pathogens that causes foodborne pathogens (Hancock et al. 1998). For example, an evaluation of a survey in 36 dairy herds showed that prevalence of Shiga toxin-positive *Escherichia coli* was related to herd management and waste management (Hancock et al. 1998). Similar to *E. coli* in dairy waste, human waste also contains many bacteria including *E. coli* (Anastasi et al. 2012), and controlling it requires implementation of waste treatment methods such as anaerobic digestion, composting, aeration, and disinfection technologies (Anastasi et al. 2013). Research have shown that *E. coli* strains can survive waste water treatment processes used in sewage waste treatment (Anastasi et al. 2012, Anastasi et al. 2013, Yu et al. 2022). Because of manure borne pathogens, soils amended with raw and improperly treated manure can pose health risks. Knowing the prevalence of pathogens in manure can assist in developing the treatment methods, and implementing the improved manure application strategies in cropland (Awasthi et al. 2020, Baker et al. 2019, Manyi-Loh et al. 2016, Mc Carthy et al. 2011).

Environmental concerns such as nutrients (nitrogen, and phosphorous), and salts have received substantial attention because of related water quality issues, however, the issues of manure borne pathogens have received relatively less attention (Pell 1997). Recent outbreaks related with *Escherichia coli* O157:H7, and their linkages with manure have received increased attention

(Bintsis 2018, Pell 1997) because of potential risks to human health. Fecal contamination from animal facilities can cause produce microbial contamination, which can affect ambient water, and soil (Bicudo and Goyal 2003, Bintsis 2018, Pandey et al. 2018, Pandey et al. 2014).

There has been a growing interest to improve our existing understanding of manure borne pathogens and associated risks to public and animal health risk (Bicudo and Goyal 2003, Han et al. 2019). Even though there are many bacteria, relatively a small population of bacteria is pathogens, which cause disease in human (Alberts et al. 2002). As an example, over 700 serotypes of *E.coli* are known, and most varieties are harmless except few, which causes diseases including diarrhea (Bean et al. 2004, Manning 2010). *E. coli* in dairy cows are considered to be an opportunistic pathogens, and most *E. coli* not necessarily cause disease (Bean et al. 2004). *E. coli* is very closely related to two types of foodborne pathogens *Shigella flexneri*, which causes bloody diarrhea, and *Salmonella enterica* that lead to food poisoning (Alberts et al. 2002). *E. coli* strains such as Shiga toxin-producing *E. coli* (STEC) can cause serious illnesses because this *E. coli* produces a toxin called Shiga, which damages the lining of small intestine that causes diarrhea (Alberts et al. 2002, Manning 2010, Paton and Paton 1998).

In dairy cows, STEC can be either serotype O157:H7 or non O157. Shiga toxins can be detected in both O157 and non-O157 STEC (Hermos et al. 2011). The STEC are a heterogeneous group of foodborne pathogens, and transmission of STEC is caused through contaminated foods and water (Ballem et al. 2020). In STEC, *stx* genes produce Shiga toxins (Stx), which are two types (Stx 1, and Stx 2) (Scheutz et al. 2012). The STEC serotype, which is most commonly linked with outbreaks is O157:H7 (Byrne et al. 2015). Cattle are one of the largest reservoirs of zoonotic STEC (Monaghan et al. 2012), which can be transmitted to human by contaminated food and by direct contact with manure.

While numerous studies have shown the prevalence of *E. coli* in dairy farm environment, the knowledge is limited to evaluate the prevalence of *Salmonella enterica* and *E. coli*, and STEC in liquid manure stored in dairy lagoons. In California, majority of dairy farms uses flush system to clean the free stall/flush lane, where manure is buildup. Manure from flush lane is removed by water flow, and flush lane drains manure water into a sediment trap where solid particles get deposited (Pandey et al. 2023, Shetty et al. 2023). Subsequently, manure water goes to liquid solid separation, where solid is separated from liquid, and liquid goes to lagoons (Pandey et al. 2018). The goal of this study is to determine the prevalence of pathogens in liquid manure stored in lagoons. The specific objectives of this research are threefold: first, to determine prevalence of *E. coli* and *Salmonella* in lagoon liquid manure using culture-based method. Second, to verify *E. coli* and *Salmonella* by polymerase chain reaction (PCR). Third, to examine the presence of STEC and *Escherichia coli* O157:H7 in lagoon liquid manure by quantitative polymerase chain reaction (qPCR) and determine the true positive and true negative samples of STEC, and *E. coli* O157:H7.

2.2 Material and Methods

2.2.1 Sample collection

More than 80% of California's dairy cows are located in Central Valley, and in this study, we randomly selected 20 dairy farms located in Central Valley based on the willingness of farmers to support this study. The approximate number of milk cows in each dairy farm varied between

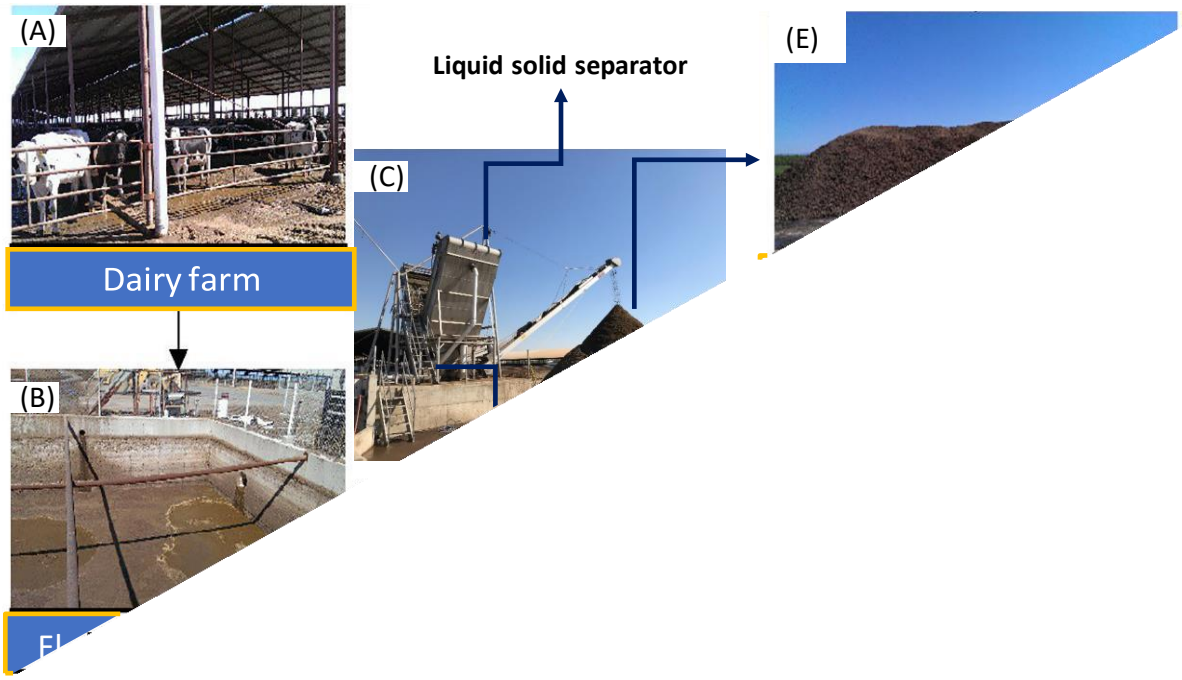


Figure 2.1: Flush manure management system, a typical practice used in manure handling in Central Valley, California. A) free stall in dairy farm, where cows are fed, and manure is collected; B) flush tank, which receives flush manure from free stall/flush lane, and mixer in this tank agitate manure before sending it to the liquid solid separator; C) mechanical liquid solid separator, which receives liquid manure from flush tank by pumps, and separates liquid from manure. After separation, liquid is sent to lagoons, and solid is stored in piles; D) solid manure pile after liquid solid separation; and E) dairy lagoon, which store the liquid manure.

700 and 1000. Cows in all dairy farms were predominantly Holstein. Previous history of pathogen presence on these farms were unknown. All dairy farms of this study are using flush system to clean the manure on free stall/flush lane. Flush manure from free stall passes through liquid solid separator, and subsequently liquid manure is stored in lagoons. In each dairy farms, manure samples were collected from two lagoons (primary and secondary lagoons), where on-farm liquid manure is stored. To collect manure samples from these lagoons, we used amber wide-mouth high density polyethylene (HDPE) plastic bottle (250 mL), which reduce UV light transmission. For each sampling location, we used sterilized bottles labeled with a simple sample ID. Bottles were filled roughly ¼ full, capped tightly, and cleaned with Milli-Q water thoroughly at each sampling

location. To collect manure samples from lagoons, we used a telescoping stainless steel rod (8 feet), and sampling bottle was attached at the end of the pole (i.e., sampling rod). Samples from dairy farms were collected in summer between May and September 2021. Dairy farm visits were planned based on the farm owner willingness to participate in the study, and agreement was made to not disclose the actual farm's name and actual geographic location of a farm. Majority of lagoons were sampled in 4-6 locations, and liquid samples were collected from top surface layer (≈ 1 feet). A total of 177 liquid manure samples were collected, and samples were kept at 4°C during transportation and until bacterial analysis completed.

2.2.2 Salmonella testing by culture based method

To test the presence of *Salmonella,* we used XLT-4 agar plates. The work flow used in testing pathogens are described in Figure 2.1 (A). For *Salmonella* testing, 1 mL of manure sample was added to a tube that contained 9 mL of buffered peptone water, and we homogenized the samples by gently mixing. Subsequently, this sample was incubated for 24 hr at 35°C, and 0.1 mL of incubated sample was transferred to a test tube containing 10 mL of Rappaport-Vassiliadis medium (RVR10). Test tubes with RVR 10 was incubated at 42°C for 20-24 hrs. The enrichment was vortexed, and 100 µL of enriched medium was streaked onto a Xylose lysine tergito 4 (XLT-4) agar plates using a sterile loop. These plates were incubated at 35°C for 24-48h. After incubation, these agar plates were examined for typical *Salmonella* colonies (red colonies with a black center). Negative samples were discarded, and 2-3 colonies were picked for confirmation by polymerase chain reaction (PCR) method, which is described in section 2.2.3.

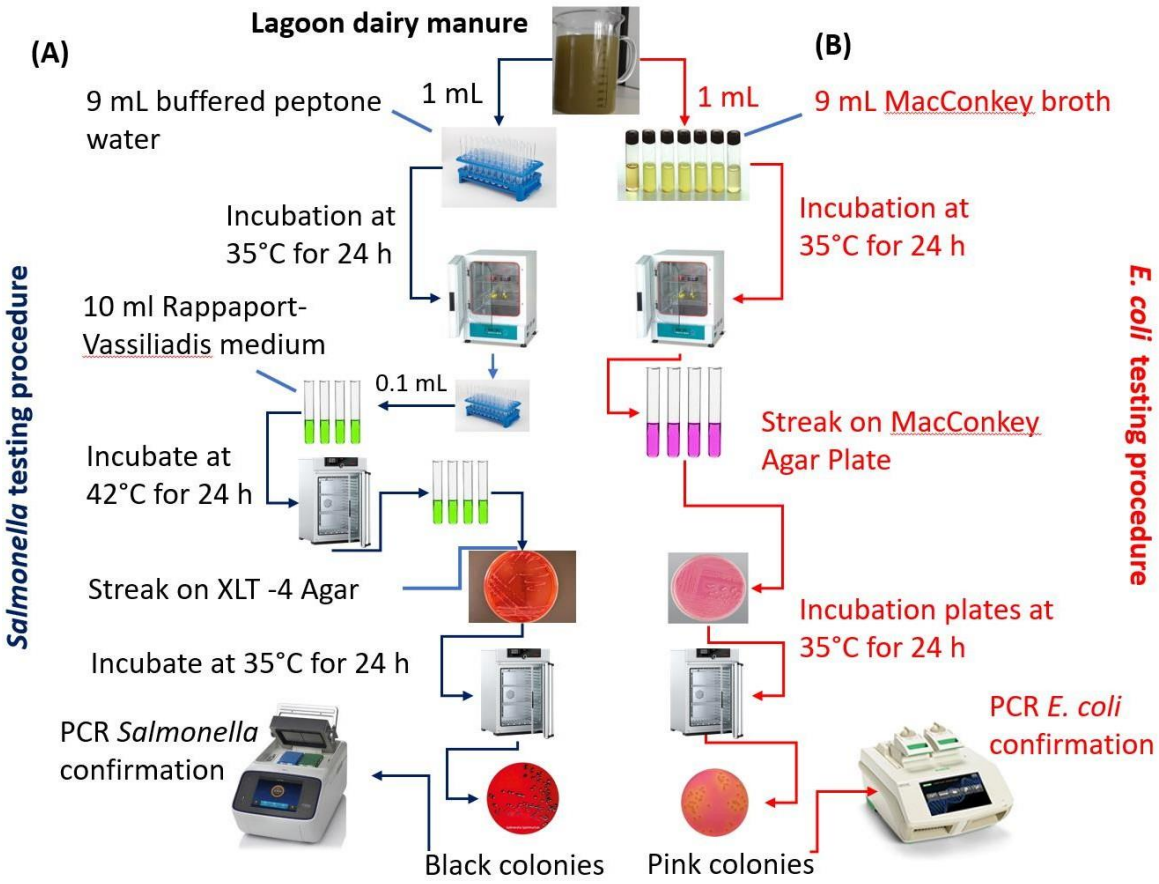


Figure 2.2: Workflow for testing *E. coli* and *Salmonella* from manure using culture based method and subsequent confirmation by PCR. Left side procedure shows the flow of testing for *Salmonella*, and right side procedure shows the flow for testing *E. coli*. The procedure involved enrichment steps for detection and isolation of *Salmonella* and *E. coli.*

2.2.3 E. coli testing by culture based method.

To test *E. coli*, we used MacConkey (MAC) agar plates. The workflow for testing *E. coli is* described in Figure 2.1 (B). For *E. coli* testing, 1 mL of liquid manure sample was added to a test tube that contains 9 mL of MacConkey. Samples were homogenized then incubated at 35°C for 24 h. One loopful from each container of MacConkey broth was streaked onto the first quadrant of a MacConkey (MAC) agar plate, and the remainder of the plate was streaked to obtain isolated colonies.. Plates were incubated at 35°C for 24 h, and each agar plate was examined for typical *E.*

coli colonies (pink to dark pink). If no growth was observed on MAC agar plates, then plate was discarded. Presumptive *E. coli* colonies on agar plates were confirmed by PCR.

2.2.4 E. coli, Salmonella, STEC and *E. coli* O157:H7 confirmation by PCR and qPCR methods

After isolating presumptive *E. coli* colonies, Genomic DNA from confirmed *E. coli* colonies was extracted using Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA) (Bowman et al. 2023), which uses a simple method for the rapid isolation of total DNA (Atwill et al. 2015). A simple procedure for genomic DNA extraction (Figure 2.3 (A)) and PCR testing is shown in Figure 2.3 (B). The use of conventional PCR method (Figure 2.3 (B)) provided the confirmation of *E. coli*. These strains were confirmed as *E. coli* by PCR amplification of the universal stress protein (Chen and Griffiths 1998). The reaction mixture included a master mix of 1,730 µl filter-sterilized, 100 µL deoxynucleoside triphosphate (dNTP; 10 mM), Milli-Q water (autoclaved), 250 µL 10 × Bioline PCR buffer, 150 µL MgCl2 (50 mM), 25 µL of forward and reverse uspA primers (50 mM) (Invitrogen), 2.0 µL of purified bacterial DNA, and 20 µL Taq polymerase (Bioline). The PCR conditions of denaturation were 5 min at 95°C followed by 30 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C; and a final extension step of 5 min at 72°C were programmed. The primer sequences used were (forward) 5′-CCGATACGCTGCCAATCA GT-3′ and (reverse) 5′-ACGCAGACCGTAGGCCAGAT-3′, which are found to be specific to the uspA gene that is expressed in *E. coli* strain, and it generates 884-bp fragment (Chen and Griffiths 1998). These amplified PCR products were used in electrophoresis on 2% agarose gels in 0.6 × Tris base-EDTA (TBE) buffer and stained with ethidium bromide. Subsequently confirmed isolates were further tested for Shiga toxin-producing *E. coli* (STEC) and *E. coli* O157:H7.

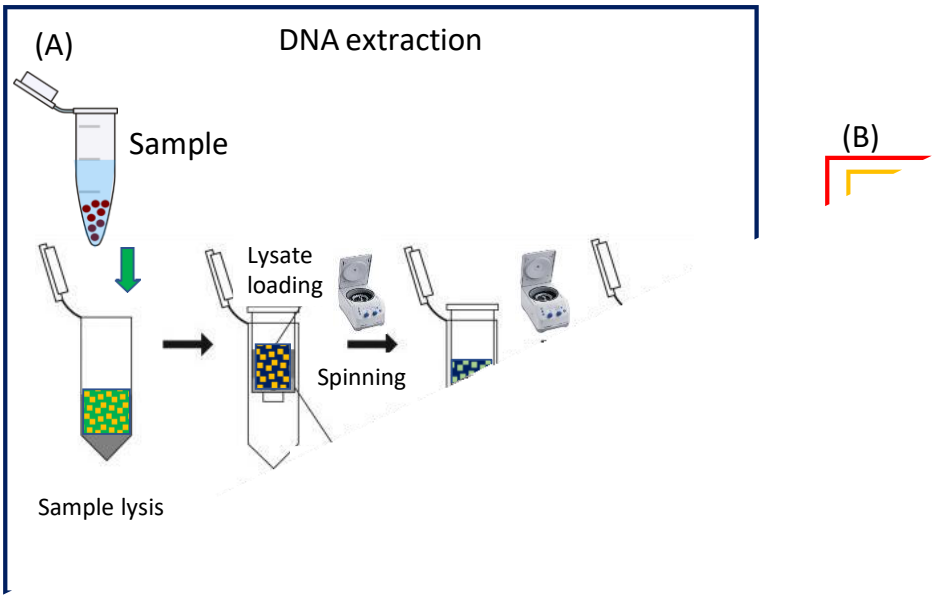


Figure 2.3: Overall workflow and procedure for genomic DNA extractions (A) from isolated colonies for testing *E. coli*, *Salmonella* by PCR (B).

The isolates, which were confirmed as *E. coli* by PCR were used for real-time PCR (qPCR) testing to examine if isolates were Shiga toxin-producing *E. coli* (STEC) and *E. coli* O157:H7. The isolated DNA of confirmed *E. coli* was screened for *stx1*, and *stx2* to identify STEC samples using the protocol described elsewhere (Baker et al. 2019, Suo et al. 2010). In qPCR testing, a Bio-Rad iCycler Optical Model was used in a 20 µL reaction [2 µL of DNA, 0.3 µM of each primer, 10 µL iTaq universal probes supermix, 0.25 µM of each probe, and 7.6 µL of sterile DNA grade water]. The conditions for qPCR were: 95˚C for 20 s, 40 cycles of 95˚C for 3 s, 60˚C for 30 s, followed by a 4˚C hold (Baker et al. 2019).

E. coli O157:H7 was tested by targeting the detection of *rfbE*, which is commonly used for determining the presence of *E. coli* O157:H7 (Baker et al. 2019, Jacob et al. 2012). Genomic DNA from previous confirmed *E. coli* was used in qPCR testing. The PCR reaction with 20 µL consisted of 2µL of DNA, 10 µL of iTaq universal probes supermix, 5 µL of sterile DNA grade water, and 0.5 µM of primers and probes. Temperature conditions of 95˚C for 10 min, 45 cycles of 95˚C for

15 s, 56˚C for 20 s, 72˚C for 40 s, followed by a 4˚C hold, were used (Baker et al. 2019, Jacob et al. 2012).

To conduct PCR for testing *Salmonella*, forward primer 51413371, SalmF-Inva 162994111 IDT [5'—ACA GTG CTC GTT TAC GAC CTG AAT—3']; T_m =58.4 ºC; MW=7,327.8; 100µM in 664 µl IDTE Buffer pH8.0 was used. The reverse primer for *Salmonella* testing was SalmR-Inva 162994112 IDT 51413372 [5'—AGA CGA CTG GTA CTG ATC GAT AAT —3']; T_m=55 ºC; MW=7,400.9; 100µM in 788 µl IDTE Buffer pH8.0. A 25 µl reaction volume included 12.5 µl dream mix, 0.1 µl *Salmonella* forward primer, 0.1 µl Salmonella reverse primer, 10.3 µl dH2O, and 2.0 µl DNA template. PCR conditions of initial denaturation at 95°C for 3 min, 30 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, followed by a final extension at 72°C for 7 min, then hold at 4°C were used.

2.2.4 Data analysis

The prevalence of *E. coli*, STEC, *Salmonella*, and *E. coli* O157:H7 was determined as the number of positive samples for each serotype divided by the total number of samples, which were used for evaluation. In addition, we created a confusion matrix (2 × 2) (Figure 2.4) using the number of positive and negative samples obtained in each approach. The results of positive and negatives samples obtained from culture based method, PCR method, and qPCR method were used to determine the true positive and negative samples, and this approach of data analysis is described elsewhere (Shental et al. 2020).

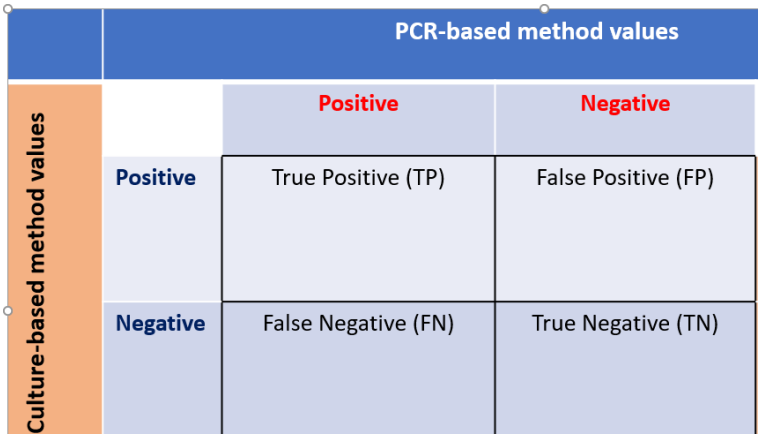


Figure 2.4: Confusion matrix for determining true positive, and true negative manure

2.3 Results and discussion

2.3.1 E. coli and *Salmonella* testing by culture-based method, PCR, and qPCR

A total of 177 liquid manure samples were collected from lagoons of 20 dairy farms (Figure 2.5 (A)). Out of these a total 88 samples were collected from primary lagoons, and 89 were from secondary lagoons (Figure 2.6). In general, equal number of samples were collected from both lagoons (primary and secondary). The second detection method was PCR, and all samples, which were found to be positive in culture based method were tested by PCR. Subsequently, qPCR method was used for detecting pathogenic *E. coli* strains, and all positive *E. coli* samples by PCR were further tested by qPCR. All positive and negative dairy manure samples from lagoon by culture-based test method are shown in Figure 2.5 (B). Out of 177, a total of 142 samples were positive for *E. coli*, and 4 samples were positive for *Salmonella* (Figure 2.5 (B)). All four samples, which were positive for *Salmonella* were also found to be positive for *E. coli.* Further testing by PCR-based method revealed that the samples (n = 4), which were positive for *Salmonella* by

culture-based method, were also found *Salmonella* positive by PCR method (Figure 2.5 (C)). In terms of *E. coli*, however, PCR based method revealed that out of 142 positive samples by culture-

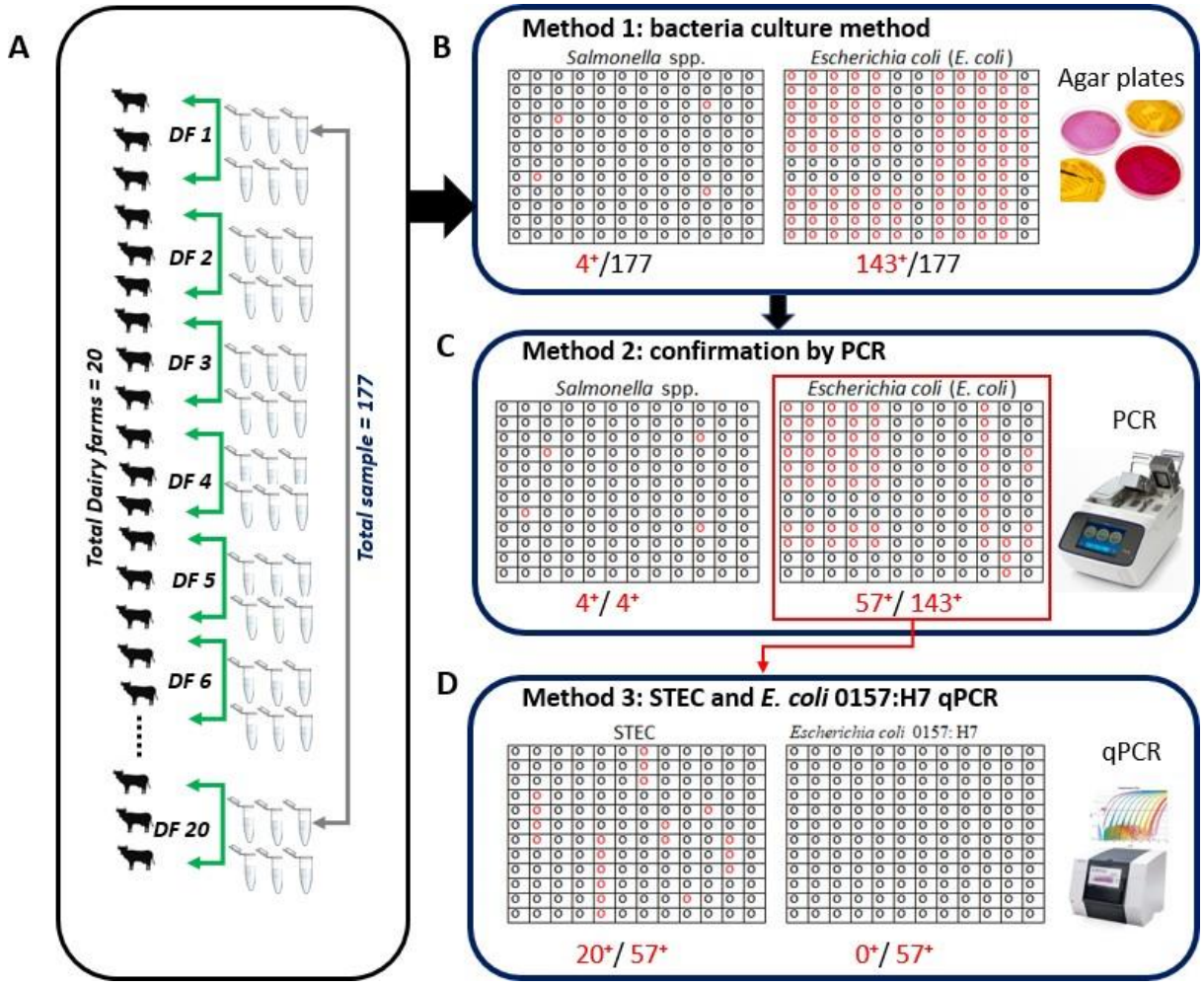
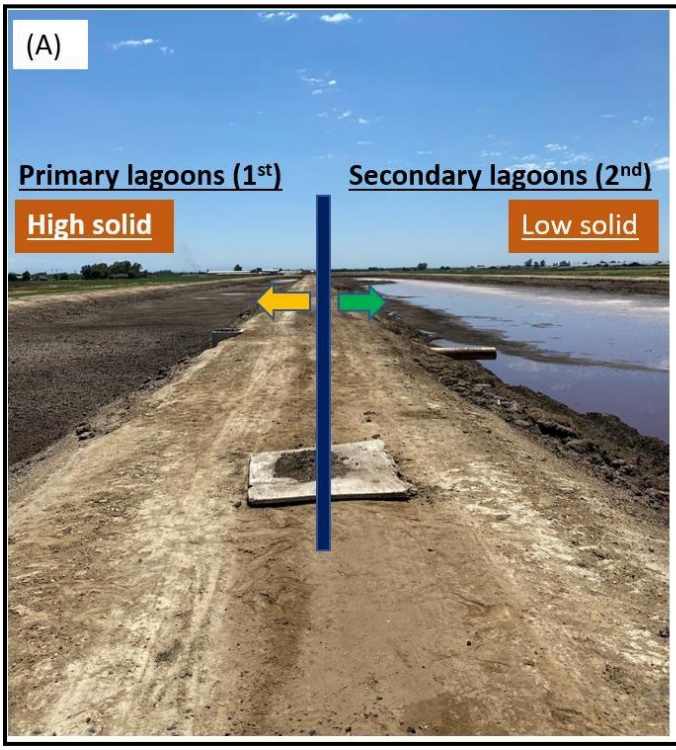


Figure 2.5: Sample number, detection methods, design, and number of positive and negative samples. A) number of dairy farms (DF) a total DF of 20, and sample number were a total of 177); B) *E. coli* and *Salmonella* detection using culture based method (Method 1) (left panel shows *Salmonella* positive and negative samples; and right panel shows *E. coli* positive and negative samples; C) confirmation of *Salmonella* and *E. coli* positive and negative samples by PCR (Method 2) (red dots indicate positive samples, and black dots indicate negative samples); and D) pathogenic *E. coli* (STEC, and *E. coli* O157:H7)detection by qPCR (left pane shows STEC positive and negative samples, and right panel shows *E. coli* O157:H7 positive and negative samples).

based method, only 57 samples were positive for *E. coli* (Figure 2.5 (C)). To determine pathogenic *E. coli* strain, we used qPCR, and results are shown in Figure 2.5 (D), which indicates that 20 samples were positive for STEC, and none of the samples were positive for *E. coli* O157:H7. The approach presented here can reduce the number of testing required during downstream confirmation process as compared to retesting each individual sample (both positive and negative) by all three methods. Results showed that number of positive samples for *Salmonella* were considerably low as compared to *E. coli.*



(B)

Dairy Farm ID	Number of liquid manure samples (n)	
	Primary Lagoon (PL)	Secondary Lagoon (SL)
D#1	6	6
D#2	6	6
D#3	6	6
D#4	6	6
D#5	6	6
D#6	6	6
D#7	6	6
D#8	6	6
D#9	2	1
D#10	3	2
D#11	2	2
D#12	2	2
D#13	2	2
D#14	2	2
D#15	2	2
D#16	6	9
D#17	6	6
D#18	6	6
D#19	5	5
D#20	2	2
Total	**88**	**89**

Figure 2.6: An image of primary lagoons and secondary lagoons (A), and sample distribution of primary and secondary lagoons (B). Depending on the system used for liquid and solid separation in dairy farms, total solid contents in manure in primary and secondary lagoons changes substantially.

2.3.2 Use of confusion matrix to determine true positive samples for *E. coli,* STEC*, and E. coli* O157:H7

In each testing method, we quantified the number of false negative (FN), false positives (FP), true negative (TN), and true positive (TP) samples, which were used to create the confusion matrix (Figure 2.7). The criteria for FN, FP, TP, and TN are shown in Figure 2.7. In brief, TP is an outcome, when both test methods (culture and PCR) produced the positive results. The TN is an outcome, when both test methods produced negative results. All positive samples from downstream testing procedure (PCR and qPCR) were compared with the culture based method. This approach of identifying the true positive and false negative samples has been found to be effective in efficient high-throughput SARS-CoV-2 testing to detect asymptomatic carriers (Shental et al. 2020). Figure 2.7 shows matrix used in determining true positive and true negative samples, and corresponding matrix results, which revealed that the number of true positive, true negative, and false positive samples varied considerably depending on the pathogens of interest. While comparing the results of *Salmonella* detection based on culture method and PCR method, 4 samples were true positive, and 173 samples were found to be true negative.

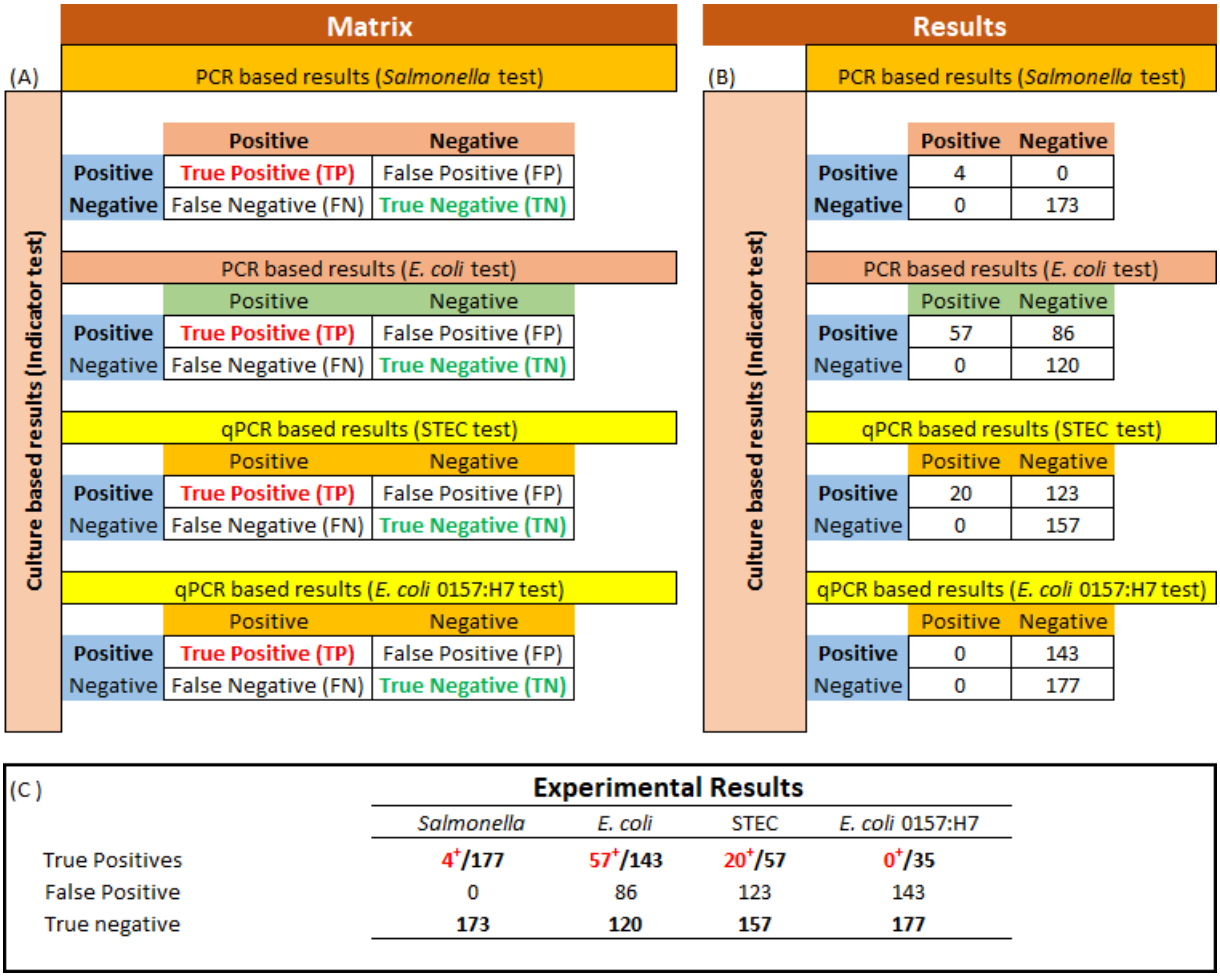


Figure 2.7: Experiment results of *E. coli*, *Salmonella*, STEC, and *E. coli* O157:H7 testing and related positive and negative samples in the form of confusion matrix. Matrix method, which compared the culture based results (*E. coli*) with PCR based results of (*Salmonella* and *E. coli*) is shown left side (A), and the results of matrix is shown on right side (B). Experimental results of true positive, false positive, and true negative are shown in bottom (C).

In downstream testing process, when we compared the culture-based results of *E. coli* with PCR based detection of *E. coli*, 57 samples were true positive for *E. coli*, and 120 samples were true negative for *E. coli*. False positive samples for *E. coli* were 85 (Figure 2.7 (B)). While comparing the results of STEC positive samples with culture based *E. coli* (i.e., indicator *E. coli*), 20 samples were true positive for STEC, and 35 were false positive, and 157 samples were true negative.

Results of qPCR based method revealed that there were no samples which were qPCR positive for *E. coli* O157:H7. All of 177 samples were negative for *E. coli* O157:H7. A total of 143 samples were positive for *E. coli* using culture based method. These *E. coli* positive samples were potentially non positive for *E. coli* O157:H7 (by qPCR). Comparative true positive, false positive, true negative samples for *Salmonella, E. coli,* STEC, and *E. coli* O157:H7 are shown in Figure 2.7 (C). The true positive and true negative samples for *Salmonella* were 4 and 173, and these values for *E. coli* based on PCR method were 57, and 120. These values for STEC were 20 and 157.

2.3.3 Positive and negative samples of primary and secondary lagoons and positivity rate

The positive and negative samples were further categorized based the samples locations (i.e., primary lagoon and secondary lagoon), and results are shown in Table 1. Out of 143 positive samples by culture-based method, 74 samples were from primary lagoons, and 69 were from secondary lagoons. Out of 4 *Salmonella* positive samples, two were from primary lagoons, and two were from secondary lagoons. There were 20 STEC positive samples, and 13 were from primary lagoons, and 7 were from secondary lagoons. None of the samples were positive for *E. coli* O157:H7 from primary and secondary lagoons. Further analysis was conducted to determine the prevalence of pathogens in terms of positivity rate (percentage of positive samples out of total tested samples), which is shown in Figure 2.8.

Table 2.1: Positive rate and number of positive and negative samples in primary and secondary lagoons.

Organisms	Number of positive & negative samples (n)		
	Primary Lagoon (PL)	**Secondary Lagoon (SL)**	**Overall (+) (PL + SL)**
E. coli pos. and neg. samples by culture method	74 (+) & 14 (-)	69 (+) & 20 (-)	143
Salmonella pos. and neg. samples by culture method	2 (+) & 86 (-)	2 (+) & 87 (-)	4
Salmonella pos. and neg. samples by PCR	2 (+) & 86 (-)	2 (+) & 87 (-)	4
E. coli pos. and neg. sample by PCR	27 (+) & 47 (-)	30 (+) & 59 (-)	57
STEC pos. and neg. by PCR method	13 (+) & 61 (-)	7 (+) & 82 (-)	20
E. coli O157:H7 pos. and neg. by qPCR	0 (+) & 88 (-)	0 (+) & 89 (-)	0
	Positive rate (%)		**Overall (%)**
	Primary Lagoon (PL)	**Secondary Lagoon (SL)**	**Overall (PL + SL)**
E. coli pos. and neg. samples by culture method	84.09%	77.53%	80.79%
Salmonella pos. and neg. samples by culture method	2.27%	2.25%	2.26%
Salmonella pos. and neg. samples by PCR	2.27%	2.25%	2.26%
E. coli pos. and neg. sample by PCR	30.68%	33.71%	32.20%
STEC pos. and neg. by PCR method	14.77%	7.87%	11.30%
E. coli O157:H7 pos. and neg. by qPCR	0.00%	0.00%	0.00%

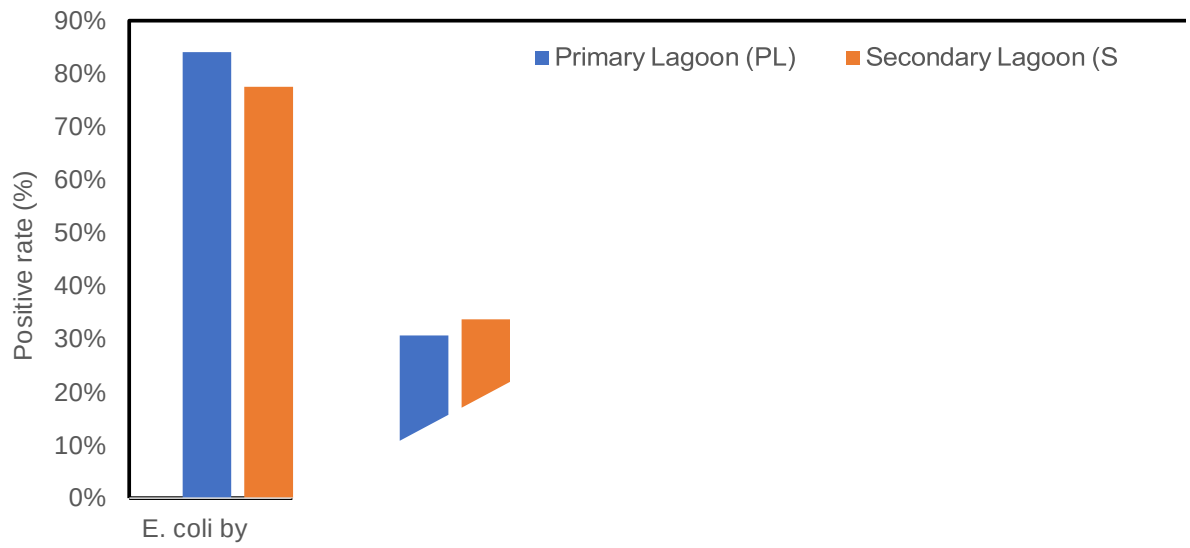


Figure 2.8: Positive rate (i.e., prevalence) of *Salmonella* and *E. coli* in primary and secondary lagoons. X-axis shows the pathogen types and corresponding test methods, and Y-axis shows the percentage of positive samples.

Based on the culture test method, 41.81% of samples from primary lagoons were positive for *E. coli*, and 38.98% of samples were positive for *E. coli* from secondary lagoons. However, the percentage of samples, which were confirmed in primary lagoons for *E. coli* by PCR was 15.25%, and 16.95% of samples were confirmed by PCR for *E. coli* in secondary lagoons. The STEC positive percentages were 7.34% in primary lagoons, and 3.94% in secondary lagoons. Only 1.13% of samples each from primary and secondary lagoons were positivity for *Salmonella.*

The primary focus of this study was to determine the prevalence of positive and negative samples in liquid manure from lagoons for *Salmonella, E. coli,* STEC, and *E. coli* O157:H7. Previous studies in this area of research were limited for California's dairy farms. Few studies in other regions, for example, a survey of animal-borne pathogens in the farm environment of 13 dairy operations in southeastern and south-central Pennsylvania evaluated the presence of pathogens

including *Salmonella enterica*, *E. coli*, and *Campylobacter* in various farm samples such as bedding, feces, milk, and soil materials. Results showed that over 50% of feces samples were positive for pathogenic bacteria, and 73% of stored manure samples were positive for pathogenic bacteria (Toth et al. 2013). However, the number of samples used in testing for each category were limited (sample number varied between 13 and 26 for various categories) (Toth et al. 2013).

Because of microbial safety issues of dairy manure fertilizer, there are concerns in terms of using manure for crops such as raspberry fruit crop and other produce crops (Sheng et al. 2019). Results showed that manure application prior to 4 months of harvesting resulted in no major impact on food safety of red raspberry (Sheng et al. 2019). In terms of impacts of manure on ambient water, extended storage of manure and application of manure several days before runoff reduce microbial loading from agricultural land substantially (Meals and Braun 2006). Other studies suggest that *E. coli* O157, *Salmonella* and *Campylobacter* survives in the soil after manure application for up to one month (Nicholson et al. 2005). In general, livestock waste is used extensively to fertilize the crop land, and improper manure management has a potential to contaminate foods and can serve as a major source of foodborne pathogens (Tabe et al. 2016). In North Dakota, for example, 136 manure samples were collected from feedlots, and 40% of samples were positive for *Salmonella*, and 18% were positive for *E. coli* O157 (Tabe et al. 2016). As reported by various studies, manure storage, age of manure, locations of farms, and weather may have substantial impacts on the prevalence of pathogens in dairy manure (Ballem et al. 2020, Chen et al. 2019, Cho et al. 2006, Franz et al. 2007). Frequent monitoring of microbial quality of manure could help in identifying the control measures for reducing microbial pollution from manure to environment and food (Some et al. 2021, Tiquia et al. 1998). Understanding the manure physical characteristics (i.e., moisture and particle size) in tandem with microbial characteristics can assist in development of manure

treatment methods which can control microbial pathogens in on-farm treatment practices. On-farm practices are essential to mitigate the pathogen risks to animal, human and environment (Ried et al. 2014, Smith et al. 2005, Spiehs and Goyal 2007, Westerman and Zhang 1997).

2.3.4 Conclusions

To determine the prevalence of *Salmonella, E. coli,* STEC, and *E. coli* O157:H7 in liquid manure stored in lagoons located in California Central Valley, this study was designed to carry out an extensive sampling plan followed by microbial testing in manure using various methods. To do that we partnered with 20 dairy farms, and over 177 liquid manure samples were collected from primary and secondary lagoons. The results suggest that the prevalence of *E. coli* O157:H7 in lagoon manure is low. Generic *E. coli* was present abundantly in manure. More than 80% liquid manure samples were positive for generic *E. coli,* which was tested in manure samples using the culture-based method, however, only 15-17% of positive manure samples by culture-based method were also found to be positive *E. coli* samples by PCR. About 2% of samples were positive to *Salmonella*. Both culture-based method and PCR based method produced similar results for *Salmonella.* About 11% of total samples (out of 177) were positive for STEC, and 35% of confirmed *E. coli* by PCR were found to be positive for STEC using qPCR. This study, and findings of this study are significant and could assist in monitoring of pathogenic bacteria in manure and help in determining the suitable methods for assessing the prevalence of pathogenic organisms in dairy manure.

CHAPTER 3

Impacts of Aeration and Ozonation on *E. coli* Inactivation in Manure Slurry

3.1 Introduction

Milk production from dairy industry is important, however, manure, which is a byproduct of dairy industry is a concern. One of the major issues related with manure is microbial pollution, and controlling risks of pathogens of manure requires implementing the improved manure treatment methods. Reduction of microbial loads in manure could reduce health risks to human, animal, and environment (Anastasi et al. 2010, Glover et al. 2023, Naranjo et al. 2020, Pandey et al. 2015, Pandey et al. 2016). Currently, dairy industry contribute to the U.S. economy substantially by providing hundreds of thousands of job, and more than $20 billion annually to its economy (Naranjo et al. 2020). Over the years, milk production and number of cows have increased (Davis et al. 2016, Naranjo et al. 2020) through intensive farming, which led to increased manure production, and consequential risk to public and environmental health (Manyi-Loh et al. 2016, Pandey et al. 2014). Needless to say that food and waterborne illnesses and outbreaks put a substantial burden on public health and economy (Toth et al. 2013), and reducing the risks to public health caused by manure requires intervention and improvement in manure treatment practices. Untreated manure application to fertilize cropland can cause microbial contamination and affect the public and environmental health. Dairy manure may contain several pathogens such as *E. coli*, *Salmonella*, *Listeria,* and *Campylobacter*, and many zoonotic pathogens from animal production systems could move into the human food and water, which causes outbreaks and illnesses (Sheng et al. 2019, Toth et al. 2013).

To reduce the contamination from manure, on-farm manure management practices such as liquid solid separation, lagoons, composting, storage, drying, and anaerobic systems are applied in many dairy farms (Biswas et al. 2018, Pandey et al. 2016, Pell 1997, Westerman and Zhang 1997). For example, studies have shown that pathogen such as *Escherichia coli* O157:H7 are reduced during on-farm composting when the temperature of the center of compost piles is higher than 50°C, however, inactivation of pathogens could be limited, and *E. coli* O157:H7 are recoverable by enrichment, and the rate of *E. coli* O157:H7 inactivation was similar to generic *E. coli* (Shepherd et al. 2007).

While testing the effect of anaerobic digestion treatment and storage, results showed a high variability of the concentration of bacteria in swine and dairy manure, and anaerobic digestion followed by storage at 18°C for 2 months reduced indicator bacteria and *Escherichia coli* O157:H7, however, clostridia was not reduced (Costa et al. 2017). A 29 weeks manure storage study showed that *E. coli* level was reduced in 14 weeks, however, *Listeria* survived more than 29 weeks (Biswas et al. 2018).

Effects of anaerobic digestion and composting process, which are the two most common on-farm manure treatment processes, on the reduction of *E. coli* on dairy manure is investigated in multiple temperature conditions such as low temperature, mesophilic conditions, and thermophilic conditions (Pandey and Soupir 2011, Shepherd et al. 2007, Smith et al. 2005). Many factors-controlled pathogen destruction during anaerobic process, and temperature was the most important factor responsible for pathogen reduction. Thermophilic anaerobic system (greater than 50°C) reduced *E. coli* and *Salmonella*, and mesophilic conditions (25-35°C) were not able to reduce pathogens completely (Pandey and Soupir 2011, Smith et al. 2005). In municipal waste treatment plants, anaerobic digestion systems are often used to reduce the load of pathogens including virus,

and parasites. However, the inactivation rate depends on volatile fatty acid, enzyme, feedstock conditions (Zhao and Liu 2019). Compare to anaerobic system, aerobic treatment of manure with diffuse air resulted in relatively faster removal of *E. coli* indicator organisms (Pandey et al. 2016). When temperature of manure pile was mesophilic (35°C), the inactivation of *E. coli* was lower than anaerobic and aerobic conditions (Pandey et al. 2016).

In general, anaerobic digesters are operated in mesophilic temperature range (25-35°C) with retention time 20-30 days, and this temperature range is not detrimental to many pathogens. Under anaerobic conditions, many aerobic pathogens such as *E. coli* growth is slowed, but anaerobic pathogens such as clostridia, which are strictly anaerobic, can survive for extended period of time under mesophilic temperature conditions (Wells and Wilkins 1996). Anaerobic bacteria such as pathogenic spore forming Firmicutes are common in animal waste, and inactivation of these anaerobic bacteria including *Clostridium spp*., and *Bacillus* spp. in mesophilic anaerobic digestion can be challenging (Subirats et al. 2022). In contrast, the treatment processes, which produce higher temperature such as composting could be more effective in reducing these pathogens, especially when thermophilic conditions (temperature > 55°C) are maintained at least six consecutive days (Pandey and Soupir 2011, Subirats et al. 2022). However, obtaining thermophilic temperature consecutively is often challenging, particularly in small-size composting, and lower temperature inside the pile slowed decomposition process and pathogen inactivation (Neugebauer and Sołowiej 2017).

Treatment methods such as Aeration is used in municipal waste treatment and results showed that Aeration assists in reduction of coliform bacteria (Arslan Topal et al. 2016, Lau et al. 1992). Additional method such as Ozonation is also found to be effective in pathogen control because ozone affects bacteria membrane (Zhang et al. 2011). However, both of these methods are rarely

used in treating liquid dairy manure, which are stored in lagoons. Previously, few studies have investigated to determine the impacts of aeration on *E. coli* inactivation at lab scale (Pandey et al. 2016, Rosso et al. 2008). The use of gaseous ozone for controlling odor is explored previously and found to be an effective ecofriendly method for sanitization (Botondi et al. 2023, Chang et al. 2022). The existing knowledge is limited in terms of using Aeration and Ozonation for controlling *E. coli* in liquid dairy manure. The goal of this study is to investigate the effects of Aeration and Ozonation on *E. coli* control from dairy manure for a broader understanding and compare the impacts of Aeration and Ozonation on manure bacteria inactivation. The specific objectives of this research are: 1) determine the impacts of Ozonation on manure *E. coli* removal, 2) examine the impacts of Aeration on manure *E. coli* inactivation in comparison with Ozonation, 3) asses the impacts of solid load in bacteria removal during Ozonation and Aeration, and 4) evaluate the change in chemical properties of manure after Aeration and Ozonation.

3.2 Material and Methods

3.2.1 Experiment setup and reactors

To determine the impacts of Ozonation and Aeration, an experiment was designed to conduct multiple experiments simultaneously that involve multiple treatments and replicates. The setup involved 24 reactor tubes (50 mL), and the experiment setup has following components: A) reaction chamber, which can distribute uniform ozone flow and concentrations to each reactor, and this chamber was attached with the flow controller to regulate the flow; B) nano ozone generator that converts oxygen (O_2) into ozone (O_3), which can generate 15 g/hr ozone from 4 LPM oxygen, and ozone concentrations could be up to 12% by weight (ATL-Series Ozone Generators, Oxidation Technologies, LLC, Inwood, IA); C) ozone analyzer (Model 205 Dual Beam Ozone Monitor, Broomfield, Colorado, USA), suitable for higher precision and faster response monitoring, and it

monitors ozone in real time during reaction; and D) compressed gases, which involved pressurized oxygen tank (2000 psi), CGA 540, equipped with a dual stage regulator, and pressurized ultra-zero grade air (2000 psi), CGA 590, equipped with a dual stage regulator (Figure 3.1). During experiments, ozone concentrations was 2.38 % (± 0.08) wt of O_2, and average pressure of injection was 1257 (± 27) mbar. The experiments were conducted at 30 (± 0.3) °C. During aeration, air was injected (20.9% oxygen) at pressure of 1257 mbar through injection nozzles dedicated for each reactor.

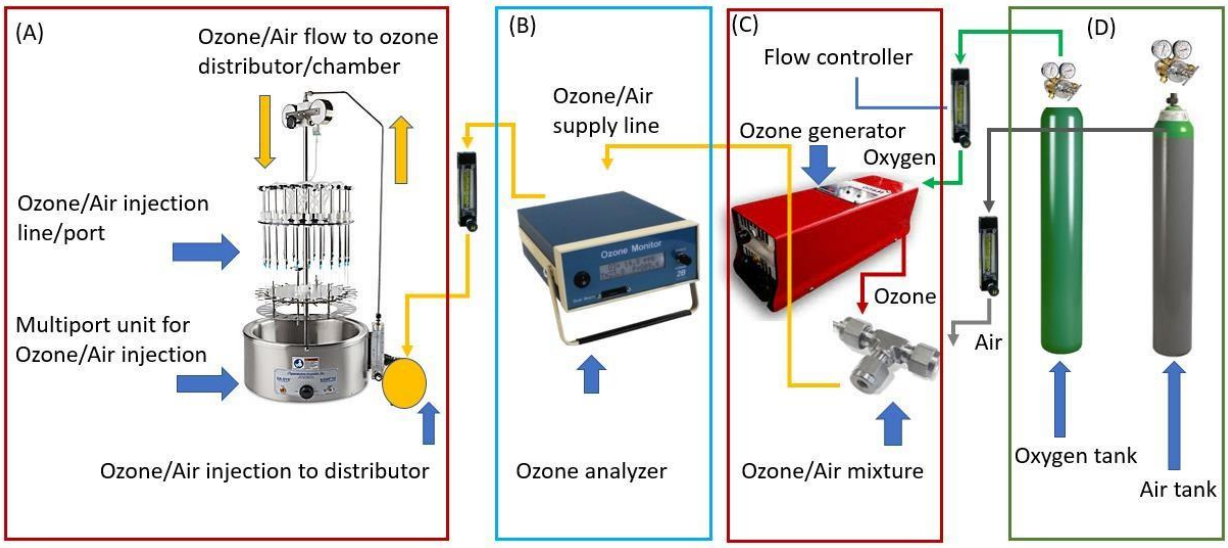


Figure 3.1: experiment setup for Ozonation (Ozo) and Aeration (Aer) experiments; A) Reaction chamber for conducting the ozone air exposure experiments; B) Ozone analyzer, which monitors ozone concentrations at real time before it goes to reaction chamber through control valve; C) Ozone generator, which converts oxygen into ozone; and D) Compressed oxygen and air gases.

3.2.2 Feedstock and experiment design

Experiment was conducted in manure collected from three commercial dairy farms, located in Central Valley California. Before starting the experiments, fresh raw manure was mixed, and manure was converted into slurry by mixing 2 kg of manure into 2 L of water. Subsequently, manure was filtered through a 800 micron sieve (8 in diameter ASTEM E11 Test Sieve) to remove

the larger particles and undigested fibers in manure. The raw manure total solid (TS) percentage was 14.95% (± 1.91%), and volatile solid (VS) percentage was 2.44%. The filtered manure TS and VS was 3.69% (± 0.25%) and 0.78% (± 0.14%). Subsequently, this filtered manure was further diluted to create six levels of total solid by adding water. These six levels of TS were: 1) TS1 (TS = 3.97%); 2) TS2 (TS = 3.57%); 3) TS3 (TS = 3.18%); 4) TS4 (TS = 2.78%); TS5 (TS = 2.38%); 5) TS6 (TS = 1.99%). All these six levels of solids were exposed to the similar flow rate of gases (ozone or gas). Experiment design is shown in Figure 3.2 describing the treatments and replicates.

Figure 3.2: Repeated measure experiment design. Experiment involved three treatments: 1) Control setup which does not receive any gas; 2) Aeration, which involved diffusion of air into manure; and 3) Ozonation, which involved diffusion of ozone into manure.

Each treatment of experiments has six levels (TS1-TS6), and each level has three replicates (R1-R3). Under control conditions, all reactors were in ambient conditions (at room temperature) without Aeration and Ozonation. During experiment, samples were collected from each reactor for repeated measurements at four-time intervals (0 min, 60 min, 120 min, and 240 min). Samples were tested for *E. coli* levels, pH, salts (%), potassium (K^+), sodium (Na^+), nitrate (NO_3^-), calcium (Ca_2^+), and electrical conductivity (EC, mS/cm). The TS and VS were measured using standard methods by American Public Health Association (APHA). Samples were measured in aluminum dish, and weight of sample and dish was measured prior to analysis. After measurements, samples were dried at 104-105°C in an oven until constant weight was achieved (16-18 hours drying). The change in weight at 104-105°C determined the TS%. To determine the VS, a muffle furnace (550°C) was used. Change in weight at 550°C was used to calculate VS% (Peces et al. 2014, Rice et al. 2012). To determine *E. coli*, EPA Method 1603 was used by applying membrane filtration system, which determines modified membrane- thermotolerant *E. coli* Agar (modified mTEC) (EPA 2002, Pandey et al. 2015, Pandey and Soupir 2011). The modified mTEC agar is a selective culture medium for chromogenic detection, and enumeration of thermo- tolerant *E. coli* in agar plate. Thermotolerant *E. coli* produces red or magenta color colonies in membrane filters (0.45 µm pore size) in agar plates after incubation at 44.5 °C (± 0.2 °C). To monitors ions and salts, we did use Horiba's highly sensitive, flat sensor technology, Standard Handheld Meters (HORIBA Advanced Techno, Co., Ltd., Minami-Ku Kyoto, Japan).

2.2.3 Data analysis and statistics

Results of *E. coli* and ions were plotted in Microsoft (MS) Excel, and GraphPad Prism (San Diego, CA, USA). To analyze data, we estimated descriptive statistics (mean, minimum, maximum, standard deviation, variance, Kurtosis, and Skewness) using MS Excel and GraphPad Prism.

Further one-way analysis of variance (ANOVA), and two-way ANOVA was carried out on the data sets using Microsoft Excel (Redmond, WA, USA). One-way ANOVA was conducted to determine the significance of the difference between mean values of *E. coli* under independent variable conditions of exposure time T_1 = 0 min, T_2 = 60 min, T_3 = 120 min, T_4 = 180 min, T_5 = 240 min) under three treatment conditions (Ozonation, Aeration, and Control). The assumption about the normality distribution was tested by determining the skewness and kurtosis values. The two-way ANOVA was conducted to determine the significant differences in *E. coli* levels under different treatment conditions (Ozone, Air, and Control) at various levels of Total Solid (TS1-TS6). We used *p-value* of 0.05 to determine if significant differences exist among treatments and solid levels.

3.3 Results and Discussion

3.3.1 Impacts of Ozonation, and Aeration on E. coli

Initial *E. coli* levels (0 min) and changes in the levels over the time for 240 min are shown in Figure 3.3. Initial *E. coli* levels in Control, Aeration, and Ozonation experiments were in 7 orders of magnitude ($\approx 5 \times 10^7$ CFU/mL). While Ozonation reduced *E. coli* levels to non-detectable levels by the end of 240 min, *E. coli* levels in Aeration and Control remained at 7 orders of magnitude. *E. coli* at a total solid of 3.97% (TS1) (shown in Figure 3.3) indicates that initial *E. coli* levels (at 0 min) was comparable in all three treatments (Control, Aeration, and Ozonation). During Ozonation, *E. coli* levels was reduced considerably in 60 min, and by the end of 240 min, *E. coli* level was not detectable. This trend in reductions of *E. coli* was similar for all solid levels [TS1 (TS = 3.97%) - TS6 (1.99%)]. From TS1 to TS3, *E. coli* levels were detectable at 120 min of Ozonation (Figure 3.3). However, when solid was reduced to 2.78% - 1.99%, *E. coli* levels were not detectable at the end of 120 min. This indicates that Ozonation was more effective at lower

solid levels. In general, Aeration also reduced *E. coli* levels compared to Control reactors, however, the reduction of *E. coli* in Aeration was limited within 240 min.

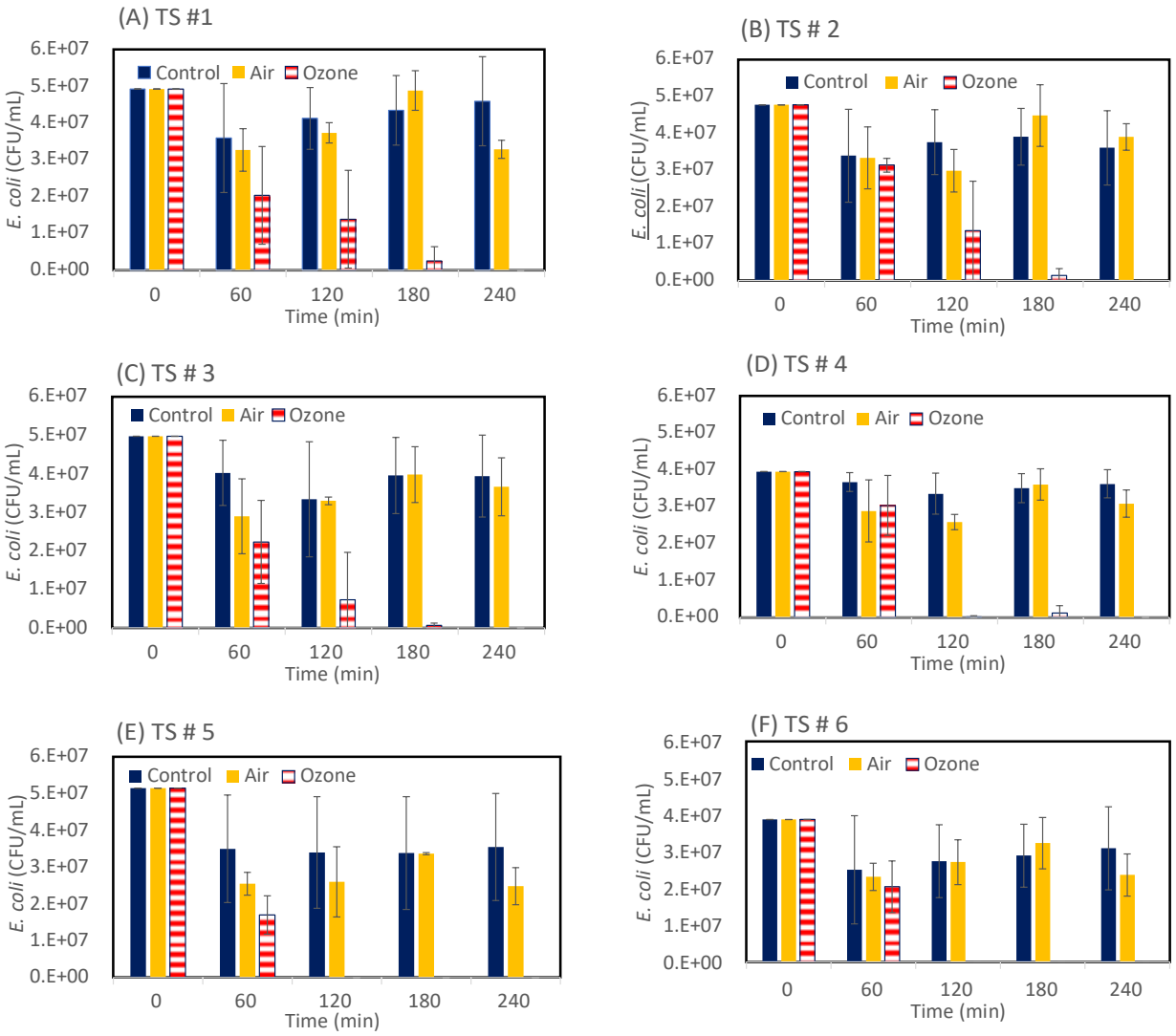


Figure 3.3: Reduction in *E. coli* levels from TS1 (total solid = 3.97%) to TS6 (total solid = 1.99%). Three treatments (Control, Aeration, and Ozonation) were performed with triplicate reactors for all solid levels (TS1-TS6). X-axis indicates time of exposure, and Y-axis indicates *E. coli* levels. Standard deviation (SD) are displayed for error bars.

While comparing Control and Aeration, *E. coli* levels in both Control and Aeration reactors were in 7 orders of magnitude throughout the experiment. Descriptive statistics of *E. coli* levels for

three treatments and 5 durations are shown in Table 3.1. The normal distribution of the variables was verified with the Shapiro-Wilk normality test, and the *p value = 0.05* was considered as the level of significance. The level of the degree to which variables were normally were determined based on the values of skewness and kurtosis shown in Table 3.1. The values of kurtosis and skewness varied within -2 and +2 (Table 3.1), which are considered to be acceptable to prove normal univariate distribution (George and Mallery 2019, Hair et al. 2010, Iwanski et al. 2019). The assumption of about normal distribution of variable *E. coli* levels was satisfied for all three treatment conditions. The significant evaluation was also conducted for *E. coli*, and results of one-way ANOVA is shown in Table 3.2.

Table 3.1. Breakdown table of descriptive statistics for *E. coli* reduction in Control, Aeration, and Ozonation.

		Variables							
Time (min)	Treatment	Mean	Stdev.	Variance	Kurtosis	Skewness	Minimum	Maximum	Count
0	Control	5.E+07	6.E+06	3.E+13	-1.8	-0.8	4.E+07	5.E+07	6
	Aeration	5.E+07	6.E+06	3.E+13	-1.8	-0.8	4.E+07	5.E+07	6
	Ozone	5.E+07	6.E+06	3.E+13	-1.8	-0.8	4.E+07	5.E+07	6
60	Control	3.E+07	5.E+06	3.E+13	3.1	-1.4	3.E+07	4.E+07	6
	Aeration	3.E+07	4.E+06	2.E+13	-1.2	-0.3	2.E+07	3.E+07	6
	Ozone	2.E+07	6.E+06	3.E+13	-1.6	0.6	2.E+07	3.E+07	6
120	Control	3.E+07	5.E+06	2.E+13	0.8	-0.1	3.E+07	4.E+07	6
	Aeration	3.E+07	5.E+06	2.E+13	-0.2	1.0	3.E+07	4.E+07	6
	Ozone	6.E+06	7.E+06	5.E+13	-2.5	0.4	0.E+00	1.E+07	6
180	Control	4.E+07	5.E+06	3.E+13	-0.3	-0.3	3.E+07	4.E+07	6
	Aeration	4.E+07	7.E+06	4.E+13	-1.3	0.6	3.E+07	5.E+07	6
	Ozone	9.E+05	9.E+05	9.E+11	0.3	0.8	0.E+00	3.E+06	6
240	Control	4.E+07	5.E+06	3.E+13	1.7	0.9	3.E+07	5.E+07	6
	Aeration	3.E+07	6.E+06	4.E+13	-1.7	-0.1	2.E+07	4.E+07	6
	Ozone	0.E+00	0.E+00	0.E+00	0	0	0.E+00	0.E+00	6

Table 3.2: Results of analysis of variance for *E. coli* degradation among Control (Con), Aeration (Aer), and Ozonation (Ozo) (one-way ANOVA)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Comparison between Control, Aeration, and Ozonation						
Between groups (Con, Aer, Ozo)	9.E+15	2	5.E+15	3.0E+01	1.E-10	3.1
Within Groups	1.E+16	87	2.E+14			
Total	2.E+16	89				
Comparison between Aeration and Ozonation						
Between Groups (Aer & Ozo)	6.E+15	1	6.E+15	29.067	1.E-06	4.01
Within Groups	1.E+16	58	2.E+14			
Total	2.E+16	59				
Comparison Between Control and Aeration						
Between Groups	1.1E+14	1	1E+14	1.9256	0.17055	4.0069
Within Groups	3.3E+15	58	6E+13			
Total	3.4E+15	59				

The inference about the significance of the difference between mean values of *E. coli* under independent variable conditions of exposure time (T_1 = 0 min, T_2 = 60 min, T_3 = 120 min, T_4 = 180 min, T_5 = 240 min) under three treatment conditions (Control, Aeration, and Ozonation) is shown in Table 3.2, which revealed that there was significant differences (at $p = 0.05$) in *E. coli* inactivation between three different treatments. Further comparison between Aeration and Ozonation revealed that there was a significant difference in *E. coli* reduction between these two treatments. However, there was no significant difference in *E. coli* between Aeration and Control conditions (Table 3.2).

3.3.2 Impacts of Ozonation, and Aeration on manure chemical characteristics

The change in electrical conductivity (mS/cm), pH, salts (%), potassium ($K^{+)}$, sodium (Na^{+}), calcium (Ca_2^{+}), and nitrate (NO_3^{-}) are shown in Figure 3.4. Among treatments, pH, sodium, and

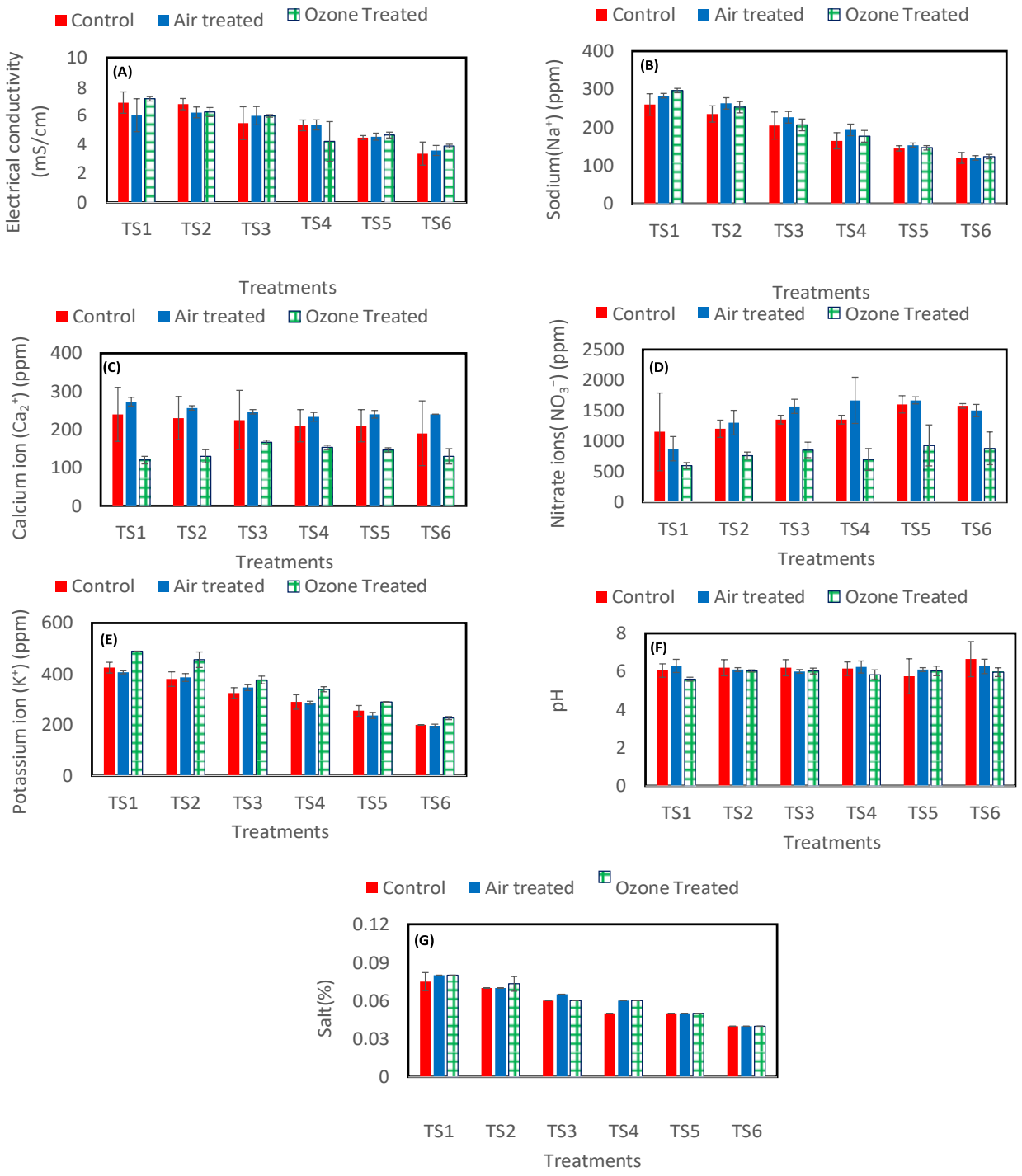


Figure 3.4: Change in ions, pH and salt at different solid concentrations (TS1-TS2) under three treatments (Control, Aeration, and Ozonation). All variables were measured in treated (final samples) and compared among the treatments. Horizontal X-axis indicates level of solids (TS1-TS6), and y-axis indicates variables (treated manure quality parameters). Standard deviation (SD) are displayed for error bars.

salt values were similar. The average salt concentrations for various solid levels varied between 0.04% and 0.08% among treatments, and pH varied between 5.75 and 6.65. The sodium concentrations varied between 120 ppm to 296 ppm. Potassium ion levels and electrical conductivity were slightly increased under Ozonation, and values for potassium and electrical conductivity varied between 196 ppm and 490 ppm, and 3.37 mS/cm and 7.15 mS/cm, respectively. After treatments, Ozonation resulted in reduced nitrate levels among treatments. Nitrate level varied in ozone between 596 ppm to 926 ppm. In Aeration, it varied between 873 ppm and 1666 ppm. The significant difference in *E. coli* levels among six various solid levels (TS1-TS6) and three treatments (Control, Aeration, and Ozonation) were estimated by two-way ANOVA, and results are shown in Table 3.3. There was no significant difference in change in *E. coli* levels among various solid levels during treatments ($p = 0.32$), however, there was significant difference in change in *E. coli* levels among treatments (Ozonation, Aeration and Control) ($p < 0.05$) (Table 3.3).

Table 3.3: Results of analysis of variance (two-way ANOVA) for *E. coli* among type of treatment (Con, Aer, Ozo), and solid concentrations in liquid manure.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Solid concentrations (TS)	9.9E+14	5	2.0E+14	1.2	0.32	2.3
Group (Con, Aer, Ozo)	9.2E+15	2	4.6E+15	27.6	1.3E-09	3.1
Interaction	1.4E+14	10	1.4E+13	0.1	1.00	2.0
Within	1.2E+16	72	1.7E+14			
Total	2.2E+16	89				

Table 3.4: Results of analysis of variance (one-way ANOVA) for chemical characteristics (EC, Ca, Na, NO3, Salt, pH, and K) ANOVA

Source of Variation (b/w Con, Aer, Ozo)	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
EC (mS/cm)	0.03	2	0.02	0.01	0.99	3.68
Ca^{2+}(ppm)	0.03	2	0.02	0.01	0.99	3.68
Na^{+}(ppm)	36565.12	2	18282.56	65.69	0.00	3.68
NO_3^- (ppm)	1519596.60	2	759798.30	15.98	0.00	3.68
Salt (%)	0.00	2	0.00	0.10	0.90	3.68
pH	0.25	2	0.13	2.92	0.09	3.68
K^{+}(ppm)	10869.44	2	5434.72	0.69	0.52	3.68

Results of ions and pH were further analyzed statistically to determine the significant difference, and one-way ANOVA resulted revealed that there was significant difference ($p < 0.05$) in nitrate (NO_3^-) during ozone treatment among all solid levels. This indicates that ozone treatment has a potential to reduce nitrate levels in liquid manure. Post-treatment, the average nitrate concentrations in ozone treatment was 785 ppm, and nitrate levels in manure under control and aeration conditions was 1370 ppm (in range of 1150 ppm – 1600 ppm). The nitrate concentration in manure samples under aeration condition was 1428 ppm (in range of 873 ppm – 1666 ppm), respectively). In previous studies, it was found that Ozonation can remove nitrate and nitrite, however, its capability of ammonia removal is limited (Lin and Wu 1996). Ozonation is also known to be highly effective in removal of bacteria because of its properties as an oxidizing agent (Schroeder et al. 2011). In recirculating aquaculture system, the use of ozone revealed that ozone is an effective disinfection agent, and efficiently improve water characteristics (Summerfelt 2003). Compare to Ozonation, aeration does not affect nitrates in liquid phase because of constrained by excess oxygen and limited carbon source, however, it removes ammonia (Huang et al. 2022).

We used linear regression to predict the *E. coli* inactivation under Ozonation, and Aeration, and results showed that a binomial model (generalized linear model) is able to predict *E. coli* (Figure

3.5) reduction during Ozonation. The coefficient of determination values (R^2) in the model was 0.99. The linear model for aeration resulted R^2 value of 0.16 and was deemed unsuitable.

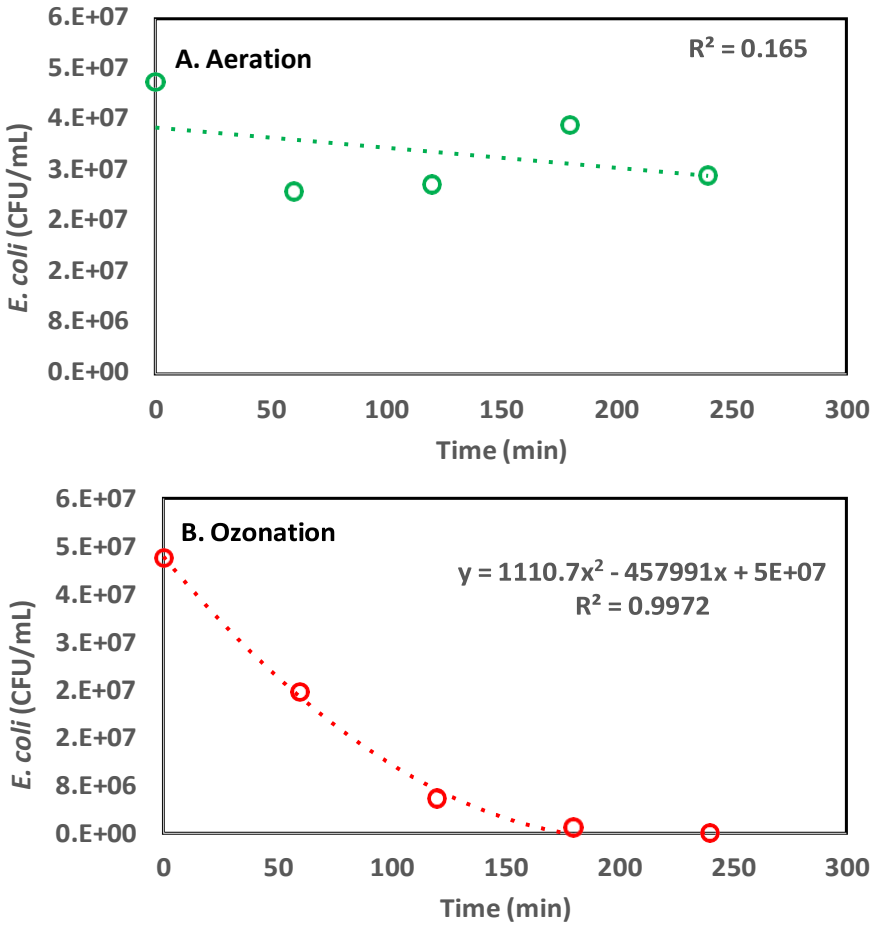


Figure 3.5: Generalized linear models to predict *E. coli* reduction in Aeration and Ozonation. A) *E. coli* inactivation in aeration; and B) *E. coli* inactivation in ozonation.

In general, ozone (O_3) gas has a detrimental effect on cell wall, lipids and DNA of bacteria and it disrupts cell viability (Rangel et al. 2021). Further, it affects enzymes and nucleic acids negatively, which causes inactivation of bacteria (Figure 3.6) (Westover et al. 2022). Reported research on *E. coli* showed that ozone causes oxidation of proteins, and damage to plasmid DNA, which results in *E. coli* inactivation (Hunt and Mariñas 1999).

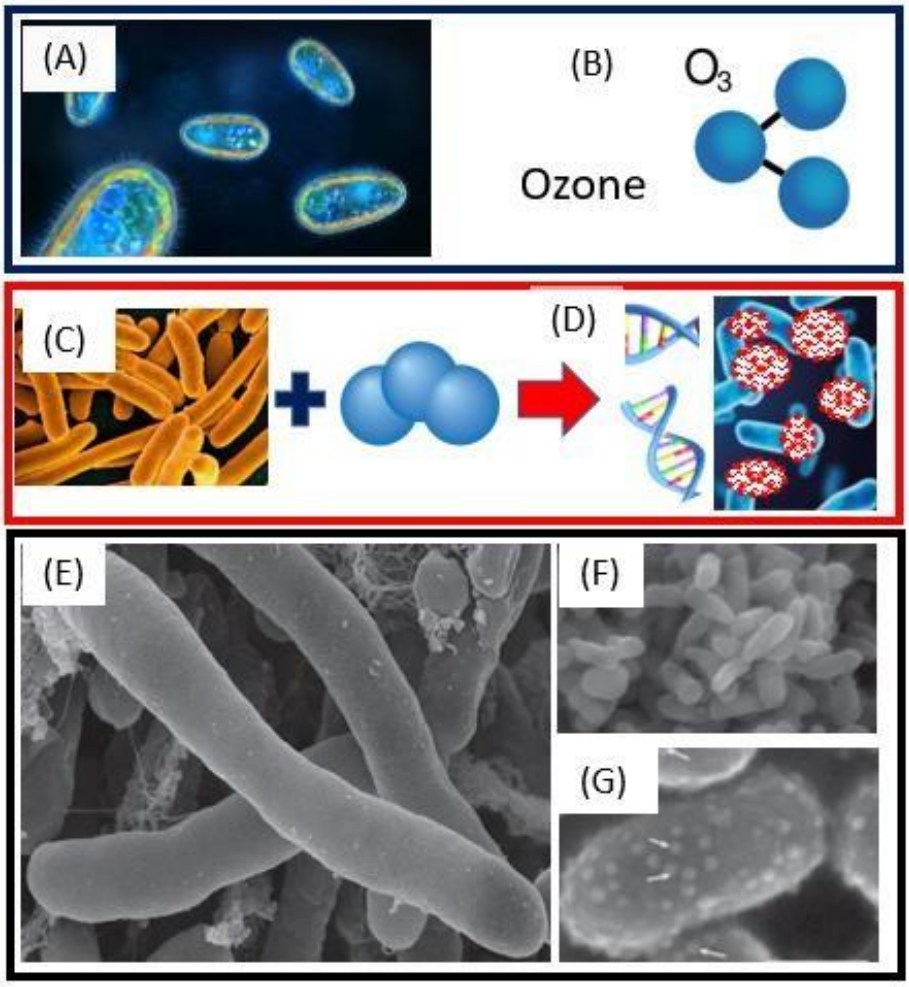


Figure 3.6: Bacteria cells (A) and their exposure to ozone (B). Increased ozone dosage and contact time affects bacteria cell wall. Healthy cell of *E. coli* (C), and the exposure of *Pseudomonas aeruginosa* cell to ozone caused vesicles on the cell surface indicating perforation of cytoplasmic membrane and release of cellular compounds from cell treated with ozone (Zhang et al. 2011). Modified online images [A, B, C, D, E, F, G]. Available from: Effects of ozone on membrane permeability and ultrastructure in Pseudomonas aeruginosa - Zhang - 2011 - Journal of Applied Microbiology - Wiley Online Library; https://americanregen.com/ozone-therapy/; https://agriculture.vermont.gov/produce-program/eye-e-coli-understanding-pathogens-concern-fruit-and-vegetable-farms; https://www.colorado.edu/today/2018/08/22/unexpected-upside-e-coli. [Accessed 3 December, 2023].

Previously Scanning Electron Microscope (SEM) has been widely used to study the damage to the bacterial cell wall caused by heat, ozone, antimicrobial agents, and pulsed electric (Figure 3.7),

and results showed that the damage to cell wall was one of the major factor for bacteria inactivation (Hartmann et al. 2010, Hunt and Mariñas 1999, Pillet et al. 2016). The morphology of bacteria is maintained by cell wall (Figure 3.7), and disruptions in cell wall causes leakage of nucleic acids and death of bacteria (Pillet et al. 2016).

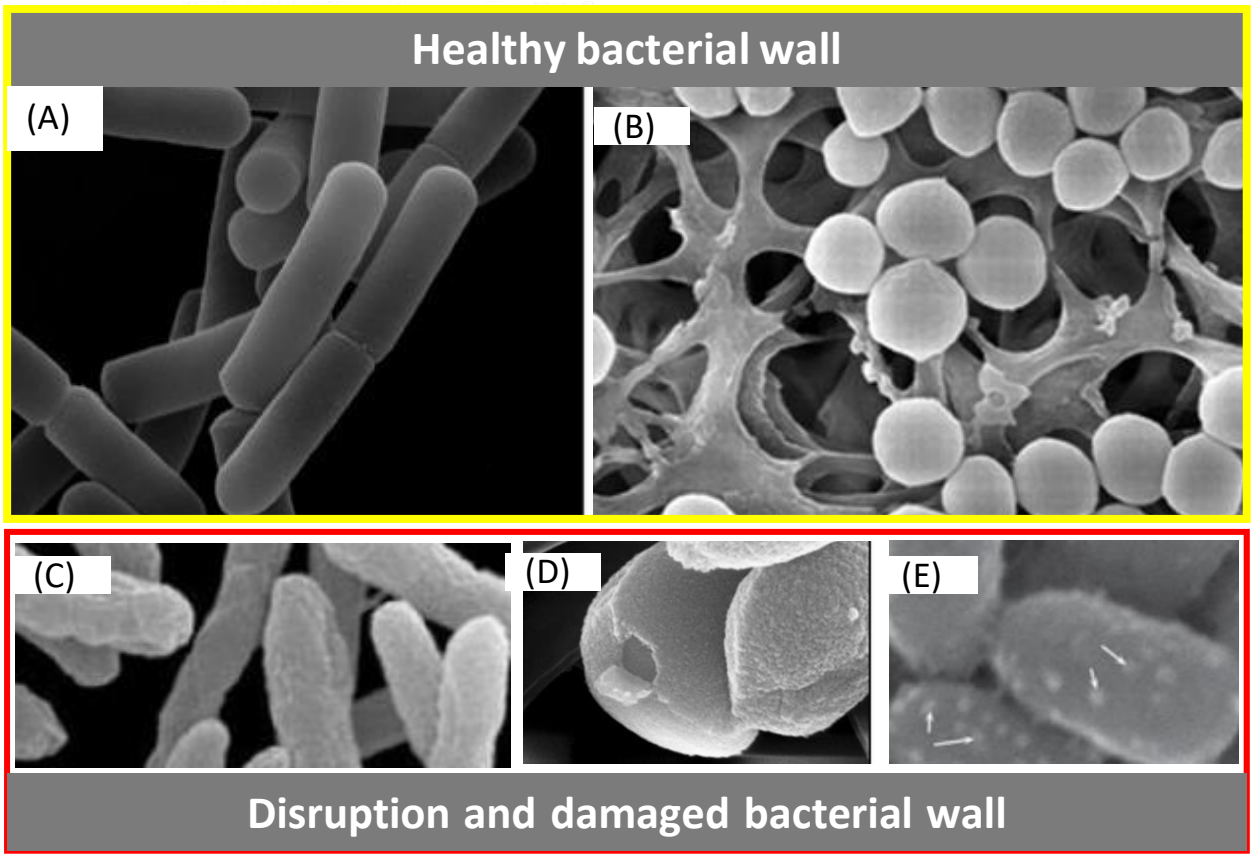


Figure 3.7: Bacterial cell and damage to bacterial cell wall. SEM image of healthy bacteria Bacillus pumilus (A) (Pillet et al. 2016) and Staphylococcus aureus cells (B) (Hartmann et al. 2010); SEM micrographs of unhealthy Staphylococcus aureus exposed to ozone (C); cell burst of Staphylococcus aureus caused by antibiotics (D) (Hartmann et al. 2010); and vesicles on cell wall caused by ozone on Pseudomonas aeruginosa (Zhang et al. 2011). Modified online images [A, B, C, D]. Available from: https://www.nature.com/articles/srep19778/figures/2; https://journals.asm.org/doi/10.1128/aac.00124-10. [Accessed 3 December, 2023].

4. Conclusions

In order to evaluate the impacts of Ozonation and Aeration on manure *E. coli*, this research focused on using air and ozone treatment, and *E. coli* inactivation was monitored. Further, the effects of ozone and air were tested on multiple ions present in manure. Results showed that Ozonation can be an effective treatment method to reduce *E. coli* levels in liquid manure. Within 240 minutes of exposure time, the *E. coli* reached to non-detectable levels regardless of solid concentrations in manure. Further, nitrate ions were reduced substantially by Ozonation. Compared to Ozonation, the impacts of Aeration was minimal in reducing bacteria within 240 minutes. The analysis of variance (one-way and two-way) releveled significant difference in *E. coli* and nitrate levels among treatments ($p < 0.05$). To predict the *E. coli* reductions in manure, a generalized linear model was developed, and the model was able to capture the degradation pattern of *E. coli* in liquidmanure (R^2 = 0.99) under ozonation. Findings of this study are important to develop on-farm dairymanure treatment methods and could help in guiding future investigation on Ozonation and Aeration for controlling *E. coli* and nitrate levels in dairy lagoons.

REFERENCES

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002) Molecular Biology of the Cell. 4th edition, Garland Science.

Alkoaik, F.N. (2009) Ozone treatment of animal manure for odor control. American Journal of Environmental Sciences 5(6), 765.

Anastasi, E.M., Matthews, B., Gundogdu, A., Vollmerhausen, T.L., Ramos, N.L., Stratton, H., Ahmed, W. and Katouli, M. (2010) Prevalence and Persistence of Escherichia coli Strains with Uropathogenic Virulence Characteristics in Sewage Treatment Plants. Applied and Environmental Microbiology 76(17), 5882-5886.

Anastasi, E.M., Matthews, B., Stratton, H. and Katouli, M. (2012) Pathogenic Escherichia coli found in sewage treatment plants and environmental waters. Applied and Environmental Microbiology 78(16), 5536-5541.

Anastasi, E.M., Wohlsen, T.D., Stratton, H. and Katouli, M. (2013) Survival of Escherichia coli in two sewage treatment plants using UV irradiation and chlorination for disinfection. Water Research 47(17), 6670-6679.

Arslan Topal, E.I., Ünlü, A. and Topal, M. (2016) Effect of aeration rate on elimination of coliforms during composting of vegetable–fruit wastes. International Journal of Recycling of Organic Waste in Agriculture 5, 243-249.

Atwill, E.R., Chase, J.A., Oryang, D., Bond, R.F., Koike, S.T., Cahn, M.D., Anderson, M., Mokhtari, A. and Dennis, S. (2015) Transfer of Escherichia coliO157:H7 from Simulated Wildlife Scat onto Romaine Lettuce during Foliar Irrigation. Journal of Food Protection 78(2), 240-247.

Awasthi, M.K., Liu, T., Awasthi, S.K., Duan, Y., Pandey, A. and Zhang, Z. (2020) Manure pretreatments with black soldier fly Hermetia illucens L.(Diptera: Stratiomyidae): A study to reduce pathogen content. Science of the Total Environment 737, 139842.

Baker, C.A., De, J., Bertoldi, B., Dunn, L., Chapin, T., Jay-Russell, M., Danyluk, M.D. and Schneider, K.R. (2019) Prevalence and concentration of stx+ E. coli and E. coli O157 in bovine manure from Florida farms. Plos one 14(5), e0217445.

Ballem, A., Gonçalves, S., Garcia-Meniño, I., Flament-Simon, S.C., Blanco, J.E., Fernandes, C., Saavedra, M.J., Pinto, C., Oliveira, H., Blanco, J., Almeida, G. and Almeida, C. (2020)

Prevalence and serotypes of Shiga toxin-producing Escherichia coli (STEC) in dairy cattle from Northern Portugal. Plos one 15(12), e0244713.

Bean, A., Williamson, J. and Cursons, R. (2004) Virulence genes of Escherichia coli strains isolated from mastitic milk. Journal of Veterinary Medicine, Series B 51(6), 285-287.

Bicudo, J.R. and Goyal, S.M. (2003) Pathogens and manure management systems: a review. Environmental technology 24(1), 115-130.

Bintsis, T. (2018) Microbial pollution and food safety. AIMS microbiology 4(3), 377.

Biswas, S., Niu, M., Pandey, P., Appuhamy, J., Leytem, A., Kebreab, E. and Dungan, R. (2018) Effect of dairy manure storage conditions on the survival of E. coli O157: H7 and Listeria. Journal of environmental quality 47(1), 185-189.

Biswas, S., Pandey, P.K. and Farver, T.B. (2016) Assessing the impacts of temperature and storage on Escherichia coli, Salmonella, and L. monocytogenes decay in dairy manure. Bioprocess and biosystems engineering 39, 901-913.

Black, Z., Balta, I., Black, L., Naughton, P.J., Dooley, J.S. and Corcionivoschi, N. (2021) The fate of foodborne pathogens in manure treated soil. Frontiers in Microbiology 12, 781357.

Botondi, R., Lembo, M., Carboni, C. and Eramo, V. (2023) The Use of Ozone Technology: An Eco–Friendly Method for the Sanitization of the Dairy Supply Chain. Foods 12(5), 987.

Bowman, M., Sharma, A., Jacobs, R., Lahoti, M. and Gordon, M. (2023) 16 Comparison of the cecal and fecal microbiome in horses administered antibiotics over a 16 d period. Journal of Equine Veterinary Science 124, 104318.

Byrne, L., Jenkins, C., Launders, N., Elson, R. and Adak, G.K. (2015) The epidemiology, microbiology and clinical impact of Shiga toxin-producing Escherichia coli in England, 2009–2012. Epidemiology & Infection 143(16), 3475-3487.

Chang, R., Pandey, P., James, P., Pandey, P., Li, Y., Zhang, R. and Weimer, B.C. (2022) Assessment Impacts of Ozone on Salmonella typhimurium and Escherichia coli O157: H7 in Liquid Dairy Waste. Applied Sciences 12(13), 6527.

Chen, J. and Griffiths, M. (1998) PCR differentiation of Escherichia coli from other Gram-negative bacteria using primers derived from the nucleotide sequences flanking the gene encoding the universal stress protein. Letters in applied microbiology 27(6), 369-371.

Chen, Z., Biswas, S., Aminabadi, P., Stackhouse, J.W., Jay-Russell, M.T. and Pandey, P.K. (2019) Prevalence of Escherichia coli O157 and Salmonella spp. in solid bovine manure in California using real-time quantitative PCR. Letters in applied microbiology 69(1), 23-29.

Chen, Z., Kim, J. and Jiang, X. (2018) Survival of Escherichia coli O157:H7 and Salmonella enterica in animal waste-based composts as influenced by compost type, storage condition and inoculum level. Journal of applied microbiology 124(5), 1311-1323.

Cho, S., Diez-Gonzalez, F., Fossler, C.P., Wells, S.J., Hedberg, C.W., Kaneene, J.B., Ruegg, P.L., Warnick, L.D. and Bender, J.B. (2006) Prevalence of shiga toxin-encoding bacteria and shiga toxin-producing Escherichia coli isolates from dairy farms and county fairs. Veterinary microbiology 118(3-4), 289-298.

Costa, A., Gusmara, C., Gardoni, D., Zaninelli, M., Tambone, F., Sala, V. and Guarino, M. (2017) The effect of anaerobic digestion and storage on indicator microorganisms in swine and dairy manure. Environmental Science and Pollution Research 24, 24135-24146.

Davis, K.R., Dunn, A.C., Burnett, C., McCullough, L., Dimond, M., Wagner, J., Smith, L., Carter, A., Willardson, S. and Nakashima, A.K. (2016) Campylobacter jejuni Infections Associated with Raw Milk Consumption — Utah, 2014. Morbidity and Mortality Weekly Report 65(12), 301-305.

EPA, U. (2002) Method 1603: Escherichia coli (E. coli) in water by membrane filtration using modified membrane-thermotolerant Escherichia coli agar (modified mTEC). United States Environmental Protection Agency Office of Water, Washington, DC.

Farrokh, C., Jordan, K., Auvray, F., Glass, K., Oppegaard, H., Raynaud, S., Thevenot, D., Condron, R., De Reu, K., Govaris, A., Heggum, K., Heyndrickx, M., Hummerjohann, J., Lindsay, D., Miszczycha, S., Moussiegt, S., Verstraete, K. and Cerf, O. (2013) Review of Shiga-toxin-producing Escherichia coli (STEC) and their significance in dairy production. International Journal of Food Microbiology 162(2), 190-212.

Fontes, B., Cattani Heimbecker, A.M., de Souza Brito, G., Costa, S.F., van der Heijden, I.M., Levin, A.S. and Rasslan, S. (2012) Effect of low-dose gaseous ozone on pathogenic bacteria. BMC infectious diseases 12, 1-6.

Franz, E., Klerks, M.M., De Vos, O.J., Termorshuizen, A.J. and van Bruggen, A.H. (2007) Prevalence of Shiga toxin-producing Escherichia coli stx1, stx2, eaeA, and rfbE genes and survival of E. coli O157:H7 in manure from organic and low-input conventional dairy farms. Appl Environ Microbiol 73(7), 2180-2190.

George, D. and Mallery, P. (2019) IBM SPSS statistics 26 step by step: A simple guide and reference, Routledge.

Glover, C.J., McDonnell, A., Rollins, K.S., Hiibel, S.R. and Cornejo, P.K. (2023) Assessing the environmental impact of resource recovery from dairy manure. Journal of environmental management 330, 117150.

Hair, J.F., Black, W.C., Babin, B.J. and Anderson, R.E. (2010) Canonical correlation: A supplement to multivariate data analysis. Multivariate Data Analysis: A Global Perspective, 7th ed.; Pearson Prentice Hall Publishing: Upper Saddle River, NJ, USA.

Han, S., Huang, Y. and Liu, Z. (2019) Bacterial indicator reduction in dairy manure using hybrid zero-valent iron (h-ZVI) system. Environmental Science and Pollution Research 26,10790-10799.

Hancock, D.D., Besser, T.E., Rice, D.H., Ebel, E.D., Herriott, D.E. and Carpenter, L.V. (1998) Multiple sources of Escherichia coli O157 in feedlots and dairy farms in the northwestern USA. Preventive veterinary medicine 35(1), 11-19.

Hartmann, M., Berditsch, M., Hawecker, J., Ardakani, M.F., Gerthsen, D. and Ulrich, A.S. (2010) Damage of the bacterial cell envelope by antimicrobial peptides gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. Antimicrobial agents and chemotherapy 54(8), 3132-3142.

Hermos, C.R., Janineh, M., Han, L.L. and McAdam, A.J. (2011) Shiga toxin-producing Escherichia coli in children: diagnosis and clinical manifestations of O157: H7 and non-O157: H7 infection. Journal of clinical microbiology 49(3), 955-959.

Hodgson, C.J., Oliver, D.M., Fish, R.D., Bulmer, N.M., Heathwaite, A.L., Winter, M. and Chadwick, D.R. (2016) Seasonal persistence of faecal indicator organisms in soil following dairy slurry application to land by surface broadcasting and shallow injection. Journal of environmental management 183, 325-332.

Horan, N.J., Fletcher, L., Betmal, S.M., Wilks, S.A. and Keevil, C.W. (2004) Die-off of enteric bacterial pathogens during mesophilic anaerobic digestion. Water Research 38(5), 1113-1120.

Huang, L., Chen, Y., Ye, J., Xiao, Y., Tao, K., Wang, Y. and Li, Y. (2022) Complete nitrogen removal from over-aeration treated black-odorous water via adding aerobic denitrifiers and iron-carbon micro-electrolysis carriers. Chemical Engineering Journal 433, 133259.

Hunt, N.K. and Mariñas, B.J. (1999) Inactivation of Escherichia coli with ozone: chemical and inactivation kinetics. Water Research 33(11), 2633-2641.

Iwanski, M.M., Chomicz-Kowalska, A. and Maciejewski, K. (2019) Impact of Additives on the Foamability of a Road Paving Bitumen. IOP Conference Series: Materials Science and Engineering 603(4), 042040.

Jacob, M.E., Shi, X., An, B., Nagaraja, T.G. and Bai, J. (2012) Evaluation of a multiplex real-time polymerase chain reaction for the quantification of Escherichia coli O157 in cattle feces. Foodborne Pathogens and Disease 9(1), 79-85.

Jiang, X., Morgan, J. and Doyle, M.P. (2003) Fate of Escherichia coli O157:H7 during Composting of Bovine Manure in a Laboratory-Scale Bioreactor. Journal of Food Protection 66(1), 25-30.

Larney, F.J., Yanke, L.J., Miller, J.J. and McAllister, T.A. (2003) Fate of coliform bacteria in composted beef cattle feedlot manure. Journal of environmental quality 32(4), 1508-1515.

Lau, A.K., Lo, K.V., Liao, P.H. and Yu, J.C. (1992) Aeration experiments for swine waste composting. Bioresource technology 41(2), 145-152.

Lin, S.H. and Wu, C.L. (1996) Removal of nitrogenous compounds from aqueous solution by ozonation and ion exchange. Water Research 30(8), 1851-1857.

Liu, Z., Sharara, M., Gunasekaran, S. and Runge, T.M. (2016) Effects of large-scale manure treatment processes on pathogen reduction, protein distributions, and nutrient concentrations. Transactions of the ASABE 59(2), 695-702.

Lung, A.J., Lin, C.M., Kim, J.M., Marshall, M.R., Nordstedt, R., Thompson, N.P. and Wei, C.I. (2001) Destruction of Escherichia coli O157:H7 and Salmonella Enteritidis in Cow Manure Composting††Florida Agricultural Experiment Station, journal series no. R-07723. Journal of Food Protection 64(9), 1309-1314.

Manning, S.D. (2010) Escherichia coli infections, Infobase Publishing.

Manyi-Loh, C.E., Mamphweli, S.N., Meyer, E.L., Makaka, G., Simon, M. and Okoh, A.I. (2016) An Overview of the Control of Bacterial Pathogens in Cattle Manure. Int J Environ Res Public Health 13(9).

Mc Carthy, G., Lawlor, P.G., Coffey, L., Nolan, T., Gutierrez, M. and Gardiner, G.E. (2011) An assessment of pathogen removal during composting of the separated solid fraction of pig manure. Bioresource technology 102(19), 9059-9067.

Meals, D.W. and Braun, D.C. (2006) Demonstration of methods to reduce E. coli runoff from dairy manure application sites. Journal of environmental quality 35(4), 1088-1100.

Megahed, A., Aldridge, B. and Lowe, J. (2018) The microbial killing capacity of aqueous and gaseous ozone on different surfaces contaminated with dairy cattle manure. Plos one 13(5), e0196555.

Monaghan, Á., Byrne, B., Fanning, S., Sweeney, T., McDowell, D. and Bolton, D.J. (2012) Serotypes and virulotypes of non-O157 shiga-toxin producing Escherichia coli (STEC) on bovine hides and carcasses. Food microbiology 32(2), 223-229.

Mukhtar, S., S. Borhan, M., Rahman, S. and Zhu, J. (2010) Evaluation of a Field-Scale Surface Aeration System in an Anaerobic Poultry Lagoon. Applied Engineering in Agriculture 26(2), 307-318.

Mulbry, W., Selmer, K. and Lansing, S. (2017) Effect of liquid surface area on hydrogen sulfide oxidation during micro-aeration in dairy manure digesters. Plos one 12(10), e0185738.

Naranjo, A., Johnson, A., Rossow, H. and Kebreab, E. (2020) Greenhouse gas, water, and land footprint per unit of production of the California dairy industry over 50 years. Journal of dairy science 103(4), 3760-3773.

Neugebauer, M. and Sołowiej, P. (2017) The use of green waste to overcome the difficulty in small-scale composting of organic household waste. Journal of Cleaner Production 156, 865-875.

Nicholson, F.A., Groves, S.J. and Chambers, B.J. (2005) Pathogen survival during livestock manure storage and following land application. Bioresource technology 96(2), 135-143.

Pandey, P., Chiu, C., Miao, M., Wang, Y., Settles, M., Del Rio, N.S., Castillo, A., Souza, A., Pereira, R. and Jeannotte, R. (2018) 16S rRNA analysis of diversity of manure microbial community in dairy farm environment. Plos one 13(1), e0190126.

Pandey, P., Kass, P., Soupir, M., Biswas, S. and Singh, V. (2014) Contamination of water resources by pathogenic bacteria. AMB Express 4 (1): 51.

Pandey, P.K., Biswas, S., Vaddella, V.K. and Soupir, M.L. (2015) Escherichia coli persistence kinetics in dairy manure at moderate, mesophilic, and thermophilic temperatures under aerobic and anaerobic environments. Bioprocess and biosystems engineering 38, 457-467.

Pandey, P.K., Cao, W., Wang, Y., Vaddella, V., Castillo, A.R., Souza, A. and del Rio, N.S. (2016) Simulating the effects of mesophilic anaerobic and aerobic digestions, lagoon system, and composting on pathogen inactivation. Ecological Engineering 97, 633-641.

Pandey, P.K., Shetty, B.D., Wickam, P., Aminabadi, P., Chen, Z., Mai, K., Stackhouse, J.W. and Jay-Russell, M.T. (2023) Physico-chemical assessment of on-farm bioconversion of organic waste in dairy farms in context to sustainability and circular bioeconomy. Environmental technology, 1-12.

Pandey, P.K. and Soupir, M.L. (2011) Escherichia coli inactivation kinetics in anaerobic digestion of dairy manure under moderate, mesophilic and thermophilic temperatures. AMB express 1, 1-10.

Paton, J.C. and Paton, A.W. (1998) Pathogenesis and diagnosis of Shiga toxin-producing Escherichia coli infections. Clinical microbiology reviews 11(3), 450-479.

Peces, M., Astals, S. and Mata-Alvarez, J. (2014) Assessing total and volatile solids in municipal solid waste samples. Environmental technology 35(24), 3041-3046.

Pell, A.N. (1997) Manure and microbes: public and animal health problem? Journal of dairy science 80(10), 2673-2681.

Pillet, F., Formosa-Dague, C., Baaziz, H., Dague, E. and Rols, M. (2016) Cell wall as a target for bacteria inactivation by pulsed electric fields. Sci Rep 6: 1–8.

Radhika, M., Saugata, M., Murali, H.S. and Batra, H.V. (2014) A novel multiplex PCR for the simultaneous detection of Salmonella enterica and Shigella species. Braz J Microbiol 45(2), 667-676.

Rangel, K., Cabral, F. and Lechuga, G. (2021) Detrimental Effect of Ozone on Pathogenic Bacteria. Microorganisms 10 (1).

Rice, E.W., Bridgewater, L. and Association, A.P.H. (2012) Standard methods for the examination of water and wastewater, American public health association Washington, DC.

Ried, A., Wang, J., Rand, W. and Fabiyi, M. (2014) Ozone to control bulking and foaming in municipal waste water treatment plant.

Rosso, D., Larson, L.E. and Stenstrom, M.K. (2008) Aeration of large-scale municipal wastewater treatment plants: state of the art. Water Science and Technology 57(7), 973-978.

Scheutz, F., Teel, L.D., Beutin, L., Piérard, D., Buvens, G., Karch, H., Mellmann, A., Caprioli, A., Tozzoli, R. and Morabito, S. (2012) Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. Journal of clinical microbiology 50(9), 2951-2963.

Schroeder, J.P., Croot, P.L., Von Dewitz, B., Waller, U. and Hanel, R. (2011) Potential and limitations of ozone for the removal of ammonia, nitrite, and yellow substances in marine recirculating aquaculture systems. Aquacultural Engineering 45(1), 35-41.

Semenov, A.V., van Bruggen, A.H.C., van Overbeek, L., Termorshuizen, A.J. and Semenov, A.M. (2007) Influence of temperature fluctuations on Escherichia coli O157:H7 and Salmonella enterica serovar Typhimurium in cow manure. FEMS Microbiology Ecology 60(3), 419-428.

Sheng, L., Shen, X., Benedict, C., Su, Y., Tsai, H.-C., Schacht, E., Kruger, C.E., Drennan, M. and Zhu, M.-J. (2019) Microbial safety of dairy manure fertilizer application in raspberry production. Frontiers in Microbiology 10, 2276.

Shental, N., Levy, S., Wuvshet, V., Skorniakov, S., Shalem, B., Ottolenghi, A., Greenshpan, Y., Steinberg, R., Edri, A. and Gillis, R. (2020) Efficient high-throughput SARS-CoV-2 testing to detect asymptomatic carriers. Science advances 6(37), eabc5961.

Shepherd, M.W., Liang, P., Jiang, X., Doyle, M.P. and Erickson, M.C. (2007) Fate of Escherichia coli O157:H7 during On-Farm Dairy Manure–Based Composting. Journal of Food Protection 70(12), 2708-2716.

Shetty, B.D., Amaly, N., Weimer, B.C. and Pandey, P. (2023) Predicting Escherichia coli levels in manure using machine learning in weeping wall and mechanical liquid solid separation systems. Frontiers in Artificial Intelligence 5, 921924.

Smith, S.R., Lang, N.L., Cheung, K.H.M. and Spanoudaki, K. (2005) Factors controlling pathogen destruction during anaerobic digestion of biowastes. Waste Management 25(4), 417-425.

Soares, A.S., Miranda, C., Teixeira, C.A., Coutinho, J., Trindade, H. and Coelho, A.C. (2019) Impact of different treatments on Escherichia coli during storage of cattle slurry. Journal of environmental management 236, 323-327.

Some, S., Mondal, R., Mitra, D., Jain, D., Verma, D. and Das, S. (2021) Microbial pollution of water with special reference to coliform bacteria and their nexus with environment. Energy Nexus 1, 100008.

Spiehs, M.J. and Goyal, S.M. (2007) Best management practices for pathogen control in manure management systems, Minnesota Extension.

Subirats, J., Sharpe, H. and Topp, E. (2022) Fate of Clostridia and other spore-forming Firmicute bacteria during feedstock anaerobic digestion and aerobic composting. Journal of environmental management 309, 114643.

Summerfelt, S.T. (2003) Ozonation and UV irradiation—an introduction and examples of current applications. Aquacultural Engineering 28(1-2), 21-36.

Tabe, N.N., Rahman, S., Tabe, E.S., Doetkott, D., Ekiri, A.B. and Khaitsa, M.L. (2016) Prevalence of Escherichia coli and Salmonella in Runoff of Two Cattle Feedlots in North Dakota. Food Protection Trends 36(1).

Tien, Y.-C., Li, B., Zhang, T., Scott, A., Murray, R., Sabourin, L., Marti, R. and Topp, E. (2017) Impact of dairy manure pre-application treatment on manure composition, soil dynamics of antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at harvest. Science of the Total Environment 581, 32-39.

Tiquia, S.M., Tam, N.F.Y. and Hodgkiss, I.J. (1998) Salmonella elimination during composting of spent pig litter. Bioresource technology 63(2), 193-196.

Toth, J., Aceto, H., Rankin, S. and Dou, Z. (2013) Survey of animal-borne pathogens in the farm environment of 13 dairy operations. Journal of dairy science 96(9), 5756-5761.

Vagheei, R. (2021) Upgrading of waste stabilization ponds using a low-cost small-scale fine bubble diffused aeration system. Water Science and Technology 84(10-11), 3104-3121.

Valldecabres, A., Wenz, J., Ferreira, F.C., Chahine, M., Dalton, J., de Haro Marti, M., Rovai, M. and Silva-del-Río, N. (2022) Perspective of dairy producers from California, Idaho, South Dakota, and Washington: Health and business implications of the COVID-19 pandemic during the second wave. Journal of dairy science 105(2), 1788-1796.

Varsaki, A., Ortiz, S., Santorum, P., López, P., López-Alonso, V., Hernández, M., Abad, D., Rodríguez-Grande, J., Ocampo-Sosa, A.A. and Martínez-Suárez, J.V. (2022) Prevalence and population diversity of Listeria monocytogenes isolated from dairy cattle farms in the Cantabria region of Spain. Animals 12(18), 2477.

Wells, C.L. and Wilkins, T.D. (1996) Clostridia: sporeforming anaerobic bacilli. Medical Microbiology. 4th edition.

Westerman, P. and Zhang, R. (1997) Aeration of livestock manure slurry and lagoon liquid for odor control: A review. Applied Engineering in Agriculture 13(2), 245-249.

Westover, C., Rahmatulloev, S., Danko, D., Afshin, E.E., O'Hara, N.B., Ounit, R., Bezdan, D. and Mason, C.E. (2022) Ozone Disinfection for Elimination of Bacteria and Degradation of SARS-CoV2 RNA for Medical Environments. Genes (Basel) 14(1).

Yadav, A., Kumar, A. and Sarkar, S. (2021) Performance evaluation of venturi aeration system. Aquacultural Engineering 93, 102156.

Yu, D., Ryu, K., Otto, S.J., Stothard, P., Banting, G., Ruecker, N., Neumann, N.F. and Zhi, S. (2022) Differential survival of potentially pathogenic, septicemia-and meningitis-causing E. coli across the wastewater treatment train. npj Clean Water 5(1), 33.

Zhang, Y., Wu, Q., Zhang, J. and Yang, X. (2011) Effects of ozone on membrane permeability and ultrastructure in Pseudomonas aeruginosa. Journal of applied microbiology 111(4), 1006-1015.

Zhang, Z., Zhu, J. and Park, K.J. (2004) Effects of duration and intensity of aeration on solids decomposition in pig slurry for odour control. Biosystems engineering 89(4), 445-456.

Zhao, Q. and Liu, Y. (2019) Is anaerobic digestion a reliable barrier for deactivation of pathogens in biosludge? Science of the Total Environment 668, 893-902.

APPENDIX A

Figure A1: Thermocycler program for PCR amplification (A) *Salmonella* (B) *E. coli*

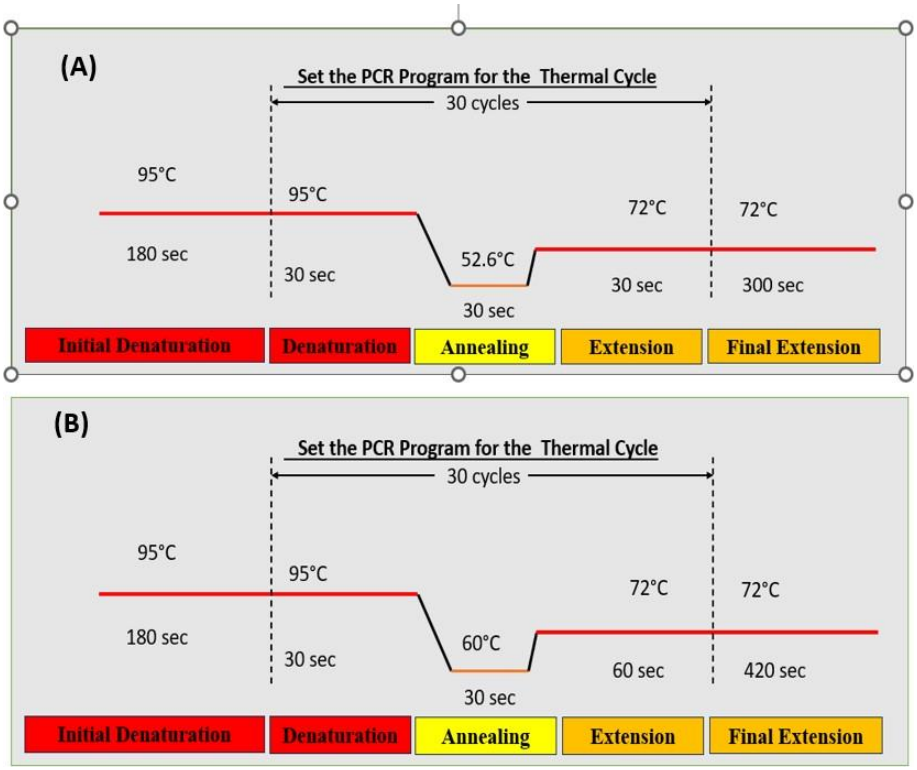


Table A1: Solid levels for different treatment (filtered manure diluted with water)

Solid Level -1	200 ml of filtered manure
Solid Level -2	180 ml of filtered manure +20 ml of water

Solid Level -3	160 ml of filtered manure + 40 ml of water
Solid Level -4	140 ml of filtered manure+40 ml of water
Solid Level -5	120 ml of filtered manure + 80 ml of water
Solid Level -6	100 ml of filtered manure + 100 ml of water

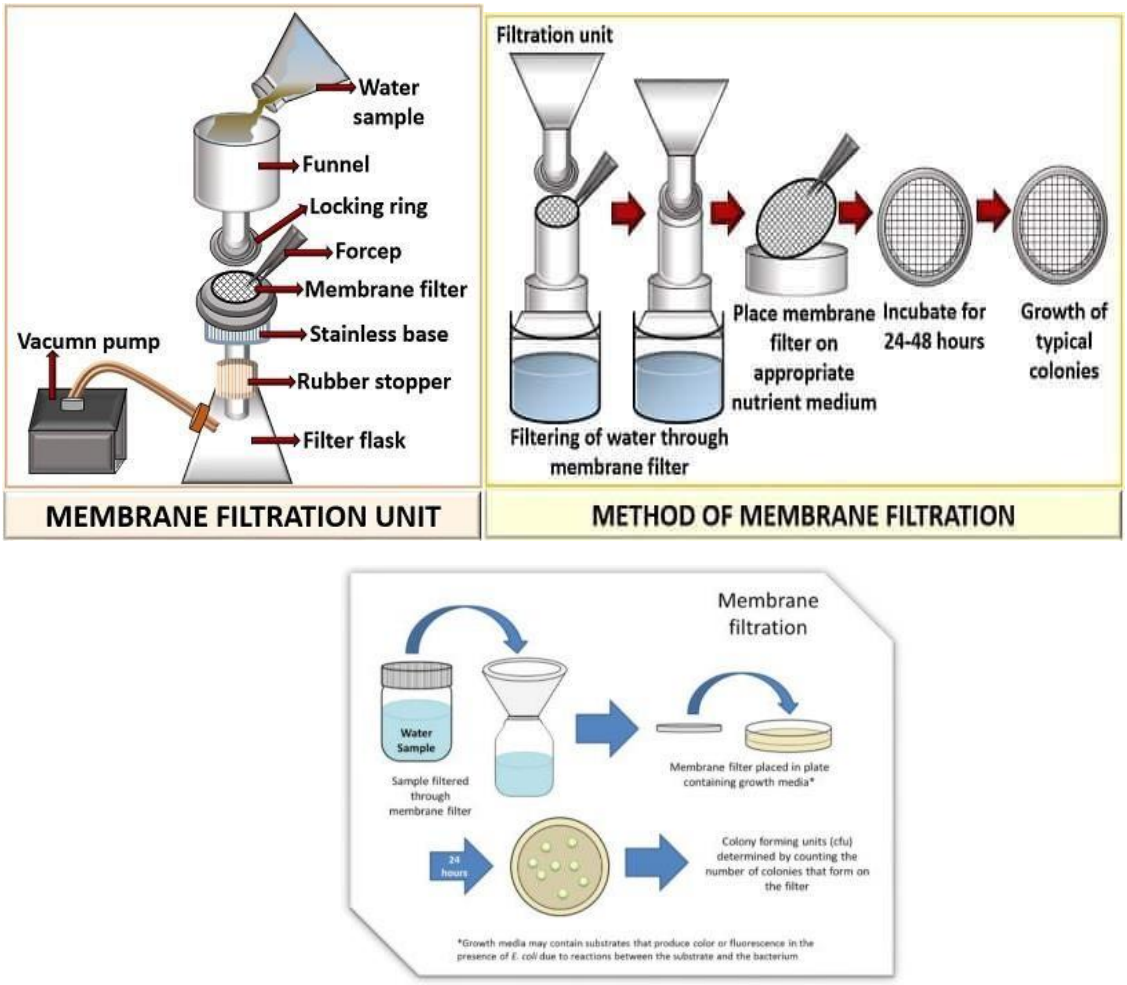


Figure A2: Membrane filtration unit (left), and method of membrane filtration (right). Online image. Available from: https://dnr.wisconsin.gov/sites/default/files/topic/LabCert/E.-coli-Test-Method-Help-Sheet-8.12.20-Final.pdf; https://www.facebook.com/102971398182044/posts/membrane-filter-technique-pharmaceuiticalmicrobiologymembrane-filters-have-a-kno/134975028315014/. [Accessed 3 December, 2023].