PATHOGEN SURVIVAL AND BIOFILM FORMATION IN IRRIGATION WATER DISTRIBUTION SYSTEMS

by

RAWANE RAAD

(Under the Direction of FAITH CRITZER)

ABSTRACT

Biofilms are known to form inside irrigation distribution lines to the point of complete blockage from the emitter holes. However, there is inadequate understanding of how foodborne pathogens may survive and what role biofilm formation may play in these conditions. The long-term objective of this research was to determine if irrigation lines can be a significant source of pathogens and not simply the source water which has been the primary focus by industry, policy makers, and researchers. Therefore, the objectives were to first, determine the microbial communities present within different irrigation water distribution lines in southern Georgia. Second, to evaluate the behavior of *Salmonella* in an irrigation drip tube system and its ability to form biofilms at different water quality parameters. Third, to evaluate control measures to prevent biofilm formation within the irrigation drip tubes. For objective one, swab samples were taken from different irrigation distribution lines (n=6 drip and n=2 center pivot). Samples were then processed for 16S rDNA sequencing for genome identification and operational taxonomical classification. For both the second and third objectives, drip tubes with no perforations were filled with surface water inoculated with a four-serotype cocktail

rifampicin resistant *Salmonella*, under different fertigation and chemigation parameters. Populations in the water and attached to the tubing were determined overtime. This study characterized the temporal and spatial dynamics of microbial communities in irrigation distribution systems used for fruit and vegetable production across various configurations, investigated the role of the foodborne pathogen *Salmonella* and biofilm development in response to commonly injected fertilizers in organic and conventional systems, and evaluated the efficacy of commonly used sanitizers in inactivating *Salmonella* biofilms within an irrigation line. These findings provide critical insights for improving irrigation system management and enhancing food safety practices in agricultural production.

INDEX WORDS: water distribution, produce safety, irrigation, 16S rDNA, chemigation, biofilms

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DEDICATION

To my father, whose boundless love and selfless sacrifices have shaped the foundation of my journey. To my mother, my unwavering supporter and greatest advocate. To my brother, my steadfast pillar of strength. And to my sister, whose courage and fearlessness continue to inspire me.

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CHAPTER 1

INTRODUCTION

Fruits and vegetables consumption in the United States has increased throughout the years (1) with the majority of produce consumed raw, or without an additional reduction, better known as “kill”, step for microorganisms. Several foodborne outbreaks associated with these products have increased correspondingly. During 2017 and 2020, the Centers for Disease Control and Prevention (CDC) reported 14,312 illnesses and 65 deaths linked to multistate foodborne outbreaks. Fruits were identified as the source of most of these solved outbreaks (8.8%). Vegetable row crops were the second most common source of multistate foodborne outbreaks (8%) (2). Updated CDC data reported that during 2022, leafy greens were identified as the source of the most solved foodborne outbreaks (3). Currently, CDC reported that six major pathogens; *Salmonella*, *Listeria monocytogenes*, *Campylobacter*, *Clostridium perfringens*, Shiga toxin-producing *Escherichia coli* (STEC), and norovirus, caused around 9.9 million domestically acquired foodborne illnesses. *Salmonella* caused 66% of multistate outbreaks and 86% of illnesses in 2022 (3).

Salmonellosis – the illness caused by *Salmonella enterica* – is the second leading cause of foodborne illnesses in the U.S. (4, 5). *Salmonella* is a Gram-negative bacterium belonging to the *Enterobacteriaceae* family (6). To date, there are over 2500 different serotypes of *Salmonella* within its two species, *Salmonella bongori* and *Salmonella enterica* (7, 8). The primary routes of *Salmonella* infection are through the ingestion of contaminated products or through fecal oral

transmission. Symptoms of human salmonellosis include acute onset of fever, abdominal pain, diarrhea, nausea and sometimes vomiting (9, 10). Historically, *Salmonella* outbreaks used to be linked to contaminated meat and poultry. However, cases of produce-linked salmonellosis are increasing. *S. enterica* is estimated to be responsible for a majority of produce-associated outbreaks in the U.S. (11).

Water is a primary input for ensuring proper growth and processing in the pre- and postharvest stages of production. Therefore, good water quality is essential in preventing contamination of crops and preventing the spread of foodborne pathogens (12-14). Sources of irrigation water include surface and groundwater. Groundwater (e.g. wells) is in aquifers beneath the earth's surface (15) while ponds or lakes are considered surface water (16). Multiple studies have reported the occurrence of *Salmonella* and other foodborne pathogens in water that could be used for growing crops (17-26). However, such surveys assessing *Salmonella* prevalence in surface water sources have generally reported low concentrations.

The production system at the farms consists of a complex combination of surface or ground irrigation water source and plastic pipes. There are several types of irrigation distribution systems; in southern Georgia both ground and surface water are used for irrigation where it is pumped from the source to the field through plastic pipes and delivered to the crop through overhead high-pressure sprinklers or near the root of plants through small perforations, commonly called drip irrigation (27). Within these systems, piping composed of metal, polyethylene, or polyvinyl chloride (PVC) is used until they are no longer fit for their purpose. Fertilizers can be added to any irrigation system through a process called fertigation. This is a common practice among growers (28). Nitrogen (N), phosphorus (P) and potassium (K) are the

most used fertilizers in conventional agriculture. Additional micronutrients such as zinc and other metals are sometimes used as well (29). According to the United States Department of Agriculture (USDA), organic cropping refers to agricultural production that relies on approved cultural, biological, and mechanical practices to promote ecological balance and conserve biodiversity, while strictly prohibiting the use of synthetic fertilizers and most synthetic substances (30). Hence, organic growers tend to use biological soil amendments of animal origin in which materials derived from animals such as manure, blood meal, bone meal, or other non-fecal animal byproducts are used to improve soil fertility and structure (30). These piping systems in which irrigation water and fertilizers are used are susceptible to microbial contamination and thus pathogens cross-contamination into irrigation water.

Along with it existing as planktonic cells, *Salmonella* often exists as multicellular forms such as biofilms. Several studies have reported the ability of different *Salmonella* serotypes to form biofilm (31-33). A biofilm is an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance (EPS) matrix (34, 35). Bacterial microorganisms have a natural propensity to attach, adhere and form biofilms on abiotic surfaces (36). Bacteria in the biofilm form are resistant to several stresses including sanitizers such as chlorine (37-39). For example, Aryal and Muriana (40) reported the resistance potential for *Salmonella* Montevideo biofilm against 200 and 1000 ppm of free chlorine after 60 minutes, however, this concentration did not completely inactivate the bacteria in the biofilm form. Peroxyacetic acid (PAA) on the other hand, reduced *Salmonella* Montevideo to undetectable levels after 30 min of treatment (40). Several factors including levels of organic matter formed, pH, water temperature and concentration of the chemical used can alter the effectiveness of the treatment against *Salmonella*

biofilms (41). It is important for the produce industry to establish control measures to prevent biofilm formation throughout their irrigation processes.

While numerous groups have studied biofilm formation on several types of abiotic surfaces (42), artificially developed biofilms of foodborne pathogens on the materials used for crop production, as they naturally exist, have not yet been investigated. Others studied the nature of irrigation system clogging and biofouling due to biofilm formation on the surfaces of irrigation materials (43, 44), biofilm formation in drinking water distribution systems (45, 46), or water quality and microbial populations in irrigation water sources (19, 47-49), but the microbial composition of natural biofilms as they exist in these distribution systems has not yet been evaluated. Few have targeted the effect of biofilms formed in irrigation system lines on the native microbial quality of the irrigation water passing (50) but without targeting the microbiome of those biofilms attached to the tubing. Information regarding these complex communities may provide a better understanding of pathogens' behavior in preharvest conditions. This could result in innovative strategies to control and intervene in the spread of pathogens via irrigation systems. Additionally, there is a need to identify likely sources of *Salmonella* contamination throughout the irrigation distribution systems. As irrigation water contains a good source of microflora and it is a likely source of fresh-produce contamination with pathogens, we hypothesize that bacteria present in these waters and travelling through irrigation pipes have the potential to form biofilms within. Pipelines remain hot and wet throughout the season and are often not cleaned nor sanitized unless clogging is observed. Establishing the need to clean and sanitize the irrigation lines constantly being used as well as providing several treatment options effective in reducing pathogens during preharvest could prevent many foodborne outbreaks each year and thus reduce hospitalization rates and save thousands of lives.

Therefore, the objective of the proposed work was to first determine the ecology, diversity and composition of the microbial communities present in different irrigation water distribution systems in southern Georgia throughout two production seasons. Second, to evaluate the behavior of *Salmonella* in irrigation lines and the ability of this pathogen to form biofilms over time, under different fertigation conditions. Third, to evaluate the efficacy of sanitation control measures which could be implemented by produce growers to manage the risk of *Salmonella* biofilms in the irrigation lines. The long-term objective was to determine if such irrigation lines can be a significant source of pathogens and not simply the source water which has been the primary focus by the industry, policy makers, and researchers. If so, it will substantiate the need to clean and sanitize irrigation pipes as a new control measure to prevent the introduction and cross-contamination of pathogens into fresh produce sold for human consumption.

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CHAPTER 2

LITERATURE REVIEW

Fresh produce consumption and increase in number of outbreaks

Consumer acceptance and consumption of fruits and vegetables have notably increased in the United States (1). Between 2015 and 2018, more than two-thirds (67.3%) of adults, aged 20 and over, consumed a fruit on a given day. Approximately three-quarters (75.3%) of children and adolescents aged 2–19 did the same (2). About 95.1% of adults consumed vegetables daily, with 26.3% consuming dark green vegetables, 79.2% consuming red and orange vegetables, 50.3% consuming starchy vegetables, and 78.8% consuming other vegetables (2). Per capita consumption of fresh tomatoes reached 19.2 pounds in 2023 (3). This rise is attributed to the growing popularity of tomatoes in salads, sandwiches, and as a key ingredient in various dishes. Additionally, the convenience and ease of preparation of fresh-cut fruits and vegetables have made them increasingly popular. The fresh-cut produce industry, valued at $12.5 billion, has experienced double-digit growth for over 15 years and remains the fastest-growing segment in the produce sector (4). Dietary guidelines emphasizing the importance of raw fruits and vegetables for a nutritionally adequate diet have further bolstered their consumption. Innovations in packaging and urban agriculture have also contributed to making fresh produce more accessible and appealing. Overall, the rising trend in fruit and vegetable consumption reflects a shift towards healthier eating habits and a demand for convenient, nutritious options.

Several foodborne outbreaks associated with these products have increased correspondingly. During 2017 and 2020, the Centers for Disease Control and Prevention (CDC) reported 14,312 illnesses and 65 deaths linked to multistate foodborne outbreaks. Fruits were identified as the source of most of the solved foodborne outbreaks (8.8%) in 2020. Vegetable row crops were the second most common source of multistate foodborne outbreaks (8%) for that year range (5). Updated CDC data reported that during 2022, leafy greens were identified as the source of the most solved foodborne outbreaks (6). Foodborne illness has widespread economic consequences, costing Americans an estimated $15.5 billion (in 2018 dollars) annually in medical care, lost productivity, and premature deaths, including those associated with secondary chronic illnesses and conditions that develop after the initial illness (7). Currently, CDC reported that over 9,000 foodborne illness outbreaks were reported between 2011 and 2022 across all 50 states with six major pathogens: *Salmonella*, *Listeria monocytogenes*, *Campylobacter*, *Clostridium perfringens*, Shiga toxin-producing *Escherichia coli* (STEC), and norovirus, causing about 10 million cases each year. *Salmonella* caused the highest number of multistate outbreaks (66%) and the majority of illnesses (86%) in 2022 (6).

Salmonella enterica

Salmonellae is one of the four leading causes of diarrheal illness worldwide (8). *Salmonella* spp. are Gram-negative – thin peptidoglycan layer surrounded by an outer membrane containing lipopolysaccharides – rod shaped bacteria (9-11). *Salmonella* belongs to the family of *Enterobacteriaceae* (12). It is a facultative anaerobe, capable of surviving and growing in the presence or absence of oxygen (10, 13, 14). It has the following characteristics: optimum temperature of 37°C with minimum 5.5°C and maximum of 45°C, pH ranges between 6.5-7.5

but can survives at lower (3.99) pH (10, 15). Its optimum water activity ranges from 0.93 to 0.97 (15-17) and it can survive at high salinity conditions; up to >2% sodium chloride (NaCl) with increasing temperatures (18). It is chemoorganotrophic; obtaining its energy from a wide range of organic substrate, with the ability to metabolize nutrients by both respiration and fermentation processes (10, 19).

Salmonella has two species: *Salmonella bongori* and *Salmonella enterica* (20, 21) with *Salmonella enterica* having 6 subspecies: I, *S. enterica* subsp. *enterica*, II, *S. enterica* subsp. *salamae,* IIIa, *S. enterica* subsp. *arizonae*, IIIb, *S. enterica* subsp. *diarizonae*, IV, *S. enterica* subsp. *houtenae*, VI, *S. enterica* subsp. *indica* (22). *S. enterica* subsp. *enterica* is the most isolated from foodborne illnesses (6, 23). *S. enterica* is further classified based on three major surface antigens: O (somatic lipopolysaccharide on the external surface of the bacterial outermembrane), H (flagellar antigens associated with flagella), and K (or Vi in the case of *Salmonella*; capsular virulence) (12). *S. enterica* subsp. *enterica* has the largest number of serovars (> 2500) and names are assigned based on geographical areas where the serovar was first isolated, or based on historical names, the illness caused, the habitat, or the host of the serovar (24).

Salmonella enterica can be spread by the fecal oral route when raw or undercooked meat is consumed, by drinking contaminated water, by food handlers who are sick, or by animals and pets (25). Common reservoirs for this pathogen include domestic and wild animals such as poultry (chicken, turkeys), livestock, reptiles, and pets. It can also be found in the environment (26-31). *S. enterica* causes a wide range of symptoms including inflammatory gastroenteritis, enteric (typhoid) fever; diarrhea (sometimes bloody), stomach (abdominal) cramps, nausea,

vomiting, chills, headache. Symptoms for the nontyphoid *Salmonella* occur a few hours to two days after ingestion of the pathogen (32). All age groups are affected by this pathogen but the newborns, infants, the elderly, and the immunocompromised most frequently experience severe symptoms. All illnesses require supportive care and hydration. Some extreme cases require hospitalization. Antibiotics can be used but are not preferred due to the emerging antibiotic resistance issue. The infectious dose; the number of bacteria that must be ingested to cause an infection, for *S. enterica* varies. It depends on many factors, including immunological status of the host, type of food consumed, the degree of virulence of the strain, and the number ingested. Some reported that for enteric fever, the infectious dose is about 10^5 by ingestion. However, other sources suggest that the infectious dose of *Salmonella* can be as low as 1-10 cells in certain conditions (33).

After *Salmonella* is ingested, it can travel through the acidic conditions of the stomach (34, 35) and can attach to the epithelial cells of the small intestine of the host. Its flagellar covered with microvilli allows the increase of surface area and hence, increase in nutrient absorption which further allows it to break through the mucus layer of the host (36-38). Once *Salmonella* invades the M cells and epithelial enterocytes, it colonizes the lymphatic tissues and travels through the lymph nodes causing gastrointestinal inflammatory response (39, 40). The detection of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (lipid A), type three secretion system (T3SS) encoded on the *Salmonella* pathogenicity island (SPI), fimbriae, and flagellin induces an inflammatory response (41). *Salmonella* uses T3SS to deliver the toxins into the host-cells. The effectors SipC, SopB, SopE, and SopE2 interact with the cellular proteins and lipids of the host to manipulate its functions (42). The latter induces cytokines and chemokines release that initiate the innate immune system and phagocytosis in the host (41). Since *Salmonella* is

intracellular, it contains vacuoles that allow it to alter the environment to a more favorable state including a dormant state. It can survive intracellularly within host macrophages by increasing the expression of some virulence genes such as acid stress (43). If *Salmonella* is not contained by the adaptive immune response, it will disseminate into the bloodstream (44).

To survive unstable and harsh environmental conditions, many foodborne pathogens, including *Salmonella*, have evolved mechanisms to respond to a wide range of environmental stresses (45). These stress responses are crucial for survival in both natural ecosystems (e.g., water, soil) and industrial or commercial settings (e.g., food processing facilities, sanitation environments), where microorganisms may encounter sudden and potentially lethal challenges (46). Among these stressors, acidic conditions are particularly significant, especially in acidified food products such as kimchi (47). During processing, cleaning, or preservation, bacteria may be exposed to low pH environments. Bacteria often employ multiple acid stress responses, such as the Acid Tolerance Response (ATR) and Acid Resistance (AR) systems. These involve general stress regulators, pH-dependent mechanisms, and enzymatic systems like acid decarboxylases that help maintain intracellular pH homeostasis (34, 45, 48, 49). Osmotic stress, another common challenge, arises from moisture loss in soil or reduced water activity in dried or processed foods particularly in dried or cured products such as jerky or flour (50, 51). In response, *Salmonella* typically accumulates compatible solutes such as amino acids and potassium ions and activates specific transport systems to restore turgor pressure. Regulatory systems like the two-component system OmpR-EnvZ play a dual role in managing both osmotic and acid stress, highlighting the interconnected nature of stress responses (10, 52, 53). Heat stress during processes like pasteurization, cooking, or hot water sanitation is managed through a network of heat-shock proteins and sigma factors (e.g., σ^E and σ^H), which are also conserved in other bacteria like *E.*

coli (10, 54). Importantly, exposure to one type of stress can induce cross-protection against others. For instance, sublethal acid exposure in marinades or fermented foods may enhance resistance to subsequent heat or osmotic stress (53). Additionally, acid or nutrient stress may upregulate genes involved in biofilm formation (e.g., *adrA*, *bapA*), which enhances resistance to multiple other environmental challenges (45, 55). The general stress sigma factor RpoS is a key regulator in this process, orchestrating a broad protective response that spans multiple stress types (56, 57) . This cross-reactivity among stress responses allows bacteria to mount a more robust defense, increasing their chances of survival in dynamic and often unpredictable environments.

Salmonellae generally produce hydrogen sulfide, orthenine, decarboxylate lysine, and do not hydrolyze urea (58, 59). Such traits have formed the presumptive biochemical identification of *Salmonella* isolates. To date, the predominant approach for the detection and isolation of *Salmonella* relies on the organism's characteristic biochemical properties. For example, the U.S. Food and Drug Administration (FDA) outlines a standardized protocol in the Bacteriological Analytical Manual (BAM), Chapter 5, which recommends a stepwise confirmation process following selective plating (60). If the isolate presents the expected reactions, it is then subjected to serological testing and molecular confirmation. For instance, the protocol suggests starting with pre-enrichment using general media broth (e.g. Buffered Peptone Water) to resuspend cells and revive injured ones, enrichment using acidic and hypersonic solutes (e.g. Tetrathionate) to inhibit background microflora followed by selective enrichment using solid agar (e.g. Xylose-lysine deoxycholate (XLD), Hektoen enteric (HE), and Bismuth Sulfite (BS) agar). *Salmonella* uses the thiosulfate enzyme to reduce the thiosulfate present in XLD and HE and reduces sulfite in BS to hydrogen sulfide (H_2S) producing black colonies. Colonies characterization occurs

using Triple sugar Irons and Lysine Iron Agar in slant positions. After confirming presumptive colonies, serological tests can be applied. However, the variability of biochemical traits between the different serovars of salmonellae is leading to the replacement of the traditional testing protocols with molecular technologies: subtyping, whole genome sequencing, and 16S identification that target the identification of stable genes or their products that are unique for *Salmonella* (61-63).

Produce Outbreaks linked to *Salmonella*

Salmonellosis – the disease caused by *Salmonella* – is estimated to cause around 93.8 million cases of gastroenteritis globally each year, with 155,000 deaths and 80.3 million of these incidents being foodborne (64). Based on data from 2000-2008 and the U.S. based population in 2006, it was estimated that *Salmonella* will cause about 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the U.S. every year with food being the source for most of these illnesses (65). According to the CDC's 2022 Foodborne Illness Source Attribution Estimates*, Salmonella* was the leading cause of domestically acquired foodborne illnesses resulting in death (238) in the U.S. More than 75% of attributed illnesses were linked to food products such as chicken, fruits, seeds vegetables, pork, nuts, beef, and turkey (6, 66). Historically, *Salmonella* outbreaks used to be linked to contaminated or undercooked meat and poultry, egg products, and raw milk in the U.S. and worldwide (25). In 1985, more than 1,500 culture-confirmed cases of *Salmonella* infection were reported in Illinois, prompting a major public health investigation. The outbreak was traced to 2% pasteurized milk. Laboratory analysis identified *Salmonella Typhimurium* - resistant to both ampicillin and tetracycline- in samples from patients as well as unopened milk cartons. State health officials later identified a

malfunctioning valve at the dairy facility that may have allowed raw, contaminated milk to mix with pasteurized milk, leading to the widespread outbreak (67, 68). Several other notable outbreaks of non-typhoidal *Salmonella* have been linked to various food products. In the U.S., a major outbreak in 1994 involving *Salmonella* Enteritidis phage type (PT) 8 affected over 224,000 people and was traced to ice cream contaminated via transport trucks previously used for raw eggs (69, 70). Another significant event occurred in 2006, when *Salmonella* Tennessee contaminated peanut butter, leading to over 600 reported cases across 47 states (71). While similar salmonellosis outbreaks continue to occur (72-78), there has been a noticeable rise in *Salmonella* infections linked to fresh produce, highlighting the evolving nature of food safety challenges.

Between 1973-1983 fruits and vegetables were reported to be the cause of 2% of the foodborne disease outbreaks in the U.S. (79). Between 1973 - 1997, the trend had risen to account 6% of all reported foodborne outbreaks with over 16,000 cases of illness identified from fresh produce and related products in the U.S. (80). Up until 2005, produce accounted for 13% of outbreaks with an identified food source (81). The most recent data indicate that produce accounted for 50% of illnesses in the U.S. with leafy greens accounting for most of the illnesses (66). The consumption of raw produce has been generally perceived as safe compared to other foods primarily of animal origin. However, vegetable production systems inherently present multiple points of vulnerability to foodborne pathogen contamination. This includes production, pre-, and postharvest practices such as using contaminated irrigation water (29, 82), contaminated compost (82) , or unhygienic harvesting practices (83). *S. enterica* is estimated to be responsible for a majority of produce-associated outbreaks in the U.S. (66). Examples of noticeable salmonellosis-produce outbreaks include the 2008 *Salmonella* Saintpaul outbreak which implicated 1,442

reported cases. The FDA traced the source of the jalapeño peppers associated with illness and reported isolation of the outbreak strain from a water sample of a holding pond used for irrigation (84). Additionally, the infamous 2015 *Salmonella* Poona outbreak linked to imported cucumbers. Investigations identified 907 case-patients from 40 states (85). Whole genome sequencing performed on both clinical isolates and cucumber samples indicated that the sequenced isolates were genetically related to one another. Although the root-cause of the outbreak was not determined, after grower inspection at the implicated farm, potential sources noted included improper waste-water management, equipment design of the flume handling system area, and improper storage of packing materials (86). More noticeable incidence occurred linked to production practices include the 2020 *Salmonella* Newport (87) and 2021 *Salmonella* Oranienburg (88) outbreaks that caused around 1,000 hospitalizations, each. FDA investigation reports stated that the source of the outbreaks was possibly due to the use of contaminated irrigation water. In 2024, a *Salmonella* Typhimurium outbreak was reported but additional serovars of *Salmonella* (Braenderup and Africana) were detected in soil or water samples collected from nearby water sheds and the farm (89). In 2025, a salmonellosis outbreak linked to cucumbers implicated with *Salmonella* Montevideo was associated with 69 cases around 21 states (90). Those findings further substantiate the environmental presence of *Salmonella* and underscore the associated risk it poses to fruit and vegetable production. While all stages of vegetable production are critical, the postharvest and distribution phases are particularly vital for preventing the proliferation of foodborne pathogens. Equally important are preharvest practices, which play a key role in minimizing cross-contamination risks throughout the farm-to-fork continuum.

Agricultural Water

Given the increasing incidence of produce-associated salmonellosis outbreaks, it is important to consider the entire production chain as critical points for food safety. Among these, the use of agricultural water during preharvest practices. Agricultural water refers to the water is used in the production of crops covered under the Produce Safety Rule [21 CFR § 112] of the Food Safety Modernization Act (FSMA). The water should intend to or more likely will be in contact with the covered produce. This includes irrigation water applied in preharvest practices, water used in harvesting, packing, and holding activities. Recognizing this, the FDA recently finalized a rule under the FSMA that revises preharvest agricultural water requirements for covered produce. The updated rule replaces previous microbial testing standards with a systems-based approach, requiring annual agricultural water assessments to identify and mitigate potential hazards that could introduce pathogens such as *Salmonella* onto produce [21 CFR § 112 Subpart E].

Globally, the agricultural sector constitutes the largest use of freshwater with irrigation withdrawals (91). Agricultural water used in irrigation settings plays a critical role in sustaining crop production. Irrigation allows farmers to supplement natural precipitation, ensuring consistent yields and crop quality throughout the growing season. Water is typically delivered through systems such as drip, sprinkler, or surface irrigation, each varying in efficiency and suitability depending on the crop and soil type (92). Sources of irrigation water include surface and groundwater. Groundwater (e.g. wells) is in aquifers beneath the earth's surface (93). Ponds, lakes, rivers, and creeks are considered surface water. Surface water has historically been the dominant source of water in the state of Georgia, nonetheless, groundwater is being heavily used for irrigation (94). Southwest Georgia relies on groundwater from the Floridan aquifer whilst coastal and northern regions depend more on surface water from reservoirs and ponds (95).

Surface water can be easily contaminated with pathogenic bacteria, parasites, and viruses which are endemic in the surrounding environment, potentially contaminating the soil, root vegetables and act as a vehicle for transferring the pathogen to the surface of the produce (82, 96-98). Improper sealing, failure of backflow prevention devices can be a cause for groundwater contamination along with the influence by surface water where rivers, lakes, or ponds are geographically close to the underground aquifers (99). Since irrigation water comes into direct contact with soil, crops, or both, maintaining high water quality is essential to ensure healthy plant growth and prevent microbial contamination. Overall sources of *Salmonella* contamination on the farm include contaminated irrigation or postharvest wash water, contaminated or inadequately composted manure, wild or domestic animals, contaminated soil, or contaminated fertilizers (100-102).

Multiple studies have reported the occurrence of *Salmonella* and other foodborne pathogens in water that could be used for growing crops (27, 103-111). However, surveys assessing *Salmonella* prevalence in surface water sources have generally reported low concentrations. In the Eastern Shore of Virginia, it was reported that *Salmonella* was present in 21.7% (26/120) of surface water samples collected between June 2016 and May 2017 (103). Researchers have reported that from October 2016-2018, there was an overall 65% (122/188) of water samples positive for *S. enterica* and MPN values ranged from 0.015 to 11 MPN/liter throughout the sampling of four surface water and two reclaimed water sites on Maryland's Eastern Shore (27). The overall mean *Salmonella* concentration in positive water samples from two ponds in southern U.S. collected throughout three growing seasons was 0.03 MPN/100 mL (110). Chevez et al. (104) reported that between February and December of 2021, the overall prevalence of *Salmonella* in water samples was 6.81% (6/88) from water samples collected from eight

irrigation ponds located in southwest of Georgia, U.S. In one of the ponds sampled by Antaki et al. (110), *Salmonella* was detected in 8.3 % (3/36) of water samples. Notably, in the associated drip irrigation system, 19.4% (14/72) of samples tested positive, indicating potential contamination and further multiplication of cells occurring along the irrigation line. Many have reported the transfer of foodborne pathogens from contaminated water and its possible link to human outbreaks (112, 113) and the occurrence of *Salmonella* in water stream from crop production (26, 29). Overall, this issue complicates efforts by researchers and industry to identify contamination sources, as the water source may not remain contaminated over time, and water distribution systems are often neglected.

Crop production system

A typical fruit or vegetable farm production system involves the complex design of components that transport water from its source (e.g. well, pond, river, reservoir, etc.) to reach the soil or directly the plant. Starting from the water source, pumps – often made of stainless steel or cast iron – are used to pressurize the water. Throughout the system, filters (often disk or sand) are installed to prevent clogging and remove large contaminants such as sticks, rocks, coarse sand, and even fish (114). Disk filters create three-dimensional filtering and allow buildup of debris on both the outside of the disks and on the surface area in between the disks. Sand filters are stainless steel tanks that are filled with fine crush silica sand. Water is pumped into the tank and downward through the media, contaminants become caught and then removed from the water (114). Pressure regulators and backflow valves are usually inserted to ensure consistent water delivery and prevent backflow water contamination. The water is then conveyed through mainlines, usually constructed from metal or plastic material such as polyvinyl chloride (PVC) or

high-density polyethylene (HDPE) (92). PVC is popular due to its durability and cost-effectiveness, making it ideal for drip irrigation and sprinkler system whilst HDPE is preferred for its high strength and flexibility (115, 116). Additionally, flexible hoses made from materials like rubber or silicone are used to connect various parts of the irrigation system, ensuring versatility and ease of use. These mainlines branch into sub-lines and laterals, which are smaller diameter pipes that distribute water closer to the crop rows. On the field, various irrigation methods are used to deliver to the soil: drip irrigation, sprinkler systems, furrow or surface irrigation. Drip irrigation uses emitters embedded in polyethylene tubing to release water slowly at the base of each plant. Sprinkler irrigation uses metal or plastic nozzles mounted on risers or pivots to spray water over the crop. Various inputs can be added to the irrigation sub-lines to boost crop yield, including fertilizers, sanitizers, and other additives like nutrients and pH adjusters.

Chemigation (or drip chemigation) is a broader term referring to the application of any chemical into or through an irrigation system including the application of fertilizers, acids, chlorine, and pesticides (117). Fertilizer application in farming depends on several factors, including the type of crops, soil conditions, and the time of year. Various techniques are used to optimize nutrient delivery. Broadcasting involves spreading fertilizer evenly across the soil surface, often with a spreader, once plants are established. Banding places fertilizer in concentrated strips near the root zone at planting, enhancing early nutrient uptake. Side dressing applies fertilizer alongside plant rows, while top dressing involves adding a nutrient-rich layer, such as compost or manure, around plants on the soil surface (118). Another common practice among growers is fertigation which includes the addition of fertilizer into the irrigation lines in the soluble form (119). The choice of fertilizers is dependent on the crop, soil, cost, and management style of the farm.

Fertilizers are often available in different forms and concentrations where formulations usually contain two or more nutrients (117). Commercial non-organic growers can either solely add synthetic or both organic and synthetic fertilizers while organic growers may only include organically approved fertilizers (120). Synthetic fertilizers, primarily the chemical form of Nitrogen (N), Phosphorus (P), and Potassium (K), are widely used in conventional agriculture, with nutrient levels indicated by numbers. For example, 4-0-8 fertilizer contains 4% N, 0% P, and 8% K [23]. Nitrogen is supplied as ammonium, urea, nitrate, or a combination of these forms; potassium is typically added as soluble potash, while phosphorus is commonly applied as phosphate compounds such as monoammonium phosphate in the form of mined rock phosphate source (121, 122). Additional micronutrients such as zinc and other metals are sometimes used as well (123). Based upon the United States Department of Agriculture (USDA), "Organic cropping" is a term indicating that the crop has been produced through approved methods of the USDA National Organic Program including cultural, biological, and mechanical practices, whilst synthetic fertilizers, sewage sludge, irradiation, and genetic engineering are not allowed (120). Growers following organic practices during fertilization aim to increase soil organic matter through the addition of biological soil amendments of animal origin (BSAAO) or providing direct nutrients to plants, through the sub-lines, using liquid organic fertilizers such as fish emulsion or blood meals (120). The chemical composition of organic fertilizers is highly complex and dependent on the main source. For example, the chemical composition of fish emulsion involves inorganic elements, mixture of essential amino acids such as histidine's, methionine, lysine, serine, tryptophan, proteins, lipids, and vitamins such as riboflavin, pantothenic acid, niacin, biotin, folacin and vitamin B-12 (124-126). Injectors are commonly used to deliver concentrated nutrient solutions into the main line of an irrigation system. The two

primary injection pumps that are commonly used during chemigation processes are venturi which use a pressure differential to draw nutrients into the irrigation line and positive displacement systems which use mechanical or electrical pumps to precisely inject the nutrients (117, 119).

Studies consistently show that both synthetic and organic fertilizers can enhance crop yields (127-132). However, when it comes to soil microbial diversity, numerous studies have demonstrated that organic fertilizers promote greater microbial richness and activity compared to conventional mineral fertilizers (131, 133-136). For example, the meta-analysis conducted by Bebber and Richards (136) provides findings on the impact of organic and mineral fertilizers on soil microbial diversity. Based on data from 37 research articles, organic fertilizers significantly enhanced both functional and taxonomic diversity of soil microbes compared to unfertilized control soils and mineral fertilizers. Specifically, organic fertilizers increased functional diversity by 7.0% compared to control soils and by 3.8% compared to mineral fertilizers, while the latter showed a modest increase of 2.8% in functional diversity compared to control soils. Another study by Abbasi et al. (128) incorporating fish emulsion into pathogen-infested soil at rates of 0.5% and 1% two weeks before planting reduced eggplant wilt incidence by 77 to 89% and decreased disease severity by 2.1 to 2.2 units compared to the untreated control, further confirming the benefits of using organic fertilizers in crop production.

Lastly, basic water distribution system components, used in agricultural fields, include valves, fittings, pumps, sprinklers, storage reservoirs, tanks, and piping materials (PVC, HDPE). These materials are recognized for providing an ideal surface that promotes bacterial colonization and biofilm formation (137). Bacteria, usually sulfur or iron forming bacteria, along with other

freshwater organisms such as diatoms can attach to the drip line and form a slime, which clogs the emitters (117, 138, 139). This phenomenon is referred to as biofouling.

Biofouling and biofilm formation

Fouling broadly describes the undesirable accumulation of materials on solid surfaces. This includes several types: (1) scaling or mineral fouling, which involves the precipitation of inorganic materials; (2) organic fouling, characterized by the buildup of substances such as oils, proteins, and humic matter; (3) particle fouling, involving the deposition of particles like silica, clay, and humic substances; and (4) biofouling, which specifically pertains to the adhesion of microorganisms and the subsequent development of biofilms (138). Biofilms are integrated multi-species cell populations that are embedded in a self-produced matrix of extracellular polymeric substances (EPS) (54). Almost all microorganisms are capable of forming biofilms on surfaces (140-142) including *Salmonella, E. coli*, *Listeria monocytogenes*, and *Pseudomonas* (143-154). For example, studies reported biofilm formation of *Salmonella* (145), *Pseudomonas*, *Lysinibacillus* (152), and *E. coli* (155) on HDPE surfaces. Gamri et al. (156) investigated how different pipe materials influence biofouling using synthetic wastewater. Their findings indicated that biofilm development was more significant at higher flow velocities (0.8 m/s). Although PVC pipes exhibited lower bacterial accumulation than polyethylene pipes, they were still vulnerable to biofilm formation. Furthermore, studies have shown that *Salmonella* can persist on various packing line materials, including stainless steel, PVC, and unfinished oak wood, for over 28 days (157).

Biofilm development begins with the attachment of bacterial communities to a surface, followed by an increase in biomass that makes the biofilm increasingly difficult to remove. This process

occurs in five key stages that allow microorganisms to attach to the surfaces and aggregate into complex, mature communities (158). It begins with an initial and reversible attachment, where free-floating cells adhere loosely to a surface (159, 160). Once bacteria are transported close to a surface, initial attachment occurs through weak intermolecular interactions, including van der Waals forces, electrostatic attractions, and hydrophobic interactions (161). Bacteria do so with the help of extracellular organelles such as flagellum and pili (148, 162). Since the attachment does not involve a differentiation series of morphological changes, it is easier for bacteria to return to its original planktonic lifestyle. Hence, simple shear forces such as rinsing will allow the removal of the cells. Bacteria communicate and coordinate their behavior through a process known as quorum sensing: a chemical signaling mechanism triggered by population density (163). In this system, bacteria release signaling molecules called autoinducers into their environment. As the bacterial population grows, the concentration of these molecules increases. Once a threshold is reached, neighboring cells detect the signals and collectively alter gene expression, enabling synchronized behaviors such as biofilm formation, virulence, and bioluminescence (164). When environmental conditions shift or bacteria experience stress, they can activate specific genes that trigger an irreversible attachment to a surface, often within minutes and is very dependent on the material (159). For example, Schwab et al. (165) reported that two *Listeria monocytogenes* strains were capable of rapid irreversible attachment within 5 min. Bacteria also use structures like pili and begin to produce EPS at this stage (158, 159). The EPS of *Salmonella* biofilm is majorly composed of curli (amyloid fimbriae), cellulose, biofilm-associated protein (Bap), O-antigen capsule, extracellular DNA, and mostly water (around 90% of the EPS matrix) (160, 166). The EPS matrix has a broad range of functions; aggregation of bacterial cells, water retention, protection, energy sink, ionic exchange, sorption of organic and

inorganic compounds, nutrient source, and exchange of genetic information (164, 167). The EPS matrix can account for more than 90 % of the dry mass of most biofilms and provides a structure for bacteria within (54, 164, 167). At the end of the irreversible attachment stage much stronger forces are required to remove the bacteria from the surface including scraping, scrubbing, or chemical cleaner. The attachment of small colonies grows into a mature biofilm with three-dimensional biofilm characteristics (158). Maturation occurs in two phases: first, the biofilm starts to develop as cells proliferate, and EPS accumulates. Second, the biofilm becomes more stable by forming channels for nutrient, waste, and genetic exchange (54, 168, 169). For example, Guillín et al. (170) studied the metabolic shift from planktonic to sessile states of *Salmonella* Enteritidis and reported that the cells were involved in a reprogramming of pathways that support biofilm formation and stress resilience. Planktonic cells were enriched in proline and phenylalanine which are precursors for essential metabolites and contribute to stress adaptation. Sessile (biofilm-forming) cells were dominated by lysine and acetylated amino acids that are associated with cellular homeostasis, stress response, and metabolic regulation (170). Biofilms commonly form as complex communities involving two or more microbial species, while single-species biofilms are relatively rare in natural environments (171). Lin et al. (172) reported that a dual-species biofilms of *E. coli* strains with or without *Salmonella* Typhimurium had significantly higher biomass, 2-6 times greater, than single-species biofilms. Dual-species biofilms showed less reduction in viable cells after chlorine treatment of 100 parts per million (ppm). Lee et al. (173) reported that a mixed-species biofilm of *Pseudomonas aeruginosa*, *P. protegens*, and *Klebsiella pneumoniae* showed higher resistance to sodium dodecyl sulfate and tobramycin that single-celled species compared to mixed species of planktonic cultures. Multiple studies have reported the role of biofilm formation in enhancing horizontal gene transfer (HGT),

particularly plasmid-mediated transfer, due to close cell proximity and increased plasmid stability in the biofilm matrix (174-183). Finally, the last step of biofilm formation is the dispersion stage, some cells detach from the biofilm to return to a free-cell state, allowing them to colonize new surfaces and repeat the cycle (158, 184). Each stage of biofilm formation is characterized by distinct gene expression and protein production profiles, as mentioned previously. In the sessile phase, bacteria can either up-or down-regulate genes, produce extracellular matrix components, and activate antibiotic resistance mechanisms such as efflux pumps, even without antibiotic exposure, while also expressing elevated levels of virulence factors (184-186). These adaptations make biofilm-associated cells more resilient than their planktonic counterparts (170, 187). Importantly, once these highly adapted cells enter the dispersal phase, they may pose an even greater threat. Having acquired enhanced resistance and virulence traits within the protective biofilm environment, dispersed cells can contaminate new niches.

The traditional five-step model of biofilm development is primarily based on in vitro flow cell experiments. However, studies of biofilms in environmental systems, as well as in vivo and ex vivo models, suggest that this model does not always reflect the dynamics observed in open systems. To address this limitation, Sauer et al. (188) proposed a simplified three-stage model that better represents biofilm development in open environments, such as natural ecosystems, where there is a continuous influx of new microbial members. The stages are : (1) aggregation and attachment, where bacteria adhere to surfaces or to each other; (2) growth and accumulation, involving expansion through cell division and recruitment; and (3) disaggregation and detachment, where bacteria leave the biofilm as single cells or clusters (188). This model more accurately represents biofilm development in systems with continuous microbial influx. In the

context of the food-to-fork chain, this mobility increases the risk of introducing resilient pathogens to previously uncontaminated surfaces or products. The ability of bacteria in the biofilm form to survive harsh conditions and evade standard sanitization measures makes them particularly dangerous during this phase, amplifying the potential for widespread contamination and infection.

Bacteria within biofilms exhibit resistance to various environmental stresses, including commonly used sanitizers (189, 190). Sanitizing agents such as chlorine (Cl); typically applied as sodium or calcium hypochlorite, and peracetic acid (PAA) are frequently used to disinfect equipment in food processing facilities (191, 192) and to treat agricultural water sources (193, 194). However, the effectiveness of these treatments against bacteria in either the planktonic or the biofilm form can be influenced by several factors, including the presence of organic matter in the solution, pH levels, water temperature, and the concentration of the sanitizing agent used (195). Both sanitizers operate through different mechanisms. Cl kills microbes by penetrating cell walls and breaking down chemical bonds within the organisms, primarily through the formation of hypochlorous acid in the water (196). It has strong oxidizing properties and the ability to generate reactive oxygen species (ROS) such as hypochlorous acid superoxide, and hydroxyl radicals (196). These ROS induce oxidative stress, damaging proteins, lipids, and DNA within cells, leading to their effective elimination (197). Cl is known to form disinfection byproducts (DBPs) such as trihalomethanes and haloacetic acids, which may pose health risks (198). Cl is cost-effective and widely applicable, but its disinfectant efficacy is significantly influenced by water quality. In turbid or organic-rich water, Cl rapidly reacts with organic matter, reducing its availability for microbial inactivation (199). PAA is a strong oxidant that disrupts microbial cell walls and cellular components of microorganism through direct oxidation,

leading to cell lysis and leakage of cellular contents (200). It is rapid-acting and does not produce DBPs on food surface or returned to the environment as it degrades quickly into oxygen and carbon dioxide (200, 201). PAA is also biodegradable in water, rapidly decomposing into acetic acid and hydrogen peroxide (200). The rapid biodegradability of PAA means that it breaks down quickly, reducing its effective concentration over time (202). The effectiveness of PAA is dependent on pH and temperature (201, 203). Nonetheless, PAA remains a practical and effective sanitizing option for both organic and conventional producers [7 CFR § 205]. Its broad applicability makes it a valuable tool across the agricultural sector, especially when paired with good agricultural practices (204). In organic production, both PAA and chlorine are allowed; however, when chlorine is used in water that comes into direct contact with crops or soil, its residual concentration must not exceed 4 ppm, in accordance with the Safe Drinking Water Act (SDWA) according to the USDA National Organic Program (NOP) (199).

Many have reported the efficacy of Cl and PAA based treatments against foodborne pathogens on food contact surfaces or in agricultural water sources (193, 194, 205-213). For example, Murphy et al. (207) evaluated the effectiveness of 150 ppm free chlorine and 80 ppm PAA against *Salmonella* and *Listeria* on stainless steel coupons under conditions simulating a packing house, using both wet and dry surfaces with contact times of 30 min and 15 h. On clean coupons, both sanitizers reduced bacterial populations by approximately 5.51 log CFU/mL, whereas on fouled coupons, the reduction was slightly lower – around 5 log CFU/mL for both *Salmonella* and *Listeria* on wet surfaces. Similarly, on dry inoculated pathogen, *Salmonella* and *Listeria* reduction was around 4 log CFU/mL and 3 log CFU/mL, respectively, on both clean and fouled coupons. Additionally, Cuggino et al. (208) evaluated the effectiveness of sodium hypochlorite at 25 ppm and PAA at 80 ppm in reducing and controlling the growth of *Salmonella* Thompson

on fresh-cut iceberg lettuce after 60 s of treatment at 4°C. Results showed that both Cl and PAA reduced populations at 2.98 log CFU/g and 2.79 log CFU/g, respectively. Etaka et al. (206) evaluated the efficacy of Cl and PAA at 200 ppm each, in reducing *L. monocytogenes* and *Salmonella* on canvas and Cordura® harvest bag materials. After 1 min of sanitizer application, PAA showed to have the most efficacy of 2.63 and 3.92 log CFU/coupon reduction of *L. monocytogenes* compared to Cl which was 1.58 and 2.74 log CFU/coupon for the canvas and Cordura®, respectively. Similarly, *Salmonella* was reduced by 3.68 and 3.21 log CFU/coupon with PAA and 1.05 and 1.29 log CFU/coupon with Cl for the canvas and Cordura®, respectively. Others (205) used longer contact time (1 or 2 min) and higher concentrations of 500 ppm and approx. 3 and 4 log reduction after 1 and 2 min of treatment on *Salmonella* inoculated wood and nylon surfaces. Nonetheless, the latter study showed that Cl and PAA were less effective on porous surfaces harboring biofilms compared to planktonic cells, reinforcing the challenge of biofilm sanitation in real-world produce operations. McCaughan et al. (210) found that Cl was more effective than PAA in reducing bacterial loads in well water, particularly at higher concentrations. A 10-min contact time consistently resulted in greater microbial reductions, while temperature variation (12°C vs. 32°C) had a minimal impact on sanitizer efficacy. At 10 ppm, chlorine achieved an average bacterial reduction of 3.48 log CFU/mL, outperforming PAA, which achieved a 2.50 log CFU/mL reduction under the same conditions. However, when targeting protozoa, PAA at 50 ppm for 10 min demonstrated superior efficacy compared to Cl, highlighting the need to tailor sanitizer selection based on the target organism. In contrast, Krishnan et al. (193) reported that at 3 and 5 ppm, PAA was more effective than Cl in reducing *Salmonella*, STEC, and *L. monocytogenes* in surface and ground agricultural water. The discrepancy in findings highlights how sanitizer efficacy against foodborne pathogens is

influenced by multiple factors, including concentration, water source, and the specific pathogen targeted.

Biofilms provide a protective barrier that can limit the penetration and effectiveness of sanitizers (54, 171, 214, 215). This barrier, composed of EPS including proteins and polysaccharides, can prevent PAA or Cl from reaching and eliminating all bacteria within the biofilm, leading to persistent contamination (216-220). The protective nature of biofilms allows bacteria to survive and potentially detach, leading to cross-contamination in untreated areas. Ivers et al. (205) used a multistrain *Salmonella* cocktail to evaluate the efficacy of sanitizers including PAA and Cl at 500 ppm each against sessile and biofilms on wood, HDPE, and nylon surfaces. Results showed that biofilms were significantly more resistant to antimicrobials than sessile cells, with a reduction of less than 3 log CFU/coupon for the three material types when treated with PAA. Cl showed limited efficacy on porous materials such as nylon and wood against the biofilm form. Jang et al. (220) showed that Cl, in the form of chlorine dioxide, at 25 ppm did not penetrate beyond a depth of 100 mm into a complex dairy process pipe biofilm that was 150–200 mm thick. Luppens et al. (217) reported that achieving a reduction greater than 4-log in *Staphylococcus aureus* monospecies biofilms formed on glass coupons required a high concentration of sodium hypochlorite – specifically, 30,000 ppm. The EPS matrix was partially degraded, which facilitated the detachment and dispersion of biofilm cells. Despite these effects, some residual clusters of biofilms remained, suggesting that while sodium hypochlorite is highly effective, it may not eliminate all biofilm-associated cells.

The complexity of the EPS and the stress responses to sanitizers further complicate the eradication of biofilms as microorganisms can adapt to these sanitizers, enhancing their

resilience (221). This highlights the need for additional or combined treatments to effectively manage biofilm formation. Biofouling has been shown to be of considerable hygienic, operational and economical relevance, for drinking (222-224) and irrigation (225, 226) water distribution systems.

Biofouling in water distribution systems

When the inner surfaces of pipelines are in contact with the water, they can become colonized by biofilm forming bacteria, leading to biofouling. The notion of biofouling still challenges water distribution systems whether in the context of microbial contamination (224), water quality degradation (223), disinfectant demand resistance (227), or operational challenges (228). Examples of pathogens found in drinking water distribution systems (DWDS) include *Vibrio cholerae*, *Salmonella*, *E. coli*, *Giardia lamblia*, *Cryptosporidium parvum*; *Mycobacterium avium*, and hepatitis viruses (229-231). Along with their resistance to disinfectants, as mentioned previously, biofilms in DWDS can be resistant to shear stress conditions (232) and predators (233). Biofilms in water distribution systems are composed of gelatinous matrix and only 2-5% of the volume is occupied by microorganisms (171, 234). Aside from the organic materials within the EPS matrix, inorganic substances such as corrosion products, suspended solids, and sand may also be incorporated in the biofilms increasing their accumulation (235). Multiple factors affect biofilm formation in water distribution systems, this includes water flow rate, organic and inorganic matter, and pipe material. Water flow significantly influences biofilm development, with different rates leading to distinct structural characteristics (236-238). For example, Cowle et al. (236) reported that microbial biomass was greater for the biofilms conditioned at lower flows, regardless of material type. In contrast, shear forces imposed by

higher flow rates inhibited biofilm development. It is known that organic matter content in water systems is a fuel for biofilm development. Simple carbohydrates, low-molecular weight proteins, and organic acids, are accessible nutrients for microbial degradation (239). Other inorganic nutrients such as phosphorus and nitrogen significantly affect biofilm formation within those systems (239). Similarly to a crop production system, a range of pipe materials are used in DWDS. Iron- (stainless steel and galvanized steel), copper-, or cement-based pipes have been used historically. Polymer-based pipes such as PVC and polyethylene are becoming increasingly popular because their cost and ease of use (239). As discussed previously, different abiotic surfaces affect biofilm formation differently (145, 152, 153, 155, 171, 236, 240-242). Hence, the choice of pipe materials could affect the development of biofilms in water distribution systems. Both dissolved and particulate iron corrosion products in DWDS can influence microbial community dynamics by supporting the growth of specific biofilm-forming bacteria. Iron-oxidizing bacteria (IOB), such as *Gallionella* spp., utilize ferrous iron (Fe^{2+}) as an energy source, oxidizing it to ferric iron (Fe^{3+}), which can contribute to the formation of stable biofilms and metal deposits on pipe surfaces (227, 243). Conversely, nitrate reducing bacteria (e.g. *Acidovorax*) and iron reducing bacteria such as *Pseudomonas* spp., can reduce ferric iron to ferrous iron altering the redox environment and contributing to the dissolution or destabilization of corrosion scales (243, 244). These microbial transformations are significant because they can promote biofilm persistence by supplying electron donors and acceptors that support microbial metabolism even in oligotrophic conditions. Additionally, the presence of iron-associated biofilms increases disinfectant demand and can shield pathogens from disinfectant exposure (227), ultimately compromising microbiological water quality and distribution system integrity. Cowle et al. (236) cultivated biofilms from custom-built flow cell reactors using four common

pipe materials: PVC, polypropylene (PP), structured wall high-density polyethylene (Str-HDPE) and solid wall high-density polyethylene (S-HDPE) pipes. Rough materials like S- and Str-HDPE provided more surface area and micro-niches for microbial attachment: 6.8 and 5.7 ng/μL, respectively in low shear forces. Bacteria were still protected on S- and Str- HDPE from high shear forces with biomass ~ 5.7 and 3.9 ng/μL, respectively, allowing biofilms to persist even when flow would otherwise inhibit growth. Biofilms were less structurally complex and produced a lower biomass on PVC and PP which had smooth surface with low roughness. Polymeric pipes, in particular, may release biodegradable volatile organic compounds (VOCs) into the water due to the leaching of additives, degradation of the polymer matrix, and by-products formed during oxidation (245). Low-molecular-weight plasticizers and residual monomers, within these compounds, can serve as nutrient sources for microorganisms, thereby supporting microbial proliferation and enhancing biofilm formation on pipe surfaces (246).

Another significant challenge is monitoring biofilm formation in DWDS. Since most pipelines are located underground, direct observation and sampling are inherently difficult. Moreover, advanced monitoring technologies and frequent sampling can be expensive. Despite these obstacles, researchers continue to develop and refine techniques to estimate the population size, spatial organization, and microbial diversity within biofilms. Whether in lab-scale studies; where researchers have direct access to test coupons or pipeline sections (223, 247), or through swabbing accessible locations within the distribution system (241, 248), characterization efforts typically focus on the direct imaging of sampled biofilm (239). During the early stages of biofilm formation, thin biomass layers are commonly visualized using epifluorescence microscopy combined with nucleic acid stains (249-252). This method allows for relatively quick and straightforward ex-situ monitoring and total cell enumeration. As biofilms mature, typically

exceeding 3 to 4 μm in thickness, confocal laser scanning microscopy (CLSM) becomes a preferred tool (248, 253-255). CLSM enables non-destructive, high-resolution optical sectioning and 3D reconstruction of biofilm architecture (256). Common nucleic acid stains used along with these technologies include DAPI (4′,6-diamidino-2-phenylindole) or SYTO dyes (255, 257), which bind to DNA or RNA and fluoresce under specific wavelengths. To distinguish viable cells from dead biomass, viability stains such as the widely used Live/Dead BacLight (Molecular Probes) are applied. This dual-stain system uses SYTO 9, which penetrates all cells and stains them green, and propidium iodide (PI), which only enters cells with compromised membranes, staining them red (254, 258). Other viability indicators include CTC (5-cyano-2,3-ditolyl tetrazolium chloride) (259-261), which fluoresces upon reduction by metabolically active cells. For mature biofilms up to 2 mm thick, optical coherence tomography (OCT) provides high-resolution imaging over a relatively large area without the need for cell staining (239, 262, 263). However, current OCT technology lacks the spatial resolution necessary to visualize individual cells (239). To investigate submicron structures within biofilms in water distribution systems, researchers increasingly use a “biofilm-friendly” version of environmental scanning electron microscopy (ESEM) (236, 264-266). This method allows for the evaluation of biofilm coverage and thickness without requiring sample dehydration as compared to the traditional scanning electron microscopy (267). Furthermore, scanning transmission X-ray microscopy (STXM) enables elemental composition mapping of macromolecules within biofilm matrices such as polysaccharides, proteins, lipids, and nucleic acids (268-270). This technique can generate detailed, correlative maps that reveal both the structural and chemical composition of biofilms within water distribution systems (270). Viability assessments are valuable for evaluating the effectiveness of disinfection strategies at various points within a water distribution network.

They help identify when and where disinfectants begin to lose their potency. A common approach to estimating microbial activity involves biochemical assays that detect specific metabolic byproducts. One widely used method in drinking water biofilm studies is the adenosine triphosphate (ATP) assay, which offers rapid and quantitative insights into the concentration of metabolically active biomass (239, 271). Although traditional culture-based methods like heterotrophic plate counts (HPC) are still used, they often underestimate viable populations due to the presence of non-culturable bacteria (266). There are molecular techniques that provide more targeted insights such as quantitative PCR (qPCR) combined with viability dyes that can selectively amplify DNA from live cells (272, 273). Together, these techniques provide a comprehensive toolkit for evaluating biofilm viability and understanding microbial dynamics in water distribution systems (239).

Continuous monitoring of biofilm development in DWDS is critical for safeguarding water quality and public health. Traditionally, biofilms are assessed by physically scraping pipe surfaces and conducting ex-situ laboratory analyses. However, because biofilms are heterogeneously distributed throughout the system, obtaining representative samples remains a significant challenge. Emerging culture-independent technologies and advances in DNA sequencing, (e.g. whole genome sequencing [WGS]) are increasingly being employed to characterize microbial communities more comprehensively (62). These methods enable the analysis of microbial populations within the biofilm as well as in the water upstream and downstream of the sampling point. By providing a more holistic view of microbial dynamics, WGS can enhance our understanding of biofilm behavior in DWDS and support the development of more effective mitigation strategies.

Microbiome research and its growing importance

Culture-independent next-generation sequencing technologies (NGS) have revolutionized researcher's understanding of the microbial world. Both targeted and untargeted metagenomic approaches have been widely applied to explore microbial communities in diverse environments such as soil, water, human skin, and food (274). Among these studies are investigations into the metagenomics and microbial communities of irrigation water and watersheds (104, 107, 275, 276), drinking water (277-279), and water distribution systems (241, 248, 279-282).

The study of microbiomes has undergone a remarkable transformation, evolving from early Sanger sequencing methods to advanced high-throughput technologies (62). Initially, microbial diversity was explored using 16S rDNA gene sequencing, a targeted approach that amplifies and sequences a conserved region of the bacterial genome to identify and classify microbes (283, 284). The advent of NGS technologies, particularly whole genome sequencing (WGS), enabled researchers to move beyond 16S to capture the full genetic repertoire of microbial communities, providing insights into functional potential and metabolic capabilities (283, 285). Among the leading platforms, Illumina sequencing is known for its high accuracy and throughput, making it ideal for large-scale metagenomic studies, though it produces relatively short reads (286-289). In contrast, Oxford Nanopore Technologies (ONT) offers long-read sequencing, which facilitates genome assembly and the resolution of complex genomic regions, albeit with a higher error rate (286, 288, 290, 291). Together, these technologies have expanded the ability to characterize microbiomes across diverse environments with unprecedented depth and precision.

The 16S rDNA gene sequencing method remains a cornerstone of microbial ecology due to its ability to identify and classify bacteria based on conserved and variable regions within the 16S

ribosomal gene (292). This technique is especially valuable in environmental studies, where it enables researchers to assess microbial diversity and community structure without the need for culturing organisms and where many microorganisms exist in a viable but non-culturable (VBNC) state (104, 241, 275, 277, 278, 280, 281, 293). The process typically involves collecting water samples, filtering them to concentrate microbial cells, extracting DNA, and amplifying the 16S rDNA gene using polymerase chain reaction with universal primers. The amplified sequences are then analyzed using bioinformatics pipelines to assign taxonomy and compare community composition across samples (283) . For example, Liu et al. (277) demonstrated how this approach can uncover the presence of VBNC bacteria in drinking water biofilms, including pathogens such as *E. coli*, *Legionella pneumophila*, *L. monocytogenes*, and *P. aeruginosa*. These organisms can enter a dormant, metabolically active state in response to environmental stressors like temperature fluctuations, chlorination, pH changes, nutrient depletion, and oxygen stress. The presence of such VBNC pathogens in DWDS poses a significant public health concern, as they can regain virulence under favorable conditions (294). Because they evade detection by conventional culturing, their population densities are often underestimated. This approach provides a cost-effective and scalable means to track microbial dynamics in response to environmental changes, pollution, or treatment processes, making it an essential tool in water microbiology and public health surveillance. Taxonomic classification of microbiome data is commonly performed by aligning sequences to reference databases such as Greengenes (295), SILVA (296), and NCBI's RefSeq (297). Standard analysis pipelines like Mothur, QIIME, and DADA2 support these methods (298). Each gene is amplified and sequenced once, increasing the risk of errors that can obscure true sequence identity. To manage this, sequences are grouped into operational taxonomic units (OTUs) based on similarity thresholds (commonly 97% or 99%)

(299, 300). These thresholds are often arbitrary and may not reflect biologically meaningful differences, varying with the gene region analyzed (283, 298).

To interpret the complexity of microbial communities revealed through sequencing, researchers commonly employ diversity analyses (298). These analyses help quantify and compare microbial richness and composition across samples and environments. Alpha diversity metrics, such as Shannon and Simpson indices, are used to assess the richness and evenness of microbial species within a single sample (301). For example, it is commonplace to compare mean species diversity between samples from environments with and without foodborne pathogens. Shannon and Simpson indices were used to assess microbial diversity across produce distribution centers, revealing that sites positive for *Listeria* often had distinct microbial profiles and lower overall diversity, suggesting that reduced microbial richness and evenness may be associated with pathogen presence (287). In contrast, beta diversity measures such as Bray-Curtis dissimilarity or UniFrac distances, evaluate differences in microbial community composition between samples or groups. They are often calculated by comparing feature dissimilarity, resulting in a distance matrix between all pairs of samples (298, 302). In the study by Townsend et al. (287), Bray-Curtis dissimilarity was used to compare microbial community composition across different surfaces and zones within produce distribution centers. This metric helped identify distinct clustering of microbial communities associated with *Listeria*-positive versus *Listeria*-negative environments, highlighting how shifts in community structure may correlate with pathogen presence and environmental conditions. These tools are essential for identifying patterns in microbial ecology, such as the impact of environmental gradients, pollution, or seasonal changes on microbial populations.

Studies using metagenomics have revealed not only bacterial diversity but also the presence of other important microbial groups, such as fungi, viruses, algae, and protists (293, 303-307). Among these, diatoms, a group of photosynthetic microalgae, are particularly significant (308). While diatoms and other microbial genera are naturally abundant in surface waters, their journey doesn't end there. During water treatment and distribution, many of these microorganisms can persist through filtration and disinfection processes. Once in the distribution system, they may adhere to pipe surfaces, especially in areas with low flow or residual nutrients (308, 309). Diatoms come in various shapes and forms, creating a physical space surrounding its cell surface – the phycosphere (308). This area facilitates exchanges between diatoms and bacteria, providing an environment for bacteria to localize and adhere to surfaces. Diatoms, with their silica-based cell walls, can serve as structural scaffolds within these biofilms, contributing to their stability and resilience. These biofilms can influence water quality by harboring pathogens, altering taste and odor, and interfering with disinfectant efficacy, making their study crucial for maintaining safe drinking water (310, 311). Just as biofilms can develop within drinking water distribution networks, similar microbial processes occur in irrigation water systems, especially those drawing from surface water sources. When irrigation water contains organic and inorganic materials, along with microbial populations such as diatoms, it creates an ideal environment for biofilm formation when put under the right conditions.

Irrigation water distribution systems

Irrigation water distribution systems, often less stringently treated than potable water supplies, can serve as reservoirs for microbial contaminants, including bacteria and algae (110, 226, 281, 282, 294, 312, 313). As previously discussed, when biofilms form inside pipes, they can harbor

pathogens such as *Salmonella*, which may be shielded from disinfectants and released intermittently into the water flow. This poses a significant risk in agricultural settings, where contaminated irrigation water can directly contact edible crops or the soil, leading to foodborne illness outbreaks. Moreover, the presence of diatoms and other microalgae in irrigation systems can contribute to biofilm complexity and clogging, affecting water delivery efficiency and crop health (139). As such, understanding microbial dynamics in irrigation infrastructure is critical for both food safety and sustainable agriculture.

Despite the clear risks posed by microbial contamination in irrigation systems, much of the existing research has concentrated on operational challenges, particularly the efficiency losses caused by biofouling and clogging (156, 226, 247, 314-318). Studies often prioritize the development of antifouling treatments, pipe material innovations, and hydraulic optimization (156, 247, 282, 319, 320) to maintain consistent water flow and reduce maintenance costs. For example, Gamri et al. (156) investigated how different pipe materials influence biofilm formation under controlled flow conditions. The researchers tested materials commonly used in water distribution systems, such as PVC, polyethylene, and stainless steel, under identical hydrodynamic conditions to isolate the effect of surface properties. Biofilm development was monitored over time using biomass measurements and microbial analysis. The results showed that pipe material significantly affected biofouling: rougher and more hydrophilic surfaces promoted greater microbial attachment and biofilm growth, while smoother materials like stainless steel exhibited lower levels of biofouling. Wang et al. (316) investigated how eight different water shear forces (ranging from 0 to 0.7 Pa) influenced biofilm formation in drip irrigation pipelines using three types of reclaimed water. Maximum biofilm growth occurred at 0.2 Pa and 0.35 Pa, indicating these shear levels promote microbial transport and nutrient

exchange. At low shear forces, biofilms were loose and unstable due to limited nutrient transport and microbial activity compared to high shear forces, biofilms were thinner but more stable due to frequent detachment and renewal. Song et al. (320) used labyrinth irrigation channels to simulate drip irrigation systems and treated them with chlorine at concentrations of 10, 20 and 30 ppm, applied at different stages of biofilm development (early, mid, and late). Biofilm samples were collected and analyzed using 16S gene sequencing to assess microbial community structure and diversity. The results showed that early chlorination at 30 ppm reduced biofilm biomass by over 80%, while late-stage treatments were significantly less effective. This highlights the importance of applying chlorination early to prevent biofilm formation, rather than attempting to remove it once it has matured in those systems. In another study by Song et al. (319), electrochemical treatment using a low-voltage direct current (1.5–3.0 V) significantly reduced biofilm formation in agricultural water distribution systems. The treatment achieved up to a 78.6% reduction in biofilm biomass compared to untreated controls. Microbial community analysis revealed a marked shift in composition: dominant biofilm-forming bacteria such as *Pseudomonas* and *Acinetobacter* were suppressed, while *Firmicutes* became more prevalent. Additionally, microbial diversity decreased, with the Shannon index dropping from 3.2 to 2.1, indicating a less complex and more controlled microbial environment. These results suggest that electrochemical methods can effectively manage biofouling by reshaping microbial communities, offering a promising strategy for biofouling control in irrigation systems. However, chemical disinfectants remain the most widely used approach in agricultural water management (193, 194, 208). Cl is commonly used in irrigation systems to control biofilms and reduce algal buildup that can clog equipment; however, there is limited information on the specific concentrations required to effectively reduce foodborne pathogens and prevent their

biofilm formation. Additionally, it remains unclear whether alternative sanitizers, such as PAA, may offer more effective or sustainable solutions for these purposes. PAA, specifically in the form of Sanidate 12.0, is currently the only EPA-registered product labeled for the reduction and control of STEC and *S. enterica* in preharvest irrigation water (EPA Reg. No. 70299-18).

While these efforts are essential for system performance, they frequently overlook the equally critical issue of microbial safety. There remains a significant gap in research focused on the presence and persistence of bacterial pathogens and foodborne contaminants within irrigation infrastructure. There is a notable lack of research addressing foodborne pathogen biofilm formation in irrigation water distribution settings, particularly in the context of current agricultural practices such as the addition of synthetic or animal-based fertilizers. This oversight is particularly concerning given the direct exposure of crops to irrigation water, which can serve as a vector for pathogens that threaten both public health and agricultural sustainability. Addressing this gap requires a more integrated approach that combines engineering solutions with microbiological surveillance and risk assessment. Mature biofilms in irrigation systems can serve as points of cross-contamination, where bacteria not only persist and proliferate but also exchange virulence genes. This phenomenon can lead to the emergence of new, more pathogenic strains, posing a significant risk to food safety through irrigation practices (109, 173, 225, 321, 322).

Conclusion

To bridge the gap between system efficiency and microbial safety in irrigation water distribution systems, future research should adopt a multidisciplinary approach that integrates microbiology, engineering, and agricultural sciences. One key direction is the comprehensive characterization

of microbial communities within irrigation infrastructure using high-resolution sequencing techniques such as shotgun metagenomics or long-read sequencing. Studies should also focus on identifying and tracking foodborne pathogens under real-world conditions, considering variables like water source, seasonal changes, and crop type. Additionally, there is a need to develop standardized protocols for microbial monitoring and risk assessment tailored to irrigation systems. Investigating the interactions between biofilm-forming organisms and pathogens could reveal mechanisms of persistence and resistance, informing more effective antifouling and disinfection strategies. Finally, integrating microbial data with system design and management practices could lead to predictive models that help mitigate contamination risks while maintaining irrigation efficiency. Therefore, in this work, we have collaborated with fruit and vegetable growers across southern Georgia to collect irrigation tubing and water samples from multiple systems over two production seasons to assess microbial quality and dynamics. Biofilms within these irrigation systems are of particular concern, as they can harbor and facilitate the persistence and transport of foodborne pathogens such as *Salmonella* to downstream crops. To address this, we evaluated *Salmonella* biofilm formation under a range of environmental and operational conditions commonly found on produce farms. Finally, we assessed the efficacy of commonly used sanitizers (Cl and PAA) in preventing biofilm formation within irrigation lines on a laboratory scale. This research has enhanced our understanding of how irrigation system design and microbial communities contribute to pathogen survival and cross-contamination risks. Ultimately, the findings can be used to assess Best Management Practices to help growers make informed decisions that protect crop safety and public health.

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CHAPTER 3

CHANGE IN MIRCOBIAL POPULATIONS IN FARM PONDS AND IRRIGATION DISTRIBUTION SYSTEMS THROUGHOUT TWO CROP PRODUCTION SEASONS IN GEROGIA COASTAL PLAINS

[1]Raad, R, Ruiz-Llacsahuanga B, Gale, M, Applon, C, Greenbaum, H, Den-Bakker, H, Vinueza, R, Singh, M, Critzer, F. **To be submitted to a peer-reviewed journal (Agricultural Water Management)**

Abstract:

Studies on the distribution of microbial populations within irrigation distribution lines are limited. We evaluated the ecology, diversity, and composition of the microbial communities present in different irrigation distribution systems in the Georgia Coastal Plains area during the 2023 (May-November) and 2024 (March-October) growing seasons. DNA samples (n=499) were collected using HiCap swabs from irrigation systems (drip systems or center pivots) in six fresh produce commercial farms and sequenced for taxonomy determination. Oxford Nanopore Technologies' 16S microbiome sequencing kit was used, followed by bioinformatics analysis using Sepia. Water samples (n=57) from irrigation sources or from the drip line were tested for physicochemical properties, total coliforms, and generic *E. coli.* Overall, there was a wide diversity of microbial populations with *Exiguobacterium*, *Thiobacillus, Pseudomonas*, *Aeromonas*, and *Bacillus* abundant in most farms throughout the two sampling years. No significant differences between alpha diversity indices were observed across the farms ($p > 0.05$), and an increase in microbial diversity at the end of the line was observed compared to the beginning. Regardless of the sampling year, all diversity indices were significantly different by month ($p < 0.001$). Bray-Curtis' beta diversity metric resulted in no significant clusters across the farm and some seasonal variation. Water quality and microbial composition across water samples revealed significant fluctuations. This work provides the first comprehensive evaluation of microbial communities within active produce farm irrigation systems, capturing their natural dynamics across seasons, water sources, and management practices. **Keywords:** microbiome, biofouling, irrigation water, produce, oxford nanopore technologies, 16S, preharvest

1. Introduction

The undesirable accumulation of materials on solid surfaces, within water distribution lines known as biofouling, is a challenge facing the agriculture industry. Biofouling specifically pertains to the adhesion of microorganisms and the subsequent development of biofilms on surfaces (Flemming, 2002). Biofilms are multi-species cell populations that are embedded in a self-produced matrix of extracellular polymeric substances (EPS) (Flemming & Wingender, 2010). Almost all microorganisms are known to form biofilms on surfaces (Fett, 2000; Henle et al., 2012; Xu et al., 2010) including *Salmonella*, *Escherichia coli*, and *Pseudomonas* (Carvalho et al., 2023; Dong et al., 2022; Giaouris et al., 2005; Ivers et al., 2024; Kim et al., 2022; Klausen et al., 2003; Manville et al., 2023; Mendez et al., 2020; Oliveira et al., 2021; Rivera-Betancourt et al., 2004; Sauer et al., 2002; Sharma, 2002). Biofilm formation occurs in five key stages that allow microorganisms to attach to surfaces and aggregate into complex, mature communities (Zhao et al., 2017). It begins with an initial and reversible attachment (Flemming et al., 2007; Palmer et al., 2007). When environmental conditions shift or bacteria experience stress, they can activate specific genes that trigger the second, irreversible attachment stage (Palmer et al., 2007). The attachment of small colonies grows into a mature biofilm, known as the biofilm matrix (Zhao et al., 2017). Multiple studies have reported the role of biofilm formation in enhancing horizontal gene transfer and exchange of genetic and virulence material (Bernier et al., 2013; Chiang et al., 2013; Cook et al., 2014; Driffield et al., 2008; Gama et al., 2020; Lecuyer et al., 2018; Liu et al., 2024; Molin & Tolker-Nielsen, 2003; Roder et al., 2021; Walters et al., 2003). Lastly, the final step refers to the dispersion stage, in which some cells detach from the biofilm to return to a free planktonic-like state (Rumbaugh & Sauer, 2020; Zhao et al., 2017).

At the farm level, drip and center pivot irrigation systems commonly use different pipe materials tailored to their specific operational needs and environmental conditions. For example, drip

systems typically utilize polyethylene (PE) pipes while center pivot systems often rely on polyvinyl chloride (PVC) or galvanized steel pipes for their main lines. Such materials are known to provide appropriate surfaces for biofilm formation (Aloraini et al., 2023; Gamri et al., 2016; Ivers et al., 2024). When the inner surfaces of pipelines are in contact with the water, biofilm-forming bacteria can attach to such surfaces, resulting in biofouling.

Current efforts prioritize the identification of biofouling in the context of irrigation efficiency, the development of antifouling treatments, pipe material innovations, and hydraulic optimization (Gamri et al., 2016; Katz et al., 2014; Shelton et al., 2013; Song et al., 2021; Song et al., 2019) to maintain consistent water flow and reduce maintenance costs. While these efforts are essential for system performance, they frequently overlook the equally important issue of microbial safety, and the microbial populations present within these systems as they naturally occur. While few have addressed the prevalence of foodborne pathogens in irrigation lines (Antaki et al., 2016), the potential for such irrigation lines to provide harborage points for antibiotic resistant bacteria (Blaustein et al., 2016), the overall effect of biofilms and biofilm structure on irrigation water quality (Pachepsky et al., 2012), and microbial populations in irrigation water sources (Gu et al., 2020; Krishnan et al., 2021; Weller, Belias, et al., 2020; Weller, Brassill, et al., 2020), there is still a gap in understanding the microbial composition of biofilms as they naturally exist in irrigation water distribution systems. Therefore, the objective of this work was to determine the ecology, diversity, and composition of the microbial communities present in different irrigation water distribution systems in southern Georgia throughout two production seasons (2023 and 2024). Swab samples collected from the irrigation lines (n=499) were processed and sequenced for taxonomic composition determination using the 16S barcode kit from Oxford Nanopore Technologies. Water samples (n=57) from irrigation sources or from the end of a drip line were

tested for pH, turbidity, electroconductivity, chemical oxygen demand, and oxidation reduction potential as well as total coliforms, and generic *E. coli.* Overall, there was a wide diversity of microbial populations with monthly variations observed in water and swab samples. This work provides a baseline for the presence of microbial communities within active produce farm irrigation systems, capturing their natural dynamics across seasons, water sources, and management practices. Information regarding these complex communities can provide a better understanding of pathogens' behavior in preharvest conditions. This could result in innovative strategies to control and intervene in the spread of pathogens via irrigation systems.

2. Methods

2.1 Sampling sites

Water and swab samples were collected from six commercial produce farms (designated A–F) located in the southern Coastal Plain region of Georgia, United States (Table 3.1). Sampling was conducted monthly during two periods: May–November 2023 and March–October 2024. No samples were collected from December through February due to winter conditions and seasonal inactivity on the farms. Five of the farms (A, B, C, D, and E) cultivated vegetable crops (e.g. cucumbers, green peppers, squash, watermelons) while Farm F specialized in small fruit production. All the farms operated conventionally, using synthetic fertilizers in the line as required and sizing between 50 – 100 acres. All farms utilized drip irrigation systems; however, Farm B employed both drip and center pivot irrigation. The source of irrigation water varied among the farms: Farms A, D, and E used surface water from nearby ponds, whereas Farms B, C, and F relied on groundwater from wells. Notably, Farm E pumped well water into an artificial pond before use; for the purposes of this study, this was classified as surface (pond) water. The

beginning and the end of emitters (Appendix 3.1) were swabbed from the same drip line for each tubing. Three lines were swabbed for each farm. Sampling logistics varied by farm and season: Farm A relocated its drip lines in 2024, and the water source was inaccessible unless the system was active, limiting water sample collection that year. Farm B installed drip lines between March and May each year and removed them afterward; thus, no drip samples were collected outside this window. Only the end of the line was swabbed in drip lines for farm B. Additionally, center pivot irrigation at Farm B was inaccessible during August and September 2023, resulting in no samples during that month. Farm D ceased irrigation activities in August 2024 and removed its drip system, so no swab or pond water samples were collected thereafter. Water samples were collected only from surface water sources when applicable (Farms A, D, and E), or from the lines when the system was active (Farms A, C, and F).

2.2 Water sampling and processing

Water samples were collected either directly from surface water sources (ponds) or from active drip irrigation lines when operational (at the end of the tubing). Due to variability in rainfall, irrigation schedules, and farm management practices, consistent access to water from active drip irrigation lines was not always feasible throughout the study period. For analysis, 1 L of water samples were collected using a water sampling dipper (Cole-Parmer, IL, USA) and transferred into sterile bottles. All samples were collected on the same day then, were immediately placed on a cooler containing ice and transported to the laboratory, where they were processed within 18 h of collection. In 2023, a total of thirty water samples were collected and in 2024, a total of twenty-seven water samples were collected from either the pond or the line (Table 3.1). Microbial water quality was assessed by quantifying total coliforms and generic *E. coli* using the

IDEXX Colilert® test in conjunction with the Quanti-Tray®/2000 system (IDEXX Laboratories, ME, USA). This method is approved by the U.S. Environmental Protection Agency (EPA) for the detection of total coliforms and generic *E. coli* in drinking water, surface water, and wastewater [40 CFR 141.402(c)(2)]. In addition to microbial testing, physicochemical parameters of the water were measured, including pH, turbidity, oxidation reduction potential (ORP), chemical oxygen demand (COD), and electrical conductivity (EC). These parameters were analyzed using the following equipment: pH, EC, and ORP: HQ4300 Portable Multi-Meter (Hach, CO, USA). Turbidity: DR900 Multiparamteter Portable Colorimeter (Hach). COD: DRB200 Digital Reactor Block for COD digestion, HR (20-1,500 mg/L COD). (Method 8000, Hach) and quantified using the colorimeter. All instruments were calibrated according to the manufacturers' instructions prior to use.

2.3 Swab sampling and processing

Swab samples were collected using HiCap sampling swabs containing 10 mL of the company's neutralizing broth (BLU-10HC, World Bioproducts, WA, USA) from irrigation equipment to assess microbial presence and distribution. Specifically, swabs were taken from the outlet of center pivot systems and from both the emitter (near the perforation points) and the end of drip irrigation lines (Appendix 3.1). This sampling strategy was designed to evaluate potential changes in microbial load along the irrigation pathway where water flow is at its highest – beginning, and lowest – end of the drip. Following collection, swabs were immediately placed on ice and transported to the laboratory, where they were processed within 18 h. In the laboratory, each swab was vortexed for 5 seconds and manually compressed to release the sample solution. The resulting liquid was transferred to sterile 50 mL conical centrifuge tubes and centrifuged at

3,600 RPM for 10 minutes at room temperature. After centrifugation, the supernatant was carefully discarded, and the pellet was resuspended in 1 mL of 1X Tris-EDTA (TE) buffer (Thermo Fisher Scientific, MA, USA). Resuspended samples were stored at 4°C for up to 24 h prior to DNA extraction.

2.4 DNA extraction and sequencing

DNA was extracted from resuspended samples using the DNeasy PowerSoil Pro Kit (Qiagen, MD, USA), following the manufacturer's protocol with minor modifications. Briefly, 250 µL of the resuspended pellet in 1X TE buffer was transferred into the PowerBead Pro Tube provided in the kit. Subsequent steps, including mechanical and chemical lysis, inhibitor removal, and DNA binding to the silica membrane, were carried out according to the kit instructions. DNA was eluted in 60 µL of Solution C6 provided by the kit. DNA concentration was measured using the Qubit™ 4 Fluorometer (Thermo Fisher Scientific) with the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific). Extracted DNA samples were stored at -80°C until further downstream analysis.

Library preparation for 16S rRNA gene sequencing was performed using the Oxford Nanopore Technologies (ONT, Oxford, UK) 16S Barcoding Kit (SQK-16S024 for the 2023 data and SQK-16S114.24 for the 2024 data) following the manufacturer's protocol. Briefly, the full-length 16S rRNA gene (~1.5 kb) was amplified using the barcoded primers provided in the kit: 27F and 1492R that contain 5' tags. Polymerase chain reaction (PCR) amplification was performed using an Eppendorf MasterCycler (Eppendorf, Hamburg, Germany), following the protocol outlined in the 16S barcode kit library preparation manual. Following amplification, PCR products were purified according to the protocol provided by ONT. Due to the low DNA concentration

observed in some environmental samples – likely resulting from minimal or absent microbial biomass, all available PCR amplicons were used for library preparation to maximize input material. The amplicons were purified and pooled for sequencing. The final concentration of the pooled, barcoded library was quantified using the Qubit™ dsDNA HS assay kit to ensure it met the recommended input concentration for ONT sequencing (~4.4 ng/µL). The final library was loaded onto a MinION™ Flow Cell (FLO-MIN106D, R9.4.1 chemistry for 2023 data and R.10.4.1 for 2024 data) and sequenced using the MinION Mk1B device. The flow cells used in 2024 differed from those in 2023 due to the discontinuation of the older sequencing chemistry by the manufacturer, necessitating the adoption of the updated version. While R9.4.1 and R10.4.1 flow cells differ in pore structure and accuracy, we deemed it was acceptable to transition to the newer chemistry mid-study, as both are compatible with standard Nanopore workflows and yield comparable results for most applications. The new sequencing was monitored and controlled using the MinKNOW software from ONT.

2.5 Bioinformatics analysis

Raw sequence reads in FASTQ format were processed and analyzed using Sepia in Rust (den Bakker & Katz, 2021). Taxonomy was assigned using an index created from the NCBI's Bacteria and Archaea: 16S ribosomal RNA project (https://www.ncbi.nlm.nih.gov/refseq/targetedloci/; downloaded January 27, 2025) and operational taxonomic unit (OTU) tables and classifications were exported to R's serialized format for further analysis with *phyloseq* v1.46.0 (McMurdie & Holmes, 2013) in R v4.3.3 (R Core Team, 2024). Metadata, including year (2023 and 2024), farm (A, B, C, D, E, or F), month (March -November), system (drip or center pivot), water source (well or pond), and drip location

(beginning or end) were also included in the *phyloseq* object. A 5% prevalence threshold was used to capture abundance taxa within each phylum and reduce those that did not appear to have a meaningful biological contribution. This cut-off was determined by plotting prevalence by total abundance of taxa within each phylum, as described in (Callahan et al., 2016). Read count thresholds were determined based on the analysis of negative PCR controls, which established the maximum background read levels. Since the ONT 16S Barcode Kit supports only 24 uniquely barcoded samples per run, thresholds were set individually for each sequencing batch according to its corresponding negative control. These thresholds ranged from fewer than 3 reads to as high as 550 reads. Alpha diversity metrics, including Shannon, Chao1, and Observed species richness, were calculated using the *phyloseq* package v1.46.0. Beta diversity was evaluated using the adonis2 function from the *vegan* v. 2.6-4 package.

2.6 Statistical analysis

Alpha diversity indices (Shannon, Observed, and Chao1) were assessed for normality using the Shapiro-Wilk test. For data that met the assumption of normality, a t-test or one way ANOVA followed by TukeyHSD *post hoc* analysis was applied. In cases where normality was not confirmed, the Wilcox test and the Kruskal-Wallis one-way ANOVA with Dunn's *post hoc* were used to compare alpha diversity across metadata variables such as year, month, farm, system, water source, and location. To evaluate beta diversity, Bray-Curtis distance matrices were computed and differences between sample groups were tested using PERMANOVA. Betadisper from the *vegan* v. 2.6-4 package was used to assess dispersion of samples and *pairwise* was used for multiple comparison if deemed appropriate after the PERMANOVA test. All statistical analyses were conducted using R v4.3.3. Spearman's rank correlation was employed in R v4.3.3

to assess relationships among water quality parameters. A value of -2.05 log CFU/mL was considered when levels were below the limit of detection (LOD: -2 log CFU/mL) for total coliforms and generic *E. coli* in water samples. The results were visualized using ordination techniques from the *vegan* package and customized plots were created with *ggplot2*.

3. Results

3.1 Taxonomic compositional analysis of microbial communities

The average relative abundance of the top 20 most abundant OTUs at genus level obtained from the sequencing analysis of marker genes are shown in Figure 3.1. *Bacillales* Family XII. Incertae Sedis' is a provisional taxon at the same level as *Enterobacteriaceae* in the NCBI taxonomy (it officially does not have a rank). It is a taxon within the order of the *Bacillales*. Across all samples, this taxon was consistently one of the most represented bacterial genera in swab samples. In 2024, 12.2% of samples were classified within this taxon, while 13.21% were classified similarly in 2023. *Thiobacillus* accounted for 5.73% of the relative abundance, *Bacillus* for 4.35%, *Aeromonas* for 3.29%, and *Pseudomonas* for 0.46 % in 2023. In 2024, *Thiobacillus* accounted for 10.08%, *Bacillus* for 3.29%, *Aeromonas* for 3.02%, and *Pseudomonas* for 1.60 %. The dominant taxa by relative abundance varied across farms and years (Figure 3.1). For instance, in Farm A during 2023, *Bacillales* Family XII. Incertae Sedis was most prevalent, comprising 24.92% of the community. However, in 2024, *Acinetobacter* became the dominant taxon at 19.25%, while *Bacillales* Family XII. Incertae Sedis dropped to 4.22%. *Thiobacillus* remained the most prevalent in farm B for both years with 10.46% and 16.50%, relative abundance in 2023 and 2024, respectively. Although present at lower relative abundances, *Pseudomonas*, *Bacillus*, and *Aeromonas* remained consistently detected across those

lines for all the farms. No unique genera were identified in only one or in just two to three of the farms, months, or years.

3.2 Alpha diversity indices: year and month effect

Regardless of month, farm, or the other parameters, there were no significant differences between microbial diversity indices of the Shannon ($p = 0.29$), but the Chao1 ($p < 0.01$) and the Observed ($p < 0.001$) index showed a significant difference across the two years of sampling. Regardless of the sampling year, all diversity indices were significantly different for the months; $p < 0.001$ (Figure. 3.2). Notably, May and August consistently exhibited lower diversity compared to several other months. Specifically, May had significantly lower Shannon diversity than March, April, June, November, October, and September ($p < 0.001$). But May had only significant differences in the Observed index with April ($p = 0.02$), march ($p = 0.04$) and October. Similarly, August had significantly lower Shannon diversity than March ($p = 0.01$), April, July, and October ($p < 0.001$). October showed significant Chao1 index differences when compared to June ($p < 0.001$) and May ($p = 0.04$). Among the diversity indices analyzed, March showed the highest median values for both the Chao1 index (579.01) and Observed species (500.0). April had the highest median Shannon index (3.75). March followed closely with a value of 3.70. In terms of variability, October displayed the widest range in Chao1 values: interquartile range (IQR) = 918.57. For the Observed index, October again had the most variability (IQR = 764.25), and for the Shannon index, May showed the greatest spread (IQR = 2.07). Several months also exhibited outlier samples, particularly in the Chao1 index, where outliers were present from June, July, August, and September. The Observed index showed

outliers from May, June, July, August, April, and September, and the Shannon index had outliers in March, June, July, and August.

3.3 Alpha diversity indices: farm effect

The Shannon indices showed no significant differences across the different farms ($p = 0.09$), but the Chao1 ($p < 0.001$) and the Observed ($p < 0.001$) indices did represent significant differences. Table 3.2 represents the range for alpha diversity indices observed throughout the different farms. In the Observed and Chao1 adjusted *post hoc* comparison, Farm A exhibited significantly higher mean values compared to all other farms (B through F), with all $p < 0.01$. The largest difference was observed between Farms A and C ($p < 0.0001$). No statistically significant differences were found among Farms B through F ($p > 0.05$).

3.4 Alpha diversity indices: drip line beginning vs end

For all the farms, except for farm B (as only the end line was sampled from that farm), the Shannon test showed no significant differences ($p = 0.03$) between the location of sampling (at the emitter vs end of the line – Appendix 3.1). Whilst the Observed ($p = 0.005$) and Chao1 ($p = 0.004$) showed significant differences with the end of the emitter having a higher observed microbial diversity ($477.12 > 369.58$) and a higher Chao1 index ($644.41 > 511.53$) than the beginning of the line.

3.5 Alpha diversity indices: distribution system and water sources

For farm B in May 2023 and April-May 2024, there were significant differences between the center pivot and drip irrigation system microbial diversity in the Shannon ($p = 0.03$), Observed

($p = 0.005$) and the Chao1 ($p = 0.004$). With Shannon index equaling 2.80 and 2.23 for drip and center pivot, respectively. The Observed index = 794.08 in the drip compared to 115.467 in the center pivot and the Chao1 was 981.89 and 213.04 for the drip and center pivot, respectively. The difference between Shannon indices between the well and the surface water source in the lines was significant at a $p = 0.01$. It was significantly different for the Observed and Chao1 ($p < 0.001$) with pond water having higher observed samples (499.09) than well water (320.97), higher Chao1 index (662.34 and 459.75), and higher Shannon index (3.31 and 3.07).

3.6 Beta diversity

The PcoA plot in Figure 3.3 provides a visual representation of the variation in microbial community composition across samples, grouped by month and farm. The first principal coordinate (Axis 1) accounts for 12.1% of the total variation, while the second principal coordinate (Axis 2) explains 9.7%, together capturing 21.8% of the overall variability in the dataset. The distribution of points across the plot indicates that samples clustered moderately by month, with some seasonal separation visible along both axes. However, overlap among farms and months indicates shared community features or environmental influences.

Analysis with PERMANOVA showed that community composition differs significantly ($p = 0.001$, $R^2 = 0.08$) between farms. *Betadisper* analysis showed that the variance is significant ($p = 0.003$). The pairwise PERMANOVA results using Bray-Curtis dissimilarity demonstrate that microbial community composition significantly differed between all farm pairs ($p < 0.001$ for all comparisons). The strongest differentiation was observed between Farms C and A ($R^2 = 0.08$). Other comparisons, such as B vs. C ($R^2 = 0.03$) and C vs. F ($R^2 = 0.03$), showed smaller but still significant differences. Community composition differed significantly ($p = 0.001$, $R^2 = 0.09$)

across months, regardless of the year. The *Betadisper* test for homogeneity showed significant difference ($p = 0.001$). Pairwise PERMANOVA also revealed statistical differences between the different pairs of months ($p < 0.05$).

3.7 Water quality parameters in farm ponds and end lines

Figure 3.4 and Figure 3.5 represent monthly variation in physicochemical properties and microbial composition of water samples across farms (2023 and 2024 combined), respectively. Water quality parameters were assessed across farms A, C, D, E, and F. For COD, values ranged from a level below the LOD in farms E and F for a maximum of 190 ppm in June and July of 2024 from the ponds of farm E and a maximum of 342 ppm of August of 2023 from the line of farm F. The maximum COD across all the farms was from September of 2023 from the line of farm A (429 ppm) while results showed a COD of 64 ppm that month from the source pond. Conductivity showed the highest range in farm A (1249.6 µS/cm), with values spanning from 109.6 to 1415.0 across all farms. ORP ranged from 125.8 to 604.4 mV, with farm F showing the highest variability (range of 435.8 mV). pH values varied widely, especially in farm D (range of 5.87), with values as low as 2.71 in November and October of 2023 in farm D and as high as 9.69 in Farm E in March of 2024. Turbidity ranged from 1.0 NTU to 216.0 NTUs, with farm A showing the highest variability (198.67 NTU) and farm E the lowest (8.0 NTU). Total coliform values ranged from levels below the LOD to 2.72 log CFU/mL, with the largest range in farm E (4.23 log CFU/mL). *E. coli* values had a large spread with the smallest range in farm F (< LOD) and the largest in farm A (3.88 log CFU/mL). A strong positive Spearman correlation (Appendix 3.2) was observed between total coliforms and generic *E. coli* ($\rho = 0.65$, $p < 0.001$). Turbidity showed moderate positive correlations with total coliforms ($\rho = 0.45$, $p = 0.001$) and COD ($\rho =$

0.42, p = 0.003). pH and EC exhibited a moderate negative correlation (ρ = -0.49, p < 0.001). Additionally, EC and *E. coli* were negatively correlated (ρ = -0.34, p = 0.007). Notably, COD and *E. coli* showed no correlation (ρ = -0.01, p = 0.413).

4. Discussion

4.1 Bacterial genera identified in the irrigation lines

Independent of the system, month, or location of sampling and across 2023 and 2024, Figure 3.1 showed that 2024 had an increase in observed abundance of OTUs for all farms (A-F) compared to 2023. The most abundant group identified was *Bacillales* Family XII. Incertae Sedis. This nomenclature refers to a taxonomic grouping within the order *Bacillales* that includes bacteria whose precise familial relationships are uncertain or unresolved. However, based on NCBI taxonomy profile, it appears to mostly belong to the *Exiguobacterium* genera (NCBI, 2025). *Exiguobacterium*, though less studied, is a psychrotolerant genus found in diverse habitats including glaciers, soil, and wastewater (Kasana & Pandey, 2018). Certain strains can grow at temperatures as low as -6°C and as high as 45°C, which explains their persistence throughout the distribution systems at the farms (Vishnivetskaya et al., 2009). Other taxon with consistent prevalence throughout the farms and months included bacterial genera commonly found in environmental and agricultural settings such as *Pseudomonas* and *Bacillus*. Both bacteria are particularly well-studied for their robust biofilm formation and ecological versatility (Abbasi et al., 2006; Dong et al., 2022; Lee et al., 2014; Logan & Vos, 2015; Shemesh & Ostrov, 2020). *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen, is notorious for forming resilient biofilms that contribute to chronic infections and resistance to antibiotics and immune responses (Heacock-Kang et al., 2017; Klausen et al., 2003). It thrives in moist environments,

including irrigation systems and hospital settings (Aloraini et al., 2023; Cowle et al., 2020; Driscoll, 2007). In contrast, *Bacillus* species, such as *B. subtilis* or *B. thuringiensis*, are Gram-positive and widely used as biocontrol agents in agriculture due to their ability to form endospores and biofilms that promote plant health and suppress pathogens (Ju et al., 2013; Szczech & Shoda, 2006). *Aeromonas* species are aquatic bacteria often associated with fish and waterborne diseases (Janda & Abbott, 2010). They can form biofilms on various surfaces, including water pipes and plant roots (Learbuch et al., 2021). *Thiobacillus*, a sulfur-oxidizing genus, is commonly found in soil and wastewater treatment systems (Robertson & Kuenen, 2006). It plays a key role in biogeochemical cycling and has been observed to form biofilms in acidic mine drainage and bioreactors, aiding in metal recovery and pollutant degradation (Barbosa et al., 2006; Robertson & Kuenen, 2006; Schrenk, 1998). Other microorganisms identified were *Priestia*, *Sphingomonas,* and *Deinococcus* (Figure 3.1). *Priestia* includes species like *P. megaterium* that are agriculturally beneficial due to their plant growth-promoting traits and moderate biofilm-forming ability (Biedendieck et al., 2021). *Sphingomonas*, known for its metabolic diversity, is prevalent in both soil and aquatic environments and contributes to the degradation of complex organic pollutants (Asaf et al., 2020). It forms biofilms that enhance its survival in nutrient-poor conditions (Bereschenko et al., 2010). *Deinococcus*, especially *D. radiodurans*, is renowned for its extreme resistance to radiation. While not a strong biofilm former, it can survive high temperatures and oxidative stress (Battista, 1997). Hence, detecting such diverse genera among irrigation lines was expected due to their high prevalence in the environment and potential use in the agricultural settings. Similar genera were observed in drinking water distribution systems where *Sphingomonas*, *Pseudomonas*, and *Bacillus* were abundant in lines (Douterelo et al., 2020).

The observed increase in overall microbial abundance across different farms from 2023 to 2024 may be attributed to several environmental and operational changes. For instance, in Farm A, the drip irrigation system used in 2023 was replaced with a new system in 2024, a change mirrored in Farm B. Such infrastructural updates could have influenced microbial colonization and dispersal. Additionally, the timing of sampling likely played a role. In Farm F, sampling began in May 2023, coinciding with the harvest season when irrigation is typically reduced, whereas in 2024, sampling started in March, a period of more active irrigation. Furthermore, 2024 experienced significantly more rainfall and storm activity in southern Georgia compared to 2023. Notably, May 2024 was the 8th wettest on record with 6.44 inches of rain (+2.73 inches above average), and September saw an average of 8.45 inches (+4.53 inches), making it the 4th wettest September on record (Environmental Protection Division, 2024). These wetter conditions, along with multiple severe weather events and increased runoff, may have altered the microbial dynamics within irrigation lines and surrounding soils, contributing to the observed shifts in community structure (Beattie et al., 2020; Le et al., 2016). No genera were found to be uniquely associated with a single farm or limited to only two or three farms, despite the geographic separation among the sites. For instance, Farm A is approximately 100-130 km from Farms B, C, D, and F; Farm F is only 13 km from Farm B; Farms C and D are just 2 km apart; and Farms C is about 20 km apart from D, and E.

4.2 Alpha diversity indices

Microbial diversity is commonly assessed using indices that capture different aspects of community structure (Galloway-Pena & Hanson, 2020). The Observed diversity index simply counts the number of unique taxa (e.g., species or OTUs) detected in a sample, providing a direct

measure of richness (Kunakh et al., 2023). In contrast, the Chao1 index is a richness estimator that accounts for unseen or rare taxa by incorporating the number of singletons and doubletons, offering a more robust estimate of total species richness (Kunakh et al., 2023). The Shannon index, on the other hand, combines both richness and evenness, reflecting not only how many taxa are present but also how evenly distributed they are (Keylock, 2005; Kunakh et al., 2023). In this study, microbial diversity did not significantly differ between 2023 and 2024 when assessed using the Shannon ($p = 0.29$) index, suggesting that overall richness and evenness remained relatively stable. However, the Chao1 ($p < 0.01$)and the Observed ($p < 0.01$) indices showed a significant difference by year, indicating that the raw count of detected taxa increased, as observed in Figure. 3.1, possibly due to the fact that in 2023, the sampling started at the end of the production season – or during harvest for most farms (May 2023) whilst in 2024, sampling started at the beginning of the production season (March – April), along with changes in environmental conditions, as mentioned previously. The *post-hoc* analysis revealed that October consistently differed significantly from several other months, particularly May and August, with $p < 0.05$. This suggests a distinct shift in microbial or ecological community structure during October. The latter could be due to temperature and weather changes as October marks the beginning of the Fall season in Georgia whilst the warm spring season starts in May and August is the hot summer. While May and August did not show statistically significant differences, they represent important seasonal transitions. May marks the beginning of summer crop production, and August typically signals the end of the summer growing season. In contrast, June and July, the mid-season or harvesting period, are characterized by standing water in irrigation lines, which may influence microbial diversity and community dynamics (Chen et al., 2023; Cowle et al., 2020; Gamri et al., 2016; Recupido et al., 2020). For example, Cowle et al. (2020) reported

that microbial biomass was greater for biofilms conditioned at lower flows, regardless of material type. In contrast, shear forces imposed by higher flow rates inhibited biofilm development. October, marking the onset of winter crop setup, exhibited the highest median diversity across all three diversity indices analyzed and the widest variability in Chao1 (IQR = 918.57) and Observed (IQR = 764.25), indicating a highly dynamic microbial environment. Interestingly, May had the greatest spread in Shannon diversity (IQR = 2.07), suggesting variability in evenness or richness during the early summer crop phase. Outliers observed across several months reflect fluctuations in microbial community structure during both transitional and peak agricultural periods. No genus was exclusive to a single month, indicating a shared diversity across the seasonal cycle, though relative abundances varied.

The lack of significant differences in Shannon diversity suggests that while the number of taxa (richness) varied significantly across farms, the distribution of abundances among those taxa remained relatively consistent. This is particularly important in microbial ecology, where a community might be rich in taxa but dominated by a few, leading to lower evenness. Farm A exhibiting significantly higher Chao1 and Observed values compared to all other farms ($p < 0.01$) suggests that this farm harbored a greater number of both observed and estimated taxa, indicating a more taxonomically rich microbial community. This elevated richness at Farm A may be linked to environmental conditions, particularly the presence of visible algal growth in the pond used for irrigation (Appendix 3.3). Although remediation efforts and sanitizers were reportedly applied, the standing water and algal blooms could have introduced or supported a wider array of microbial taxa, including transient or opportunistic bacteria (Jang et al., 2011; Liu et al., 2025). These conditions likely inflated both the Observed and Chao1 indices, which are sensitive to the presence of rare or low-abundance taxa. As seen with other farms, despite this

variability in richness, Shannon diversity did not differ significantly, reinforcing the idea that evenness was not substantially affected, perhaps due to the dominant taxa maintaining similar relative abundances across farms.

Across all farms (excluding Farm B, which only had end-line samples), the Shannon diversity index showed no significant differences between sampling locations ($p = 0.03$), suggesting that evenness in microbial communities remained relatively stable along the irrigation lines. However, both the Observed richness ($p = 0.005$) and Chao1 estimated richness ($p = 0.004$) were significantly higher at the end of the irrigation lines compared to the beginning of the line. Specifically, the Observed index increased from 477.12 to 369.58, and the Chao1 index rose from 644.41 to 511.53. This pattern can be explained by hydraulic dynamics within irrigation systems. Water flow is typically strongest at the emitter, where pressure is highest and movement is continuous during irrigation events. In contrast, water tends to stagnate at the end of the line, where pressure is the lowest or when the system is not flushed regularly. This stagnant water can persist for extended periods, days to months, between irrigation or crop cycles, creating an ideal environment for microbial accumulation and growth. Moreover, biofilms are more likely to form in these stagnant zones, where nutrients, organic matter, and microbes can settle and proliferate (Cowle et al., 2020). Once established, multi-species biofilms tend to form more robustly than single-species counterparts, as they can support a broader range of microorganisms which contributes to the higher microbial richness observed in diversity indices (Lee et al., 2014; Pang et al., 2017). The lack of significant difference in Shannon diversity suggests that while more taxa are present at the end of the line, their relative abundances may still be dominated by a few key species, maintaining similar evenness to the emitter samples. These findings underscore the importance of regular flushing and maintenance of irrigation systems, particularly at the terminal

ends, to prevent microbial buildup and potential biofilm-related issues that could affect crop health or water quality.

The comparison between drip and center pivot irrigation systems at Farm B during May 2023 and April–May 2024 revealed significant differences in microbial diversity across all three alpha diversity indices. These results indicate that drip irrigation systems support a significantly richer and more diverse microbial community than center pivots. This difference can be attributed to the hydraulic and structural characteristics of the two systems. In drip irrigation, water is delivered slowly and directly to the root zone through emitters and low flow rates (Harrison, 2022), water can accumulate and remain stagnant in the lines, as discussed earlier. In contrast, center pivot systems are designed for rapid and uniform water distribution over large areas (Harrison, 2022). The water in these systems tends to dry quickly between irrigation events, especially in warm months like August, reducing the opportunity for microbial communities to establish and persist. However, the presence of non-zero diversity indices in center pivot systems during August; average Observed = 420, Chao1 = 595.89, Shannon = 3.26, demonstrates that microbial communities can persist. This persistence may be due to resilient or spore-forming taxa (Sanchez et al., 2014; Shemesh & Ostrov, 2020). These findings emphasize the ecological impact of irrigation design on microbial dynamics. Additionally, the different material type within those lines may impact biofilm formation differently. For example, researchers reported that PVC pipes exhibited lower bacterial accumulation than polyethylene pipes under reclaimed water conditions (Gamri et al., 2016). Drip systems, while efficient for water delivery, may require more frequent maintenance and sanitation to manage microbial buildup. Conversely, center pivot systems may naturally limit microbial proliferation but still harbor persistent communities that could influence water safety. Because center pivot irrigation systems often

apply water directly to the harvestable and edible portions of produce, it is important to minimize microbial biofilm formation within these systems to reduce the risk of direct contamination.

The comparison between groundwater (from wells) and surface water (from ponds) as irrigation sources revealed significant differences in microbial diversity across all three alpha diversity indices. These results indicate that pond water supports a more diverse and taxonomically rich microbial community than well water. This is expected, as surface water bodies are more exposed to environmental inputs, including runoff, wildlife, decaying vegetation, and atmospheric deposition (Murphy et al., 2022; Murphy, 2023; Page, 1981; Ritter et al., 2002). In contrast, well water is typically drawn from deeper, confined aquifers, which are less exposed to surface contaminants (Wunderly, 2021). Nonetheless, groundwater tends to show more persistent contamination due to slower movement and less natural filtration. Hence, contamination occurs through infiltration, leaching from soils, and poorly constructed wells (Ritter et al., 2002). Surface water is more susceptible to short-term microbial fluctuations due to seasonal changes, runoff events, and dilution (Page, 1981), whereas groundwater typically harbors more stable, long-term microbial communities that are often more difficult to remediate (Ritter et al., 2002). This can be critical for irrigation distribution lines. If surface water contaminated with a foodborne pathogen, such as *Salmonella*, (Acheamfour, 2021; Antaki et al., 2016; Murphy, 2023) and it enters these systems, the pathogen can become embedded within existing biofilms (Pachepsky et al., 2012; Pang et al., 2017). These biofilms can provide a protective environment, allowing the pathogen to persist, multiply, and potentially form resilient layers (Sha et al., 2013; Steenackers et al., 2012). Even after the surface water source is no longer contaminated, the pathogen may still be released during subsequent irrigation events (Chua et al., 2014; Gonzalez-Machado et al., 2018). This persistence complicates efforts to trace the original contamination

source and poses significant challenges for managing foodborne pathogen risks in agricultural water systems. Although groundwater is not entirely risk-free, it may pose a lower microbial risk for contamination of fresh produce with foodborne pathogens, hence, it still requires monitoring and treatment. These findings underscore the importance of water source selection and management in agricultural systems.

4.3 Beta diversity analyses

The PCoA ordination plot (Figure 3.3.) reveals both temporal and spatial patterns in microbial community structure. In typical ordination plots, one would expect clear clustering by factors such as month or farm management style. However, in this case, the samples show high variability with no distinct clustering patterns, except for some seasonal shifts in farm A. The latter could be since this farm had completely changed their drip system in-between seasons. Month was not a significant driver of microbial community composition within or between farms. Even when comparing the farms using either groundwater or surface water for irrigation, no strong grouping was observed. This suggests that the microbiomes within the distribution system remain relatively stable across seasons and management practices. Despite the consistent statistical significance in PERMANOVA across all farm comparisons ($p < 0.001$), the R^2 values ranged from 0.034 to 0.09, suggesting that farm identity explains between 3.4% and 8.0% of the total variation in microbial communities. This relatively modest explanatory power implies that other unmeasured factors, such as irrigation frequency or microclimatic conditions**,** likely contribute to the remaining variation. The combination of low p-values and R^2 values is common in ecological studies, where complex, multifactorial systems often yield statistically significant but diffuse patterns of variation (Zuur, 2007). Similarly, when analyzing microbial community

composition across different months, the PERMANOVA results revealed statistically significant differences with R^2 values ranging from 0.03 to 0.09. This indicates that seasonal shifts account for 3% to 9% of the total variation in microbial communities. Nonetheless, the statistically significant *Betadisper* results indicate that differences in microbial community composition between farms and months may stem not only from shifts in group centroids (as shown by PERMANOVA), but also from varying levels of within-group heterogeneity, with some farms or months exhibiting more diverse microbial communities than others (Simpson, 2016). These findings highlight the complex and multifactorial nature of microbial ecosystems, where both spatial (farm-level) and temporal (month-level) factors contribute meaningfully, but not exclusively, to shaping community composition.

4.4 Water quality parameters in farm ponds and end lines

Water quality physicochemical parameters such as COD, EC, pH, ORP, and turbidity, are used to characterize waters, identify trends over time and recognize emerging problems (Environmental Protection Agency, 2025). COD measures the amount of oxygen required to chemically oxidize organic matter in water (Geerdink et al., 2017), indicating potential microbial food sources; however, in this study, COD showed no meaningful correlation with *E. coli* (ρ = -0.01), suggesting that not all organic content supports microbial growth equally. Turbidity, which reflects the presence of suspended particles (Sadar, 2017; Zaman et al., 2018) , showed moderate positive correlations with both total coliforms (ρ = 0.45) and COD (ρ = 0.42), implying that turbid water may harbor more microbes and organic debris. Conductivity, which measures the ionic strength of water (Zaman et al., 2018), was negatively correlated with both pH (ρ = -0.49) and *E. coli* (ρ = -0.34). This suggests that higher salt or ion concentrations may suppress

microbial presence, possibly due to osmotic stress or chemical treatments. For example, in Farm F, chlorine gas was actively injected within the irrigation lines, which likely inhibited microbial growth while increasing conductivity due to the presence of residual ions. Additionally, EC becomes particularly important when fertilizers, such as synthetic salts, are added to irrigation systems. These salts can influence microbial dynamics; on one hand, elevated salt concentrations may induce osmotic stress, prompting bacteria to form biofilms as a protective mechanism (Burgess et al., 2016). On the other hand, high salinity can also inhibit or reduce the survival of certain pathogens, such as *Salmonella*, depending on the serotype and environmental conditions (Lewis et al., 2019). This dual effect underscores the complexity of interpreting EC in relation to microbial safety and highlights the need for a comprehensive review that integrates both physicochemical and microbiological perspectives. ORP is also used to measure the electron activity of water and usually relates to the sanitation state of the water (Steininger, 1996). ORP values across the sampled farms ranged from 125.8 to 604.4 mV, reflecting considerable variability in redox conditions likely influenced by farm-specific management practices. Notably, Farm F exhibited the highest ORP variability, particularly in water sampled from the end of the distribution line. This observation aligns with prior findings suggesting the use of chlorine gas in Farm F's irrigation system, which likely contributed to elevated and fluctuating ORP levels. The observed correlations between ORP and other parameters, such as conductivity ($\rho = 0.6$), turbidity ($\rho = 0.4$), and COD ($\rho = 0.2$), further support the presence of dynamic redox environments shaped by both chemical and microbial activity. pH represents the acidity of the water, and it is particularly important when chemicals such as chlorine are added. Farm D had noticeably pH values as low as 2.71 in November and October of 2023. The pH level was particularly low for surface water (McEgan et al., 2013; Murphy et al., 2022; Weller, Brassill, et

al., 2020); the pond water at the time of sampling had a visible high algae content and may have undergone chemical treatment. Nonetheless, it was not being used for irrigation at the time as the sampling occurred in between the crop seasons. These findings underscore the complex interplay between physicochemical and microbiological parameters in irrigation water systems and highlight the need for multi-parameter monitoring rather than reliance on a single metric to assess water quality and microbial risk.

While the observed water quality parameters provide valuable insights into the potential for microbial contamination, they represent only part of the picture. Biofilms in irrigation lines are influenced not only by the source water but also by the infrastructure and flow dynamics within the irrigation system. Before water reaches the final delivery point (e.g., drip emitters or center pivot nozzles), it typically passes through multiple stages, including pumps, filtration units, chemigation systems, layflat hoses, and finally polyethylene or PVC lines (Granberry et al., 2023). Each of these components can alter the water's physicochemical properties and introduce or support microbial communities. Therefore, direct comparisons between source water quality and microbial diversity within irrigation lines may be misleading. Future studies should aim to characterize microbial shifts across these system components, providing a more holistic understanding of how water quality and infrastructure interact to shape microbial risks in agricultural irrigation.

5. Conclusion

This study provides the first comprehensive characterization of microbial communities within active produce farm irrigation systems, capturing their natural dynamics across seasons, water sources, and management practices. Despite seasonal shifts and periods of low activity,

microbial communities persisted, demonstrating resilience and survival even during non-production months. The presence of biofilms and potential for biofouling across systems underscores the need for deeper investigation into microbial interactions, including horizontal gene transfer, which could facilitate the spread of resistance or virulence traits. Future studies should also explore the impact of agricultural inputs such as fertilizers and sanitizers, not only for their role in limiting biofouling but also for their potential to influence foodborne pathogen persistence and cross-contamination, particularly during biofilm dispersion phases. While this study offers valuable insights, it is not without limitations. Water quality parameters were measured at single time points, which may not capture temporal fluctuations. The use of 16S rRNA gene sequencing, while effective for broad community profiling, lacks resolution for species-level identification and pathogen detection. Additionally, the ONT platform, though powerful for long-read sequencing, is prone to higher error rates, partially mitigated by trimming, which may also lead to data loss. Future research should focus on targeted detection of foodborne pathogens, their interactions with native microbial communities, and how these dynamics evolve under real-world agricultural conditions. This foundational work lays the groundwork for such studies and emphasizes the importance of monitoring microbial ecosystems in produce farms as they adapt to environmental changes and operational demands.

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Tables and Figures

Table 3.1: Farm Sample IDs, Number (#) of Samples Collected (Water and Swabs), and Number of Swab Samples Sequenced from irrigation lines and ponds from produce farms located in Georgia coastal plain areas. All farms were conventional production.

Farm ID	System	Water Source	# Water Samples Collected (1 L)	# Total Swabs Collected	# Total Swab Samples Sequenced	Crop produced
A	Drip	Pond	12	87	79	Vegetable
B	Drip and Center Pivot	Well	0	96	77	Vegetable
C	Drip	Well	5	90	80	Vegetable
D	Drip	Pond	13	72	70	Vegetable
E	Drip	Pond	15	90	86	Vegetable
F	Drip	Well	12	64	57	Small Fruit
	Total		**57**	**499**	**449**	

Table 3.2: Alpha diversity indices range separated by sampling farms across all months and years of sampling from irrigation lines in produce farms located in Georgia coastal plain areas.

Farm	Observed Range	Chao1 Range	Shannon Range
A	14 – 1853	29 – 1930.0	0.070 – 5.132
B	3 – 1874	3.5 – 1950.7	0.286 – 5.199
C	1 – 1156	1.0 – 1411.0	0.000 – 5.116
D	1 – 1865	1.0 – 1865.0	0.000 – 4.874
E	29 – 1881	60.3 – 1954.9	0.993 – 5.138
F	7 – 1773	15.0 – 1847.3	0.680 – 5.705

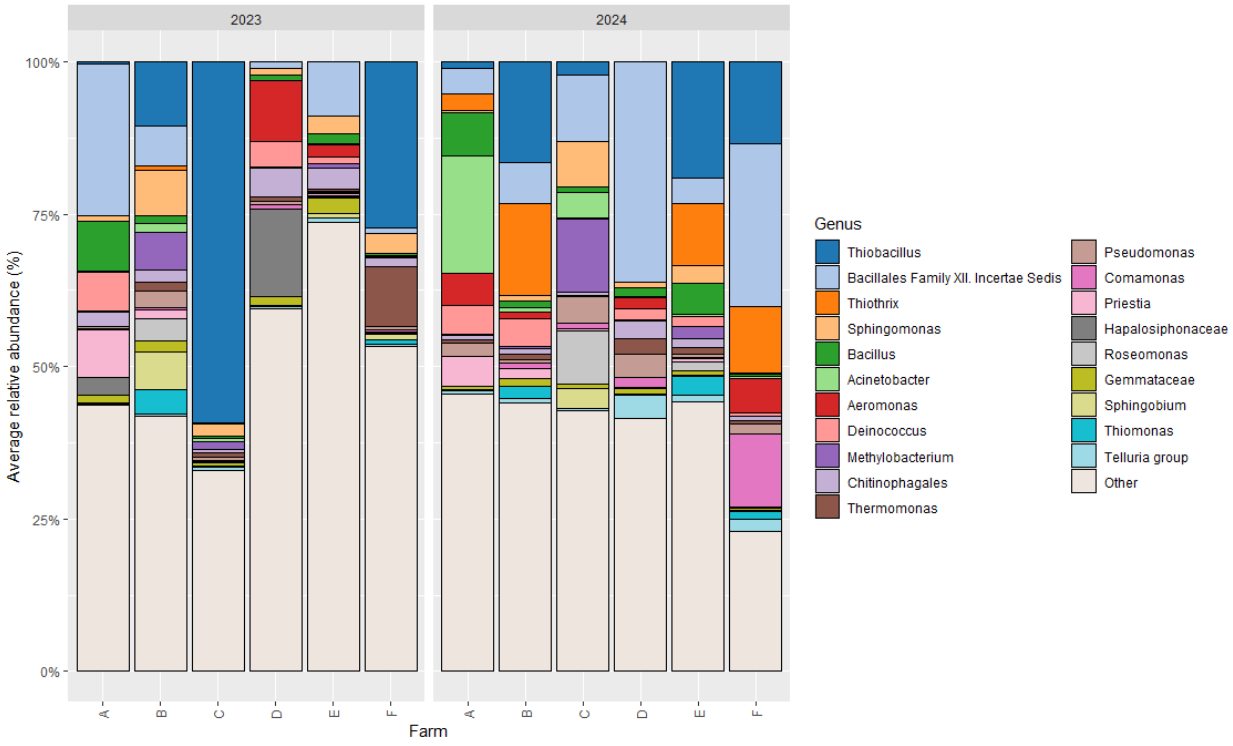

Figure 3.1: Average relative abundance (%) of the top 20 bacterial genera across southern Georgia crop farms (A–F) between two sampling years 2023 and 2024.

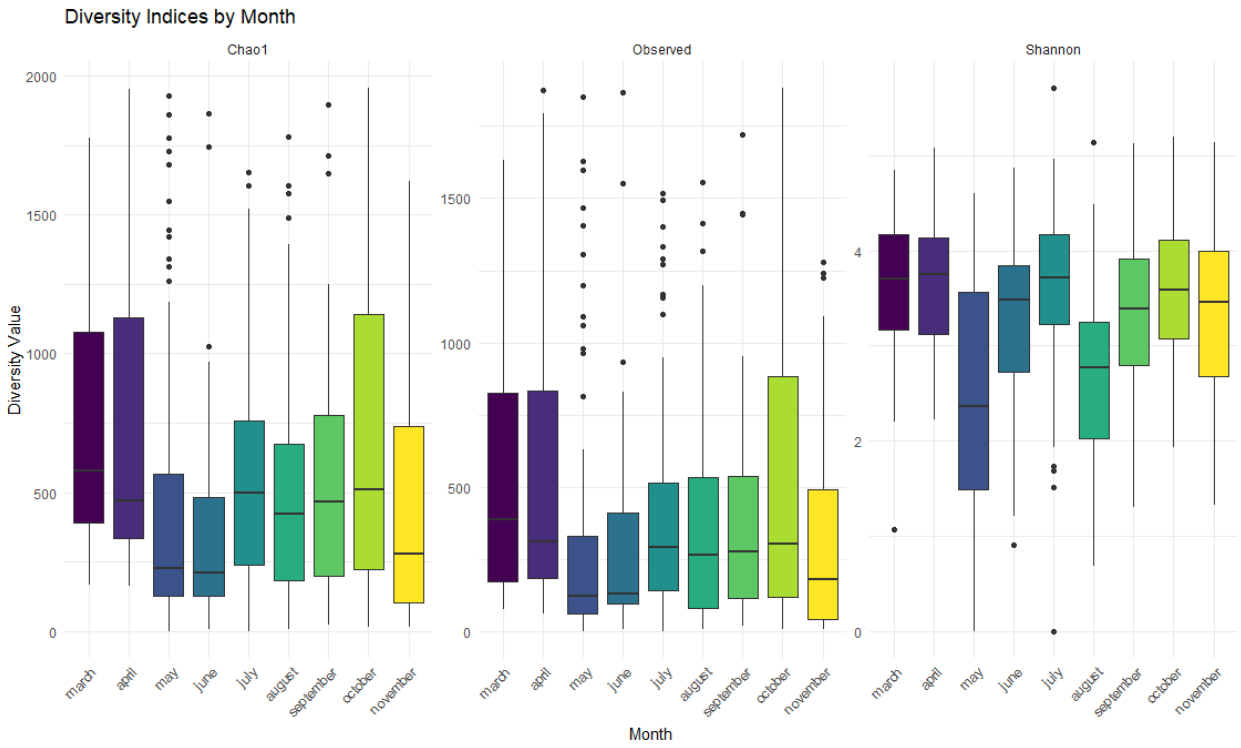

Figure 3.2: Boxplot of alpha diversity indices of swab samples collected from produce farms from southern Georgia separated by collection month.

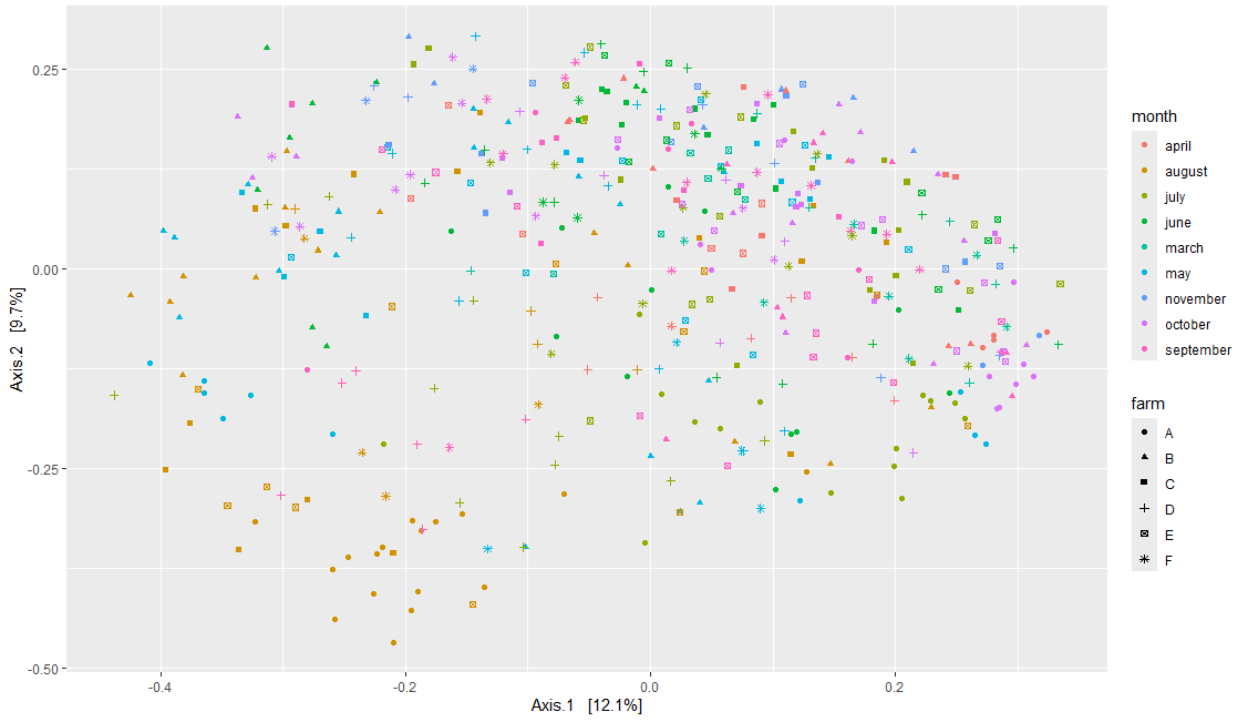

Figure 3.3.: Principal coordinates analysis (PCoA) of beta diversity using Bray-Curtis distance matrix of swab samples collected from irrigation lines from produce farms (A-F) in southern Georgia grouped by collection month. No clusters were identified.

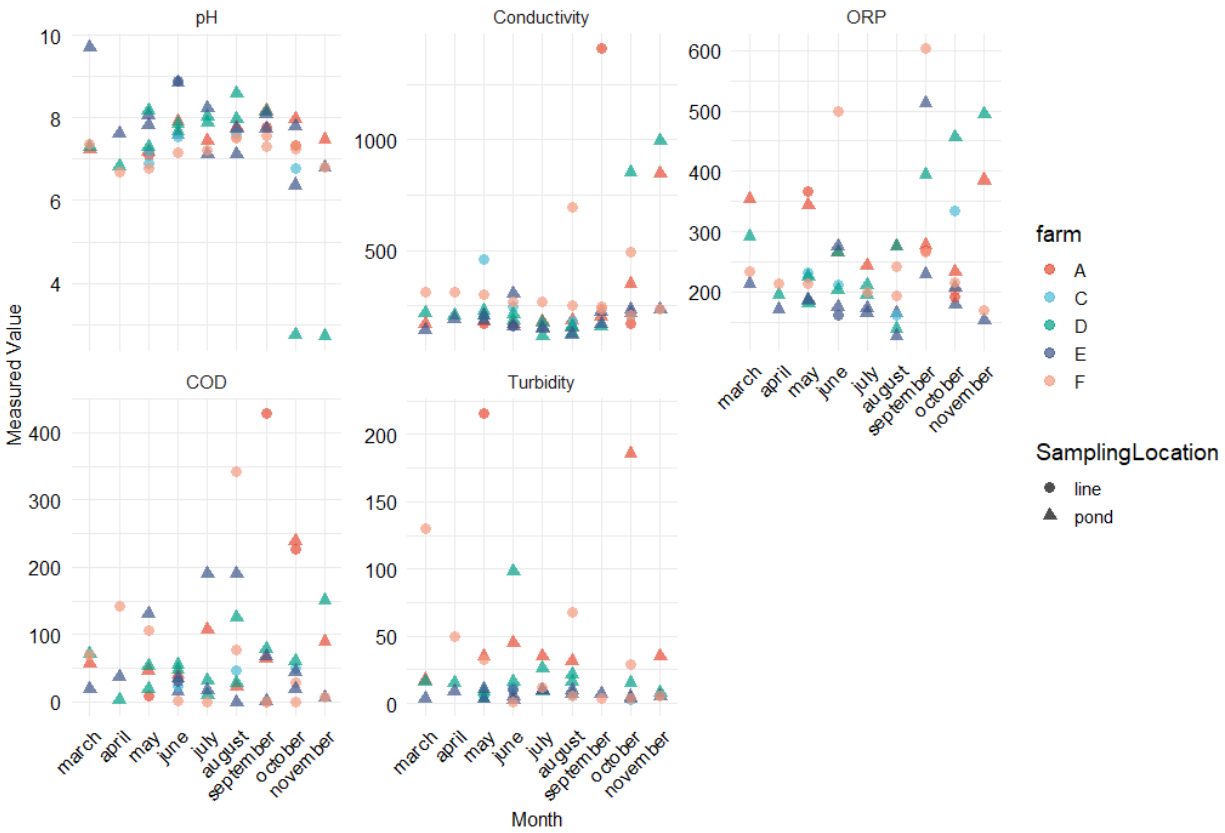

Figure 3.4: Monthly variation in water physicochemical parameters: chemical oxygen demand (COD; ppm), pH, turbidity (NTU), oxidation reduction potential (ORP; mV), and conductivity (µs/cm) across surface water and end line sources in farms A, C, D, E, and F.

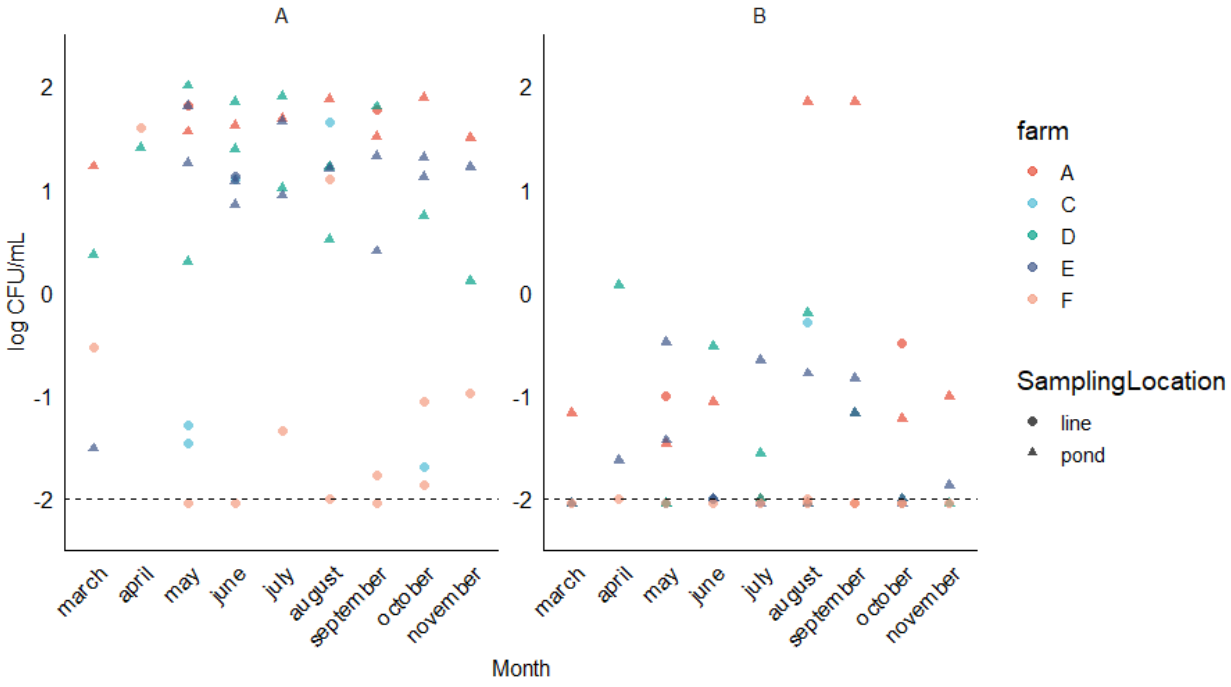

Figure 3.5: Monthly variation in microbial populations (mean in log CFU/mL): (A): total coliforms (B) generic *E. coli* across pond water and end line sources in farms A, C, D, E, and F. Dashed line represents the limit of detection (-2 log CFU/mL).

CHAPTER 4

EFFECT OF SYNTHETIC AND FISH EMULSION FERTILIZERS IN THE SURVIVAL AND BIOFILM FORMATION OF *SALMONELLA* IN IRRIGATION DISTRIBUTION LINES

[2]Raad, R, Ruiz-Llacsahuanga, B, Appolon, C, Daniel, J, Coolong, T, Critzer, F. **Submitted to Journal of HortScience Friday, 06/20/2025**

Abstract

Biofilms in irrigation water distribution systems such as drip tubing or drop hoses from center pivot systems may play an essential role in spreading pathogens into downstream irrigated crops and thus should be evaluated as a potential harborage point. In this study, we evaluated the formation of *Salmonella* biofilms in irrigation lines in the presence of synthetic liquid (4-0-8) or fish emulsion (2-4-1) fertilizers in a lab setting. Drip tubing (without emitters) were filled with 100 mL of either pond water (PW) with no fertilizer (NoFert), 1% (v/v) synthetic fertilizer (S), or 0.1% (v/v) fish emulsion (O). The drip tubes filled with water were inoculated with 1 log CFU/mL population of a rifampicin-resistant *Salmonella* mixture, and tubing were incubated at 21.1°C for 21 days. Water was replaced with non-inoculated PW, with its respective fertilizer condition on days 7, 10, 14, 17, and 21 to mimic irrigation events. *Salmonella* populations in the water and attached to the tubing were determined on days 0, 7, 14, and 21. Biofilm formation on the drip tubing was observed using Scanning Electron Microscopy (SEM) at each sampling day. From day 0 to 7, *Salmonella* populations in inoculated water significantly increased by 4 log CFU/mL in O ($p = 0.0004$), remained constant in water with NoFert, and decreased below the limit of detection (LOD: -1 log CFU/mL) with S. Populations on the O tubing significantly increased by 6 log CFU/tube from day 0 to 7 (p-value = 0.001) and remained constant until day 21. For the NoFert treatment, the populations in tubing significantly increased to 0.58 log CFU/tube (p-value= 0.02) and then decreased to 0.08 log CFU/tube on day 21. The population remained below the LOD: -0.84 log CFU/tube in S for all sampling events. For the O and NoFert treatments, cross-contamination from the drip tube to the non-inoculated PW and biofilm formation was observed. This work suggests that fertilizers and contaminated stagnant irrigation water can affect the formation of *Salmonella* biofilms in drip tubes, and in certain instances, be a

vector for cross-contamination in subsequent irrigation events. **Keywords:** irrigation lines, crop, *Salmonella*, fertilizer, produce safety

Introduction

Foodborne outbreaks related to consumption of fresh produce are a known risk to fruit and vegetable producers. For example, the 2008 *Salmonella* Saintpaul outbreak implicated 1,442 reported cases. The U.S. Food and Drug Administration (FDA) traced the source of the jalapeño peppers associated with the cases and reported isolation of the outbreak strain from a holding pond used for irrigation (Centers for Disease Control and Prevention 2008). Other outbreaks linked to fresh produce include the *Salmonella* Newport (U.S. Food and Drug Administration 2020b) and *Salmonella* Oranienburg (U. S. Food and Drug Administration 2021) that caused around 1,000 hospitalizations, each, and were possibly associated with the use of contaminated irrigation water based upon the FDA traceback investigation. In 2023 a Shiga-toxigenic producing *Escherichia coli* (STEC) outbreak was reportedly responsible for illness in children playing with garden hoses and splashing, pressurized, municipal irrigation water (Osborn 2023). The latter indicates the potential for water contamination from pathogen persistence in water distribution lines in the form of aggregated cells, or biofilms.

Water is a primary input for ensuring proper growth and processing in the preharvest stage of fruit and vegetable production. Therefore, good water quality is essential in preventing contamination of crops and the spread of human pathogens. While both ground and surface water sources are routinely used for irrigation throughout the U.S., surface water can be easily contaminated with pathogenic bacteria, which are endemic in the surrounding environment, potentially contaminating the surface of the produce when direct contact occurs (Islam et al.

2004; Jacobsen and Bech 2012; Steele 2004; Van Haute et al. 2020). Further, pathogens such as STEC and *Salmonella enterica* originating from irrigation water sources can be internalized by growing fruits and vegetables (Cooley et al. 2003; Deering et al. 2012; Solomon et al. 2002). Multiple studies have reported the occurrence of *Salmonella* and other foodborne pathogens in water that could be used for growing crops (Acheamfour 2021; Chevez et al. 2024; Gorski 2022; Gu et al. 2020; Li et al. 2014; Micallef et al. 2012; Murphy 2023; Truitt et al. 2018). Groundwater (e.g., wells) can also become contaminated for reasons such as improper sealing, failure of backflow prevention devices, or become influenced by surface water where rivers, lakes, or ponds are geographically close to underground aquifers. Other sources of *Salmonella* contamination include contaminated or inadequately composted manure, wild or domestic animals and/or contaminated soil as well as fertilizers (Baker et al. 2019; Dunn et al. 2022; Miller et al. 2013).

The production system at the farms consists of a complex combination of irrigation water sources and irrigation infrastructure that is made of a wide range of metal or plastic materials such as low-density or high-density polyethylene, or polyvinyl chloride (PVC). These materials are continuously being reused by growers until they are no longer fit for their purpose. Growers use both overhead and drip irrigation depending on the crop production system (bareground or plastic mulch), cost, and water availability (Harrison 2002). Water soluble fertilizers can be added to any irrigation system through a process called fertigation, which is a common practice among commercial growers (Miles et al. 2010). Soluble fertilizers containing of nitrogen (N), phosphorus (P) and potassium (K) are most commonly utilized for fertigation, though many mixtures may contain macronutrients such as calcium or sulfur and micronutrients such as zinc or iron (United States Environmental Protection Agency 2022). While most conventional farmers

utilize synthetic fertilizers, organic farmers may utilize natural fertilizer sources for fertigation. While most organic fertilizers such as feather meals or pelletized poultry litters are not readily soluble and cannot be used for fertigation, others such as Chilean nitrate (sodium nitrate) or liquid fish emulsions may be used by certified organic farmers to provide supplemental nutrition to crops (Boyhan et al. 2022; USDA AMS 2011).

Distribution systems in which irrigation water and fertilizers are used are susceptible to microbial contamination and thus pathogens cross-contamination into subsequent irrigation water may occur. Few studies reported the effect of biofouling in produce irrigation distribution systems on the microbial quality of water (Antaki et al. 2016; Blaustein et al. 2016; Pachepsky et al. 2012). However, the ability of foodborne pathogens to form biofilms within these systems has not yet been fully reported. Additionally, biofilms within water distribution systems can play an essential role in supporting the growth and subsequent distribution of foodborne pathogens into downstream irrigated crops and thus should be evaluated to assess under what conditions cross-contamination may occur.

The input of common fertilizers may affect the potential for *Salmonella* to attach, grow and form biofilms in the water piping system as it can later contaminate the subsequent irrigation event. Therefore, the objective of this study was to evaluate the behavior of *Salmonella* in stagnant irrigation water and its ability to form biofilms over time under varying water quality conditions. This study seeks to provide a deeper understanding of the potential risks associated with irrigation infrastructure. It focuses on increasing knowledge about the role of irrigation systems and the conditions that favor the growth of *Salmonella*, leading to potential cross-contamination of irrigation water. Such insights could inform industry practices, influence policy development,

and guide future research efforts towards more holistic approaches in managing pathogen contamination in agricultural settings.

Materials and Methods

Bacterial Culture. A four-serotype mixture of *Salmonella enterica* was used in this study: *S. enterica* Enteritidis (2020AM-1539 –2020 Peach outbreak), *S. enterica* Newport (2020AM-0919 – 2020 Onion outbreak), *S. enterica* Montevideo (ATCC BAA-710 – 1993 Tomato outbreak), *S. enterica* Poona (ATCC BAA-3139 – 2010 Cucumber outbreak). All strains were adapted to 80 parts per million (ppm) rifampicin and stored at -80°C in glycerol stocks. Prior to inoculation, 10µL of each strain was transferred consecutively three times and grown individually in tryptic soy broth with rifampicin (TSBR; Difco, Becton Dickinson Co., Sparks, MD, USA) then incubated at 37°C for 24 h. After the third transfer, tryptic soy agar with rifampicin (TSAR; Difco, Becton Dickinson Co) was used to create a bacterial lawn: 250µL of each strain was inoculated onto the TSAR plates of and incubated at 37°C for 24 h. Bacterial cells were harvested by flooding each plate with 10 mL buffered peptone water (BPW; Difco, Becton Dickinson Co.). Cells were dislodged with a cell spreader, then equal volumes (3 mL) of each serotype were combined to create the four-serotype mixture which was used for inoculation. To determine the starting cell population, the combined cell mixture was serially diluted in 0.1% (w/v) peptone water (Difco, Becton Dickinson Co), plated on TSAR, and incubated at 37°C for 24 h.

Irrigation Water Collection and Inoculation. Surface water was collected using a peristaltic pump (GEOPUMP2, Geotech Environmental Equipment, Inc., CO, USA) from a pond that is used for irrigating crops in southern Georgia Tift County, over the summer of 2023 and used for all challenge studies. Once collected, water jugs were placed on ice and transported to the lab to

be frozen at -20°C until usage. Water was thawed in 4°C one week prior to use. Pond Water (PW) was either injected with 1% (v/v) synthetic liquid 4N-0P-6.64K fertilizer (S; R.W. Griffin, Ty Ty, GA, USA; Appendix 4.1) or with 0.1% (v/v) 2-4-1 fish emulsion (O; 2N-2.2P-0.8K; Ocean Crest Seafoods Inc., Gloucester, MA, U.S.). PW with no fertilizers (NoFert) was tested as a control. Each type of water was inoculated with a 1 log CFU/mL cocktail of rifampicin-adapted *Salmonella* cultures described previously by diluting the mixture in 9 mL of the surface pond water to reach a final concentration of ~1 log CFU/mL in the sample water.

Drip Tube Preparation. Polyethylene drip tubing (70 cm length;1.27 cm internal diameter; NDS Inc., Lindsay, CA, USA) with no perforations were aseptically cut and filled with 100 mL of each type of inoculated water mentioned previously, using 50 mL serological pipettes, and incubated at 21.1°C (70°F) for 21 days. The ends of each tubing were capped using 1/2" figure-8 end closure (Gardrip, Amazon, Seattle, WA, USA) to prevent leakage. To mimic static water conditions found after an irrigation event, the inoculated water was purged on day 7. A 100 mL of non-inoculated PW, from the same source, was then added. PW (100 mL) was replaced on days 7,10, 14, and 17 after the initial inoculation day (day 0), to mimic a crop production month at the farm (Figure 4.1). To capture cross-contamination of bacteria from the tubing to the water on day 0, three drip tubes were randomly selected on day 0 in which inoculated PW was purged and a new batch of PW was circulated and tested before cutting the tubing (Figure 4.1).

***Salmonella* Enumeration.** *Salmonella* populations in water and attached to the tubing were determined on days 0,7, 14, and 21. After purging the water from its respective drip tube, it was serially diluted in 0.1% (w/v) peptone water (Difco, Becton Dickinson Co), and spiral plated (EDDY JET2, v1.0, IUL Instruments, Barcelona, Spain) in duplicate on xylose lysine tergitol

4+rifampicin (XLT4R; Difco, Becton Dickinson Co.). Additionally, 10 mL of each sample was filtered using 0.45µm membrane filters (MilliporeSigma, Burlington, MA, USA) and plated on XLT4R. Populations were determined following incubation at 37°C for 48 h and the limit of detection of water (LOD) was -1 log CFU/mL. The remaining populations attached to the drip tubes were determined by randomly selecting three tubing from each treatment combination and aseptically cutting to four equal parts (approx. 3 cm length). Each cut part was washed with 25 mL of sterilized deionized water to remove any planktonic cells. Biofilms were dislodged using HiCap swabs (BLU-10HC, World Bioproducts, Woodinville, WA, USA). The swab solution was later serially diluted in 0.1% (w/v) peptone water, and spiral plated in duplicate on XLT4R at 37°C for 48 h. The remaining swab solution (7 mL) was also filtered using 0.45µm membrane filters (MilliporeSigma, Burlington, MA, USA) and plated on XLT4R at 37°C for 48 h. LOD of tubes: -0.84 log CFU/tube.

Scanning Electron Microscopy. Undisturbed tubing was imaged each sampling day by aseptically cutting square sections (0.25 cm^2) and analyzed by scanning electron microscopy (SEM) imaging to determine the biofilm structure throughout the production process. To fix the biofilms on the surface, 200µL of 10% formalin (w/v) (Fisher Scientific, Pittsburgh, PA, USA) was added to the cut tubes for 10 min. After 10 min, the tubes were washed with 500µL of sterilized deionized water. Samples were kept at 4°C until examination. Fixed tubes were then sputter-coated with gold at the following settings WD 12.5 mm, 60 s, 15 mA (SPI sputter coater, Structure Probe, Inc., West Chester, PA, USA) and examined with a scanning electron microscope acceleration voltage of 500 V–30 kV, at working distance 10 mm (FE-SEM Thermo Fisher Teneo, Waltham, MA, USA).

Data Analysis. A completely randomized design was used with three samples per biological replicate analyzed for each treatment combination. Each experiment per treatment combination was repeated three times (N=9). To capture low levels of inoculated or attached *Salmonella* in the water or tubing, respectively, two methods were used: i) enumeration by plating and ii) membrane filtration, simultaneously for the water inoculated, drip tubes, and water non-inoculated for all samples at each time point. Counts determination followed guidelines from the Food and Drug's Bacteriological Analytical Manual (Chapter 3) for spiral plates use (Maturin 2001a) and standard guidelines for filter enumeration (Maturin 2001b). Based on Shapiro-Wilk test, the distribution of the *Salmonella* populations across the different treatments was not normally distributed, therefore the microbial enumeration data was log-transformed before they were statistically analyzed with R *v4.3.3*. A Kruskal-Wallis test followed by a Steel-Dwass *post-hoc* analysis was used to compare differences in means between treatments and between each treatment across days. A Wilcoxon rank-sum test was used when comparing two independent groups. P-values below 0.05 were considered significant. When *Salmonella* was not detected by plating or membrane filtration, a value of -1.05 log CFU/mL or -0.89 log CFU/tube was assigned to each water or tubing sample, respectively, for data analysis. Figures were created with ggplot2 in R *v4.3.3*.

Results and Discussion

***Salmonella* populations at the initial point of water contamination**. On day 0, the target *Salmonella* population inoculated was 1 log CFU/mL. Populations were significantly different between S and O (p-value =0.001) and S and NoFert (p-value= 0.003) but not between the O and the NoFert samples (p-value = 0.404). *Salmonella* populations were approximately 1 log

CFU/mL (Table 4.1) for the O and NoFert treated samples, as expected. However, once the 4-0-8 fertilizer in the S treatment was added, it decreased the population to 0.51 log CFU/mL. Population in the O water significantly (p-value =0.0004) increased from day 0 to 7 by 4 log CFU/mL (Table 4.1) whilst it significantly decreased in the S (p-value =0.0001) samples to levels below the LOD and to 0.88 log CFU/mL for the NoFert water samples (p-value = 0.001).

The use of fish emulsion in this study provided a condition that is favorable for *Salmonella* growth. In contrast, the significant decrease in the S treated samples to undetectable levels implies that the synthetic fertilizer (4-0-8) inhibited *Salmonella* survival overtime. This indicates that the 4-0-8 synthetic fertilizer used in this study had an immediate detrimental effect on *Salmonella* populations in the water samples. Multiple studies reported that the slow release of nitrogen in the form of ammonia or urea limits the survival of *Salmonella* species, whether in poultry litter samples (Gutierrez and Schneider 2022), manure-treated soil (Holley et al. 2006), or other types of soil (Dincă et al. 2022). The N within the 4-0-8 synthetic fertilizer is largely composed of either nitrate -N only, or a mix of ammonic-N and nitrate-N depending on the supplier. In this study, the fertilizer used contained 4.18% of nitrate-N and 0.32% of ammonium-N (Appendix 4.1). When ammonic nitrogen dissolves in water it undergoes a dissolution reaction that may form nitrite (NO_2), nitric oxide (NO), and other gaseous forms of N (Canfield 2010; Rhodes et al. 2017; Stief et al. 2022; Zheng et al. 2023). NO_2 has been shown to have antimicrobial effects on *Salmonella enterica* in food products and broilers (Bedale et al. 2016; Jung et al. 2003; Majou and Christieans 2018; Prior et al. 2009). Additionally, Wang et al (2022) explored how NO_2 in surface waters, when exposed to UVA light, undergoes photolysis to generate reactive nitrogen species (RNS) demonstrating its inactivation of pathogenic microorganisms such as *Salmonella*. NO is currently being explored as a major bacteriostatic

(Fang and Vazquez-Torres 2019; Williams and Boon 2019). It has been shown that NO is involved in the regulation of bacterial quorum sensing (Hossain et al. 2017), a communication mechanism between bacteria that is interconnected with biofilm formation (Flemming et al. 2016). NO can induce biofilm dispersal in *Pseudomonas aeruginosa* (Barraud et al. 2006), *E.coli*, and *Salmonella* (Marvasi 2014). NO reacts with superoxide as well, to form peroxynitrite, a highly potent oxidant (Soodaeva et al. 2020). Peroxynitrite can damage microbial cells by oxidizing proteins, DNA, and lipids making it a significant contributor to cellular injury (Hurst 1997; McLean et al. 2010; Wang et al. 2022). For example, Hurst et al. (1997) reported that peroxynitrite is particularly effective at inactivating *E. coli* due to its ability to cross membranes and cause widespread oxidative damage. Additionally, when fertilizers, such as synthetic salts, are added to irrigation systems, microbial dynamic can be influenced. On one hand, elevated salt concentrations may induce osmotic stress, prompting bacteria to form biofilms as a protective mechanism (Burgess et al. 2016) On the other hand, high salinity can inhibit or reduce the survival of certain pathogens, such as *Salmonella*, depending on the serotype and environmental conditions (Lewis et al. 2019). Electroconductivity (EC) measures the ionic strength of water, hence as the amount of salt increases, EC increases (Zaman et al. 2018). The addition of 1% (v/v) 4-0-8 synthetic fertilizer in this study had greatly elevated the EC of the water at various times (Appendix 4.2). For example, after 1 min of injection of the corresponding fertilizer, the EC was 241 and 287 µs/cm for PW only and PW with 0.1% of the fish emulsion (2-4-1), respectively. When 1% of the synthetic liquid 4-0-8 fertilizer was added, the EC was 5180 µs/cm. Many have reported the irreversible correlation of bacterial concentrations and EC (Gonzalez et al. 2012; McEgan et al. 2013; Smet et al. 2015) and it is reported that the minimum water activity (a_w) values required for growth of *S. Typhimurium* are 0.94 (International

Commission on Microbiological Specifications for Foods 1996).For example, Smet et al. (2015) reported that planktonic and surface colonies of *S. Typhimurium*, decreased over a wide range of salt (NaCl) concentrations 0-6% (w/v) and did not grow at pH 6.0 and 8% (w/v) NaCl. McEgan et al. (2013) reported the highly significant inverse correlation (p=0.0001) with *E.coli* concentrations as conductivity level increases. Elevated EC levels caused by the addition of the 4-0-8 fertilizer used in this study may have alerted osmotic conditions and potentially increased microbial stress, thereby affecting their viability. Nonetheless, in practical irrigation systems, EC is not static. It typically increases following fertilizer application due to the influx of dissolved salts and nutrients and subsequently decreases as the system is flushed. This dynamic fluctuation in EC underscores the importance of understanding how synthetic fertilizers, especially under continuous or cyclic flushing regimes, influence foodborne pathogens ecology in irrigation lines.

Multiple studies reported the enhanced soil microbial diversity when they are treated with organic fertilizers compared to conventional mineral fertilizers (Bebber and Richards 2022; Jiangwei et al. 2020; Ouyang et al. 2020; Pan et al. 2014). The effect of biological soil amendments of animal origin (BSAAO) on the growth and survival of pathogens including *Salmonella* was reported by multiple authors (Gu et al. 2018; Miller et al. 2013; Sharma and Reynnells 2016). Fish emulsion is a BSAAO made from a combination of hydrolyzed fish, molasses, and other ingredients such as seaweed, and humic acid, depending on the supplier. These amendments may provide a rich source of nutrients creating a favorable environment for pathogens like *Salmonella*. Furthermore, the method and timing of the BSAAO application can impact pathogen persistence, with applications close to harvest time posing a higher risk of contamination (Benjamin et al. 2013). Miller et al. (2013) reported the growth of pathogens such as *Salmonella* and *E. coli* O157:H7 when introduced to the fertilizers, noting that both pathogens

increased by about 1 log CFU/g within one day of incubation in both plant-based and fish emulsion-based composts.

The reduction in population within the NoFert water samples indicates that the absence of fertilizer also limited *Salmonella* growth, though not completely as the 4-0-8 treatment. Populations remained detectable in NoFert, indicating the possibility of biofilms forming when surface water is used without additional inputs. This underscores the need for further research to elucidate the mechanisms by which this specific synthetic fertilizer combination interacts with *Salmonella*. The regrowth potential of foodborne pathogens in the presence of fish-based fertilizers emphasizes the need for careful monitoring and management of the distribution systems to ensure food safety.

***Salmonella* biofilm formation in drip tubes.** For the drip tube samples, *Salmonella* populations in all fertilizer treatments were significantly different across sampling days. In O, the *Salmonella* population significantly increased (p-value = 0.001) to 6.10 log CFU/tube on day 7 and steadily increased to reach 6.50 log CFU/tube until day 21 (Figure 4.2). Figure 4.3A shows the progressive biofilm formation on the tubing. For the NoFert treated tubing, *Salmonella* populations significantly increased (p-value = 0.021) to 0.58 log CFU/tube on day 7, increased to 0.65 log CFU/tube on day 14, and started to decrease to reach 0.08 log CFU/tube on day 21 (Figure 4.2). Figure 4.3B illustrates the start of a biofilm formation within the NoFert tubing with minimal adhesion evident in the cell structure. On day 0, *Salmonella* populations were recovered from 33% (3/9) of the S tubing samples using at least one of the plating methods**.** Levels then decreased below the LOD from day 7 onwards for all the remaining samples (9/9) (Figure 4.2). The SEM images in Figure 4.3C show that no biofilms were forming throughout the sampling.

The ability to enumerate *Salmonella* at high levels and the clear adhesion structure forming within the O treated tubing samples confirms biofilm formation and the presence of culturable cells on the tubes when water is injected with fish emulsion. Biofilms pose a significant challenge in micro-irrigation systems, leading to bioclogging in emitters. Few studies highlight how emitter design and water quality impact biofilm development, which can result in clogging and reduced irrigation efficiency (Batte 2003; Fu et al. 2021; Gamri et al. 2013). Biofilms are found on moist surfaces such as water pipelines (Batte 2003; Chan et al. 2019; Gamri et al. 2013). The release of biofilms in PVC pipes within drinking water distribution systems (Batte 2003; Chan et al. 2019) suggests that similar issues could occur in irrigation water distribution systems. The growth mode of a biofilm involves the adhesion to a surface and is usually made up of many bacterial genera. Biofilm formation occurs in five key stages that allow microorganisms to attach to the surfaces and aggregate into complex, mature communities (Zhao et al. 2017). The process begins with an initial attachment, where planktonic cells (free-floating) adhere loosely to the surface (Flemming et al. 2007; Palmer et al. 2007). This is followed by irreversible attachment, where bacteria attach more firmly using structures like pili and begin producing extracellular polymeric substance (EPS) (Palmer et al. 2007; Zhao et al. 2017). There are two maturation phases: first, the biofilm starts to develop as cells proliferate, and EPS accumulates. Second, the biofilm becomes more stable by forming channels for nutrient, waste, and genetic exchange (Cvitkovitch 2004; Flemming and Wingender 2010). Finally, the last step is the dispersion stage; some cells detach from the biofilm to return to a sessile state, allowing them to colonize new surfaces and repeat the cycle (Zhao et al. 2017). Mature biofilms in irrigation systems can serve as points of cross-contamination, where bacteria not only persist and proliferate but also exchange virulence genes. This phenomenon can lead to the emergence of

new, more pathogenic strains, posing a significant risk to food safety through irrigation practices (Blaustein et al. 2016; Chua et al. 2014; Lee et al. 2014; Li et al. 2014; Pang et al. 2017).

The significant increase in population by day 7, within the NoFert treatments, suggests that the microorganisms initially adapted well to the NoFert environment, finding sufficient nutrients or favorable conditions to support their growth. The constant population from day 7 to day 14 indicates that the microorganisms reached a stable phase. This could be due to the depletion of readily available nutrients or the establishment of a steady-state environment. Finally, the decrease in population by day 21 suggests that the conditions in the NoFert treated tubing became less favorable over time. On the field, this could occur due to several factors, such as nutrient depletion, accumulation of waste products, or changes in environmental conditions that can negatively impact the microorganisms' survival. Figure 4.3B illustrates the potential for biofilm formation within the tubing line; however, only a low abundance of cells was observed, with minimal adhesion evident in the cell structure – compared to when pond water was used in combination with injected fish emulsion. The observed trends highlight the importance of nutrient availability and environmental conditions in supporting microbial populations. In agricultural or environmental contexts, this suggests that the absence of fertilizers (NoFert) may initially support microbial growth, but over time, the lack of nutrients can lead the biofilm to reach its dispersion phase. Once the cells are attached, they may not be able to form a mature biofilm. However, based on evidence from this study, the irrigation tubing can still harbor *Salmonella* over time. Other factors, such as changes in temperature, soil intrusion, and drip system breakdowns that may occur in the field, can also affect this process. This indicates the importance of constantly maintaining the drip tubes, even when no fertilizers are added. The low recovery of *Salmonella* within the S drip tubes on day 0 and the later decrease to levels below the

LOD for the remainder of the experimental days, indicates the inability of *Salmonella* cells to form an irreversible attachment in the tubing when water is injected with the 4-0-8 synthetic fertilizer used in this study. The SEM images in Figure 4.3C show very close similarity to a drip tube image with no treatment (Appendix 4.3), confirming no bacterial attachment.

Cross-contamination of non-inoculated water. When tubes were washed with a batch of non-inoculated pond water with O fertilizer and NoFert, it resulted in the cross-contamination of this pond water. On day 0, *Salmonella* concentrations were -0.22 and -0.54 log CFU/mL for the NoFert and O treatments (p-value = 0.08), respectively. Levels were below the LOD for the S treatment; p-value = 0.001 and p-value = 0.01 for O and NoFert water samples, respectively (Figure 4.4). In the O treated samples, *Salmonella* populations in the water at day 7 significantly increased (p-value = 0.002) to reach 4.44 log CFU/mL, population then gradually decreased to 4.33 log CFU/mL on day 14 and 4.09 log CFU/mL on day 21 (Figure 4.4). For the S treated samples, *Salmonella* populations decreased below LOD in the non inoculated PW compared to the O and NoFert treatments. Levels in the S non inoculated PW then decreased below the LOD throughout all the remaining sampling days. For the NoFert water samples, 0.19 log CFU/mL were recovered from the water on day 7, then decreased to -0.35 log CFU/mL on day 14. *Salmonella* was recovered from 33% (3/9) of the NoFert water samples using at least one of the plating methods on day 21 (Figure 4.4).

The initial *Salmonella* inoculation level in this study was 1 log CFU/mL, which is higher than levels typically encountered in real-world agricultural settings. However, this population concentration was required to allow for bacterial enumeration in a lab setting. Surveys assessing *Salmonella* prevalence in surface water sources have generally reported low concentrations

(Antaki et al. 2016; Chevez et al. 2024; Murphy et al. 2022; Murphy 2023). For example, Chevez et al. (2024) reported that the overall prevalence of *Salmonella* in water samples was 6.81% (6/88) from water samples collected from February to December 2021 from eight irrigation ponds located in Southwest Georgia, USA. Murphy et al. (2023) reported 21.7% (26/120) of surface water samples positive for *Salmonella* from the Eastern Shore of Virginia. Antaki et al. (2016) reported that the overall mean *Salmonella* concentration in positive water samples from two ponds in the southern U.S. was 0.03 MPN/100 mL. Nonetheless, this study demonstrated that even a single contamination event within the irrigation line can lead to subsequent biofilm formation –whether an animal-based fertilizer (fish emulsion) is applied or no fertilizer is used, which could pose a risk for ongoing cross-contamination of irrigation water. Current monitoring protocols primarily focus on the sanitary quality of the source water, yet they often overlook the potential for irrigation tubing itself to become a reservoir for pathogens, contributing to contamination in future irrigation events. Many have reported the transfer of foodborne pathogens from contaminated water and its possible link to human outbreaks (Centers for Disease Control and Prevention 2019; U.S. Food and Drug Administration 2020a) and the occurrence of *Salmonella* and *E. coli* in water stream from crop production (Benjamin et al. 2013; Strawn et al. 2013). Overall, this issue complicates efforts by researchers and industry to identify contamination sources, as the water source may not remain contaminated over time, and water distribution systems are often neglected. The EPS layer protects the biomass from environmental stresses such as shear forces and antimicrobials added to the line. Consequently, biofilm formation within irrigation lines poses a significant challenge to the produce industry, as it can degrade the quality of irrigation water and promote bacterial contamination. There is a lack of studies concerning the prevalence of bacterial pathogens within the irrigation water

distribution systems. Nonetheless, the few that have reported it such as Antatki et al. (2014), they reported that in one of the ponds sampled, *Salmonella* was detected in 8.3 % (3/36) water samples. Notably, in the associated drip irrigation system, 19.4 % (14/72) samples tested positive, indicating potential contamination and further multiplication of cells occurring along the irrigation line.

Surface water contains a wide range of organic and inorganic materials, along with microbial populations, including diatoms – single-celled algae. Multiple studies have reported the relationship between bacterial and diatom biofilm formation, and their effect on biofouling in aquatic environments (Amin et al. 2012; Khandeparker et al. 2013). Diatoms come in various shapes and forms, creating a phycosphere, which is the physical space surrounding the diatom's cell surface where nutrients and exudates are concentrated (Amin et al. 2012). This area facilitates exchanges between diatoms and bacteria, providing an environment for bacteria to localize and adhere to surfaces. Bacteria use chemotaxis to find nutrients, detecting molecules in their surroundings to determine swimming direction, either toward attractants or away from repellents with *E. coli* and *Salmonella* exhibiting this behavior (Olsen 2013; Szurmant and Ordal 2004). Bacteria can benefit diatoms by providing more available nutrients or protecting them from other opportunistic microorganisms. When irrigation water contains organic and inorganic materials, along with microbial populations such as diatoms, it creates an ideal environment for biofilm formation under the right conditions. Diatoms, with their phycosphere, facilitate the attachment and colonization of bacteria on surfaces. This interaction can lead to the development of biofilms that harbor pathogenic bacteria like *Salmonella* as reported in this study (Appendix 4.4 and 4.5).

As biofilms grow and mature, they can clog irrigation pipes and emitters, reducing water flow and irrigation efficiency, as mentioned previously. Additionally, pieces of biofilm can detach and spread throughout the irrigation system, subsequently contaminating the water, as observed by this study. Many authors have reported the role of the dispersion stage in biofilm in releasing viable cells into the environment (Chan et al. 2019; Chua et al. 2014; Gonzalez-Machado et al. 2018; Sha et al. 2013). For example, in the study by Sha et al. (2013), *Salmonella* populations in biofilms formed on submerged surfaces of ceramic tiles reached densities exceeding 10^7 cells/cm^2 during early colonization. Over a four-week period, these biofilm-associated populations gradually declined to around 10^4 cells/cm^2, yet viable *Salmonella* cells continued to disperse into the surrounding water (Sha et al. 2013). This poses a significant risk to food safety as detachment or significant disruption of these biofilms, within the irrigation lines, could trigger a sudden release of pathogens, potentially in quantities that exceed the infectious level. Therefore, understanding and controlling biofilm formation in irrigation distribution systems is crucial to preventing the spread of *Salmonella* and other pathogens onto irrigation water. Implementing effective water treatment methods and regular maintenance of irrigation systems can help mitigate these risks and ensure the safety of irrigated crops.

Conclusion

This study compared the effects of no fertilizer, synthetic (4-0-8), and fish emulsion on microbial populations of *Salmonella* and biofilm formation in drip tubing. In the absence of fertilizer, microbial populations attached to the drip tubing survived within and transferred to the circulating water, with free cells attached after 21 days. Synthetic (4-0-8) fertilizer hindered *Salmonella* survival and growth in distribution lines. In contrast, fish emulsion resulted in

microbial populations attaching to the tubing and surviving, forming mature biofilms, and transferring to water during the 21 days of sampling. This highlights the distinct impact of a biologically based fertilizer such as fish emulsion on promoting biofilm formation and microbial survival compared to the use of a synthetic (4-0-8) fertilizer or no fertilizers in the irrigation line. Based on the findings, the industry should prioritize regular maintenance of drip irrigation systems, even when fertilizers are not being added, to prevent the buildup of biofilms and microbial populations that can lead to cross-contamination. Regular monitoring of water quality is essential to detect microbial contamination early, including pathogens like *Salmonella*. Frequent inspections of drip systems are necessary to identify and repair any breakdowns promptly, ensuring the system remains free from contaminants

This study has several limitations that should be considered. These include the presence of stagnant water in increased capacity, which may not accurately reflect field conditions. In future studies on the effect of fertilizers on the survival of *Salmonella* and other microbiomes, several additional factors should be considered. These include variations in environmental conditions such as temperature, water flow and pressure conditions, and the input of commonly used sanitizers on the field. The quality of irrigation water, interactions with other microorganisms, and the specific composition of fertilizers can also influence microbial dynamics. Addressing these factors will provide a more comprehensive understanding of microbial survival and biofilm formation in irrigation water distribution settings.

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Tables and Figures

Table 4.1: *Salmonella* population (log CFU/mL) mean±standard deviation in pond water inoculated for 7 days, at different fertilizer conditions: 0.1% v/v fish (2-4-1) emulsion (O), 1% v/v liquid synthetic (4-0-8) fertilizer (S), or pond water only (NoFert).

Day	O	S	NoFert
0	1.28± 0.04 aA	0.51±0.60 aB	1.24±0.08 aA
7	5.10± 0.31 bA	-1.05± 0.00 bB	0.88±0.23 bC

Limit of detection: -1 log CFU/mL

Lowercase letters represent significant differences after 7 days incubation in drip tubes at 21.1°C within each treatment.

Uppercase letters represent significant differences across treatments for the same day

N = 9 per treatment combination

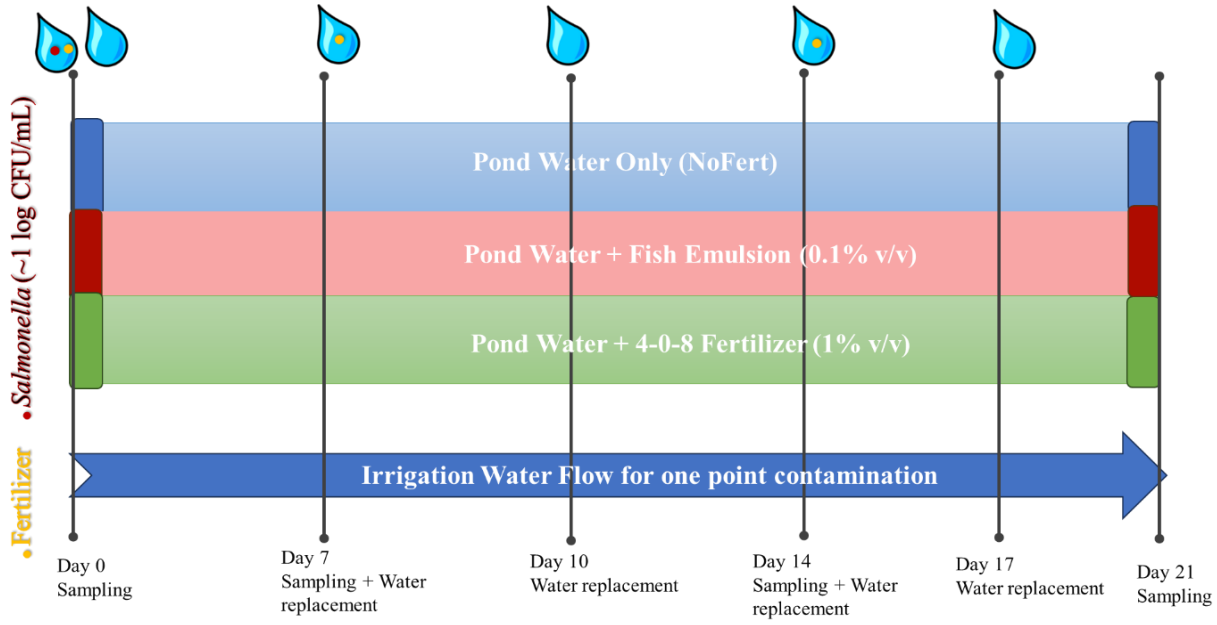

Figure 4.1: Timeline of experimental procedure for drip tubing filled with 100 mL of pond water (PW), on day 0, with no fertilizer (NoFert), PW injected with 0.1% (v/v) fish emulsion, or PW injected with 1% (v/v) 4-0-8 synthetic fertilizer, during 21 days of sampling. Drip tubes were filled with 100 mL inoculated water on day 0 and incubated for 7 days at 21.1°C. Water (100 mL) was constantly replaced with non-inoculated PW on days 7, 10, 14, and 17. Sampling of tubing and water took place on days 0, 7, 14, and 21.

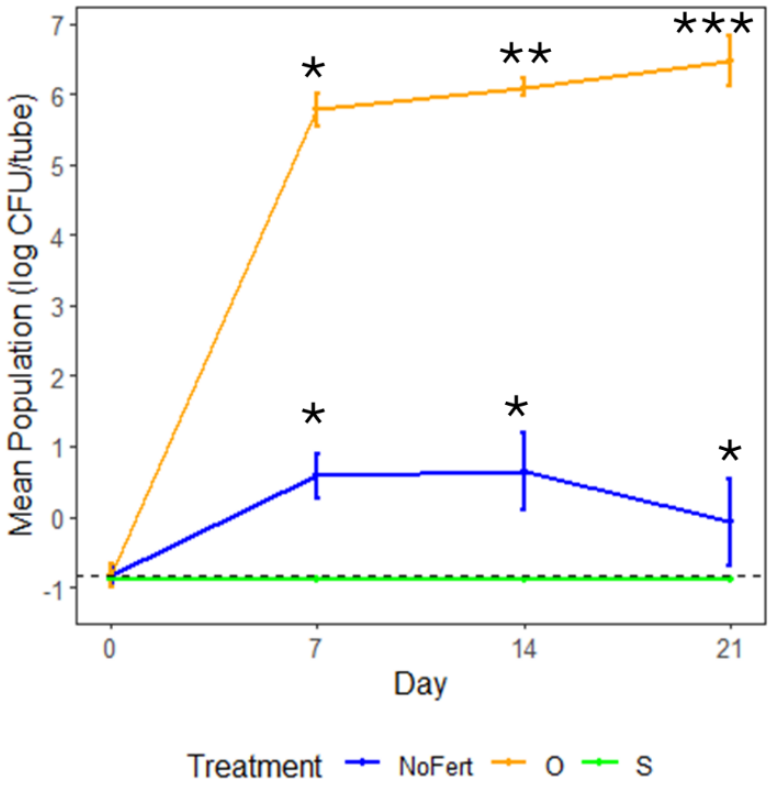

Figure 4.2: *Salmonella* populations in drip tubing (1.27 cm internal diameter; log CFU/tube) Samples were treated with no fertilizer (NoFert), 0.1% (v/v) fish emulsion (O), or 1% (v/v) 4-0-8 synthetic liquid fertilizer (S). Error bars represent standard deviation from the mean. Asterisks [*] represent significant differences across days for the same treatment. Dashed black lines represent the limit of detection: tube: -0.84 log CFU/tube.

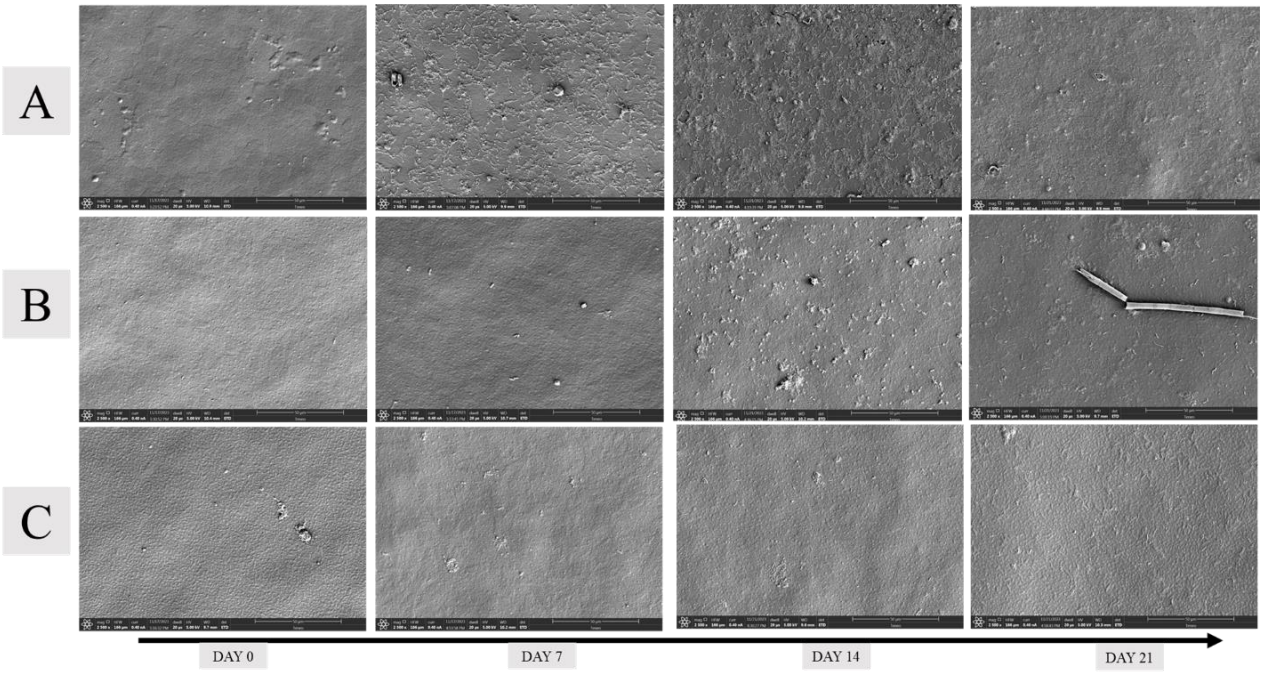

Figure 4.3: Scanning Electron Microscopy images of drip tubes (0.25 cm^2) treated with (A) pond water + 0.1% (v/v) fish emulsion, (B) pond water only, and (C) pond water + 1% (v/v) 4-0-8 synthetic liquid fertilizer on days 0, 7, 14, and 21. Magnification 2500x. Scale bar: 50 µm**.**

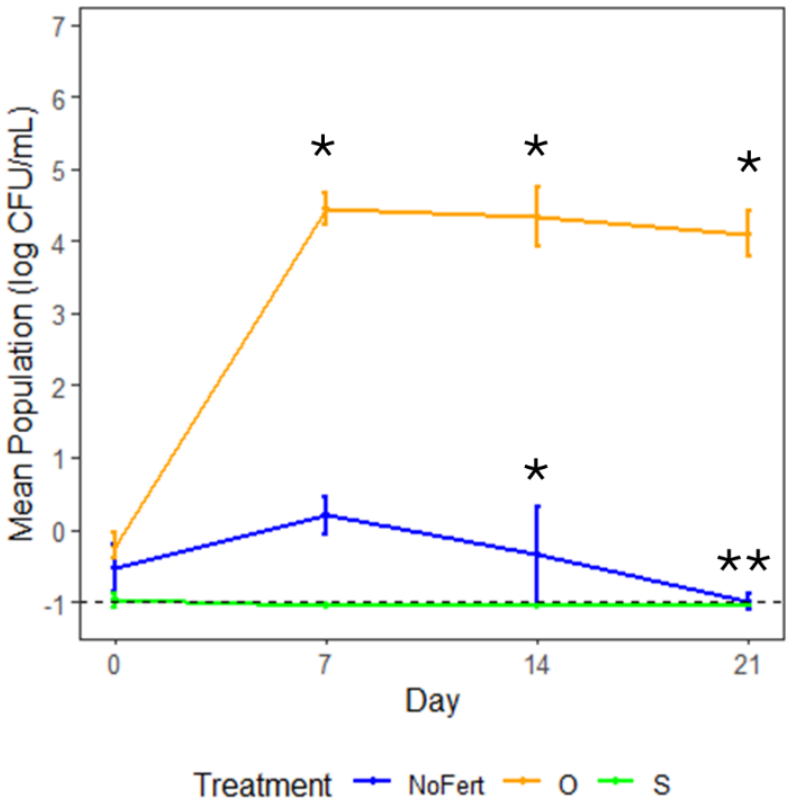

Figure 4.4: Transfer of *Salmonella* to uninoculated pond water (log CFU/mL) from day 0 to day 21. Samples were treated with no fertilizer (NoFert), 0.1% (v/v) fish emulsion (O), or 1% (v/v) 4-0-8 synthetic liquid fertilizer (S). Error bars represent standard deviation from the mean. Asterisks [*] represent significant differences across days for the same treatment. Dashed black lines represent the limit of detection: water: -1 log CFU/mL.

CHAPTER 5

EFFICACY OF PERACETIC ACID AND CHLORINE IN MANAGING *SALMONELLA* BIOFILMS IN IRRIGATION LOOP SYSTEMS

[3]Raad, R, Ruiz-Llacsahuanga, B, Appolon, C, Greenbaum, H, Vinueza, R, Critzer, F. **To be submitted to a peer reviewed journal (Applied and Environmental Microbiology)**

ABSTRACT

Biofouling presents significant challenges to the crop production industry, notably reducing irrigation efficiency and potentially dispersing pathogens to irrigated crops. This study evaluated the efficacy of peracetic acid (PAA) and chlorine (Cl) against *Salmonella* biofilms in irrigation lines with or without fertilizers. Pond water (PW) with 2-4-fish emulsion (O), PW with 4-0-8 synthetic liquid fertilizer (S), or PW with no fertilizer (NoFert) was inoculated with 2 log CFU/mL of a rifampicin-resistant *Salmonella* cocktail. Inoculated water was then circulated through polyethylene loop irrigation system for a month. *Salmonella* populations both in the water and attached to the tubing were determined. Data showed that a single point of contamination from the water resulted in a biofilm formation with O and NoFert, but not the S treatments, after 3 days. Both PAA and Cl effectively reduced *Salmonella* populations for all fertilizer treatments in water samples. However, when no sanitizer was introduced to the line, biofilm dispersion resulted in the contamination of a subsequent irrigation event for the O treatments. In contrast to the S and NoFert treatments which presented no microbial proliferation. Our findings suggest that O treatments may result in persistent biofilm formation that could lead to contamination of irrigation water when no sanitizers are introduced. These studies provide insight into the behavior of foodborne pathogens in irrigation distribution systems. **Significance:** The accumulation of bacteria in water distribution systems due to biofouling can lead to contamination, making it crucial to evaluate and implement effective mitigation measures to prevent these issues and ensure safe and efficient irrigation practices. The use of the 2-4-1 fish emulsion in-line may support the establishment of *Salmonella* biofilms and subsequent cross-contamination of irrigation water if not fully flushed from the system. This study demonstrates that PAA and Cl effectively reduce *Salmonella* contamination in water but

will not eliminate populations in-line once biofilms are established. **Keywords:** irrigation water, distribution lines, *Salmonella*, fertilizer, sanitizer, crop production

INTRODUCTION

Current data indicate that produce accounted for 50% of illnesses in the U.S with *Salmonella enterica* being responsible for a majority of those outbreaks. (1). The consumption of raw produce has been generally perceived as safe compared to other foods primarily of animal origin. However, vegetable production systems inherently present multiple points of vulnerability to foodborne pathogen contamination. This includes production and pre-harvest practices such as using contaminated irrigation water (2, 3). Noticeable incidence which occurred linked to production practices include the 2020 *Salmonella* Newport (4) and 2021 *Salmonella* Oranienburg (5) outbreaks that caused around 1,000 hospitalizations, each. FDA investigation reports stated that the source of the outbreaks was possibly due to the use of contaminated irrigation water, however, the outbreak strains were not identified in those water sources.

Biofouling refers to the undesirable accumulation of biotic matter on a surface such as water inlets, pipes, and grates resulting in the clogging of such materials (6). Biofouling has been shown to be of considerable hygienic, operational and economical relevance, for drinking water (7, 8) and irrigation water distribution systems (9, 10). Basic water distribution system components include valves, fittings, pumps, sprinklers, storage reservoirs, tanks, and the piping materials used in agricultural fields. This includes polyvinyl chloride (PVC) which is popular due to its durability and cost-effectiveness, high-density polyethylene (HDPE) which is favored for its high strength and flexibility, making it suitable for diverse irrigation applications (11, 12). These materials are recognized for providing an ideal surface that promotes bacterial colonization and biofilm formation (6).

Biofilms are integrated multi-species cell populations that are embedded in a self-produced matrix of extracellular polymeric substances (EPS) (13). Multiple studies reported biofilm formation of *Salmonella* (14), *Pseudomonas* (15), and *Escherichia coli* (16) on HDPE surfaces. Gamri et al. (2016) examined the impact of pipe materials on biofouling using synthetic wastewater. The study found that biofilm growth was more pronounced at a velocities of 0.8 m/s and that while PVC pipes were less prone to bacterial accumulation compared to polyethylene pipes, they were still susceptible to biofilm formation (17). Additionally, *Salmonella* has been shown to persist in packing line materials such as stainless steel, PVC, and unfinished oak wood for an extended period of time (over 28 days) (18). The formation of biofilms in agricultural water distribution systems is unavoidable and can lead to several problems, including reduced irrigation efficiency due to pipeline blockages and compromised water safety (19).

Microbial contamination, including *Salmonella*, of crop production sources encompasses contaminated irrigation or postharvest water, inadequately composted manure, wild or domestic animals, contaminated soil, and fertilizers (20-22). Irrigation water sources include groundwater and surface water. Groundwater resides in aquifers beneath the earth's surface, while surface water includes ponds, lakes, or rivers. Surface water is commonly used in irrigation systems due to its accessibility and abundance in the southern U.S. However, it can easily be contaminated with endemic bacteria from the environment, potentially transferring pathogens to the soil or crop (2, 23-25).

Fertilizers are often added to irrigation systems through fertigation, a common practice among commercial growers (26). Synthetic fertilizers, primarily Nitrogen (N), Phosphorus (P), and Potassium (K), are widely used in conventional agriculture, with nutrient levels indicated by

numbers (e.g., 4-0-8 fertilizer contains 4% N, 0% P, and 8% K) (27). In contrast, organic farms adhere to the U.S. department of Agriculture's National Organic Program (NOP) guidelines, where biological amendments of animal origin such as fish emulsion, blood meals are used as fertilizers (28).

When the inner surfaces of pipelines are in contact with contaminated water, they can become colonized by biofilm forming bacteria, which may highly impact water safety and quality during irrigation events. Bacteria in the biofilm form are resistant to several stresses such the use of sanitizers (29, 30). This includes chlorine (Cl) in the form of sodium or calcium hypochlorite and peracetic acid (PAA). These chemicals are often used for sanitation of equipment in food processing facilities (31, 32) or to treat agricultural water (33, 34). Several factors including levels of organic matter formed, pH, water temperature and concentration of the chemical used can alter the effectiveness of a treatment against *Salmonella* biofilms (35). It is important for the produce industry to establish control measures to prevent biofilm formation in the irrigation pipelines throughout their production. PAA and Cl are both effective disinfectants used to reduce microbial contamination, but they operate through different mechanisms. PAA is a strong oxidant that disrupts microbial cell walls through direct oxidation, leading to cell lysis and leakage of cellular contents (36). Cl, on the other hand, kills microbes by penetrating cell walls and breaking down enzymes within the cells, primarily through the formation of hypochlorous acid (HOCl) in water (37). However, Cl is known to form DBPs such as trihalomethanes and haloacetic acids, which may pose health risks if present in significant concentrations (38).

Cl is commonly used in drinking water distribution systems to resolve biofouling issues due to its availability and low cost (6). PAA, specifically in the form of SaniDate 12.0, is currently an

EPA-registered product labeled for the reduction and control of Shiga toxin-producing *E. coli* (STEC), and *Salmonella enterica* in preharvest irrigation water (EPA Reg. No. 70299-18). Both disinfectants are influenced by factors such as concentration, contact time, and water characteristics, but PAA tends to dissipate quickly, while chlorine's effectiveness is impacted by water pH and temperature (37, 39). Despite a few studies reporting the effectiveness of various disinfectant strategies in reducing clogging and bioaccumulation in piping systems (40, 41), there is a notable lack of research addressing foodborne pathogen biofilm formation in irrigation water distribution settings, particularly when synthetic fertilizers or fish emulsion are involved. Irrigation water has been the main focus as a source of contamination, but very little attention is paid to the sanitation of irrigation tubing to ensure that any organisms remaining in the line do not become a source of contamination. Therefore, the objective of this study was to evaluate the efficacy of sanitation control measures (Cl or PAA) which could be implemented by produce growers to manage the risk of *Salmonella* biofilms in an in-lab irrigation loop piping system with the presence of synthetic (S) or fish emulsion (O) fertilizers.

This research focuses on increasing knowledge about the role of common sanitizers used for preharvest water in preventing the conditions that favor the biofilm formation of *Salmonella* in irrigation lines, leading to potential cross-contamination of the water. The insights gained could inform industry practices and guide future research efforts towards more holistic approaches in managing pathogen contamination in agricultural settings.

RESULTS

Injecting the line with fish emulsion resulted in *Salmonella* attachment and growth in drip tubes over time.

Across all treatment combinations, *Salmonella* populations in water remained consistent after 1 h of inoculated water circulation in the drip tubes on day 0 ($p = 0.23$; Table 5.1). It is important to note that sanitizers were introduced on day 3, not on day 0 (Figure 5.1). On day 0, cross-contamination from the inoculated water to the drip tubes was evident in the 0.1% *v/v* fish emulsion treatments (O) regardless of sanitizer condition: No sanitizer: -0.31 log CFU/tube, PAA: -0.08 log CFU/tube, and Cl: 0.18 log CFU/tube. Similarly, cross-contamination was evident for the synthetic1% *v/v* 4-0-8 (S) fertilizer treatment with no sanitizer: -0.68 log CFU/tube, PAA: -0.57, and Cl: -0.68 log CFU/tube. Whilst levels for the no fertilizer (NoFert) treatment were -0.26 CFU/tube for the no sanitizer, -0.51 log CFU/tube for the PAA, and -0.55 log CFU/tube for the Cl treatment combinations (Table 5.2). Regardless of the treatment, *Salmonella* populations attached to the drip tubes on day 0 were significantly different between the O and the NoFert ($p = 0.01$) and the O and the S ($p \leq 0.001$) but not between the S and the NoFert treatments ($p = 0.07$).

Populations in the tubing significantly increased in the O treatments without sanitizers ($p \leq 0.001$) on day 7, reaching 5.45 log CFU/tube. They remained constant on day 14 (5.49 log CFU/tube), then gradually decreased on day 40 to 4.29 log CFU/tube (Table 5.2). Biofilm formation was evident based on the scanning electron microscopy (SEM) images, which illustrate the thickness and maturity of biofilms formed on the drip tubes (Figure 5.2 and Appendix 5.1). In the O – PAA treatment combination, populations significantly increased (p-value = 0.002) to 1.49 log CFU/tube on day 7. It remained constant at 1.51 log CFU/tube, then decreased to 0.40 log CFU/tube on day 40 (Table 5.2). The start of a biofilm formation was evident in the PAA treated samples with O fertilizers. However, no clear EPS structure was observed (Figure 5.3 and Appendix 5.2). Similarly, in the O – Cl treatment combination,

Salmonella levels increased to 0.43 log CFU/tube then gradually decreased to 0.09 and -0.75 log CFU/tube on days 14 and 40, respectively (Table 5.2). Figure 5.4 and Appendix 5.3 illustrate a substantial aggregation of cells.

The NoFert treatment with no sanitizer-treated pond water (PW) showed a dynamic change in *Salmonella* levels on the tubing over the experimental period. Initially, *Salmonella* levels slightly increased to -0.04 log CFU/tube on day 7 ($p = 0.89$), followed by a gradual decrease to -0.47 log CFU/tube by day 14, and eventually reaching levels below the limit of detection (LOD) on day 40 (Table 5.2). Freshwater microorganisms such as diatoms are illustrated in the SEM images for the NoFert – no sanitizer treatment combinations throughout the days with no clear bacterial attachment represented (Figure.5.2). In the NoFert – PAA treatment group, *Salmonella* populations significantly decreased ($p = 0.001$), falling below the LOD at day 7. Approximately 33% (3/9) of the NoFert – PAA tubing samples still showed at least one colony-forming unit (CFU) of *Salmonella* using either enumeration methods; however, by day 40, *Salmonella* was undetectable in all samples (Table5.2). SEM analysis revealed that diatoms and freshwater microorganisms adhered to the tubing throughout the experiment, along with dispersed rod-shaped cells; however, a mature *Salmonella* biofilm development appeared limited or absent throughout (Figure 5.3). For the NoFert – Cl treatment combination, *Salmonella* levels were below the LOD for all sampling days. SEM analysis showed diatoms and free cells attached to the tubing, but no clear EPS and subsequent biofilm formation was shown (Figure 5.4). In the S treated samples with and without any sanitizer, *Salmonella* levels on the tubing remained below the LOD throughout the experimental days (Table 5.2). No clear biofilm formation or attachment of other microorganisms was observed on the SEM images (Figure 5.2, 5.3, and 5.4).

Salmonella biofilms in drip lines contaminated later irrigation events when no sanitizers are injected.

When the drip tubes were subsequently exposed to a new batch of PW on day 3 to simulate another irrigation event, it resulted in the cross-contamination for the water samples in the O (4.52 log CFU/mL) and the NoFert (0.97 log CFU/mL) treatments. Whilst it did not result in the contamination of water with tubing injected with S (levels below LOD; Table 5.3). *Salmonella* populations in the water for the O with no sanitizer treatment were significantly higher on day 3 (4.52 log CFU/mL; $p = 0.0003$) than population levels on day 0 (2.32 log CFU/mL). Bacterial concentrations decreased to 3.73 log CFU/mL and 3.86 log CFU/mL on days 7 and 10, respectively. Populations continued to decrease reaching 3.4 log CFU/mL on day 17 in the PW circulated in the O with no sanitizer treated tubing with or without fertilizer throughout the experimental days (Figure 5.1, Table 5.3). *Salmonella* remained detectable in the water samples for the NoFert treatments with no sanitizers on day 3 (0.97 log CFU/mL) and day 7 (-0.40 log CFU/mL) but then decreased to levels below the LOD for the remainder of the sampling days. *Salmonella* populations significantly decreased ($p \leq 0.0001$) on day 3 to levels below the LOD and populations remained undetectable throughout the experimental procedure for the S treatments with no sanitizer (Table 5.3).

PAA and Cl at 20 ppm effectively prevented cross-contamination of _Salmonella_ from the tubing to the subsequent irrigation water but did not completely eliminate the biofilms already formed in the tubing

On day 3, both PAA and Cl effectively reduced *Salmonella* populations to levels below the LOD for all fertilizer treatments in water samples (Table5.3). The observed effect was consistent

across days 10 and 17. However, on days 7 and 14, the O and PAA treatments resulted in detectable counts of 1.54 log CFU/mL and 1.15 log CFU/mL, in water samples, respectively. The O and Cl treatment combination exhibited similar behavior with detectable levels on days 7 and 14 at -0.26 and -0.65 log CFU/mL, respectively (Table 5.3). The S and NoFert treatments with PAA and Cl resulted in levels below the LOD on days 7 and 14.

DISCUSSION

The increase in recovery of bacterial cells from the drip tubes on day 0 for the O compared to the S and NoFert may be attributed to the non-uniform bacterial attachment on the tubing and the ingredients found in the fish emulsion, including hydrolyzed fish. Numerous studies have shown that soil microbial diversity is significantly enhanced when treated with organic fertilizers, as opposed to conventional mineral fertilizers (42-45). For example, the meta-analysis conducted by Bebber and Richards (45) reported data from 37 research articles and revealed that organic fertilizers significantly enhance both functional and taxonomic diversity of soil microbes compared to unfertilized control soils and mineral fertilizers. Many have reported the role of strains, surface type, temperature, and other ecological features in the formation of biofilms on abiotic surfaces (14, 46-48). For example, Contreras-Soto et al. (46) reported that *Salmonella* biofilms exposed to river water exhibited significant structural variations compared to those formed in laboratory conditions. The structural variations observed through SEM indicated that environmental factors in river water, such as organic matter and nutrients, contribute to the non-uniform formation of biofilms on surfaces. Additionally, the fish emulsion treatment, in this study, promoted greater bacterial attachment to the tubing after one hour of circulation compared to the synthetic 4-0-8 fertilizer or surface water alone. This enhanced attachment may be

attributed to the higher carbon content in the fish emulsion, which could have initiated the early, reversible stages of biofilm formation (48-50). The sustained increase in *Salmonella* populations on the tubing in the O and no-sanitizer treatments over time suggest that continuous water circulation within the drip lines – regardless of fertilizer presence – supported *Salmonella* survival and attachment. Moreover, the minimal decline in *Salmonella* levels in the O – no sanitizer treatment, despite nearly 20 days without water input, indicates robust biofilm formation on the tubing surface. This is further supported by the SEM images, which illustrate the thickness and maturity of biofilms formed on the tubes (Figure 5.2 and Appendix 5.1).

The decrease in *Salmonella* populations within the tubing observed in the NoFert – no sanitizer treatment combination indicates that, with the absence of water circulation (from day 17 – 40), *Salmonella* biofilms were unable to sustain themselves by the end of the experimental period. This observation aligns with findings from previous studies, which have demonstrated that biofilm formation by *Salmonella* on abiotic surfaces can be highly variable and influenced by multiple conditions. The production of EPS is crucial for biofilm persistence, but various factors such as nutrient availability, microbial competition, and environmental factors such as temperature and relative humidity can significantly impact biofilm stability (51-53). Figure 5.2 further supports this concept by illustrating the presence of other diatoms and non-rod-shaped materials on the tubing. This suggests that the microbial community within the tubing was diverse and included various freshwater organisms. The presence of these organisms implies potential competition for resources, which could inhibit *Salmonella* biofilm formation. Studies have shown that microbial interactions, including competition and cooperation, play a critical role in shaping biofilm structure and stability (54, 55). For example, Behringer et al. (54) highlighted the strong conservation of bacterial communities associated with diatoms, suggesting

that these interactions can influence biofilm formation and persistence. Hence, the inability of *Salmonella* to form sustained biofilms in the NoFert treatment can be attributed to the complex interplay of environmental factors and microbial competition. Understanding these interactions is essential for developing effective strategies to control biofilm formation in natural and engineered systems.

In this study, *Salmonella* populations dropped below the LOD in both tubing and water samples when no sanitizer was applied, in presence of the 4-0-8 synthetic fertilizer (Table 5.1 and 5.2). This clearly indicates that synthetic fertilizers inhibit *Salmonella* attachment and subsequent growth in the system. SEM images showed that no organisms, besides algae-like filaments from the PW have attached to the tubing within the S treatments overtime (Figure 5.2). Many crop production researchers have reported that long-term N fertilization significantly alters the microbial environment, leading to shifts in microbial community composition and diversity. These changes disrupt the natural balance of microbial communities, affecting nutrient cycling and microbial interactions (56, 57). Studies have reported that the gradual release of N in the form of ammonia through synthetic fertilization can suppress the growth and survival of *Salmonella* species (58-60). The N content in the 4-0-8 synthetic fertilizer used in this study consists of 4.18% nitrate-N and 0.32% ammonium-N (Appendix 5.4). When ammonium-N dissolves in water, it undergoes a chemical transformation that can produce nitrite (NO_2), nitric oxide (NO), and other gaseous forms of N (61-64) that can have antimicrobial effects against *Salmonella enterica* (65-68). Its antimicrobial activity is largely due to the generation of reactive nitrogen species, such as nitric oxide and superoxide. Nitric oxide can react with superoxide to form peroxynitrite, a highly reactive oxidant (69). Peroxynitrite can damage microbial and host cells by oxidizing proteins, DNA, and lipids, making it a potent agent of cellular injury (70-72).

Additionally, microbial dynamics can be influenced when synthetic salts from fertilizers are added to the irrigation systems, it can either induce osmotic stress, prompting bacteria to form biofilms as a protective mechanism (73), or inhibit the survival of certain pathogens, such as *Salmonella* (74). Water electroconductivity (EC) measures the ionic strength of water; hence as the amount of synthetic salt increases, EC increases (75). The addition of 1% (v/v) 4-0-8 synthetic fertilizer in this study greatly elevated the EC of the water at various times (Appendix 5.2). To add, many have reported the irreversible correlation of bacterial concentrations and EC (76-78). For example, McEgan et al. (76) reported the highly significant inverse correlation ($p=0.0001$) with *E. coli* concentrations as EC level increases. Osmotic conditions may have been altered due to the elevated EC levels caused by the addition of the 4-0-8 fertilizer used in this study, potentially increasing microbial stress, thereby affecting their viability. In practical irrigation systems, EC is not static. It typically increases following fertilizer application due to the influx of dissolved salts and subsequently decreases as the system is flushed. This study aimed to simulate continuous flushing to understand its impact on EC dynamics and pathogen dynamics. However, water pressure may also play a critical role in this process, potentially influencing the effectiveness of flushing and the distribution of synthetic fertilizers. These insights underscore the need to consider both flushing regimes and hydraulic conditions when evaluating the behavior of foodborne pathogens in fertigation systems. Finally, material type of the irrigiation line may have impacted the attachment and subsequent biofilm formation of *Salmonella*. For example, Ivers et al. (14) reported that *Salmonella* attachment and biofilm characteristics were significantly impacted by surface type and incubation time where the highest biofilm formation was observed on nylon surfaces across all time points (2, 24, and 96 hours) whereas the least biofilm formation was observed on HDPE surfaces.

Bacterial concentrations remained constant in water circulated in the O with no sanitizer treated tubing with or without fertilizer throughout the experiment (Table 5.2). This indicates that the continuous water circulation in the line supported the stable survival and attachment of *Salmonella*. The consistency in bacterial concentrations indicates that *Salmonella* have formed biofilms on the tubing surfaces, corroborating the previously discussed SEM tubing data for the O treatment. Biofilms are protective layers that bacteria create, making them more resistant to environmental stress and treatments. Based on Appendix 5.1 presenting *Salmonella* biofilm formation with O treatment on day 40, a visible crack is present in the tubing within the biofilm, which was not observed on day 14. This suggests that *Salmonella* survived over time in the tubing, even in periods of non-use. This finding is particularly relevant in agricultural settings, where irrigation lines are often reused across multiple growing seasons in which it may remain inactive in-between crop production stages. The reintroduction of water and nutrients could promote new biofilm formation (8, 48, 79, 80) and potentially reintroduce *Salmonella* contamination in subsequent seasons. The switch between the O fertilizer (days 7 and 14) and no fertilizer injection in the water (days 3, 10, and 17) suggests that the biofilm could thrive regardless of nutrient availability from the constant addition of fertilizers.

Salmonella remained detectable in the water samples for the NoFert treatments with no sanitizers on day 3 and day 7 but then decreased to levels below the LOD for the remainder of the sampling days indicating that the conditions in the NoFert treated tubing became less favorable over time. Nutrient depletion, accumulation of waste products, or changes in environmental conditions can negatively impact the microorganisms' survival, all of which could occur on the field. Figure 5.2, Figure 5.3, and Figure 5.4 illustrate the potential for cellular attachment within the tubing line when no fertilizer is injected; and only a low abundance of cells was observed,

with minimal adhesion evident in the cell structure – compared to when pond water was used in combination with injected fish emulsion. In agricultural or environmental contexts, this suggests that the absence of fertilizers (NoFert) may initially support microbial growth, but over time, the lack of nutrients can lead to planktonic cells to reproduce but become incapable of sustaining themselves and then dying-off. Once the cells attached, they may not be able to form a mature biofilm. However, based on evidence from this study, the line can still harbor *Salmonella* over 14 days.

On days 3, 10, and 17 both PAA and Cl effectively reduced *Salmonella* populations to levels below the LOD for all fertilizer treatments in water samples (Table 5.2). This indicates that water treated with 20 ppm of either PAA or Cl can limit cross-contamination of *Salmonella* from tubes to water. However, on days 7 and 14, the O-PAA and O-Cl treatments resulted in detectable counts in water samples compared to the NoFert and S fertilizer treatments with either PAA or Cl. Biofilm formation was evident in the PAA-treated samples with O fertilizers in Figure 5.3 and Appendix 5.2. This indicates that while PAA may have limited cross-contamination from the drip tubes treated with fish emulsion to the circulated water treated with the sanitizer, it did not eliminate the bacteria attached to the tubing. Consequently, cross-contamination from the dispersed biofilms occurred in the water circulated with the fertilizer that was not treated. PAA is known for its strong oxidizing properties, which can disrupt cell walls and cellular components of microorganisms (36, 39). However, biofilms provide a protective barrier that can limit the penetration and effectiveness of PAA (13, 51). This barrier, composed of EPS including proteins and polysaccharides, can prevent PAA from reaching and eliminating all bacteria within the biofilm, leading to persistent contamination. The protective nature of biofilms allows bacteria to survive and potentially detach, leading to cross-contamination in untreated areas. PAA is also

biodegradable, rapidly decomposing into acetic acid, hydrogen peroxide, and water (39). The rapid biodegradability of PAA means that it breaks down quickly, reducing its effective concentration over time (81). As PAA decomposes, the residual acetic acid and hydrogen peroxide may not be sufficient to completely eradicate biofilms, as noticed by the results of this experiment. The chemical stability of biofilms, combined with the rapid degradation of PAA, means that biofilms can persist within the irrigation loop system in this study, even after PAA treatment.

The O and Cl treatment combination exhibited similar behavior, albeit to a lesser extent. This indicates that chlorine was more effective in inhibiting *Salmonella* growth, likely due to its strong oxidizing properties and ability to generate reactive oxygen species (ROS) such as hypochlorous acid superoxide, and hydroxyl radicals (37). These ROS induce oxidative stress, damaging proteins, lipids, and DNA within *Salmonella* cells, leading to their effective elimination. Nonetheless, Figure 5.4 and Appendix 5.3 illustrate a more substantial biofilm formation- aggregation of cells - compared to Figure 5.3 and Appendix 5.2 – which shows non-uniform biofilm formation. This suggests that Cl was more effective in inhibiting *Salmonella* growth, but not biofilm formation along with other cocci and rod-shaped bacteria potentially present in freshwater when fish fertilizers are present in-line. Stress responses to PAA and Cl further complicate the eradication of biofilms as microorganisms can adapt to these sanitizers, enhancing their resilience (82). This highlights the need for additional or combined treatments to effectively manage biofilm formation.

This study demonstrated the effectiveness of PAA and Cl in limiting biofilm formation within irrigation lines in a closed-loop system, simulating 40 days of irrigation production. However,

several limitations should be noted. The experiment was conducted on a small scale in a laboratory setting, which does not fully replicate field conditions, particularly the high-water volumes and pressures typically used in agricultural operations (300-600 gal/min). Therefore, field-based studies are necessary to evaluate the performance of these sanitizers under real-world conditions. The initial inoculation level in this study was 2 log CFU/mL, which is higher than levels typically encountered in real-world agricultural settings, however such levels were used in the lab study to enumerate and observe sanitizers effects on microbial load overtime. Additionally, the chemical composition of different fertilizers may influence biofilm development and should be further investigated. Environmental factors such as elevated field temperatures, which were not replicated in this room-temperature study, may also impact biofilm formation and should be explored in future research.

CONCLUSION

This study has demonstrated that fish emulsion fertilization enhances biofilm growth in a loop setting over time. While PAA and Cl decrease *Salmonella* population and cross-contamination during irrigation, they are less effective afterward when biofilm is formed. Cl has a longer-lasting bactericidal effect than PAA in drip tubes treated with fish fertilization. PAA and Cl are effective at reducing population and biofilm formation when no fertilizer is added to the water. Regardless of sanitizer treatment, the 1% of 4-0-8 synthetic fertilizer treatment limited *Salmonella* growth and survival of other freshwater organisms in the line. Biofilm formation is not continuously formed on the tubing, and the presence of diatoms may provide locations for bacterial aggregation and biofouling. The incomplete elimination of bacteria in the NoFert tubing resulted in cross-contamination in the water circulated with no sanitizer. The residual bacteria in

the biofilm can detach and contaminate the circulated water, especially when untreated fertilizers are introduced. Overall, these findings underscore the need for effective sanitation practices in irrigation systems to prevent the biofilm formation and spread of pathogenic bacteria such as *Salmonella*. Understanding these dynamics can help in developing better strategies for managing microbial contamination in agricultural settings.

MATERIALS AND METHODS

Bacterial Culture

For this study, a four-serotype cocktail of *Salmonella enterica* was used: *S. enterica* Enteritidis (2020AM-1539 –2020 Peach outbreak), *S. enterica* Poona (ATCC BAA-3139 – 2010 Cucumber outbreak), *S. enterica* Newport (2020AM-0919 –2020 Onion outbreak), *S. enterica* Montevideo (ATCC BAA-710 –1993 Tomato outbreak). Strains were adapted to 80 parts per million (ppm) rifampicin and stored at -80°C in glycerol stocks. Before inoculation, 10µL of each strain was transferred individually and grown in tryptic soy broth with rifampicin (TSBR; Difco, Becton Dickinson Co., Sparks, MD, USA) for 24 h at 37°C three times consecutively. To create a bacterial lawn, after the third transfer, 250µL of each strain was inoculated onto tryptic soy agar plates with 80 ppm of the rifampicin (TSAR; Difco, Becton Dickinson Co) then incubated at 37°C for 24 h. Bacterial cells were harvested by flooding each plate with 10 mL buffered peptone water (BPW; Difco, Becton Dickinson Co.) and dislodging cells with a cell spreader. Equal volumes (3 mL) of each serotype were combined to create the four-serotype cocktail which was used for inoculation. To determine the initial *Salmonella* populations in TSB, the combined cocktail was serially diluted in 0.1% (w/v) peptone water (Difco, Becton Dickinson Co), plated on TSAR, and incubated at 37°C for 24 h prior to enumeration.

Irrigation Water Collection and Inoculation

Surface water was collected using a peristaltic pump (GEOPUMP2, Geotech Environmental Equipment, Inc., Denver, CO, USA) from a pond that is used for irrigating crops in southern Georgia Tri County area over the summer of 2024 and used for all challenge studies. Once collected, water jugs were placed on ice and transported to the lab to be frozen at -20°C until usage. Water was stored at -20°C until it was thawed in 4°C for immediate usage. Pond Water (PW) was either injected with 1% (v/v) synthetic liquid 4N-0P-6.64K fertilizer (S; R.W. Griffin, Ty Ty, GA, USA; Appendix 5.5) or with 0.1% (v/v) fish emulsion (O; 2N-2.2P-0.8K; Ocean Crest Seafoods Inc., Gloucester, MA, USA). PW with no fertilizers (NoFert) was used as a control. Each type of water was inoculated with a 2 log CFU/mL cocktail of rifampicin-adapted *Salmonella* cultures mentioned previously by diluting the cocktail in 9 mL of PW to reach a final concentration of ~ 2 log CFU/mL in the sample water.

Drip line preparation in a loop system

Each set of polyethylene drip tubes (1.27 cm internal diameter; NDS Inc., Lindsay, CA, USA) with no perforations, was connected to a 110V magnetic pump which had a water flow of 19 L/min [5 gal/min] (YaeKoo, Amazon, Seattle, WA, USA) and a pre-assembled one-gallon jug (LocknLock, Amazon) with a ½ inch spigot kit (Rain Barrel Spigot Kit with Bulkhead Fitting, Amazon). These components were placed in a large black 26 x 18 x 12", heavy-duty tote (Uline Mighty Totes, Home Depot, Atlanta, GA, USA) to control the water flow and prevent any leakage since pathogens were used for this study. The drip tubes were aseptically cut to approximately 8.5 inches (22 cm) each and then connected using in-line couplers (1/2" Drip Irrigation Coupling Fitting, Amazon). Each treatment combination included one magnetic pump

connected to a pre-cut tube and a water jug drilled and attached to a ½ inch spigot with a barbed valve. To connect the tube set with the magnetic pump, a ½ inch female fitting was attached to each side of the pump using ½ inch female threaded PVC fittings (Yahenda Amazon), ensuring secure attachment. Then, a ½ inch drip line coupling was connected to the female PVC thread on each side, ensuring tight connections. On the upper side, the drip tube leading to the entire set of tubing was connected, and on the lower side, a ~3-inch drip tube was connected to the barbed valve from the jug (Appendix 5.6). This in-lab irrigation loop system enabled us to evaluate irrigation events using water inoculated with biosafety level II pathogens

Irrigation Water Circulation In-Line

On day 0, each jug was filled with 1.5 L of inoculated PW, tailored to its respective fertilizer condition. The spigot was then turned on to initiate water circulation for 1 h, simulating a continuous irrigation event. This setup ensured that surface water was the first and only source of contamination on that day. To simulate post-irrigation water conditions, the inoculated water was removed from the jugs. Non-inoculated PW from the same source was then continuously replaced according to specific schedules: with its respective sanitizer condition on days 3, 10, and 17, or with its respective fertilizer condition on days 7 and 14. This process began after the initial inoculation on day 0, mimicking a typical crop production month on the farm (Figure 5.1).

Sanitizer Treatments

For each treatment combination set, the water was either treated with 20 ppm of peracetic acid (PAA; Sanidate 12.0, BioSafe Systems, East Hartford, CT, USA), 20 ppm of chlorine (Cl; 65% Granular Cal Hypo, Aqua Org, Amazon), or left as untreated surface pond water to serve as a

control. Sanitizer concentrations were determined based on common practices and manufacturer's recommendations and were assessed by using the FAS-DPD Chlorine/Bromine test kit (LaMotte, Chestertown, MD, USA) and Peracetic Acid Test Kit (Thermo Fisher Scientific, Waltham, MA, USA) for free chlorine and PAA, respectively, before each circulation day. The water was circulated in-line for 5 minutes, followed by a 1 h period of stagnant contact time (Figure 5.1).

Processing and Microbial Determination

Populations in water were determined on days 0,3,7, 10, and 17 whilst populations in tubes were determined on days 0, 7, 14, and 40 per the following: Twenty milliliters of water were collected from each jug onto 50 mL conical tube. The latter was repeated three times to indicate 3 technical replicates for each treatment/day combination. On day 0, water samples were collected at time 0 and after 1 h of circulation then processed for microbial testing. Whilst the remainder of the days, water samples were collected after the irrigation stopped (after 1 h). For the days when the circulated water was treated with sanitizers, 0.2 mL of neutralizer was added to the conical tubes. The neutralizer was prepared by dissolving 28g of 97% granular sodium metabisulfite (Fisher Scientific, Pittsburgh, PA, USA) in 1 L of deionized water. Samples were then mixed and diluted in 0.1% (w/v) peptone water (Difco, Becton Dickinson Co) as needed and spiral plated (EDDY JET2, v1.0, IUL Instruments, Barcelona, Spain) in duplicate on xylose lysine tergitol 4+rifampicin (XLT4R; Difco, Becton Dickinson Co.). Populations were determined after incubation at 37°C for 24-48h. Simultaneously, 10 mL of each sample was filtered using 0.45µm membrane filters (MilliporeSigma, Burlington, MA, USA) and plated on XLT4R for 24-48h. Limit of detection (LOD) of water samples: -1 log CFU/mL.

The remaining populations attached to the drip tubes were determined by randomly selecting three tubing from each treatment combination and aseptically cutting it to four equal parts. Each cut part was washed with 25 mL of sterilized deionized water to remove any planktonic cells. Biofilms were dislodged using HiCap swabs (BLU-10HC, World Bioproducts, Woodinville, WA, USA). The swab solution was later diluted in 0.1% (w/v) peptone water, as needed, and spiral plated in duplicate on XLT4R at 37°C for 24-48h. The remainder of the swab solution (6 mL) was filtered using 0.45 µm membrane filters (MilliporeSigma) and plated on XLT4R at 37°C for 24-48h (LOD of tube samples: -0.78 log CFU/tube).

Scanning Electron Microscopy

Undisturbed tubing was imaged each sampling day by aseptically cutting square sections (0.25 cm^2) and analyzed by SEM imaging to determine the biofilm structure throughout the production process. To fix the biofilms on the surface, 200 µL of 10% formalin (w/v) (Fisher Scientific) was added to the cut tubing for 10 min. After 10 min, the tubing was washed with 500µL of sterilized deionized water. Samples were kept at 4°C until examination. Fixed tubes were then sputter-coated with gold at the following settings WD 12.5 mm, 60 s, 15 mA (SPI sputter coater, Structure Probe, Inc., West Chester, PA, USA) and examined with a scanning electron microscope acceleration voltage of 500 V–30 kV, at working distance 10 mm (FE-SEM Thermo Fisher Teneo, Waltham, MA, USA).

Data Analysis

A completely randomized design was used with three samples per each three biological replicates analyzed (N=27) for each treatment combination. To capture low levels of inoculated

or attached *Salmonella* in the water or tubes, respectively, both enumeration by plating and membrane filtration methods were used simultaneously. Plate counts were deemed acceptable following guidelines for spiral plates use according to the Food and Drug's Chapter 3 of the Bacteriological Analytical Manual (83) and standard guidelines for filter enumeration (84). Based on Shapiro-Wilk test, the distribution of the *Salmonella* populations across the different treatments was not normally distributed, therefore a Kruskal-Wallis test followed by a Steel-Dwass *post-hoc* analysis was used in R v4.3.3 (85) to compare differences in means between treatments and between each treatment across days. A Wilcoxon rank-sum test was used when comparing between two independent groups. P-values below 0.05 were considered significant. When *Salmonella* was not detected by plating or membrane filtration, a value of -1.05 log CFU/mL or -0.78 log CFU/tube was assigned to each water or tube sample, respectively, for data analysis.

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Tables and Figures

Table 5.1: *Salmonella* population (log CFU/mL) mean ± standard deviation in pond water inoculated from time 0 to 1 h, at different fertilizer conditions: 0.1% v/v fish 2-4-1 emulsion (O), 1% v/v synthetic liquid 4-0-8 fertilizer (S), or pond water only (NoFert). Each separated based on their sanitizer condition: Peracetic Acid: (PAA), Chlorine (Cl) at 20 ppm each, or No Sanitizer which served as control.

Fertilizer	**Sanitizer**	**Time (hour)**	
		0	1
O	PAA	2.33 ± 0.10	2.31 ± 0.04
	Cl	2.35 ± 0.15	2.34 ± 0.05
	No Sanitizer	2.33 ± 0.15	2.32 ± 0.08
S	PAA	2.25 ± 0.13	2.13 ± 0.17
	Cl	1.81 ± 0.69	2.09 ± 0.32
	No Sanitizer	1.89 ± 0.45	2.06 ± 0.22
NoFert	PAA	2.34 ± 0.09	2.12 ± 0.73
	Cl	2.35 ± 0.12	2.34 ± 0.10
	No Sanitizer	2.31 ± 0.18	2.33 ± 0.12

Limit of detection: -1 log CFU/mL
N= 9 per treatment combination

Table 5.2: *Salmonella* population (log CFU/tube) mean ± standard deviation in tubing samples over time at different fertilizer conditions: 0.1% v/v fish 2-4-1 emulsion (O), 1% v/v synthetic liquid 4-0-8 fertilizer (S), or pond water only (NoFert). Each separated based on their sanitizer condition: Peracetic Acid: (PAA), Chlorine (Cl) at 20 ppm each, or No Sanitizer which served as control. Limit of detection (LOD): -0.78 log CFU/tube.

Fertilizer	**Sanitizer/Day**	**0**	**7**	**14**	**40**
O	No Sanitizer	-0.31 ± 0.45 Aa *	5.45 ± 0.82 Ba	5.49 ± 1.00 Ba	4.29 ± 1.52 Ba
	PAA	-0.08 ± 0.43 Aa *	1.49 ± 1.09 Bb	1.51 ± 1.35 Bb	0.40 ± 1.22 ABb
	Cl	0.18 ± 0.53 Aa *	0.43 ± 0.65 Ab	0.09 ± 0.68 Ab	-0.75 ± 0.15 Bb
S	No Sanitizer	-0.68 ± 0.27 Aa **	-0.82 ± 0.00 Ba	-0.82 ± 0.00 Ba	-0.82 ± 0.00 Ba
	PAA	-0.57 ± 0.23 Aa **	-0.82 ± 0.00 Ba	-0.82 ± 0.00 Ba	-0.82 ± 0.00 Ba
	Cl	-0.68 ± 0.31 A **	-0.82 ± 0.00 Ba	-0.82 ± 0.00 Ba	-0.82 ± 0.00 Ba
NoFert	No Sanitizer	-0.26 ± 0.44 Aa **	-0.04 ± 0.58 Aa	-0.47 ± 0.36 ACac	-0.82 ± 0.02 BCa
	PAA	-0.51 ± 0.31 Aa **	-0.82 ± 0.00 ACb	-0.66 ± 0.26 ACbc	-0.82 ± 0.00 Ba
	Cl	-0.55 ± 0.34 Aa **	-0.77 ± 0.05 Bb	-0.82 ± 0.00 Bb	-0.82 ± 0.00 Ba

Uppercase letters represent significant differences between days in drip tubes within each treatment (fertilizer/sanitizer) combination.
Lowercase letters represent significant differences across treatment combinations for the same day and fertilizer.
Asterisks (*) indicate significant differences among the fertilizer groups on day 0.
N=9 per treatment combination

Table 5.3: *Salmonella* population (log CFU/mL) mean ± standard deviation in water samples over time at different fertilizer conditions: 0.1% v/v fish 2-4-1 emulsion (O), 1% v/v synthetic liquid 4-0-8 fertilizer (S), or pond water only (NoFert). Each separated based on their sanitizer condition: Peracetic Acid: (PAA), Chlorine (Cl) at 20 ppm each, or No Sanitizer served as control. Limit of detection (LOD): -1.00 log CFU/mL.

Fertilizer	**Sanitizer/Day**	**0**	**3**	**7**	**10**	**14**	**17**
O	No Sanitizer	2.32 ± 0.08 Aa *	4.53 ± 1.43 Bca	3.76 ± 0.36 ABCa	3.86 ± 1.13 BCa	3.39 ± 0.08 ABCa	3.44 ± 1.07 ABCa
	PAA	2.31 ± 0.05 Aa *	-1.04 ± 0.02 Bb	1.54 ± 0.65 Ab	-1.05 ± 0.00 Bb	1.15 ± 0.83 Ab	-1.05 ± 0.00 Bb
	Cl	2.34 ± 0.05 Aa *	-0.97 ± 0.21 BCDb	-0.27 ± 0.78 ADc	-1.05 ± 0.00 BDb	-0.65 ± 0.44 ACc	-1.05 ± 0.00 BDb
S	No Sanitizer	2.06 ± 0.22 Aa *	-1.05 ± 0.00 Ba	-1.05 ± 0.00 Ba	-1.05 ± 0.00 Ba	-1.05 ± 0.00 Ba	-1.05 ± 0.00 Ba
	PAA	2.13 ± 0.17 Aa *	-1.05 ± 0.00 Ba	-1.05 ± 0.00 Ba	-1.05 ± 0.00 Ba	-1.05 ± 0.00 Ba	-1.05 ± 0.00 Ba
	Cl	2.09 ±0.32 Aa *	-1.05 ± 0.00 Ba	-1.05 ± 0.00 Ba	-1.05 ± 0.00 Ba	-1.05 ± 0.00 Ba	-1.05 ± 0.00 Ba
NoFert	No Sanitizer	2.33 ± 0.12 Aa *	0.97 ± 0.24 Aa	-0.40 ± 0.47 Bb	-0.85 ± 0.23 Ba	-1.04 ± 0.02 Ba	-1.05 ± 0.00 Ba
	PAA	2.12 ± 0.73 Aa *	-1.05 ± 0.00 Bb	-1.05 ± 0.00 Bb	-1.05 ± 0.00 Bb	-1.05 ± 0.00 Bb	-1.05 ± 0.00 Bb
	Cl	2.34 ± 0.10 Aa *	-1.05 ± 0.00 Bb	-1.05 ± 0.00 Bb	-1.05 ± 0.00 Bb	-1.05 ± 0.00 Bb	-1.05 ± 0.00 Bb

Uppercase letters represent significant differences between days in drip tubes within each treatment (fertilizer/sanitizer) combination.
Lowercase letters represent significant differences across treatment combinations for the same day and fertilizer.
Asterisks (*) indicate significant differences among the fertilizer groups on day 0.
N=9 per treatment combination

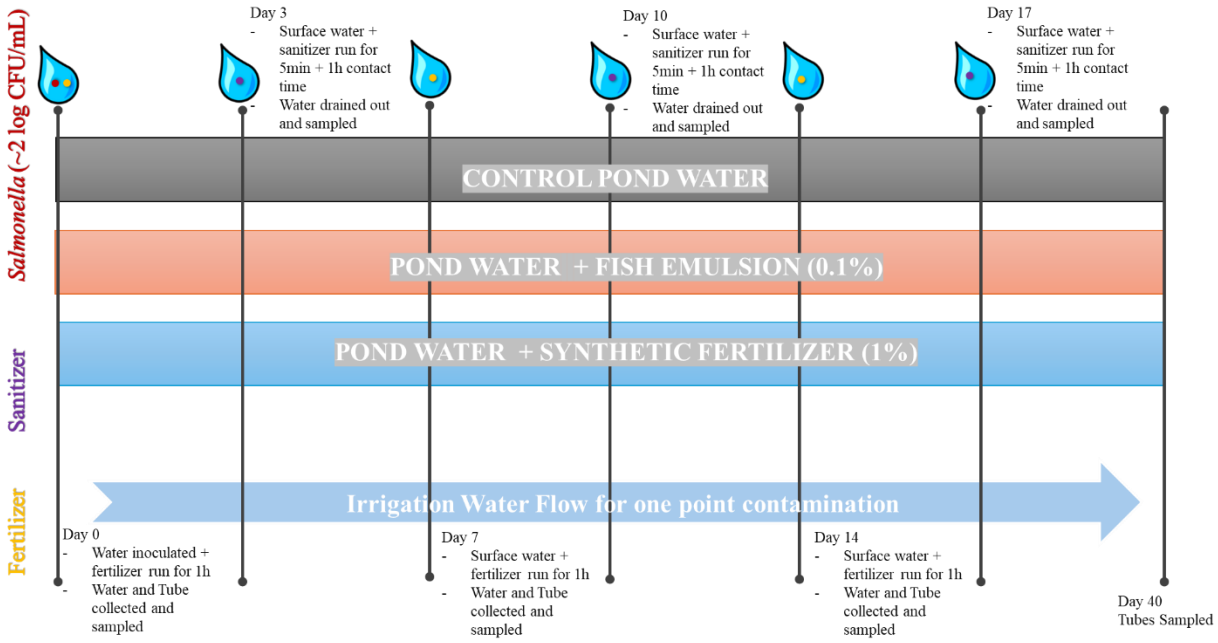


Figure 5.1: Timeline of experimental procedure for tubing circulated with either Pond Water (PW) injected with 1% (v/v) synthetic (4-0-8) fertilizer, PW injected with 0.1% (v/v) fish emulsion; or PW with no fertilizer (control pond water) during 40 days of sampling. Inoculated water was circulated on day 0 then drained. On days 3, 10, and 17, PW was injected with sanitizers: peracetic acid or chlorine at 20 ppm each. On days 7 and 14 water was injected with its respective fertilizer. Tubes were kept until day 40 for sampling to mimic a crop production month.

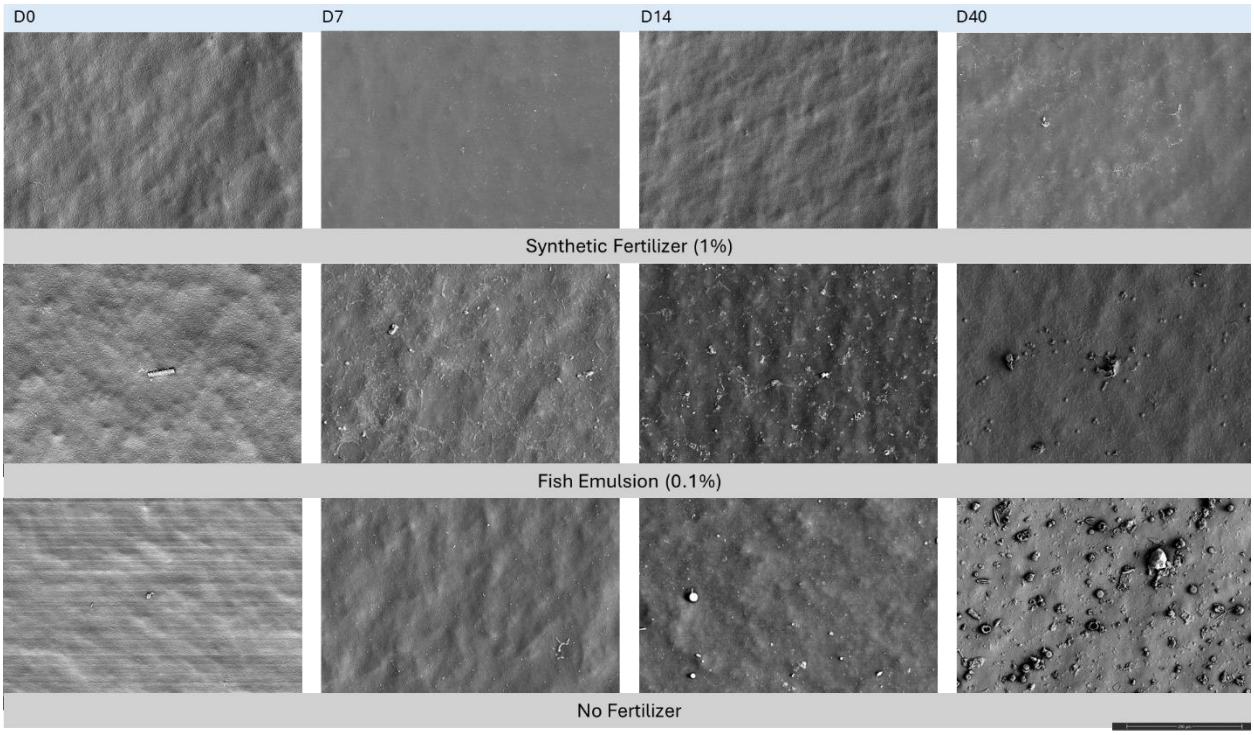


Figure 5.2: Scanning Electron Microscopy images of drip tubes (1.27 cm internal diameter - 0,25 cm^2) treated with pond water + 1% (v/v) synthetic fertilizer, pond water + 0.1% (v/v) fish emulsion, and pond water only on days 0, 7, 14, and 40 **without sanitizer** treatment. Pictures taken at magnification of: 800x. Scale bar: 200 µm

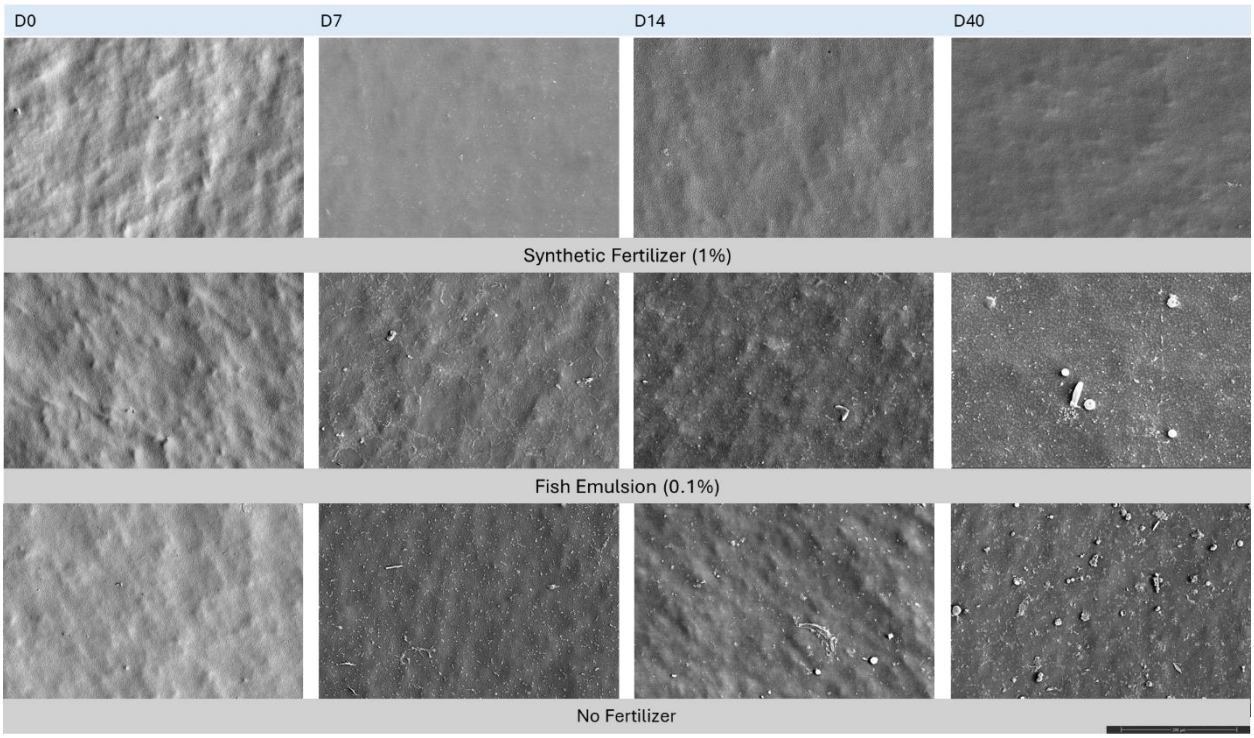


Figure 5.3: Scanning Electron Microscopy images of drip tubes (1.27 cm internal diameter - 0,25 cm^2) treated with pond water + 1% (v/v) synthetic fertilizer, pond water + 0.1% fish emulsion, and pond water only on days 0, 7, 14, and 40 with 20 parts per million of **peracetic acid** as a sanitizer treatment. Pictures taken at magnification of 800x. Scale bar: 200 µm

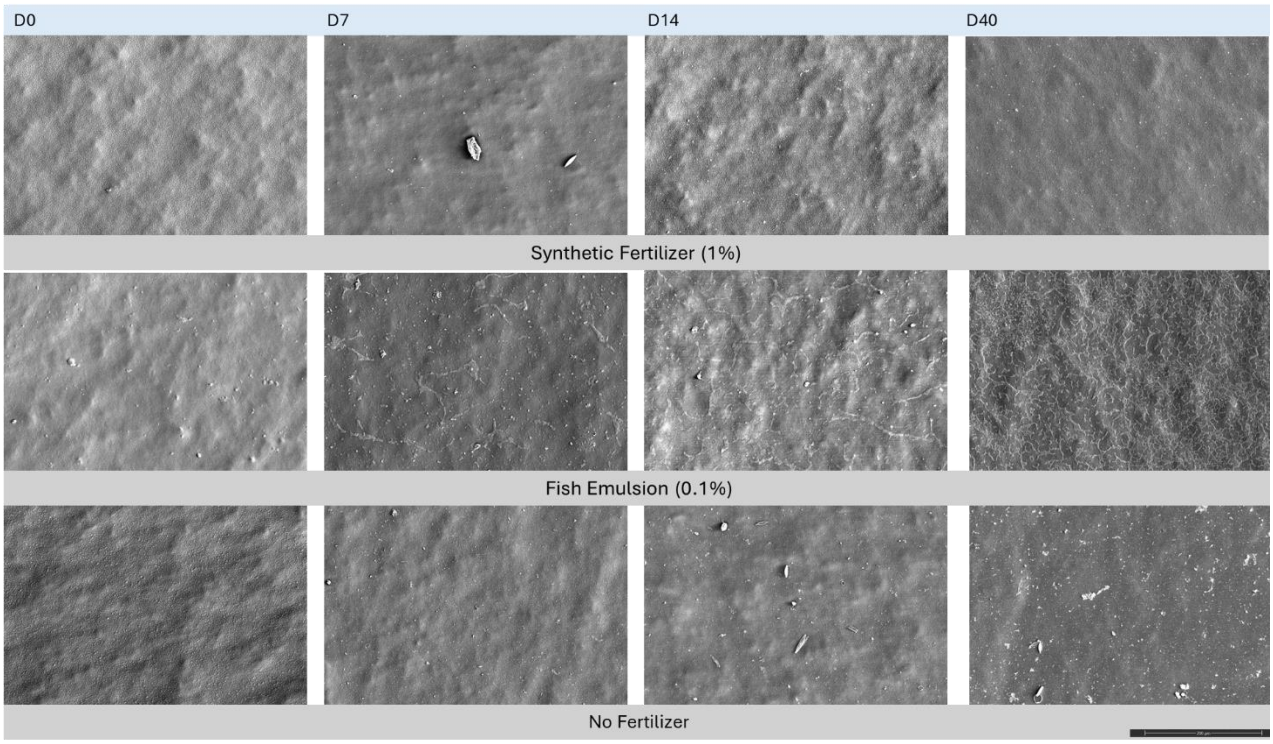
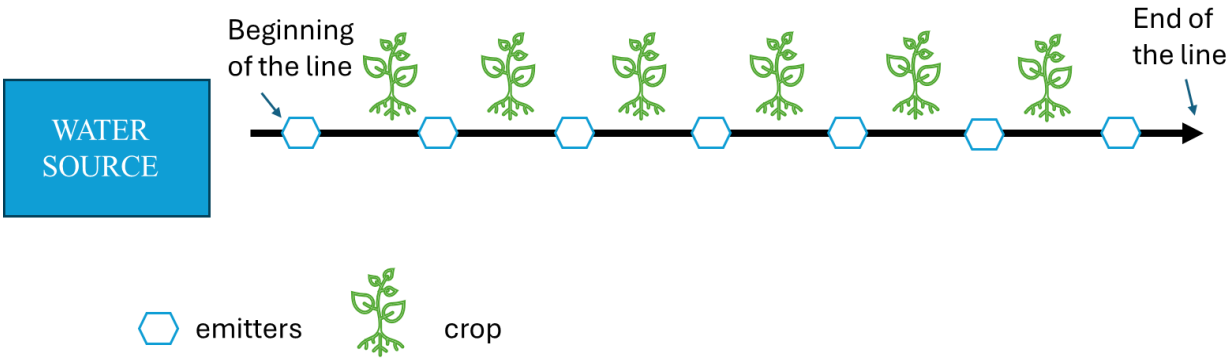


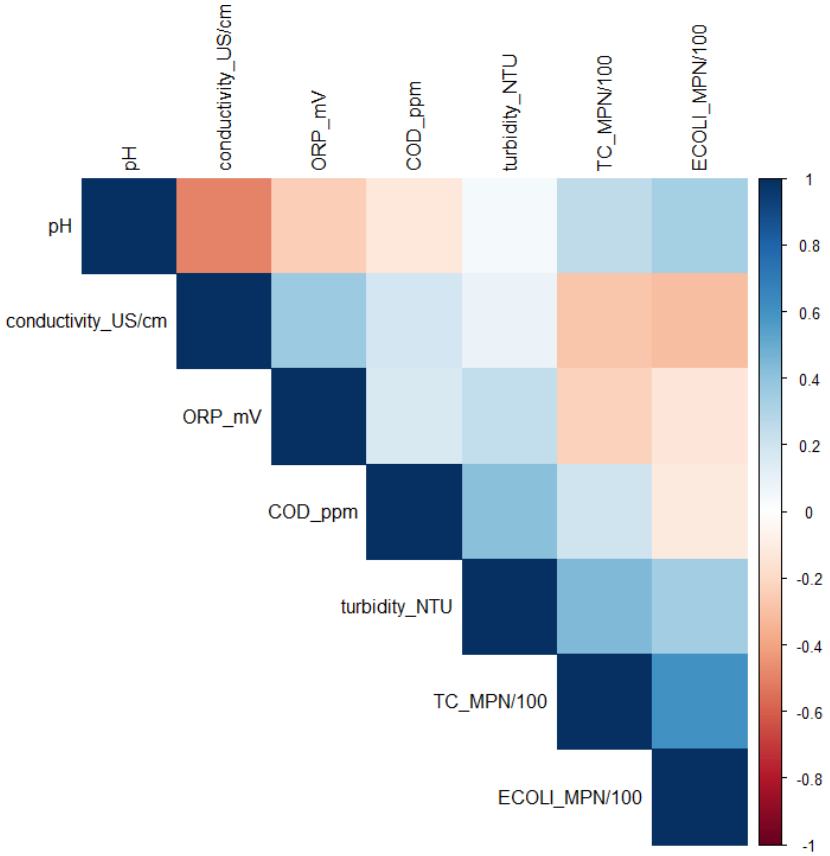
Figure 5.4: Scanning Electron Microscopy images of drip tubes (1.27 cm internal diameter - 0,25 cm^2) treated with pond water + 1% (v/v) synthetic fertilizer, pond water + 0.1% fish emulsion, and pond water only on days 0, 7, 14, and 40 with 20 parts per million of **chlorine** as a sanitizer treatment. Pictures taken at magnification of 800x Scale bar: 200 µm

APPENDICES

Appendix 3.1: Illustration of swabbing locations from drip lines comparison between emitter beginning and end points



Appendix 3.2: Spearman correlation plot across the different water quality parameters: pH, turbidity (NTU), oxidation reduction potential (ORP; mV), conductivity(µs/cm), chemical oxygen demand (COD; ppm) and microbial data: total coliforms (TC; MPN/100 mL), *E. coli* (MPN/100mL) collected from either surface water samples or water from the line.



Appendix 3.3: Illustration of an onset of algal bloom in a formerly active irrigation pond.

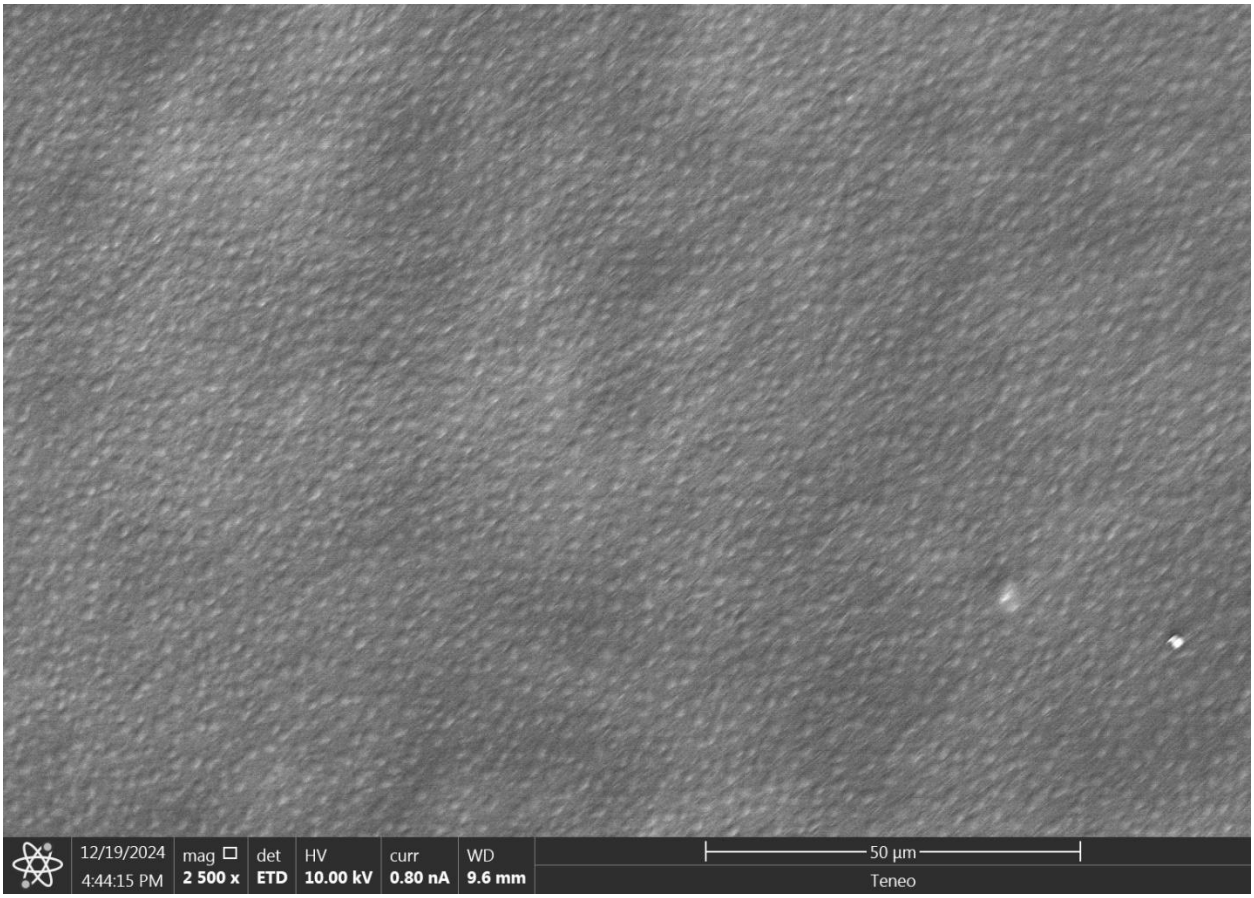
Appendix 4.1: Liquid fertilizer component analysis used for this study. Sample analyzed by Water Agricultural Labs Inc. 257 Newton Rd, Camilla, GA 31730-1653

Analyte	Result (%)
Nitrogen - Total	4.50
Nitrate - Nitrogen	4.18
Ammonium - Nitrogen	0.32
Urea - Nitrogen	0.00
P_2O_5 - Total	0.00
K_2O - Total	9.38

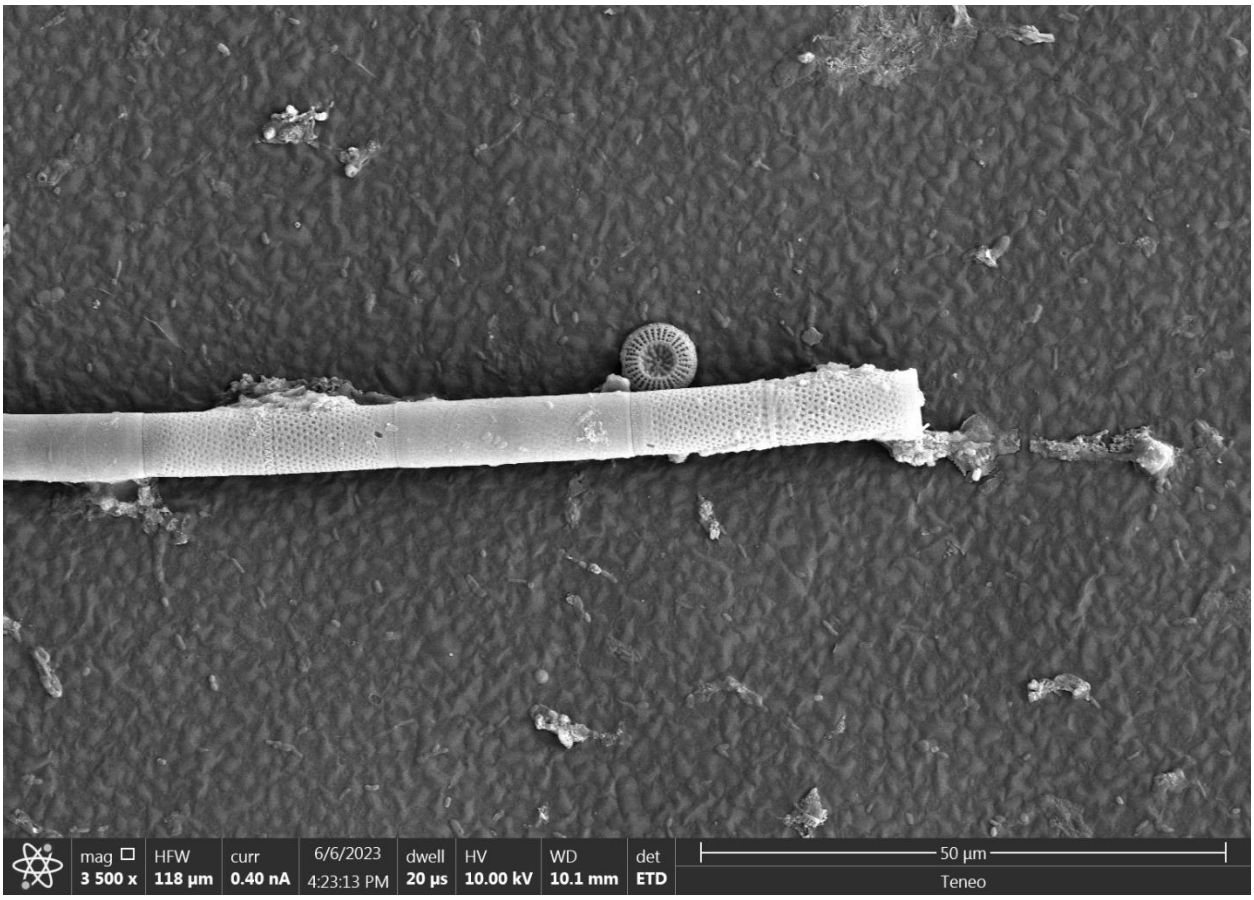
Appendix 4.2 Conductivity (µs/cm) and pH measurement of pond water only, pond water with (+) 1% synthetic 4-0-8 liquid fertilizer, or pond water with 0.1% fish 2-4-1 emulsion after 1 min and 5 min of injection. Conductivity and pH were measured using HQ4300 Portable Multi-Meter (Hach, Loveland CO, USA).

Sample	Contact time with water	pH	Conductivity (µs/cm)
Pond Water - Only	1 min	7.56	241
Pond Water + 1% of 4-0-8	1 min	7.13	5180
Pond Water +0.1% of 2-4-1	1 min	6.76	287
Pond Water + 1% of 4-0-8	5 min	6.89	5240
Pond Water +0.1% of 2-4-1	5 min	6.47	197.4

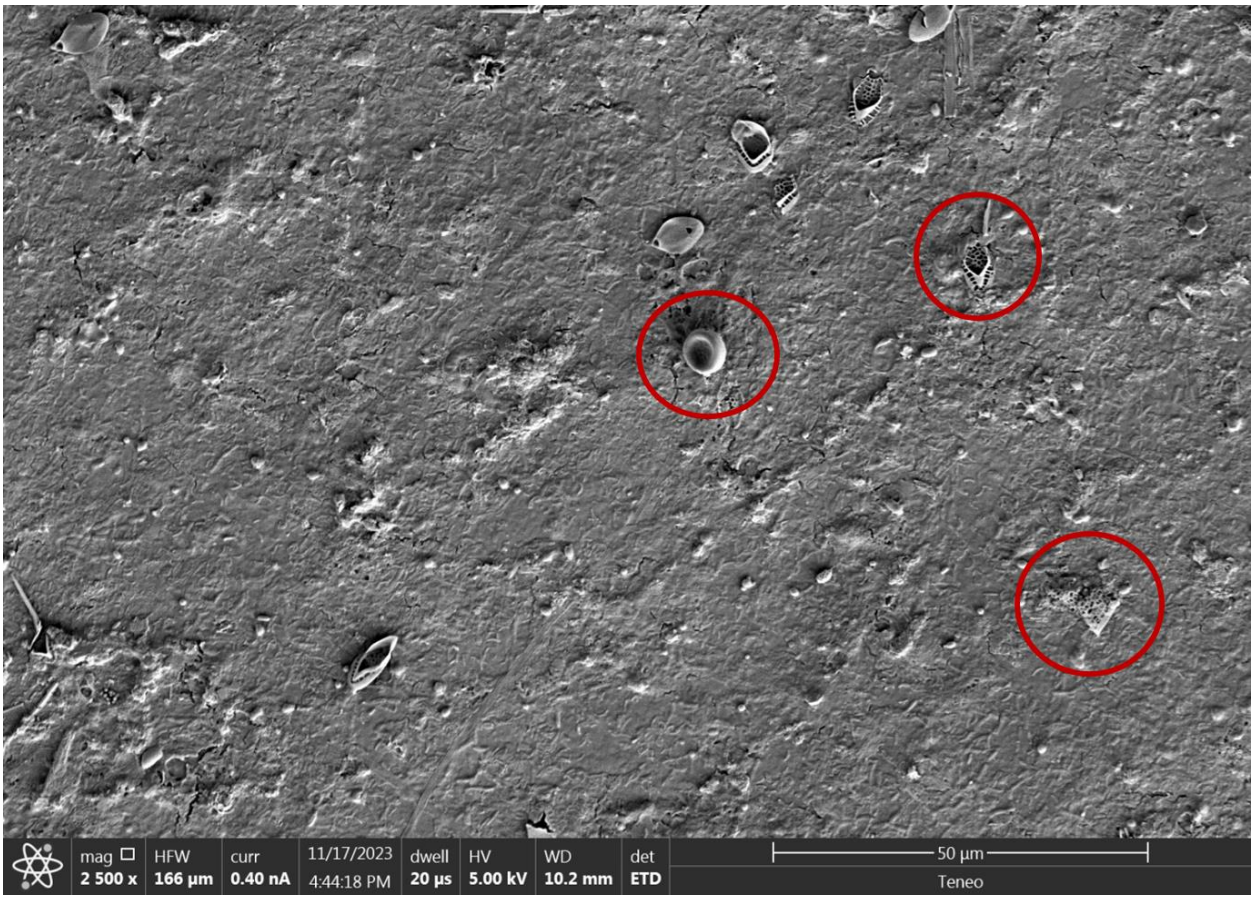
Appendix 4.3: Scanning Electron Microscopy images of the inside surface of a drip tube (0.25 cm^2) that has not been used for any irrigation or water event. Figure shows the natural curvature of drip tube polymers despite showing a smooth surface under the naked eye. Magnification 2500x.



Appendix 4.4: Scanning Electron Microscopy images of the inside surface of a drip tube (0.25 cm^2) when diatoms have adhered and attached along with the starting formation of a *Salmonella* biofilm surrounding it. Magnification 3500x.



Appendix 4.5: Scanning Electron Microscopy images of the inside surface of a drip tube (0.25 cm^2) when different shaped diatoms (circled) and bacterial biofilms have formed. Magnification 2500x.



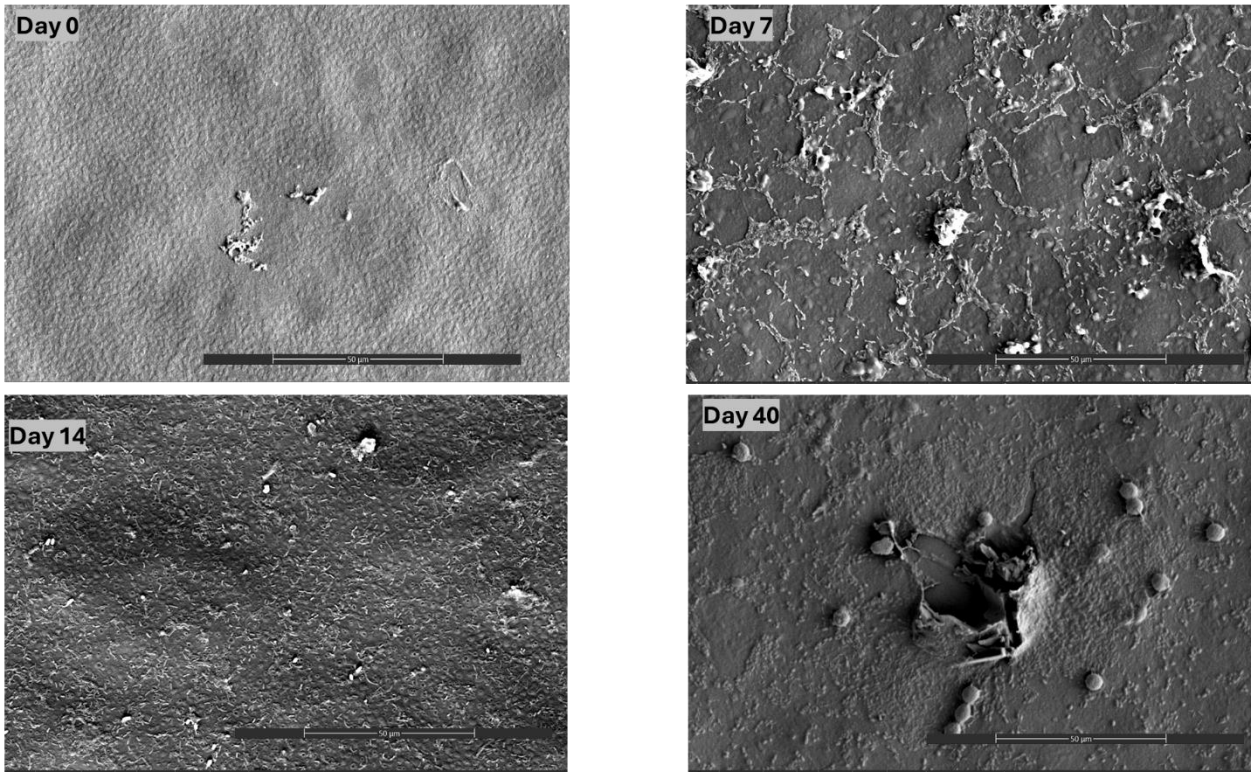
Appendix 5.1: Synthetic liquid fertilizer component analysis used for this study. Sample analyzed by Water Agricultural Labs Inc. 257 Newton Rd, Camilla, GA 31730-1653

Analyte	Result (%)
Nitrogen - Total	4.50
Nitrate - Nitrogen	4.18
Ammonium - Nitrogen	0.32
Urea - Nitrogen	0.00
P_2O_5 - Total	0.00
K_2O - Total	9.38

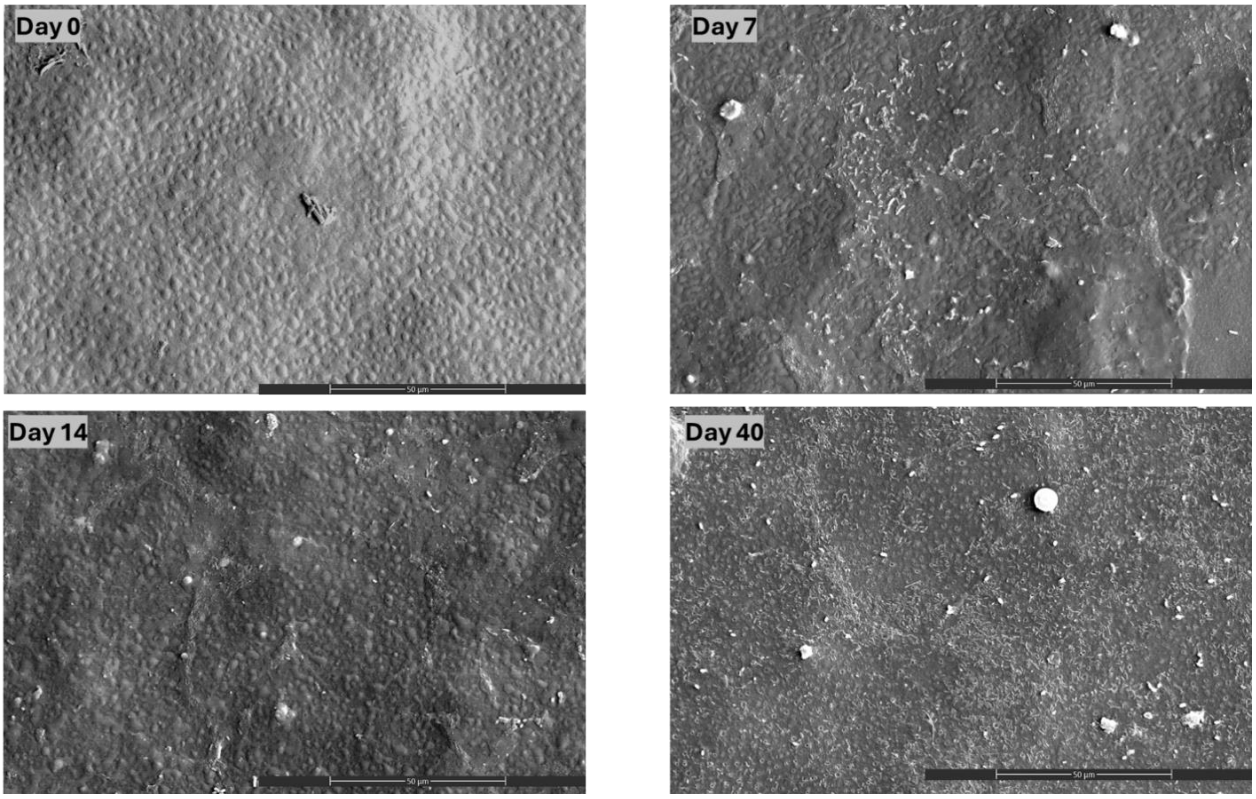
Appendix 5.2: Conductivity (µs/cm) of pond water only, pond water with 1% synthetic 4-0-8 liquid fertilizer, or pond water with 0.1% fish 2-4-1 emulsion after 1 min and 1 h of injection. Conductivity and pH were measured using HQ4300 Portable Multi-Meter (Hach, Loveland CO, USA).

Sample	Contact time with water	Conductivity (µs/cm)
Pond Water - Only	1 min	241
Pond Water + 1% of 4-0-8	1 min	5180
Pond Water +0.1% of 2-4-1	1 min	287
Pond Water + 1% of 4-0-8	1 h	5040
Pond Water + 0.1% of 2-4-1	1h	243

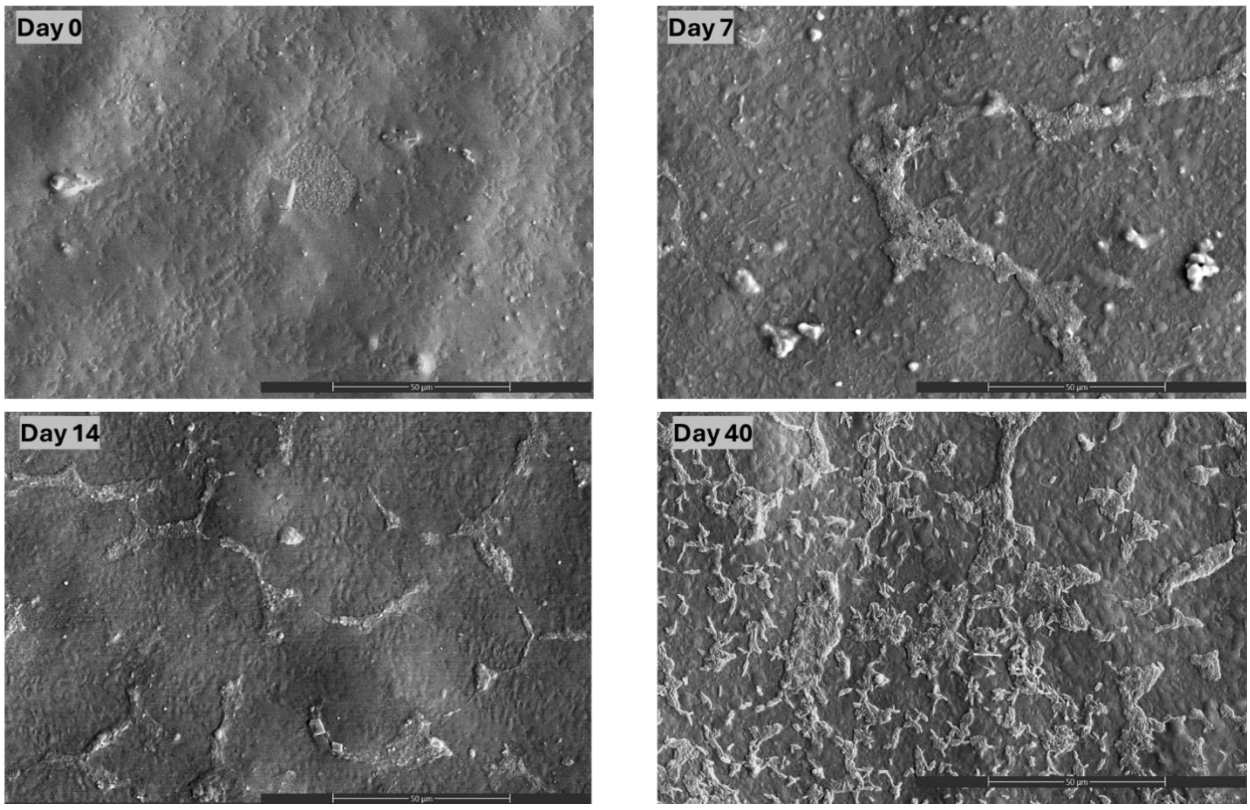
Appendix 5.3: SEM image of the inside surface of a drip tube (1.27 cm internal diameter – 0.25 cm^2) treated with pond water + 0.1% fish emulsion on days 0, 7, 14, and 40 **without sanitizer treatment.** Pictures taken at magnification of 2500x. Scale bar: 50 µm. Biofilm formation and growth is evident over time.



Appendix 5.4: SEM image of the inside surface of a drip tube (1.27 cm internal diameter – 0.25 cm^2)) treated with pond water + 0.1% fish emulsion on days 0, 7, 14, and 40 with **peracetic acid** treatment. Pictures taken at magnification of 2500x. Scale bar: 50 µm.



Appendix 5.5: SEM image of the inside surface of a drip tube (1.27 cm internal diameter – 0.25 cm^2) treated with pond water + 0.1% fish emulsion on days 0, 7, 14, and 40 with **chlorine** treatment. Pictures taken at magnification of 3500x. Scale bar: 50 µm



Appendix 5.6: Example Setup of polyethylene drip tubing (without perforations) and pump used for water circulation in a single treatment combination. PVC= polyvinyl chloride