Evaluation of *Escherichia coli* and Coliforms in Aquaponic Water for Produce Irrigation

by

Jennifer Mae Dorick

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Approved by

Tung-Shi Huang, Chair, Professor, Poultry Science
Daniel Wells, Assistant Professor, Horticulture
Emefa Monu, Assistant Professor, Poultry Science

Abstract

With the increase of commercialized aquaponics, concerns associated with pathogens in aquaculture water transferring to the produce have increased. The FDA Produce Safety Rule states water used for irrigation purposes that is likely to come into contact with the edible portion of the fruit and vegetables must not exceed a defined limit of *Escherichia coli* in the water. It requires a geometric mean (GM) and a statistical threshold (STV) of 126 or less and 410 CFU or less of generic *E. coli*/100 mL of irrigation water, respectively. Even though aquaponics has not been included in this guideline, it creates a baseline for aquaponic facilities to reference if monitoring the water.

A one-year evaluation was completed to identify points in the aquaponics system in which the microbial profile changed and to determine whether the water used on produce followed the FDA Produce Safety Rule. Water was sampled and analyzed at six points in the system in which the *E. coli* and coliforms profile was likely to change. The GM and STV were calculated based on the irrigation source, determining the water collected from February 1 to May 31, 2019 had *E. coli* populations below the FDA limit and from June 1, 2019 to January 31, 2020, the *E. coli* populations were above the FDA limit. From this study it was concluded that from June to January water must be monitored more closely in an aquaponics system to ensure safety of the produce.

A microbial analysis was performed on a nutrient film technique (NFT) system using aquaponic water over an initial 16-d growth cycle of butterhead lettuce. Three sump tanks contained aquaponic water and one contained a hydroponic control that was applied to the lettuce roots continuously. Water samples were collected on d 0, 4, 8, 12, and 16 followed by microbial

isolation for *E. coli* and coliforms. The *E. coli* and coliforms populations decreased as holding time increased and the *E. coli* population was within the FDA Produce Safety Rule on d 8. From these results, in order to ensure proper reduction of *E. coli*, the water must be held for at least 8 d and can be help up to 16 d before changing the water out.

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List of Abbreviations

AE	Aquaculture effluent
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
DO	Dissolved oxygen
DWC	Deep water culture
EPA	Environmental Protection Agency
FDA	United States Food and Drug Administration
FSMA	Food Safety Modernization Act
GAP	Good agricultural practices
GHP	Gallons per hour
GM	Geometric mean
PW	Peptone water
NFT	Nutrient film technique
STV	Statistical threshold value
USDA	United States Department of Agriculture
VRBA	Violet red bile agar

Introduction

Aquaponics is a sustainable integration of aquaculture and hydroponics using nutrient rich effluent from aquaculture to fertigate produce production (Goddek et al., 2016). The expansion and manipulation of aquaponics to fit each farmer's needs, systems, and designs have evolved over the years. An aquaponics system can consist of a coupled or decoupled structure and produce can grow with or without a medium. A coupled system is when the water is returned back to the fish following plant irrigation and a decoupled system is when the water does not return back to the fish following plant irrigation. More specific designs include open aquaponics, domestic systems, demonstration aquaponics, commercial aquaponics, and large-scale systems (Goddek et al., 2016; Palm et al., 2018)

The nutrients in aquaculture byproduct are similar to those added in the hydroponics system for produce production (Rakocy, 2012). In a traditional fish tank, the removal of ammonia and solid waste excreted from the gills and feces is necessary, as high ammonia levels are toxic to fish. Ammonia can easily build up to toxic levels if not removed, but in a recirculating system, the toxic ammonia is removed and microorganisms in the byproduct convert ammonia to nitrite and eventually to nitrate, the preferred form of nitrogen for produce to grow (Rakocy, 2012).

Aquaponics provides several benefits that a separate aquaculture or hydroponic unit could not utilize. Nutrients dissolved in aquaculture water would normally be discarded, but aquaponics transports this byproduct to plants extending the usage of the water which reduces water exchange rate reciprocating reduced operating costs, cost of monitoring water quality, and daily feed replaces lost nutrients in the water. In a hydroponic unit, water would need to be

exchanged as nutrients deplete, but in an aquaponic system, the nutrients are continuously replenished (Rakocy, 2012).

Chapter I: Literature Review

Food Safety Concerns Associated with Aquaponics

1.1 Introduction

There are concerns related to food safety with aquaponics due to limited research, but it has slowly increased in the past 5 years (Stivers, 2016). In 2019, Canada had concerns with produce safety in aquaponics, therefore farms are unable to obtain certifications through the CanadaGAP program due to the lack of food safety information with the potential of up taking foodborne pathogens and chemical hazards (CanadaGAP, 2019). The CDC estimated that 48 million people got sick, 128,000 hospitalized, and 3,000 died from foodborne diseases each year in the United States with produce attributing nearly half (46%) of illnesses and 23% of deaths from 1998 to 2008 (CDC, 2019; Painter et al., 2013). Concerns with aquaponics stem from the uncertainty of potential sources of contamination as it is difficult to pinpoint where in the system foodborne pathogens enter, making it difficult to come up with methods to reduce contamination. This includes but is not limited to water sources, humans, fish, environment, and equipment. Produce can become contaminated with pathogenic microorganisms by contact with soil or improperly composted manure, irrigation or post-harvest washing with contaminated water, or contact with infected food handlers (Beuchat and Ryu, 1997).

Produce irrigation utilizes a plethora of water sources, including ground water, surface water, rainwater, and municipal water (Lennard, 2017). Each one has a different concern in relation to foodborne pathogens contaminating the produce, and many food outbreaks have been linked to the water sources used on plants during growth. This concern has been addressed in the

Food Safety Modernization Act (FSMA) Produce Safety Rule developed by the FDA (FDA, 2019b). The FDA identified origins of potential contamination in agriculture including the use of water and soil amendments, work training and hygiene, and equipment and sanitation practices used in production for fruits and vegetables (Steele and Odumeru, 2004). Through the monitoring of indicator microorganisms in irrigation water, it can assist in the prevention of the likelihood of foodborne pathogens coming into contact with the produce.

Coupled aquaponics is a potential concern because the water is never released from the system, providing a perfect environment for continuous microbial growth. Currently, there are no specific food safety guidelines established about aquaponics systems possibly due to the lack of research and therefore the FDA Produce Safety Rule can be used as a reference if the water is likely to come into contact with the edible portion of the produce and the farm has at least an annual monetary value of $25,000 (Stivers, 2016). More recently, the USDA established a pilot Aquaponic Good Agricultural Practices program as a trial to test whether aquaponics units can be certified under the Harmonized Produce GAP or Harmonized GAP Plus+ audit effectively (AMS, 2020). The purpose of this review is to have a deeper understanding of the three components of aquaponics including fish, plants, and water, and how food safety can impact each component. This is followed up with how the FDA Produce Safety Rule can impact aquaponics and possible mitigation methods utilized to ensure produce safety.

1.2 Aquaponic Fish

One component of aquaponics is aquaculture, in which fish break down feed and excrete nutrients for plants utilization. Some systems utilize the fish in addition to harvesting produce as a source of income while other systems are at a low stocking density to provide nutrients to the

plants and the fish are not harvested for a profit. There have been many types of fish utilized in aquaponics and found successful based on various factors including climate and demand. Some fish species currently used in aquaponics are Nile tilapia (*Oreochromis niloticus*), hybrid tilapia (*Oreochromis urolepis hornorum and Oreochromis mosambicus*), koi carp (*Cyprinus carpio*), hybrid carp (*Ctenopharyngodon idella and Aristichthys nobilis*), hybrid striped bass (*Morone chrysops* and *Morone saxatilis*), and goldfish (*Carassius* spp.) (Selock, 2003). Rainbow trout (*Oncorhynchus mykiss),* Australian barramundi (*Lates calcarifer*), and Murray cod (*Maccullochella peelii peelii*) as well as crustaceans such as red claw crayfish (*Cherax quadricarinatus*) have also been utilized in aquaponic systems (Adler et al., 2000; Diver and Rinehart, 2000). In a national survey, 55% of fish raised in aquaponic farms were tilapia and 48% of fish were ornamental, koi, goldfish, and tropical fish; 27% of farms raised two species of fish, 18% raised three or more species of fish, and 81% of farms preferred raising at least one edible species of fish as it provided an income source (Love et al., 2014). Tilapia is the most commonly used fish as they are able to survive in poor water qualities, handle easily, and grow in high densities (Love et al., 2014; Popma and Lovshin, 1996). Since tilapia is the most commonly used fish, the majority of research has been performed on it. Consequently, there is limited research using crustaceans or other fish and their impacts on the system (Love et al., 2014). As there is a vast number of fish species used in aquaponics, it is difficult to conclude that aquaponics overall is safe from pathogens introduced by fish as some are more likely to store enteric microorganisms in their intestines for extended periods of time.

1.2.1 Freshwater Fish Associated with Foodborne Pathogens

Freshwater fish historically have not been associated with human foodborne pathogens, but recently there have been concerns of foodborne pathogen with freshwater fish that could potentially increase pathogens in water for aquaponic purposes (Wang, 2020; Greenlees et al., 1998). Bacterial pathogens are associated with environmental factors such as temperature, water salinity, organic and inorganic load in water, and stocking density (Greenlees et al., 1998). Tilapia is a cold-blooded animal and therefore does not have similar gut microflora as warm bodied animals. Warm bodied animals often harbor *E. coli*, commonly nonpathogenic, in their natural gut microflora and excreted through feces (Al-Harbi, 2003; WHO, 2018). The common gut microflora of Nile tilapia *Oreochrmis niloticus* was found to consist of *Aeromonas hydrophilia*, *Chromobacterium violacem*, *Escherichia coli*, *Flavimonas oryzhaitns*, and *Plesiomonas shigelloids* in a semi intensive system (Molinari et al., 2003). There has been an association with water contaminated with *E. coli*, *Staphylococcus* spp., *Salmonella* spp., and *Vibrio* spp., which leads to high populations of these enteric pathogens in the intestine of tilapia (Marie Kaktcham et al., 2017), but zero or low populations in the flesh of the fish (Mhongole et al., 2016).

A preliminary study was performed on an aquaponics facility, no detectable *E. coli* O157: H7 or *Salmonella* spp. was found in the muscle of freshwater tilapia (Fox et al., 2012). In São Paulo, Brazil, skin, gut, muscle, and fillets samples of market tilapia were tested for *Salmonella* spp. and *Staphylococcus* spp. There were no *Salmonella* spp. detected but *Staphylococcus* spp. were identified, containing 1.0×10^2 CFU/g and 2.3×10^3 CFU/g in two samples (Junior et al., 2014). In a similar study, *Listeria* spp. and *Aeromonas* spp. were isolated from local market freshwater fish (sea trout, redfin perch, and European chub) on the gills and skin in Ankara, Turkey. Nine of 30 samples were positive for *Listeria* spp. with *Listeria monocytogenes* being

the most common and three out of 30 samples were positive for *Aeromonas* spp. (Yucel and Balci, 2010). *Aeromonas* spp., specifically *Aeromonas hydrophilia* have been commonly found in freshwater fish causing opportunistic illnesses with the fish and occasional cases of foodborne illness in humans (Smith et al., 2009). In a study performed on an aquaponics system, fish feces were found positive for STEC indicating fish are able to carry STEC from a contaminated source to a new system living in the intestines of the fish (Wang et al., 2020). These finding have caused a concern with aquaponics as even though pathogens have not been prominent in the flesh of the fish, they have been found in the gut microflora leading to contaminated water that can be passed through the system.

1.3 Produce in Aquaponics

Plants shown to thrive in aquaponics include tomatoes, cucumbers, peas, squash, lettuce, cabbage, peppers, and basil. There are three methods to growing aquaponic produce: drip irrigation, nutrient film technique (NFT), and deep-water culture (DWC) (Cooper, 1979; Palm et al., 2018; Saaid et al., 2013). Drip irrigation method emits water at a specific time onto plants through rate-controlled drippers using a computerized system. Produce is often grown in Beto or Dutch buckets, which are buckets containing a medium and a drainage hole positioned in the bottom of the bucket to prevent overwatering (Palm et al., 2018). Produce that grows with drip irrigation are often vining crops like tomatoes and cucumbers and bush crops like peppers, squash, and beans. NFT is a method in which plants are placed in a channel and their roots are submerged in a shallow stream of recirculating water (Cooper, 1979). DWC consists of 20-30 cm deep tank(s) constructed and waterproofed with polyethylene film. The tank is filled with water and floating rafts, constructed of foam, are placed on top of the water (Saaid et al., 2013). The

tank can either recirculate water as a coupled system or hold the same water for the entire plant growth period and be removed once produce is removed (Maucieri et al., 2019). Lettuce and basil are common plants grown in NFT and DWC, but must be closely monitored due to the accessibility of water in conjunction to the edible plant leaves. If the water contains pathogens and comes into contact with the leaves, bacteria can flourish within the leaves of the lettuce (Elumalai et al., 2017; Steele and Odumeru, 2004). In this section there will be an expansion on the association of plants and plant systems with foodborne pathogens.

1.3.1 Produce Production Associated with Foodborne Pathogens

Fruits and vegetables are an excellent harborage site for microorganisms due to available high nutrient concentrations and the ease of microbial attachment on the exterior (Yadav and Chugh, 2016). There has been a shift in common foodborne pathogens associated with specific foods because of market globalization, increasing consumption, aging population, and possibly changing climate (Salazar et al., 2016; Tirado et al., 2010). Common sources of foodborne pathogens in aquaponics on produce are based on the method produce is grown; such as contamination from water, soil, biological amendments, wild animals, and human contact (Goodburn and Wallace, 2013; Martinez-Vaz et al., 2014; Nuesch-Inderbinen and Stephan, 2016; Olaimat and Holley, 2012; Warriner et al., 2009). Since produce is often consumed as a ready-to-eat (RTE) product with no cook step and if prevention is not implemented, such as GAP procedures, foodborne pathogens are likely to contaminate the produce and in the end, intervention must transpire (Nuesch-Inderbinen and Stephan, 2016). Common foodborne pathogens in produce include Shiga toxin producing *E. coli* (STEC), *Salmonella* spp., *Listeria monocytogenes*, but outbreaks with Norovirus, *Vibrio* spp., *Shigella* spp., *Giardia*,

Cryptosporidium, *Cyclospora*, *Toxoplasma gondii*, and Hepatitis A virus have also been associated with produce (CDC, 2019; Johnson, 2019; Murray et al., 2017).

There are a few studies that have been performed on the common foodborne pathogens found in an aquaponic system. This allowed researchers to understand which foodborne pathogens could potentially cause a risk to human health in a commercialized aquaponic system. In a study on one system, produce was sampled over a year and found that *E. coli* O157:H7 and *Salmonella* spp. were negative or below the detectable limit of the assay. The population of generic *E. coli* were at low levels, <3.0 *E. coli* MPN/25 g (Fox, 2012). In another study of comparing foodborne pathogens on lettuce grown in aquaponic, soil, and hydroponic settings, found *E. coli* was <10 CFU/25 g and no data of *Listeria* spp. was reported. (Barnhart, 2015). From these two studies, the likelihood for produce to contain pathogenic microorganisms is low and GAP procedures are suggested in producing safe produce.

1.3.2 Plant Internalization of *E. coli*

There is controversy as to whether plants are able to internalize foodborne pathogens through their roots, and some recent studies have been completed to explore this issue. There are several factors such as produce type, cultivar, physiological state of the plant, and type of pathogen that influence the colonization on or in produce (Critzer and Doyle, 2010). Natural microflora, *Pseudomonas fluorescens*, *Aeromonas hydrophila,* and *Pseudomonas fluorescens*, of minimally processed produce, like lettuce, was inhibitory to *E. coli* O157:H7, *Salmonella montevideo*, *Listeria monocytogenes*, and *Staphylococcus aureus* as many isolates had inhibitory activity against all four pathogens (Schuenzel and Harrison, 2002). In a study on the internalization of *E. coli* O157:H7 in cucumbers, it was found that once fruit came in contact

with *E. coli* O157:H7, the cucumber stored *E. coli* in the stomata and wrinkles on the epidermis of the cucumber (Sun et al., 2019). Additional studies have shown that pathogens are able to enter into plant if there is a puncture or opening, but the main concern in aquaponics is whether plants are able to uptake foodborne pathogens through the roots affecting the produce as water could contain pathogens. Produce is likely to come in contact with pathogens through the roots, but if GAP protocol is followed the fruit is not likely to come into contact with pathogens (Critzer and Doyle, 2010).

E. coli O157: H7 was found to internalize within plant tissue and leaves. Macarisin et al. (2014) discovered hydroponically grown spinach is able to uptake *E. coli* O157:H7 when continuously exposed to this bacterium at 7 log CFU/mL but little internalization occurred at 5 log CFU/mL indicating that the internalization relates to microbial population. Frantz et al. (2007) found significantly greater *E. coli* O157:H7 internalization in soil than in a hydroponic system though the roots. An additional study found when high amounts of *E. coli* were introduced into the system, *E. coli* was present in the leaves after 1, 3, and 5 days (Takeuchi and Frank, 2000). It can be concluded from these three studies that in order for *E. coli* to enter into the plant system through the roots it must be at high populations and if internalized populations are very low inside the plant.

When *E. coli* is present in low populations, it is less likely for produce to uptake the pathogen. In an aquaponic system study, *E. coli* and coliforms were found in the system's water at less than 1 log CFU/ mL. At this *E. coli* population in the water, there was no detectable *E. coli* found in the lettuce indicating that there were not enough bacteria for roots to uptake (Moriarty et al., 2018). An additional study was conducted on an aquaponics system and lettuce, basil, and tomatoes, where STEC was present in the water, fish feces, and on the root surface but

was not found in the internal root, leaf surface, or internal leaf of lettuce and basil. Additionally, all the same areas were tested for *Salmonella* and *Listeria monocytogenes* and they were below the detectable limit (Wang et al., 2020). Aquaponic water is likely to contain low amount of *E. coli* but has not been found to internalize within the plant.

Plant microbiota interactions can play a critical role in colonization or inhibition of enteric pathogens in the rhizosphere and phyllosphere of fresh produce (Critzer and Doyle, 2010). Lettuce roots and leaves contaminated with *E. coli* O157:H7 were studied to analyze the interactions it had between two epiphytes, *Wausteria paucula* and *Enterobacter asburia* (Cooley et al., 2006). Competition was observed between *E. asburiae* and *E. coli* O157:H7 in the rhizosphere and determined both microorganisms utilized the same nitrogen and carbon sources in the rhizosphere; therefore, it was suggested that *E. asburiae* outcompeted *E. coli* in the soil. A different mechanism may exist between *E. coli* and *W. paucula* as commensalism was not observed in the rhizosphere or plant exudate (Cooley et al., 2006). There are many studies being conducted on this topic as each cultivar of a plant will react differently and contain different epiphytes in their rhizospheres causing them to act differently towards enteric pathogens.

1.4 Aquaponic Water

In an aquaponic system, water quality has a significant impact on food safety and nutrient concentration, as water is a highly variable input. If compromised, water quality would significantly change causing the death of fish, plants, and microflora. Water quality can be affected chemically, physically, or biologically and it needs to be monitored to meet the criteria of use. The water source has an effect on these factors, as it can contain high quantities of minerals impacting fish growth and microflora of the fish tank. Knowing the water chemistry can

support nutrient management and manipulation of the water for fish growth and produce production (Lennard, 2017). Monitoring biological content, specifically *E. coli*, is suggested by the FDA Produce Safety Rule as aquaponic water without solids is viewed as agricultural irrigation water and sampling is required if the water can come into contact with the edible produce eaten in raw, which is an indicator of being contaminated by foodborne pathogens (FDA, 2019; Stivers, 2016).

1.4.1 Pathogen Transmission Through Water

Agricultural irrigation water has been identified as a risk factor for fresh produce contamination with foodborne pathogens during production and especially in recirculating aquaculture systems (EFSA, 2014). Water utilized in an aquaponic system is often either ground water, municipal water, or surface water; rainwater is also often used as a supplemental source (Lennard, 2017). There has been recent investigation on foodborne pathogens in irrigation water on produce and its potential effects, specifically a study found lettuce and cabbage was contaminated with foodborne pathogens that was irrigated with sewage-contaminated water (Ackers et al., 1998; Ceuppens et al., 2015; Decol et al., 2017; Wachtel et al., 2002). Many foodborne pathogens thrive in water, as it provides nutrients, neutral pH, and high available water needed for metabolism and cellular function allowing bacteria, viruses, and protozoa to grow in the environment (Craun et al., 2003). According to the EPA, pathogens are the leading cause for contamination in 480,000 km of rivers and shorelines, and 2 million ha of lakes (EPA, 2010). A wide array of foodborne pathogens has been found in ground water environments including *Salmonella* spp., *E. coli* and other fecal coliforms, and *Staphylococcus aureus* whereas surface water environments have contained *Yersinia entericolitica*, *E. coli*, *Cryptosporidium*,

Clostridum perfringence, *Campylobactor* and *Salmonella* spp. The most common microorganisms found in ground water and surface water include *E. coli* and fecal coliforms (Pandey et al., 2014). Indicator organisms like fecal coliforms and generic *E. coli*, have been used to estimate pathogen loads in ambient bodies of water. But by using indicator organisms, it becomes difficult to identify the source of contamination and therefore prevention cannot be performed (Pandey et al., 2014). All three types of water sources, ground water, municipal water, or surface water, and are utilized in aquaponics, therefore monitoring the water is essential in identifying the likelihood of contaminating produce.

1.4.2 Water Sources Associated with Foodborne Pathogens

As mentioned in the previous section, many water sources used in an aquaponics include ground water, municipal water, surface water, and supplemented with rainwater. Ground water and rainwater are the most suitable in aquaponics as they are less likely to have high amounts of minerals that could impact fish growth and survival, while low in pathogens (Lennard, 2017; Rakocy et al., 2004). In a survey conducted on the water sources in aquaponics across the United States, 90% of the facilities use potable water, well water, or piped water due to its accessibility. Of those facilities, 39% of producers use drinking water supplemented with rainwater. Surface water was used by 8% of producers (Love et al., 2014; Rakocy et al., 2004). Other water sources can be used, but should be tested prior to usage to ensure they do not contain high amounts of minerals, salts, and pathogens (Lennard, 2004).

Aquaponic water requires a balance of microflora to transform ammonia while combating input of foodborne pathogens. Since surface water is open to the environment, it can become easily contaminated with foodborne pathogens carried by birds or mammal, this includes *E. coli*,

Salmonella spp., *Vibrio*, and *Shigella* (Cabral, 2010). By utilizing surface water, these microorganisms could contaminate the system producing unsafe produce for consumers. In addition to foodborne pathogens, water allows for high microbial loads of other microorganisms that could affect the overall fish tank microbiota and nitrogen cycle.

Studies have been performed on the pathogens in the water used for irrigation purposes. In one study, fecal microorganisms such as *E. coli* and presumptive positive *Salmonella* spp. were found in surface water and reclaimed water, leading to a risk factor to the produce (Lopez et al., 2008). Additionally, there was a multistate outbreak with tomatoes contaminated with *Salmonella* Newport in the United States due to contaminated irrigation pond water (Greene et al., 2008). When determining the irrigation water microbial quality application method should be kept in mind as water directly applied to the plant has been found to easily contaminate the produce, but when applies to specifically the roots, the water is less likely to contaminate the edible part (Xiao et al., 2015).

Ground water is defined by the EPA as rainwater or melted snow that travels through the ground and rocks and is stored in pores, just under the water table (EPA, 2018). Groundwater can become contaminated by polluted water which seeped into the ground. A hazardous substance can soak through soil and rocks, as dissolved contaminants can be carried along in water and are small enough to travel through soil and rocks. Once ground water is contaminated, it is difficult to remove contaminants because the water systems are often vast and it is challenging to identify points of contamination (EPA, 2018). It has been recommended that recirculating aquaponics utilize ground water as a lower amount of water is used in aquaponics and not released back into the ground. Traditional produce agriculture often over pumps ground water causing aquifers to become dry and runoff water from irrigation could contaminate the

water returned back into the ground. (Ehrlich and Harte, 2015). Recently, the EPA issued a Ground Water Rule (GWR) to monitor foodborne pathogens by collecting many samples of water to test for fecal contamination, but this new rule does not apply to ground water that is used for irrigation purposes as that water is monitored by the FDA Produce Safety Rule (EPA, 2006; FDA, 2019b).

Municipal water can be used in an aquaponic system but could have limiting factors. The high standards set by the EPA means pathogens are not a concern in this type of water (EPA, 2006). The apprehension with municipal water is the potential for high concentrations of chlorines and chloramines added to the water, which could cause fish to die and an improper pH for plant and fish growth; therefore, these chemicals must be removed before using this water (Sallenave, 2016). The chlorines and chloramines can be removed through evaporation, activated carbon, ultraviolet radiation, or sodium sulfite (Seegert and Brooks, 1978).

Surface water is defined as water in ponds, rivers, lakes and estuaries not soaked into the ground. It is riskier than ground water or municipal water for irrigation purposes as it can become contaminated with waste and wastewater from animals which commonly contain pathogens harmful to humans or fish (Truchado et al., 2018). Not only can surface water be high in harmful microorganisms, but its quality is variable and it is difficult to ensure consistency (Sallenave, 2016). Surface water used in aquaponics must be monitored closely due to these factors and many producers apply a treatment to reduce potential pathogens from contaminating produce (Love al., 2104; FDA, 2019b).

1.4.3 Aquaponic Water Treatment

The most popular method for controlling microorganisms of aquaponic water is UV, but ozone and hydrogen peroxide are also used for water treatment (Glaze et al., 1987). Water treatments are still being researched as there are many challenges to overcome for ensuring water safety while preserving the beneficial microorganisms and nutrients required for a successful aquaponic system. Through the USDA Aquaponics Agricultural Practices Pilot water likely to contaminate the edible portion of the produce must undergo a water treatment, such as UV treatment, chlorination, or ozonation (AMS, 2020).

Ozone is commonly used in the water industry as a method to disinfect drinking water. In 1975, the FDA recognized ozone treatment as a good manufacturing practice for the bottled water industry, with the minimum treatment concentration of 0.1 ppm of ozone in water solution in an enclosed system for at least 5 minutes (FDA, 2019c). Ozone is an unstable O^3 molecule which easily breaks down, deactivating microorganisms. Ozone disinfects water through direct reaction with the ozone molecule and indirect reaction with the radical species formed when ozone is decomposed in water (Glaze et al. 1987). Once ozone oxidizes the microorganisms, it transforms into oxygen resulting in a safe byproduct to the water and environment. Ozone can also be used as a hurdle with UV or hydrogen peroxide as a more effective of treatment at lower concentrations.

UV treatment is the most common form of water disinfection in aquaponics with the target microorganism being *E. coli*. UV light acts as an antimicrobial agent by penetrating bacteria and damaging its DNA to the point of inactivation. In an aquaponic system, UV treatment requires a balance of exposure to the water to inactivate *E. coli* but does not exceed exposure to the point of damaging beneficial bacteria that assist in the nitrogen cycle (Rico et al., 2007).

Moriarty et al. (2018) tested whether UV treatment had a significant effect as an antimicrobial on coliforms, that were often the indicators of fecal contamination and the presence of *E. coli*. When used in water with 90-95% transmittance, the UV light is able to deliver a dose of UV radiation between 180 mJ/cm^2 at 26 L/min and 30 mJ/cm^2 at 170 L/min. The results had a significant reduction of coliforms in the water used in an aquaponic system containing lettuce (Moriarty al., 2018). In an additional study performed by Elumalai et al., there was no significant difference between a UV and non-UV treated model aquaponics system in accordance to coliforms and aerobic plate counts, and there was no detected *E. coli* in either system (2017). The main concern with UV treatment in aquaponic water is whether UV is able to penetrate into the water. If there are too many solid particles in the water, it will inhibit UV light from being able to penetrate into the water for disinfection.

Chemical treatment such as chlorine or chloramine is common in disinfecting water used for municipal purposes (CDC, 2015). Similar treatment has been suggested to be used on the water in aquaponics systems by the USDA before applying it to the plant to kill potential pathogens in the water (AMS, 2020). This method cannot be used in a coupled aquaponics system as the chlorine and chloramine could be potential fatal to the fish but could be used in a decoupled aquaponics system at low concentrations as it could also be detrimental to the plants (Sallenave, 2016). Limited research has been performed on this method as there are many negative effects that could occur with the addition of chlorine. Hydrogen peroxide is a viable chemical that could be added into the water as it has been found to be effective in reducing to low amounts of *E. coli* (Glaze al., 1987). Hydrogen peroxide is often used together with other antimicrobials as a hurdle to increase the efficiency of other disinfectants (EPA).

1.5 Microbial Water Testing Parameters and Methods

As aquaponics is still a novel method for produce production, guidelines are limited on water quality monitoring. There has been much discretion as to how aquaponic water is viewed, either as biological amendment or agricultural irrigation water. If the solids are removed from the system, it can be determined that the water is no longer manure and therefore agricultural irrigation water (Stivers, 2016). The FDA created the Final Rule on Produce Safety as part of FSMA in 2016. It details a minimum standard for the microbial quality of water used for agricultural irrigation purposes (FDA, 2019b). As long as the water is not likely to come into contact with the edible portion of the produce it does not need to be monitored, but if water is likely able to splash on the edible portion of the leafy greens, the water must be monitored (Stivers, 2016).

In the FSMA Produce Safety Rule, states two criteria must be upheld. The water must contain no generic *E. coli* that would be transferred from direct or indirect contact with the produce, including water used on food contact surfaces, water used to directly contact produce during or after harvest, and water used for sprout irrigation. Therefore, untreated surface water cannot be used for any of these purposes. The second criteria being agricultural irrigation water that is directly applied to the growing produce (other than sprouts) must have a geometric mean (GM) of 126 or less and a statistical threshold (STV) of 410 or less CFU of generic *E. coli* per 100 mL of water (FDA, 2019b). If water does not meet this criterion, corrective actions must be made within one year. Generic *E. coli* must be measured throughout a year using one of the approved methods (FDA, 2019b). Ground water initially must be sampled four times in the first year and once annually thereafter and surface water initially must be sampled 20 times over 2 to 4 years and a minimum of 5 times after the standard has been set. This difference is due to the

higher risk and potential contamination of surface water compared to ground water. These rules are not as strict for water that is transported onto the plant through drip irrigation where it does not come into contact with harvestable portion of the plant, but there is no set limit for irrigation water used in this aspect (FDA, 2019b).

E. coli and coliforms are used as indicator microorganisms to indicate the likelihood of other pathogens being present in the water without the cost of testing an entire panel of pathogens. Total coliforms are commonly tested in drinking water, present in the environment, and generally harmless. Fecal coliforms are a subgroup of total coliforms and are found in the intestines and feces of humans and animals. *E. coli* is an even smaller subgroup of fecal coliforms, many strains are considered harmless, but a few can cause illness to humans through consumption (DOH, 2016).

In 2017, the FDA compiled a list of methods used to test *E. coli* and coliforms in the water which suggested using the EPA Method 1603: *Escherichia coli* in Water by Membrane Filtration Using Modified membrane-Thermotolerant *Escherichia coli* Agar (Modified mTEC), but also approved other EPA Methods including: 1103.1, 1604, 9213 D, 9222 B, D 5392-93 (FDA, 2019b). In our study, the EPA Method 1604 was found to work well and accurately for *E. coli* detection in aquaponics water, as there tends to have a large number of solids in the water and when used the EPA Method 1603, the solids hindered *E. coli* isolation.

1.6 Reducing Risks of Foodborne Pathogens in Aquaponics

The CDC estimates that each year, 48 million people get sick from a foodborne illness, 128,000 are hospitalized, and 3,000 die; the government has provided many guidelines and resources to reduce the risk before it leads to an outbreak (CDC, 2018). Reducing the risk of

produce contamination can be done though the Produce Safety Rule, food safety plan, and GAP in addition to water monitoring and treatment as mentioned in previous sections. A survey study was conducted in Hawaii on aquaponic growers in 2018 and it indicated that the growers were knowledgeable in food safety, as they were HACCP certified for fish production, but few were GAP certified and half of the participants showed unawareness of contamination sources, practices to prevent contamination, and ways to control it (Castro, 2019). Key areas that could cause potential spread of foodborne pathogens are as follows: human hygiene, harvesting produce safely, managing warm-blooded animal feces, water sources for fish and produce, zoonoses prevention, disposing of the systems wastewater, and all of which should be mentioned in a Produce Safety Rule, food safety plan, or GAP (Hollyer et al., 2009; Barnhart, 2015).

One of the major risks to produce is coming into contact with hands as they can transport harmful bacteria (Hollyer et al., 2009; Barnhart, 2015). Proper hand washing, gloves usage, and washing harvesting equipment should be performed any time in handling produce (Ovissipour et al., 2019). Using proper harvesting techniques is essential in ensuring a safe product, especially with produce that could potentially come into contact with water containing foodborne pathogens. Even though fish do not necessarily carry foodborne pathogens; pests, small rodents, and animals in the nearby environment may contaminate the water and produce. Proper control of wild pests through traps is important along with signage indicating no domestic animals are near or around the facility (Hollyer et al., 2009). As a preventative measure, many facilities have implemented UV sterilization on the water before it is applied to the plant system. This reduces the potential spread of pathogens from the fish to plants (Moriarty, 2018). All of these methods in addition to water monitoring will ensure the safety and quality of produce protecting consumers.

In 2020, the USDA Agriculture Marketing Service (AMS) Specialty Crop Inspection (SCI) announced a pilot audit program for aquaponic units (AMS, 2020). This would allow for aquaponic units to gain GAP certification for them to sell their produce to certain markets. During this pilot period of one-year, facilities can enter into the program if their facility meets the following criteria: water likely to come into contact with edible portion of the plant must undergo water treatment, water must be tested monthly, and facility must undergo standard operating procedures to prevent cross contamination from the fish to the plants (AMS, 2020). This Aquaponics Good Agriculture Practice Pilot will allow for an evaluation to be completed by the USDA to ensure produce can be produced in aquaponics in a safe manner without the contamination of pathogens from the water.

1.7 Conclusion

Aquaponics is still a novel method for growing fish and produce and with that comes many concerns on the safety of food being produced in this system. Though proper monitoring of the water and water source in addition to GAP and a food safety plan, it will provide enough support to the industry to ensure the produce is safe to consume. More research is needed to determine the validity of the safety of aquaponics, but through studies and surveys more information will assist the government to make appropriate decisions for producer or processors to produce food.

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Chapter II

Evaluation of *Escherichia coli* and Coliforms in Water Used in a Decoupled Aquaponics System

2.1 Abstract

There is a concern of foodborne pathogens, specifically *Escherichia coli*, transferring in an aquaponic system from water containing Nile tilapia to produce. Furthermore, there are few research studies performed on aquaponics in relation to food safety. The purpose of this one-year study was to identify the introductory points of *E. coli* and understand whether *E. coli* populations are within the limits of the FDA Produce Safety Rules for irrigation water. Over the one-year span of the experiment, four rounds of 14 cucumber plants and three rounds of 14 tomato plants were planted in perlite while being watered automatically in 30 min intervals for 3 min by aquaponic water. Water samples (250 mL) were collected every two weeks in triplicates from six locations within the system, for a total of 598 samples. Microbial isolations in the samples were performed using EPA Method 1604 with modifications and the filters were incubated on MI agar and VRBA for *E. coli* and coliforms identification, respectively. Temperature was measured and recorded from each sample immediately after sample collection. The water temperature throughout the entire system ranged between 12.6 °C and 32.8 °C with the average of 24.24 °C. The Produce Safety Rule requires a geometric mean (GM) and a statistical threshold (STV) of 126 or less CFU of generic *E. coli*/100 mL and 410 CFU or less of generic *E. coli*/100 mL of irrigation water, respectively. The GM and STV were calculated based on the irrigation source *E. coli* populations. From February 1 to May 31, 2019, the GM

and STV were below the FDA limits. From June 1 to July 31, 2019, the GM was below the limit and the STV was above the limit and from August 1, 2019 to January 31, 2020, the GM and STV were above the limit. The coliforms remained around the same population throughout the entire year. This study showed that there is a need to monitor *E. coli* populations more closely from June to January and correct the microbial quality of irrigation water if necessary.

2.2 Introduction

The agricultural industry is discovering novel ways to produce local, sustainable food in the safest way possible. An aquaponics system reduces start up, operating, and infrastructure costs of the aquaculture and horticulture sides thus reducing water usage and waste discharge to the environment (Tyson et al., 2011). While discovering more sustainable methods of producing food, food safety should be considered to ensure ready-to-eat (RTE) produce is unlikely to come in contact with potential foodborne pathogens. The CDC estimates 48 million people get sick, 128,000 are hospitalized, and 3,000 die from foodborne diseases each year in the United States with produce attributing nearly half (46%) of the illnesses and 23% of deaths from 1998 to 2008 (Painter et al., 2013). Major foodborne pathogens associated with produce are *Listeria monocytogenes, Escherichia coli* O157:H7, *Salmonella* spp., and Norovirus (Hu and Gurtler, 2017; Johnson, 2019; Painter et al., 2013). There are limited outbreaks associated with vining crops like cucumbers or tomatoes due to water being applied to roots and unlikely to come into contact with produce, but in 2013-2015 three outbreaks were associated with cucumbers and one outbreak in 2006 associated with tomatoes (CDC, 2020). Therefore, precautionary methods should be taken to prevent or reduce future outbreaks related to these foods.

In the past 40 years, the number of small-scale aquaponics facilities has increased, as it creates an additional source of income for aquaculture farmers (Rakocy, 2012). More recently commercial aquaponic units have been established with an increased concern for food safety, as there is limited research on potential foodborne pathogens transferred from fish and aquaponic water to plants (Rakocy, 2012). Aquaponics is the symbiotic relationship between aquaculture, horticulture, and microorganisms either in a coupled or decoupled system to maximize nutrient uptake in a sustainable way by minimizing the use of nonrenewable resources (Goddek et al., 2015; Rakocy, 2012; Somerville et al., 2014; Tyson et al., 2011). Aquaponic produce can be grown in an in-soil or soil-free system, based on the plant and its growing conditions (Somerville et al., 2014). Fish waste excreted from gills, feces, and urine is broken down into ammonia and converted to nitrites and eventually nitrates which serve as a nitrogen source for plant growth (Goddek et al., 2015; Rakocy, 2012; Tyson et al., 2008; Tyson et al., 2011).

The FDA Food Safety Modernization Act (FSMA) Produce Safety Rule states key requirements of agriculture water microbial quality for direct contact produce irrigation must have a geometric mean (GM) and a statistical threshold (STV) of 126 or less CFU of generic *E. coli* per 100 mL and 410 CFU of generic *E. coli* per 100 mL of irrigation water, respectively (FDA, 2019b). Keeping this in mind, aquaponics growers are advised to follow the Produce Safety Rule and irrigation water guidelines as there are no specific regulations for aquaponics they must follow (FDA, 2019b).

The objectives of this study were to: (1) identify the points in which *E. coli* is introduced into the system; (2) investigate the changes of *E. coli* populations traveling through the system; and (3) understand whether the *E. coli* population was within the limits of the FDA Produce Safety Rule on agriculture irrigation water. Overall this experiment aimed to have a better

understanding of the aquaponic water microbial profile utilizing surface water to provide a base line for possible mitigation methods.

2.3 Materials and Methods

2.3.1 Experimental Design

This experiment was conducted at Auburn University, E. W. Shell Fisheries Aquaponics Unit (lat. 32° N, long. 85° W). A decoupled aquaponic system (Figure 2.1) consisted of a large covered fish tank (27,000-L) and two clarifying tanks (1,500-L) which led to a plant greenhouse (9.1m x 29.3 m). Nile tilapia, *Oreochromis niloticus*, were grown at a capacity of about 5,000 on rotation for 10 years and harvested weekly. A hydrated lime slurry was used to adjust the fish tank if the pH was below 6.5. The dissolved oxygen was maintained between 5.0-7.0 ppm through aeration. Ammonia content was maintained at a safe level for fish and the quantity of water inflow into the system was recorded.

Four growth cycles of Deltastar Cucumbers (Paramount Seeds, Stuart, FL) and three growth cycles of Climstar Truss Tomatoes (Paramount Seeds, Stuart, Fl) were grown in 11-L Dutch buckets two per bucket (CropKing, Lodi, Ohio) containing horticultural-grade perlite (Sungro, Agawarm, MA) over a one year span in a double polyethylene-covered greenhouse with a N to S orientation. The tomatoes and cucumbers were placed into four rows, two sets of cucumbers and two sets of tomatoes, containing 7 buckets each and randomly placed in the greenhouse for a total of 28 tomato plants and 28 cucumber plants per growing season.

The plants were watered using aquaculture effluent (AE) controlled by an irrigation controller (Sterling 30, Superior Controls, Torrance, CA). They were watered every 30 min at a rate of 1 GPH through drip irrigators for 12 h each day. An integrated pest management was

established to control white flies and aphids; Mycotrol™ was applied as needed throughout the year when the pests had a significant impact on the plants.

2.3.2 Sample Collection

Six major points were identified within the aquaponic system for water collection in which the microbial profile, this included, water source, fish tank, clarifier, solid waste, drip irrigators and the sump tank. Water samples from these points were collected in triplicate every two weeks over a one-year period (February 11, 2019 to January 27, 2020). Samples were collected in sterilized 250 mL polypropylene bottles as described in the USEPA Microbiology Methods Manual, Part II, Section A (Bordner et al., 1978). As each sample was collected, temperature was recorded from each bottle. Samples were immediately placed in a cooler containing ice and transported to Auburn University for microbial testing within 6 h.

2.3.3 *Escherichia coli* and Coliforms Detection

The water sampling method followed the EPA Method 1604 with modifications. MI agar was used to measure *E. coli* populations and Violet Red Blue Agar (VRBA) was used to measure coliforms. Media were prepared on the previous day of sample collection along with sterile peptone water (PW) in dilution bottles and 30 mL rinse tubes.

The sample was vigorously shaken 25 times and dilutions were made using the sterile PW dilution bottles. Appropriate dilutions were made for bacterial isolation and numeration. The 100 mL diluted sample was filtered through a sterilized vacuum filtration unit using a sterile 0.45 μm filter paper (grid side up). The apparatus was rinsed with 30 mL PW twice after sample filtration to ensure the entire sample was filtered. After sample filtration, the filter paper was removed from the apparatus and rolled onto a 9 x 50 mm petri dish containing the medium,

ensuring there were no air bubbles trapped in between the filter paper and medium. Petri plates were inverted and incubated at 35 ± 2 °C for 24 h.

After 24 h incubation, the target bacterial colonies were enumerated. On MI plates, blue colonies were counted and recorded as *E. coli* and on VRBA plates, the pink colonies were counted and recorded as coliforms.

The limit of detection (LOD) was 1 CFU/100 mL and no detectable colonies were recorded as 1 for log transformation. Pure *E. coli* culture was used as a positive control for the MI agar and *Klebsiella pneumoniae* was used as a positive control on the VRBA on each sampling day.

2.3.4 Statistical analysis

Microbiological data were transformed to log CFU/100 mL of water. A one tailed t-test ($p < 0.05$) was ran for log transformed *E. coli* populations every two months with the FDA limit GM as the population parameter ($\mu < 126$) using SAS studio (Cary, NC).

2.4 Results

The average *E. coli* populations in 100 mL of effluent at each source were recorded and presented in Figure 2.2. Throughout the entire year, there were always less than 10 CFU/100 mL of *E. coli* in the water source except May 20, 2019, July 29, 2019, and November 18, 2019. This indicated that a low amount of *E. coli* entering into the aquaponics system from the water source. The solids exchange had the highest *E. coli* population in every sampling with a range of 0 to 6.85 log CFU/100 mL. The fish tank, clarifier, and emitter had similar *E. coli* populations in each sampling with a range of 0 to 5.32 log CFU/100 mL, 0 to 5.26 log CFU/100 mL, and 0 to 5.13 log CFU/100 mL, respectively. They were always lower in the final sump tank, with the

exception of July 1, 2019. *E. coli* populations in the final sump tank ranged from 0 to 4.34 log CFU/100 mL, decreased from August 29, 2019 to October 21, 2019, while the fish tank, clarifier, solids exchange and emitter increased in *E. coli* populations. Overall, the *E. coli* populations increased during the summer when the water was warmer and decreased in the winter and spring when the water temperature was cooler.

The GM and STV were calculated bimonthly based on the *E. coli* populations in the emitter water, according to the formulas provided in Geometric Means, Statistical Threshold Values, and Microbial Die-Off Rates published by the Produce Safety Alliance (Bihn et al., 2017). The GM was calculated by averaging the log-transformed results and converting it to anti-log. The STV was calculated by using the following formula and the final values were converted to antilog.

$$\log(\text{STV}) = \text{avg}(\log \text{value}) + 1.282 \times \text{std}(\log \text{value})$$

A one tailed t-test was conducted to compare the bimonthly data set to the FDA Produce Safety Rule GM limit. The GM was significantly lower ($p < 0.05$) than the FDA limit from February 1 to July 31, 2019. Table 2.1 showed the GM and STV were higher than the limit established by the FDA Produce Safety Rule from June 1, 2019 to January 31, 2019. From February 1, 2019 to May 31, 2019, the GM and STV were below the regulatory limits. From June 1, 2019 to July 31, 2019, the GM was below the limit at 13.3 CFU/100 mL but the STV was above the limit at 439 CFU/100 mL. The following months, August 1, 2019 to January 31, 2020, both the GM and STV were above the limit and between October 1, 2019 to November 31, 2019, the GM and STV were the highest at 12,800 CFU/100 mL and 111,000 CFU/100 mL of water, respectively.

The coliforms had no trend over time from each sampling point (Figure 2.3). Similar to *E. coli*, coliforms population were the lowest from the water source ranging from 1 CFU/100 mL to 15,133 CFU/100 mL of water and the highest from the solids exchange ranging from 49,000 CFU/100 mL to 14,600,000 CFU/100 mL of water. There was a large decrease on July 15, 2019, followed by an increase on July 29, 2019 in coliforms population from each source except the solids exchange. There was no association between coliforms and *E. coli* populations overall or in each source.

The average temperature from each sampling site is shown in Figure 2.4. Over the year, the temperature increased during the summer months and decreased in the winter months. Towards the later winter months, temperature fluctuated due to heating the fish greenhouse to ensure the water temperature was warm enough for tilapia survival and growth. The overall average temperature of the water was 24.24 °C ranging from 12.6 °C to 32.8 °C. The quantity of water inflow from the water source to the fish tank is shown in Figure 2.5. During the low temperature, the demand of water for produce production deceased, resulting in a decrease of water pumped into the system.

2.5 Discussion

The major concern associated with produce grown in an aquaponics system is the safety due to potential pathogen contamination from aquaponic water. The purpose of this study was to establish a base line of the microbial profile in an aquaponics system over a one-year span by identifying introductory points of *E. coli* and the population change throughout the system. In addition, this study provides information on whether mitigation measures need to be taken before

the AE is applied to the plants based on the microbial quantity of agriculture water for produce production standard in the FDA Produce Safety Rules.

In July 2019, the generic *E. coli* populations had increased from each source and continuously increased until mid-December (Figure 2.2). Before this time, the *E. coli* populations were low, with occasional spikes throughout the year. This increase in *E. coli* could be due to an increase in production and water usage; therefore, a higher input of water was entered into the system (Figure 2.5). Results showed there was a slight increase in *E. coli* populations from the water source in the summer months. Since the water source is from open surface water, it is likely to become contaminated with mammal and bird feces (Lennard, 2017). Warm blooded animal feces are likely to carry pathogenic and non-pathogenic *E. coli*, contaminating an open water source like surface water (FDA, 2019a). Figure 2.5 shows the surface water that was put into the system dramatically increased in the warmer months. This could increase the numbers of microorganisms and pathogens in the system. The sample collected was only 100 mL of the surface water inflow, but at the peak of the summer and growing period, 53,903 gallons were put into the system in a month. Therefore, even though only a few colonies were identified in 100 mL of the input water, when put in perspective, thousands of *E. coli* colonies could have been pumped into the system since over 1,000 gallons water was being pumped in daily.

Once the fish tank is contaminated from the water input, it is difficult to decrease or remove the *E. coli* in the water for irrigation if no mitigation steps are established. Additionally, an aquaponic system has an ideal environment for microbial growth, e.g. temperature, pH, oxygen, and nutrients (Hou et al., 2017). This allows *E. coli* to grow throughout the system once it is introduced into the fish tank. One way to reduce the microorganisms in the system is

through solids removal. By removing the solids, microorganisms are also removed resulting from the microbial attachment on the solids (Wu et al., 2019). In addition to the solids removal, there is also a decrease in *E. coli* and coliforms in the final sump tank after the water is used for produce irrigation. This could be because of the plant root microbiome outcompeting with microorganisms introduced though the emitters for similar nutrients in the AE (Cooley et al., 2006; Critzer et al., 2010).

The calculated experimental GM and STV were higher than the limit values provided by the FDA Final Produce Safety Rule from June 2019 to January 2020, concluding that this system should be closely monitored during these months (FDA, 2019b). Therefore, the microbial quality of the water source should be closely monitored for use in an aquaponics system. Usually, well water or city water are less likely to be contaminated by outside sources and if possible, used instead of surface water (Lennard, 2017). In addition, a mitigation step should be installed, like UV or ozone, and utilized in the summer months if the microbial population is too high (Elumalai et al., 2017; Glaze et al., 1987). A final step of monitoring water microbial quality should be included in the FDA Produce Safety Rule for aquaponics systems. Monitoring should be performed extensively in the beginning to establish a base line for irrigation water and help design an appropriate aquaponics system that meets regulatory guidelines (Castro, 2019).

2.6 Conclusion

With the increase in commercial aquaponics, there is a need for a better understanding of the potential foodborne pathogens that could enter into the system. As long as the harvestable portion of produce is not likely to come into contact with aquaponic water the FDA Produce Safety Rule does not necessarily apply to aquaponics systems, but should be used as a reference

rather than a guideline (Stivers, 2016). By analyzing aquaponic water at many different points over a one-year span it allowed us for a better understanding of how the microbial quality changed throughout the system. From the results, aquaponic water should be closely monitored from June to January to ensure that the population of *E. coli* does not exceed the regulation limit to enter the system for produce production. Utilization of a different water source, like well water or treated water, could reduce the likelihood of pathogens to enter into the system that surface water could transmit.

Future research is needed including the study of microbial quality from different water sources in various aquaponics systems and the potential microbial contamination sources from the system, such as fish, water exposure to the air, equipment, employees, etc.

2.7 Tables and Figures

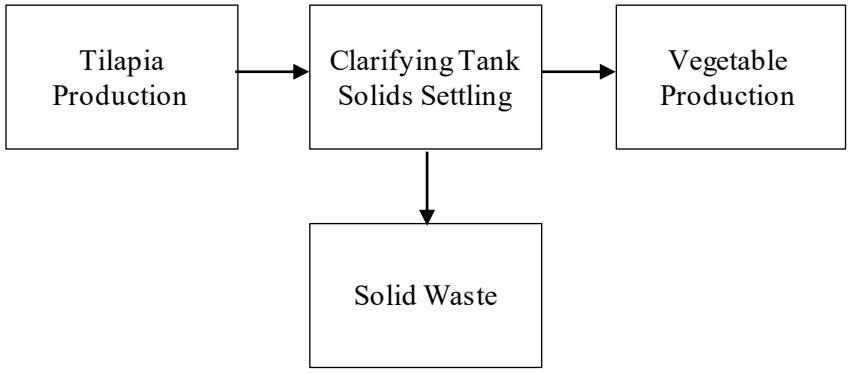

Figure 2.1 Representation of decoupled aquaponic system

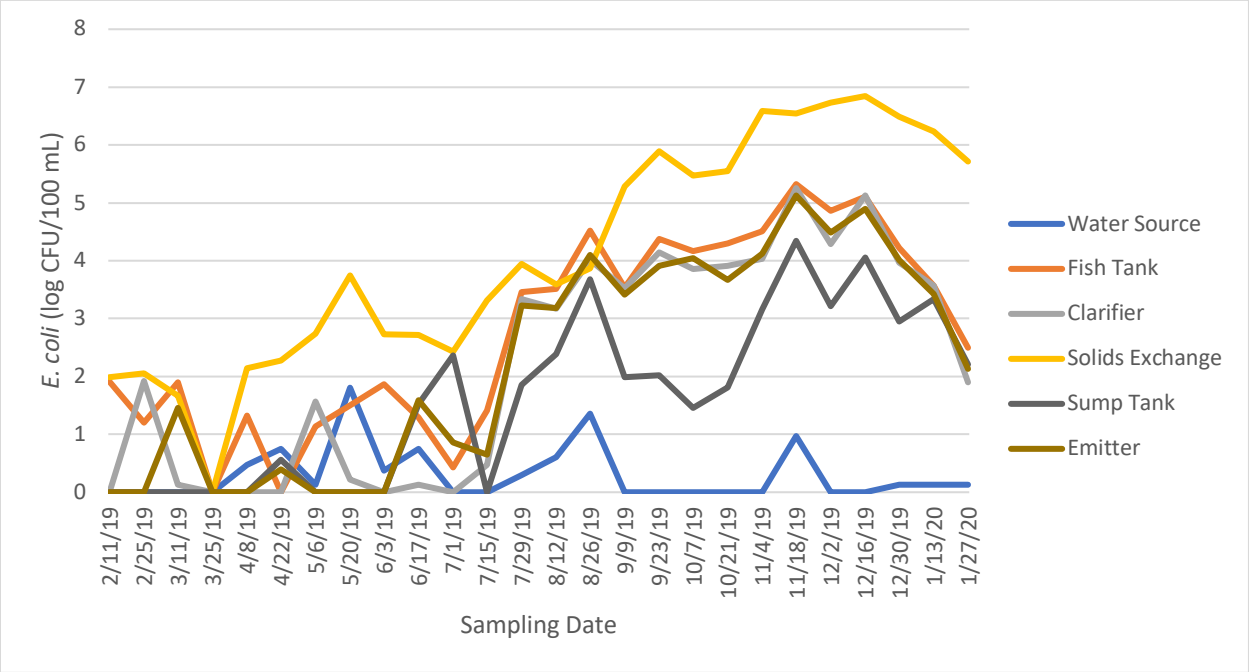

Figure 2.2 *E. coli* populations at each sampling point of a decoupled aquaponic system on MI agar using membrane filtration. The culture plates were incubated at 37 ± 2 °C for 24 h (February 2019- January 2020).

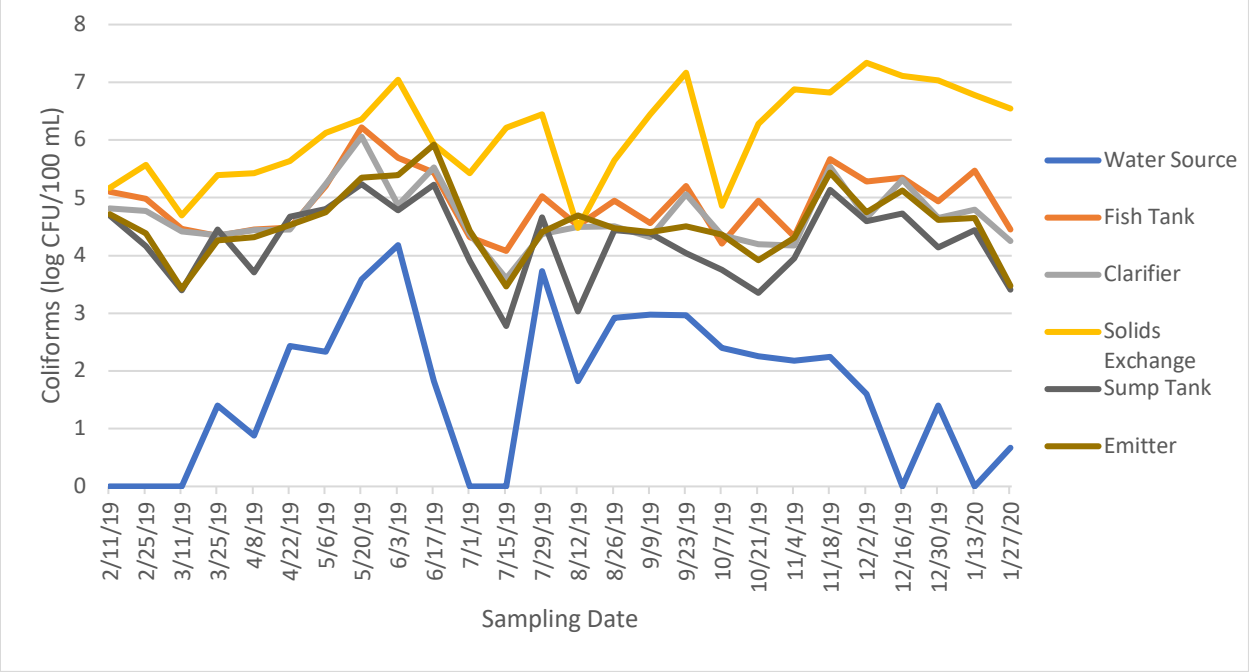

Figure 2.3 Coliforms population at each sampling point of a decoupled aquaponic system on VRBA using membrane filtration. The culture plates were incubated at 35 ± 2 °C for 24 h (February 2019- January 2020).

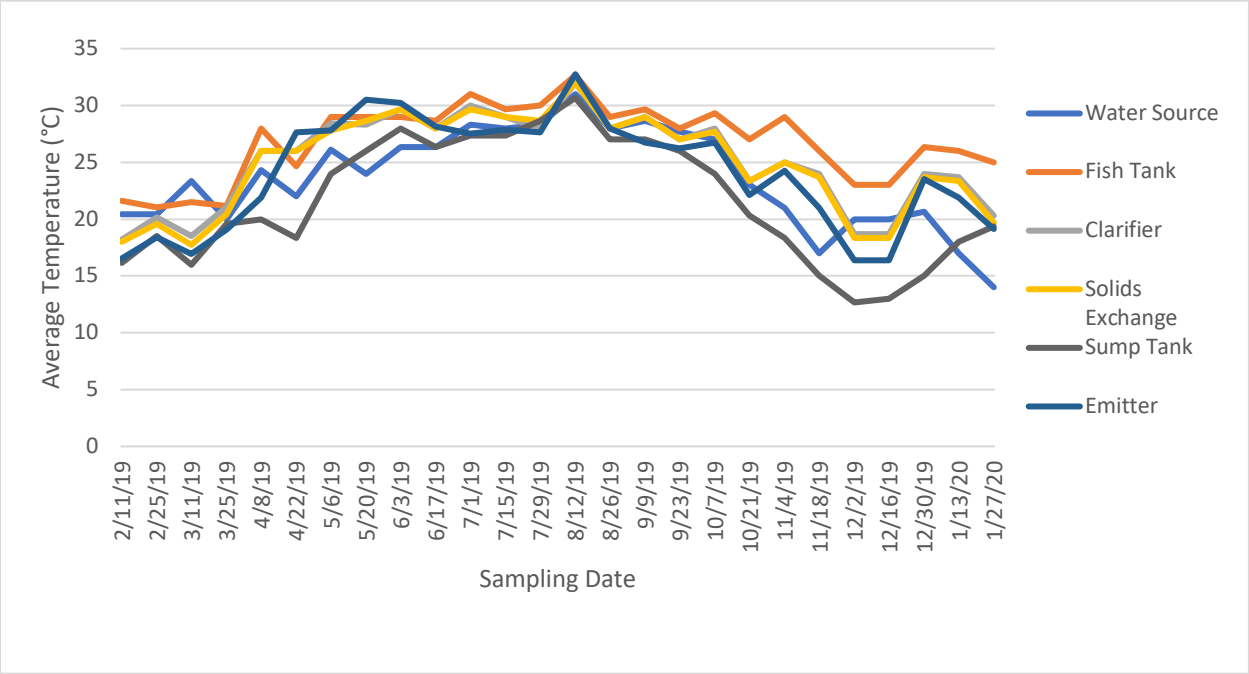

Figure 2.4 Water temperature at each sampling point of the decoupled aquaponics system over 1 year (February 2019- January 2020).

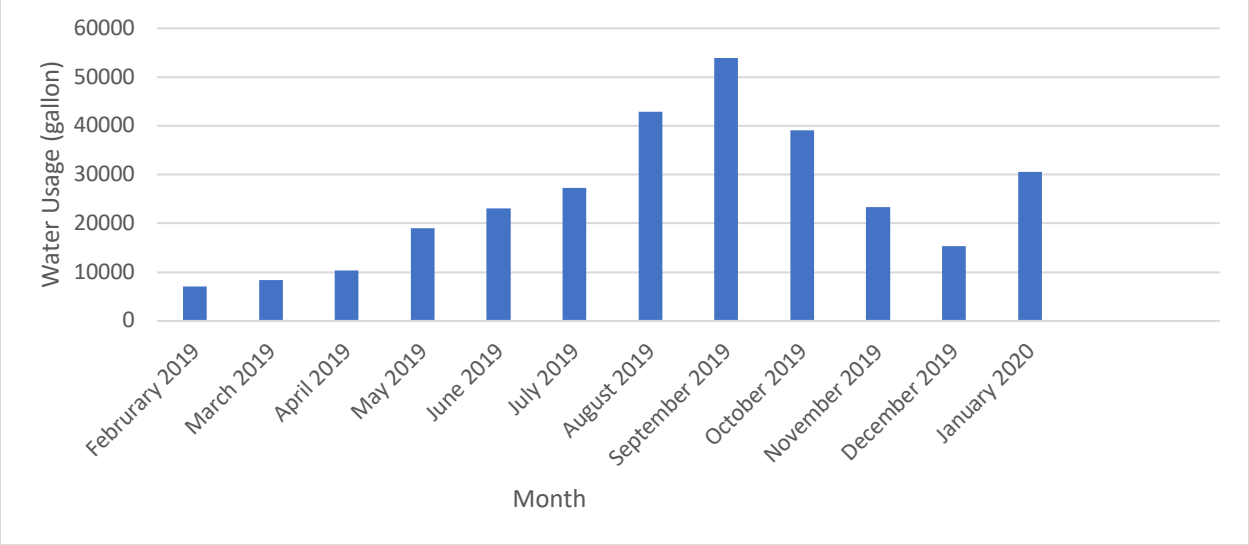

Figure 2.5 Monthly input in gallons of surface water pumped into the fish tank over one year (February 1, 2019- January 31, 2020).

Table 2.1 Bimonthly geometric mean (GM) and statistical threshold (STV) of *Escherichia coli* in the water from the aquaponics system emitter and limits established for irrigation water on produce stated in FSMA.

	GM (CFU/100 mL)[a]	STV (CFU/100 mL)[b]
Feb 1 – Mar 31	1.83a[d]	9.72
Apr 1 – May 31	1.26a	2.29
Jun 1 – Jul 31	13.3a	439
Aug 1- Sep 31	4,030n.s.	14,900
Oct 1 – Nov 31	12,800n.s.	111,000
Dec 1 – Jan 31	5,790n.s.	108,000
FSMA Limit[c]	<126	<410

[a,b] Calculated using document and formulas provided by Produce Safety Alliance (Bihn, 2017).
[c] Values established in the Food Safety Modernization Act Final Produce Safety Rule (FDA, 2019b).
[d] a indicates statistical significance ($p < 0.05$) of *E. coli* populations for the sampling period as determined by a one tailed t-test in SAS.

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Chapter III

Evaluation of *Escherichia coli* and Coliforms in Aquaponic Water Used in an NFT System Related to Time

3.1 Abstract

Studies have shown pathogenic *Escherichia coli* was found in aquaponic water. There is a concern of foodborne pathogens transferring in an aquaponic system from water containing Nile tilapia to the plants, furthermore there is a lack of research performed on aquaponics in relation to food safety. This 16-d study utilized nutrient film technique (NFT) containing 96 butterhead lettuce for microbial isolation. Three tanks held aquaponic water and one control tank of hydroponic water, each tank representing a trial. Water samples were taken from each trial on d 0, 4, 8,12, and 16 in triplicate for *E. coli* and coliform population analysis. Microbial isolations in the samples were performed using EPA Method 1604 with modifications and the filters were incubated on MI agar and VRBA for *E. coli* and coliforms, respectively. The water temperature ranged between 16.6 °C and 23.5 °C during the 16-d trial. The population of *E. coli* and coliforms reduced as time increased, starting on d 0 at 3.07 log CFU/100 mL and 4.49 log CFU/100 mL and ending on d 16 at 0.34 log CFU/100 mL and 1.96 log CFU/100 mL, respectively. This study showed that *E. coli* and coliforms populations were reduced in the water used for lettuce production in an NFT system at the first 16 d and was ultimately within the guidelines of the Produce Safety Rule of microbial quality of irrigation water by d 8.

3.2 Introduction

The CDC has estimated 1 in 6 Americans become sick and approximately 3,000 die from foods and beverages contaminated with foodborne pathogens (CDC, 2018). The trend to healthier, convenient, and sustainable food lifestyles is increasing, forcing companies to develop new products, methods, and packaging to fill these demands (Garrett, 2002). From 2004 to 2012, the CDC has found that Norovirus, *Salmonella* spp., *E. coli*, *Campylobacter*, and *Cyclospora* spp. were the leading causes of foodborne outbreaks in produce in the United States and therefore, development of methods to prevent these outbreaks is major concern in the food production industry (Callejon et al., 2015).

Aquaponics has been a growing industry in the past 40 years, evolving from research based to commercialized farming. Fish farmers are utilizing nutrients in the water that otherwise would be a byproduct and providing those nutrients to produce as a form of sustainable farming (Love et al., 2014; Rakocy, 2012). An aquaponic system can either be coupled or decoupled, water is either recirculated within the entire system or exclusively within the plant system once it is removed from the fish system (Rakocy, 2012). The plants can be grown in either medium-based grow buckets through drip irrigation or without medium such as nutrient film technique (NFT) or deep water culture (DWC) (Goddek et al., 2015). These growing methods could impact the spread of potential foodborne pathogens from the water to edible parts of the produce. The growth of commercialization of aquaponics leads to concerns of possible foodborne pathogens and how it could impact the safety of the produce.

Aquaponics is a system joining conventional aquaculture and horticulture together in a symbiotic relationship between fish, plants, and microorganisms. Freshwater fish excrete nutrients comprised of soluble and solid organic compounds including nitrogen, phosphorus, and potassium, through their gills, urine, and feces. These compounds are dissolved in the water in

ionic form which allows the plant to uptake them easily (Goddek et al., 2015). The microbial community within aquaponics has an impact on the fish and produce. The nitrogen cycle, with the assistance of beneficial microorganisms, is the driving force in aquaponics to ensure ammonia does not reach toxic levels and the nitrogen is converted to an available form for plants to uptake. Ammonia is released from the gills of fish and nitroso- bacteria break down the ammonia converting it to nitrite (Tyson et al., 2008). Nitro- bacteria then transform nitrite to nitrate, which is less toxic to fish and a main nitrogen source for plant growth (Goddek et al., 2015; Graber and Junge, 2008; Rakocy, 2012).

NFT is a soilless system consisting of many narrow channels containing holes in the top of the channels for the plant to be placed into the hole. This allows for roots to be partially submerged in the continuous flow of water while preventing the edible leaves from coming into contact with the water which could carry foodborne pathogens, e.g. *E. coli* (Goddek et al., 2015)

Pathogenic *E. coli* is a persistent foodborne pathogen commonly found in ready-to-eat fruits and vegetables. Over the past 5 years, 3 out of the 13 *E. coli* outbreaks were associated with lettuce (CDC, 2020). FDA instituted a new regulation to help control *E. coli* in water used for agricultural irrigation purposes, Food Safety Modernization Act (FSMA) Final Rule on Produce Safety also known as the Produce Safety Rule (FDA, 2019). The new standards for produce irrigation are the measurements of generic *E. coli* populations in irrigation water, which include the geometric mean (GM) less than 126 and statistical threshold (STV) less than 410 of generic *E. coli* per 100 mL of irrigation water (FDA, 2019). The objectives of this experiment were to analyze the microbial growth of aquaponic water in an NFT system between 4, 8, 12, and 16 d and determine the duration aquaponic water can be recirculated in an NFT system before reaching the FDA limit.

3.3 Materials and Methods

3.3.1 System Design

This experiment was conducted at Auburn University, E. W. Shell Fisheries Aquaponics Unit (lat. 32° N, long. 85° W) and Auburn University, Paterson Greenhouses Greenhouse 1 (lat. 32° N, long. 85° W). The decoupled aquaponic system (Figure 2.1) consisted of a large covered fish tank (27,000-L) and two clarifying tanks (1,500-L). Nile tilapia, *Oreochromis niloticus*, fish were grown at a capacity of about 5,000 which have been growing in the tank on rotation for about 10 years and harvested at about one-pound size. The pH was kept at about 6.5 by adding a hydrated lime slurry when the pH fell below 6.5. The dissolved oxygen was maintained between 5.0- 7.0 ppm through aeration. Ammonia was maintained at a safe level.

A decoupled NFT system was built containing 8 - 4.625" x 10' channels with 12 1" x 1" square holes in each channel. Two channels flowed into one sump tank for a total of 4 sump tanks. Three aquaponic trials and one hydroponic control were pumped using a smartpond 155-GPH Submersible fountain, 120V 60 HZ 0.1A (Mooresville, NC) at a rate of 155 GPH to the beginning of each channel. The NFT system had a slope gradient of 1:40.

3.3.2 Experimental Design

120 butterhead lettuce, *Lactuca sativa,* were seeded on January 6th, 2020 in Oasis Horticubes (0.75" x 0.875" x 1.5") (Kent, OH) in Paterson Greenhouse 1 on a Rediheat plant propagation mat and temperature thermostat (Earth City, MO) was adjusted to 86°F. The seedlings were grown out for 16 d. On January 21st, 2020, water was removed from the clarifier at E. W. Shell Fisheries Aquaponics Unit and transported in two sterilized Uline (Pleasant Prairie, WI) 55-gallon plastic drums to Paterson Greenhouse 1. Twenty-five gallons of aquaponic

water were pumped from the drums into one sump tank three times. The sump tanks were randomized within the NFT system indicating 3 replicates. The hydroponic control was filled with 25 gallons of Auburn City water (Auburn, AL) with nitrogen, phosphorus, potassium mix, magnesium sulfate, and calcium nitrate. Water samples were collected as described in the following section for d 0. Oasis Horticubes containing seedlings were separated, and 12 seedlings were placed into each channel for a total of 96 seedlings. The system was turned on and kept on continuously for the 16-d trial period. The system was monitored daily to ensure there were no defects within the electrical system and all the pumps were flowing properly. In addition, Onset HOBO 8K waterproof temperature data loggers (Bourne, MA) were placed into the bottom of each sump tank and set to record the temperature hourly.

3.3.3 Sample Collection

Water samples were taken from each sump tank on the transplant day (0 d) and every 4 days afterwards up to 16 days (4, 8, 12, 16). Water (750 mL each) was collected in triplicate from each sump tank, 3 trials and 1 hydroponic control, in sterilized Nalgene bottles on the specific sampling day. The samples were placed in a cooler with ice immediately and transported to Auburn University for microbial analysis within 6 h.

3.3.4 *Escherichia coli* and Coliforms Detection in Water Samples

The water sampling method was followed the EPA Method 1604 with modifications. MI agar was used to measure the *E. coli* and Violet Red Blue Agar (VRBA) was used to measure coliforms populations. The media were prepared on the previous day of sample collection along with sterile peptone water (PW) in dilution bottles and 30 mL rinse tubes.

The sample was vigorously shaken 25 times and proper dilutions were made using the sterilized PW dilution bottles. Appropriate dilutions were made for bacterial isolations and

numeration. The 100 mL diluted sample was filtered through a sterilized vacuum filtration unit using a sterile 0.45 μm filter paper (grid side up). After sample filtration, the apparatus was rinsed twice with 30 mL PW. The filter paper was removed from the apparatus and rolled onto the medium grid side up, ensuring there were no air bubbles trapped in between the filter paper and medium. Each sample was done in duplicate. Petri plates were inverted and incubated at 35 ± 2 °C for 24 h.

After 24 h incubation, the target bacterial colonies were enumerated. On the MI plates blue colonies were counted and recorded as *E. coli* and on the VRBA plates the pink colonies were counted and recorded as coliforms.

The LOD was 1 CFU/100 mL water and no detectable colony were recorded as 1 for log transformation. Pure *E. coli* culture was used as a positive control on the MI agar and *Klebsiella pneumoniae* was used as a positive control on the VRBA on each sampling day.

3.3.5 Statistical Analysis

The three replicates and control were organized in a completely randomized design. After the data were collected, ANOVA and lsmeans ($p < 0.05$) was ran using the GLIMMIX procedure and type III sum of squares in SAS studio (Cary, NC).

3.4 Results

Figure 3.1 summarizes the *E. coli* and coliforms populations over the 16-d trial. As holding time of the aquaponic water increased, the number of *E. coli* and coliforms decreased. The aquaponic water started with an *E. coli* population of 3.07 log CFU/100 mL and coliforms population of 4.49 log CFU/100 mL of water. The final populations on d 16 were 0.33 log CFU/100 mL and 1.96 log CFU/100 mL for *E. coli* and coliforms population, respectively. The

E. coli and coliforms populations were significantly different for each sampling d (0, 4, 8, 12, 16), except d 16 for coliforms population during the 16-d study ($p > 0.05$). The hydroponic control indicated that there were no *E. coli* or coliforms population in the water at any collection d throughout the trial.

In Table 3.1, the GM and STV were calculated based on the mean of *E. coli* population for each sampling day according to the formulas provided in Geometric Means, Statistical Threshold Values, and Microbial Die-Off Rates published by the Produce Safety Alliance (Bihn et al., 2017). The GM were calculated by averaging the log-transformed results and converting it to anti-log. The STV were calculated by using the following formula and the final values were converted to antilog.

$$\log(\text{STV}) = \text{avg}(\log \text{value}) + 1.282 \times \text{std}(\log \text{value})$$

The initial GM and STV were above of the FDA regulation limits of 126 and 410 or less CFU/100 mL, respectively, with a GM of 1,142.25 CFU/100 mL and STV of 1,290,311.90 CFU/100 mL of water. By d 4, *E. coli* populations were within specification as described in the Produce Safety Rule with the GM being 57.22 CFU/100 mL but were not within the limits for the STV. By d 8, the *E. coli* population was within the GM and STV limits which the GM was 11.46 CFU/100 mL and the STV was 188.30 CFU/100 mL of water. The GM and STV were not calculated for coliforms as coliforms are not regulated under the Produce Safety Rule.

The water temperature was recorded in each sump tank every hour using HOBO waterproof data loggers (Figure 3.2). The temperatures of all sump tanks followed the same trend of the increase and decrease of the greenhouse temperature. The water temperature of the sump tanks was 20.17 ± 0.82 (mean ± SD).

3.5 Discussion

There is a concern with *E. coli* and other foodborne pathogens coming into contact with the edible parts of produce. Lettuce is susceptible to pathogens as the edible leaves are in close proximity to the water applied to the plant. By monitoring the *E. coli* in the water, the likelihood of pathogens being present in produce can be predicted (FDA, 2019). The purpose of this study was to understand how the microbial growth of *E. coli* and coliforms populations changed over a 16-d growing period in a decoupled recirculating aquaponic system and predict the duration water can be held in the system based on the FDA Produce Safety Rule regulatory limit.

A traditional NFT system containing a hydroponic sump tank exchanges the water approximately every two weeks to maintain the optimal pH and nutrient level in the water (Cooper, 1979). After two weeks, nutrients diminish which can cause other parameters to fluctuate (Resh, 2012). Therefore, water was sampled at 4, 8, 12, and 16 d to determine how long the water can be recirculated in the system based on *E. coli* populations to determine if this was a factor to determine the holding time of aquaponic water in a decoupled system. *E. coli* and coliforms populations significantly decreased on each sampling day, except the coliforms population on d 16. The hydroponic control ensured *E. coli* and coliforms were not introduced into the system from another input like the plants, employees, environment, etc.

Aquaponic water sustains ideal conditions for mesophilic microorganisms to grow, such as temperature, DO, available nutrients, pH, and moisture (Hoagland et al., 2018; Shadbolt et al., 2001). The change in water temperature did not affect the growth of microorganisms, as the temperature remained in a range of 16.62 °C to 23.5 °C and an average of 20.2 °C. These conditions are cool for *E. coli* to grow rapidly in, as the ideal temperature range for *E. coli* is between 30 °C and 42 °C, with 37 °C optimal for growth (Doyle and Schoeni, 1984). If *E. coli* is

provided with adequate nutrients, it can grow at a slower rate at 25 °C and even lower rate at 15 °C (Lee et al., 2019). Therefore, the *E. coli* in the system would have been able to grow in 20 °C, but at a suppressed rate. At an average of 20 °C, not only does *E. coli* and coliforms grow but other mesophilic microorganisms grow in similar conditions.

Plants and non-pathogenic microorganisms could be utilizing essential nutrients in competition with *E. coli*. The rhizosphere and microbiota of the lettuce roots can impact the growth of *E. coli*. Based on the cultivar of the lettuce, there have been finding of resistance to *E. coli* near the roots of the lettuce plants, but not necessarily universal with all lettuce cultivars (Quilliam et al., 2012). In an additional study, an epiphyte found in the plant rhizosphere utilized the same nitrogen and carbon source followed by the survival of the epiphyte and decrease in *E. coli* O157:H7 (Cooley et al., 2006). The plant root microbiota could be a contributing factor to the decrease of *E. coli* and coliforms in the study.

The GM and STV were calculated based on the *E. coli* data of each treatment, and based on the calculations, the water must be held in the system for at least 8 d. This ensures if high populations of *E. coli* are in the system there is a die off period for *E. coli* to decrease to a safe level (FDA, 2019). After 8 d, the water can either be held for up to 16 d or replaced if the nutrients had significantly decreased.

Studies on aquaponic systems have found that *E. coli* and pathogenic microorganisms are less likely to spread to edible produce and ultimately to the consumer by performing the following practices: cleaning and sanitizing containers, environmental controls, hand washing, and the use of clean irrigation water (Barnhart et al., 2015; Saylor, 2018).

3.6 Conclusion

Through the completion of this study, we have found that aquaponic water can be held at least 8 d and up to 16 d within an NFT system before replacing it based on the GM and STV FDA limits of *E. coli* populations. As long as the water is being monitored properly and corrective actions taken as necessary, there should not be a concern with generic *E. coli* levels in the water. Future studies based on this research include sampling the leaves of the lettuce in addition to the water of an NFT system to ensure no *E. coli* was internalized or came into contact through splashing of the water.

3.7 Tables and Figures

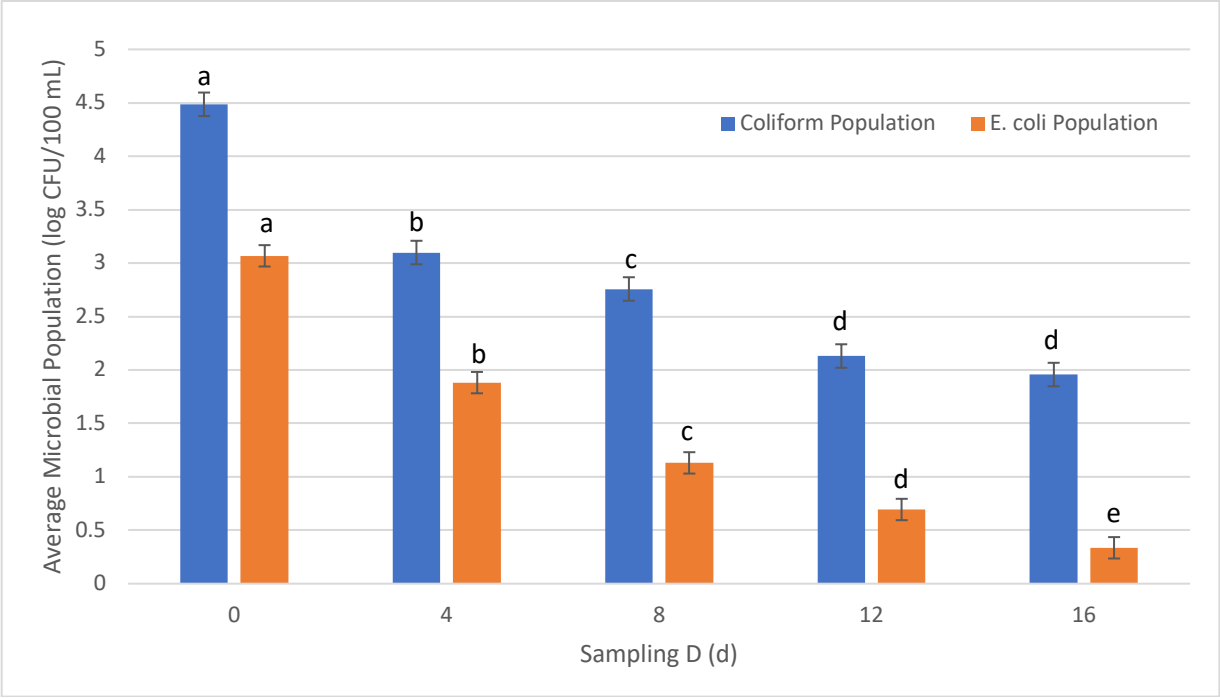

Figure 3.1 *Escherichia coli* and coliforms population in an NFT system using aquaponic water to grow butterhead lettuce for each sampling day for up to 16 d. *E. coli* and coliforms populations were sampled by utilizing membrane filtration and on MI agar and VRBA, respectively.

[a b c d e] Different letters indicate significant differences ($p < 0.05$) within the specific microorganism for the sampling period as determined by analysis of variance and lsmeans using the GLIMMIX procedure and type III sum of squares in SAS.

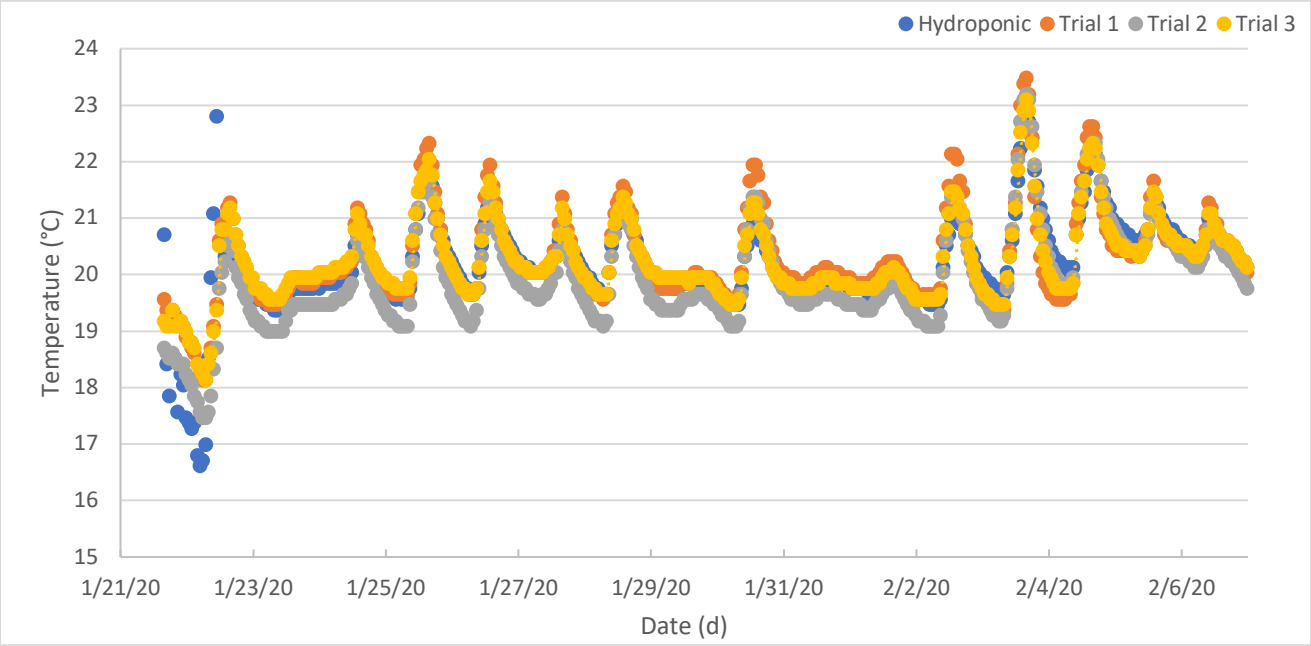

Figure 3.2 Temperature of each aquaponic sump tank trial and hydroponic control over the 16-d trial collected on HOBO waterproof data loggers every hour, January 21, 2020 to February 6, 2020.

Table 3.1 Geometric mean (GM) and statistical threshold (STV) of *Escherichia coli* in the recirculating NFT water on each analysis day.

Sampling Day (d)	GM (CFU/100 mL)[a]	STV (CFU/100 mL)[b]
0	1,142.25	1,290,311.90
4	57.22	9,157.64
8	11.46	188.30
12	3.97	17.15
16	2.07	3.83
Overall Average	22.80	26,090.85
FDA Limit[c]	<126.00	<410.00

[a,b] Calculated using formulas provided by Produce Safety Alliance (Bihn, 2017).
[c] Values established in the Food Safety Modernization Act Final Produce Safety Rule (FDA, 2019b).

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