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# Effect of Synthetic and Fish Emulsion Fertilizers in the Survival and Biofilm Formation of *Salmonella* in Irrigation Distribution Lines

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***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 injected with synthetic liquid (4N–0P–8K) or fish emulsion (2N–4P–1K) fertilizers. Drip tubing (without emitters) were filled with 100 mL of either pond water (PW) with no fertilizer (NoFert), 1% (v/v) synthetic fertilizer (SynFert), or 0.1% (v/v) fish emulsion (FishFert). The drip tubes filled with water were inoculated with 1 log colony-forming unit (cfu)/mL population of a rifampicin-resistant *Salmonella* mixture, and tubing was incubated at 21.1 °C for 21 days. Water was replaced with noninoculated 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 at each sampling day. From days 0 to 7, *Salmonella* populations in inoculated water increased by 4 log cfu/mL in FishFert ($P$ value = 0.0004), remained constant in water with NoFert, and decreased below the limit of detection (LOD: −1 log cfu/mL) with SynFert. Populations on the FishFert tubing increased by 6 log cfu/tube from days 0 to 7 ($P$ value = 0.001) and remained constant until day 21. For the NoFert treatment, the populations in tubing 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 SynFert for all sampling events. For the FishFert and NoFert treatments, cross-contamination from the drip tube to the next irrigation batch was observed along with the subsequent biofilm formation on the tubing. This work suggests that fertilizers and contaminated stagnant irrigation water can affect the formation of *Salmonella* biofilms in drip tubes and, in certain instances, can be a vector for cross-contamination in subsequent irrigation events.**

Foodborne outbreaks related to consumption of fresh produce are a known risk to fruit and vegetable producers. For example, a 2008 outbreak of *Salmonella* Saintpaul linked to 1442 cases was traced by the US Food and Drug Administration (FDA) to contaminated jalapeño peppers. The FDA identified the outbreak strain in a holding pond used for irrigation, confirming the source of contamination (Centers for Disease Control and Prevention 2008). Other outbreaks linked to fresh produce include the *Salmonella* Newport (US Food and Drug Administration 2020b) and *Salmonella* Oranienburg (US Food and Drug Administration 2021), which caused around 1000 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 2024). 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 United States, 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 and Odumeru 2004; Van Haute et al. 2020). Further, pathogens such as STEC and *Salmonella enterica* sources can be internalized by growing fruits and vegetables (Cooley et al. 2003; Deering et al. 2012; Solomon et al. 2002). However, this phenomenon remains debated, with evidence suggesting that internalization is highly dependent on the type of produce, inoculum level, and environmental conditions (Coleman et al. 2017; Erickson et al. 2010). Multiple studies have reported the occurrence of *Salmonella* and other foodborne pathogens in water that could be used for growing crops (Acheamfour et al. 2021; Chevez et al. 2024; Gorski et al. 2022; Gu et al. 2020; Li et al. 2014; Micallef et al. 2012; Murphy et al. 2023; Truitt et al. 2018). Groundwater (e.g., wells) can also become contaminated for reasons such as improper sealing and failure of backflow prevention devices or can 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 (bare ground 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 used for fertigation, although many mixtures may contain macronutrients such as calcium or sulfur and micronutrients such as zinc or iron (US Environmental Protection Agency 2022). While most conventional farmers use synthetic fertilizers, organic farmers may use 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; US Department of Agriculture, Agricultural Marketing Service 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. Thus, this occurrence 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 toward more holistic approaches in managing pathogen contamination in agricultural settings.

## Materials and Methods

*Bacterial culture.* A four-serotype mixture of *S. enterica* was used in this study: *S. enterica* Enteritidis (2020 AM-1539, 2020 peach outbreak), *S. enterica* Newport (2020 AM-0919, 2020 onion outbreak), *S. enterica* Montevideo [American Type Culture Collection (ATCC) BAA-710, 1993 tomato outbreak], and *S. enterica* Poona (ATCC BAA-3139, 2010 cucumber outbreak). All strains were adapted to 80 ppm rifampicin and stored at −80 °C in glycerol stocks. Before inoculation, 10 µL of each strain was transferred consecutively three times and grown individually in tryptic soy broth with rifampicin (Difco, Becton Dickinson Co., Sparks, MD, USA) and 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: 250 µL 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 of buffered peptone water (BPW; Difco, Becton Dickinson Co.). Cells were dislodged with a cell spreader, and 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., Denver, 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 laboratory to be frozen at −20 °C until usage. Water was thawed in 4 °C 1 week before use. Pond water (PW) was injected either with 1% (v/v) synthetic liquid 4N–0P–8K fertilizer (SynFert; 4N–0P–6.64K; R.W. Griffin, Ty Ty, GA, USA; Supplementary Material 1) or with 0.1% (v/v) 2N–4P–1K fish emulsion (FishFert; 2N–2.2P–0.8K; Ocean Crest Seafoods Inc., Gloucester, MA, USA). PW with no fertilizers (NoFert) was tested as a control. Each type of water was inoculated with a 1 log colony-forming unit (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 d. The ends of each tubing were capped using 1/2-in figure-eight 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, and 100 mL of noninoculated 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 (Fig. 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 (Fig. 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, version 1.0; 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 0.45-µm membrane filters (Millipore-Sigma, Burlington, MA, USA) and plated on XLT4R. Populations were determined following incubation at 37 °C for 48 h, and the limit of detection (LOD) of water 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 (∼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 0.45-µm membrane filters (Millipore-Sigma) and plated on XLT4R at 37 °C for 48 h. The LOD of tubes was −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, 200 µL 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 500 µL of sterilized deionized water. Samples were kept at 4 °C until examination. Fixed tubes were then sputter-coated with gold at the following settings: working distance 12.5 mm, 60 s, 15 mA (SPI sputter coater; Structure Probe, Inc., West Chester, PA, USA). The tubing were later examined with a scanning electron microscope acceleration voltage of 500 V to 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: (1) enumeration by plating and (2) membrane filtration,

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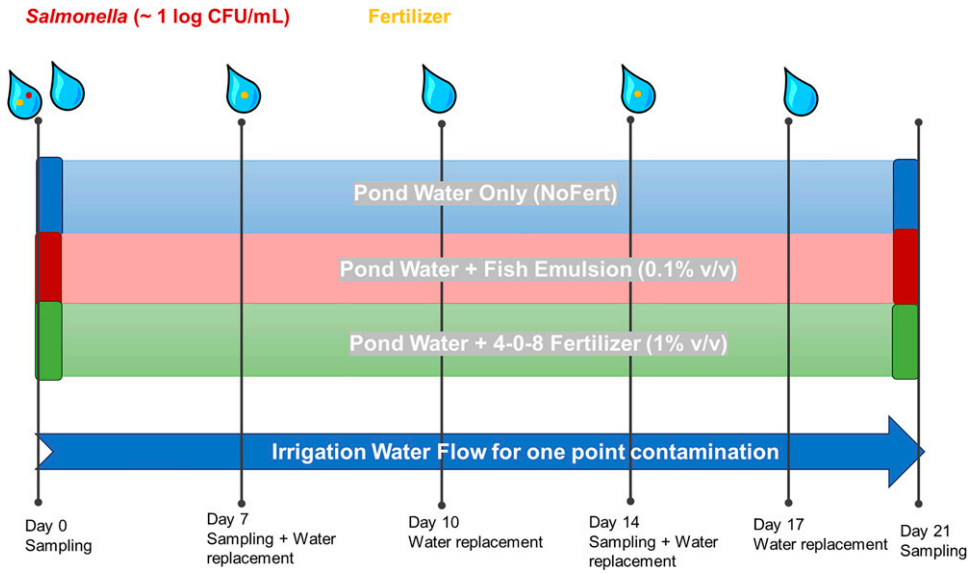


Fig. 1. Timeline of experimental procedure for drip tubing filled with 100 mL of pond water (PW), on day 0, with no fertilizer (NoFert), PW injected with 0.1% (v/v) fish emulsion, or PW injected with 1% (v/v) 4N–0P–8K synthetic fertilizer, during 21 d of sampling. Drip tubes were filled with 100 mL of inoculated water on day 0 and incubated for 7 d at 21.1 °C. Water (100 mL) was constantly replaced with noninoculated PW on days 7, 10, 14, and 17. Sampling of tubing and water took place on days 0, 7, 14, and 21. cfu = colony-forming unit.

simultaneously for the water inoculated, drip tubes, and water noninoculated for all samples at each time point. Counts determination followed guidelines from the Food and Drug's Bacteriological Analytical Manual for spiral plates use (Maturin and Peeler 2001a) and standard guidelines for filter enumeration (Maturin and Peeler 2001b). Based on the Shapiro–Wilk test, the distribution of the *Salmonella* populations across the different treatments was not normally distributed; therefore, the microbial enumeration data were log-transformed for statistical analysis with R version 4.3.3. 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. A $P$ value below 0.05 was considered significant. When *Salmonella* was not detected by plating or membrane filtration, a $P$ 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. The figures were created with ggplot2 in R version 4.3.3.

## 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 SynFert and FishFert ($P$ value = 0.001) and between SynFert and NoFert ($P$ value = 0.003) but not between the FishFert and the NoFert samples ($P$ value = 0.404). *Salmonella* populations were ∼1 log cfu/mL (Table 1) for the FishFert- and NoFert-treated samples, as expected. However, once the 4N–0P–8K fertilizer in the SynFert treatment was added, it decreased the population to 0.51 log cfu/mL. The population in the FishFert water significantly ($P$ value = 0.0004) increased from day 0 to 7 × 4 log cfu/mL (Table 1), while it decreased in the SynFert ($P$ 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 favored *Salmonella* growth. In contrast, the decrease in the SynFert-treated samples to undetectable levels implies that the synthetic fertilizer (4N–0P–8K) inhibited *Salmonella* survival overtime. This indicates that the 4N–0P–8K 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 4N–0P–8K 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 (Supplemental Material 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 et al. 2010; Rhodes et al. 2017; Stief et al. 2022; Zheng et al. 2023). $NO_2$ has been shown to have antimicrobial effects on *S. 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 ultraviolet A light, undergoes photolysis to generate reactive nitrogen species, 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 et al. 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 and Lymar 1997; McLean et al. 2010; Wang et al. 2022). For example, Hurst and Lymar (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, the microbial

Table 1. *Salmonella* population (log cfu/mL) mean ± standard deviation in pond water inoculated for 7 d, at different fertilizer conditions: 0.1% (v/v) fish (2N–4P–1K) emulsion (FishFert), 1% (v/v) liquid synthetic (4N–0P–8K) fertilizer (SynFert), or pond water only (NoFert).

| Day | FishFert | SynFert | 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 |

The limit of detection was −1 log cfu/mL (N = 9). Lowercase letters represent significant differences after 7-d incubation in drip tubes at 21.1 °C within each treatment. Uppercase letters represent significant differences across treatments for the same day.

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) 4N–0P–8K synthetic fertilizer in this study had greatly elevated the EC of the water at various times (Supplemental Material 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 (2N–4P–1K), respectively. When 1% of the synthetic liquid 4N–0P–8K 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 *S. Typhimurium* population decreased over a wide range of salt (NaCl) concentrations 0% to 8% (w/v). McEgan et al. (2013) reported the highly significant inverse correlation ($P = 0.0001$) with *E. coli* concentrations as the conductivity level increases. Elevated EC levels caused by the addition of the 4N–0P–8K 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 enhanced microbial diversity in soils when they are treated with organic fertilizers compared with 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 affect 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 1 d 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, although not completely as within the SynFert 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 different across sampling days. In FishFert, 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 (Fig. 2A). Figure 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 (Fig. 2A). Figure 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 of 9) of the SynFert tubing samples using at least one of the plating methods. The levels then decreased below the LOD from day 7 onwards for all the remaining samples

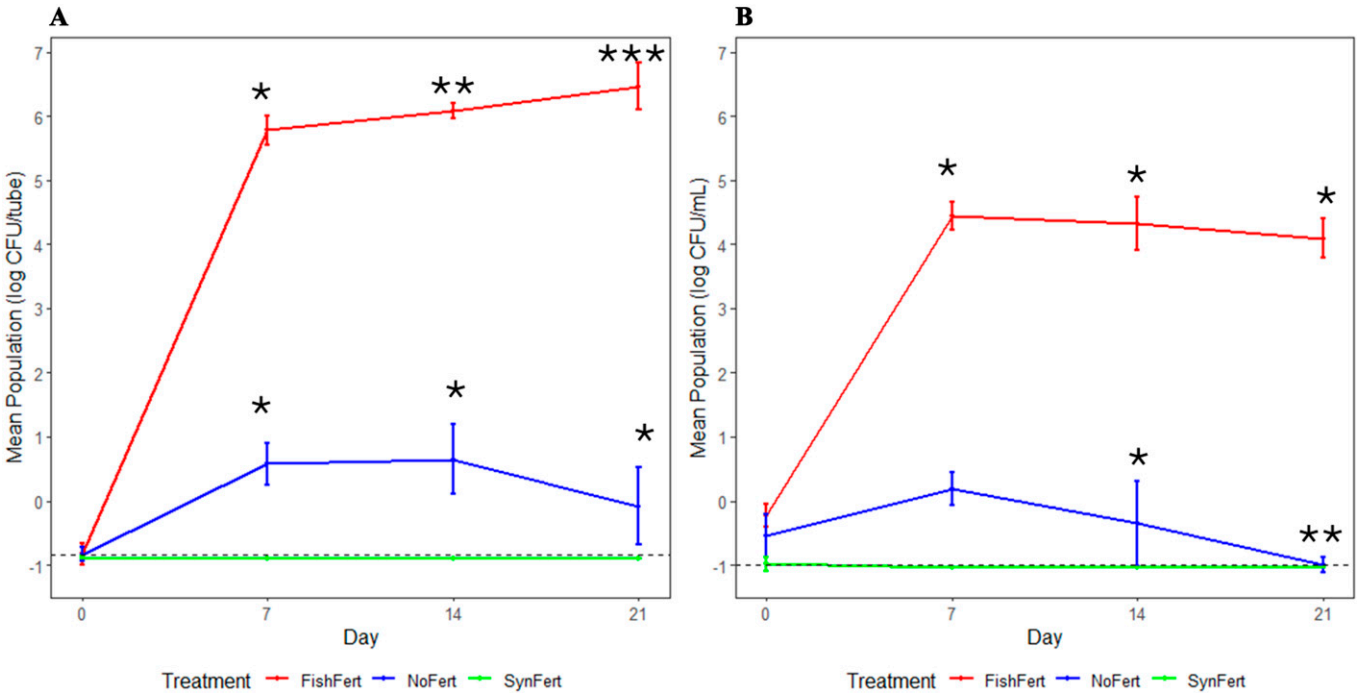


Fig. 2. *Salmonella* populations in drip tubing [1.27 cm internal diameter; log colony-forming unit (cfu)/tube] (**A**) and transfer of *Salmonella* to uninoculated pond water (log cfu/mL) (**B**) from day 0 to day 21. Samples were treated with either 0.1% (v/v) fish emulsion (FishFert), 1% (v/v) 4N–0P–8K synthetic liquid fertilizer (SynFert), or no fertilizer (NoFert). The 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 (LOD). LOD tube: −0.84 log cfu/tube, LOD water: −1 log cfu/mL.

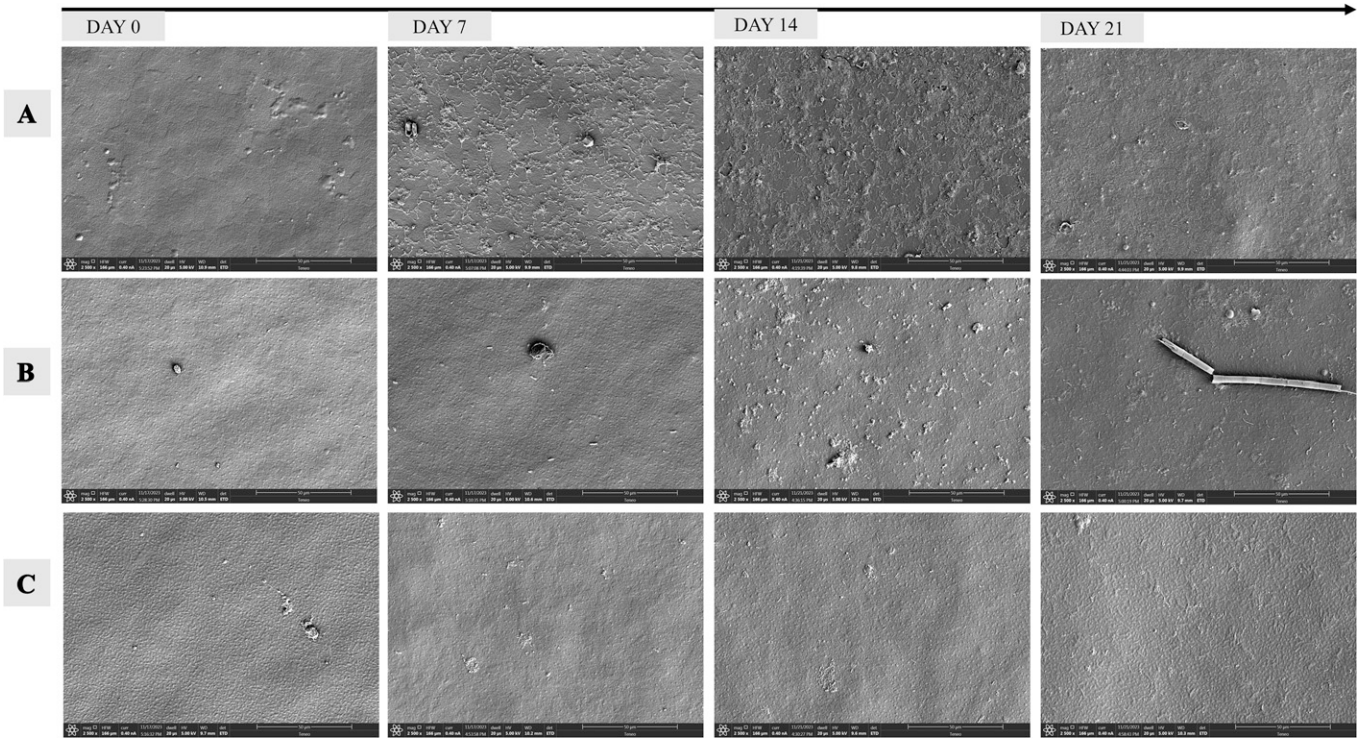


Fig. 3. Scanning electron microscopy images of drip tubes (0.25 $cm^2$) treated with pond water + 0.1% (v/v) fish emulsion (**A**), pond water only (**B**), and pond water + 1% (v/v) 4N–0P–8K synthetic liquid fertilizer (**C**) on days 0, 7, 14, and 21. Magnification, 2500×. Scale bar = 50 µm.

(9 of 9) (Fig. 2A). The SEM images in Fig. 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 FishFert-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 microirrigation systems, leading to bioclogging in emitters. Few studies highlight how emitter design and water quality affect biofilm development, which can result in clogging and reduced irrigation efficiency (Batte et al. 2003; Fu et al. 2021; Gamri et al. 2014). Biofilms are found on moist surfaces such as water pipelines (Batte et al. 2003; Chan et al. 2019; Gamri et al. 2014). The release of biofilms in PVC pipes within drinking water distribution systems (Batte et al. 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, in which planktonic cells (free-floating) adhere loosely to the surface (Flemming et al. 2007; Palmer et al. 2007). This is followed by irreversible attachment, in which 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 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 affect the microorganisms' survival. Figure 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 with 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 SynFert drip tubes on day 0 and the later decrease to levels below the LOD for the remainder of the experimental days indicate the inability of *Salmonella* cells to form an irreversible attachment in the tubing when water is injected with the 4N–0P–8K synthetic fertilizer used in this study. The SEM images in Fig. 3C show very close similarity to a drip tube image with no treatment (Supplemental Material 3), confirming no bacterial attachment.

*Cross-contamination of noninoculated water.* When tubes were washed with a batch of noninoculated pond water with FishFert 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 FishFert treatments ($P$ value = 0.08), respectively. The

levels were below the LOD for the SynFert treatment; $P$ value = 0.001 and $P$ value = 0.01 for FishFert and NoFert water samples, respectively (Fig. 2B). In the FishFert-treated samples, *Salmonella* populations in the water at day 7 increased ($P$ value = 0.002) to reach 4.44 log cfu/mL, the population then gradually decreased to 4.33 log cfu/mL on day 14 and 4.09 log cfu/mL on day 21 (Fig. 2B). For the SynFert-treated samples, *Salmonella* populations decreased below LOD in the noninoculated PW compared with the FishFert and NoFert treatments. Levels in the SynFert noninoculated 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 and then decreased to −0.35 log cfu/mL on day 14. *Salmonella* was recovered from 33% (3 of 9) of the NoFert water samples using at least one of the plating methods on day 21 (Fig. 2B).

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 laboratory 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, 2023). For example, Chevez et al. (2024) reported that the overall prevalence of *Salmonella* in water samples was 6.81% (6 of 88) from water samples collected from Feb to Dec 2021 from eight irrigation ponds located in southwest Georgia, USA. Murphy et al. (2023) reported 21.7% (26 of 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 United States was 0.03 most probable number/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; US 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 Antaki et al. (2014), have reported that in one of the ponds sampled, *Salmonella* was detected in 8.3% (3 of 36) of water samples. Notably, in the associated drip irrigation system, 19.4% (14 of 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 et al. 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 (Supplemental Materials 4 and 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 in 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; González-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 4-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 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.

## Conclusions

This study compared the effects of no fertilizer, synthetic fertilizer (4N–0P–8K), 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 d. Synthetic (4N–0P–8K) 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 d of sampling. This highlights the distinct impact of using fish hydrolysates with promoting biofilm formation and microbial survival in the irrigation line. To prevent initial microbial attachment associated with fish emulsion use, its application should be carefully managed within irrigation lines. Mitigation strategies such as flushing with approved sanitizers should be implemented immediately following exposure. Further field-based studies are needed to appropriately inform and optimize mitigation strategies under practical agricultural conditions. Based on the overall 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. For example, static water in the line in increased volumes and the inoculation with high bacterial concentration (1 log cfu/mL) do 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. Finally, it is important to recognize that freeze–thaw cycles could influence microbial

activity and alter community composition. These changes could have affected the background microbiota and nutrient conditions in this study, potentially introducing variability. Addressing these factors will provide a more comprehensive understanding of microbial survival and biofilm formation in irrigation water distribution settings.

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