ABSTRACT

Title of Thesis:	SURVIVAL OF *ESCHERICHIA COLI* AND CHANGES IN PHYSICOCHEMICAL PARAMETERS IN AQUAPONIC SYSTEMS DURING BASIL AND LETTUCE PRODUCTION
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Aquaponics (APs), a soilless production system, integrates aquaculture and hydroponics to provide local fresh produce while conserving natural resources. The absence of soil in APs eliminates one potential food safety risk present in typical soil-based production systems, but APs may become contaminated from a variety of sources. *Escherichia coli* TVS 354 long-term survival was evaluated in bench-scale, deep-water APs units. In addition, pathogen presence on basil and lettuce at the time of harvest and changes in the population density of mesophilic aerobic bacteria in APs were measured. Results showed *E. coli* populations significantly decreased 24 h post-inoculation in water samples and remained undetectable by day 1 post-inoculation. Lettuce harvested on day 60 had detectable *E. coli* on lettuce leaves and roots at harvest. These results provide new insight on *E. coli* survival in harvested plants, indicate potential risks for foodborne illnesses, and unreliability of water testing as a monitoring tool.

SURVIVAL OF *ESCHERICHIA COLI* AND CHANGES IN PHYSICOCHEMICAL PARAMETERS IN AQUPONIC SYSTEMS DURING BASIL AND LETTUCE PRODUCTION

by

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List of Abbreviations

dpi	Days post-inoculation
TSB-r	Tryptic soy broth with Rifampicin
MACR	MacConkey agar-Rifampicin
APC	Aerobic Plate Count
RAS	Recirculating Aquaponic System
AP(s)	Aquaponics
HP(s)	Hydroponics
DW	Distilled water
UN	Uninoculated control
IBCW	Aquaponic effluent with distilled water
IBC	Aquaponic effluent
RIBC	Replenished aquaponic effluent
LD	Low dose treatment (low *E. coli* inoculum concentration)
HD	High dose treatment (high *E. coli* inoculum concentration)
h	Hours
EC	Electrical conductivity
SE	Standard error
DF	Degrees of Freedom

Chapter 1: Introduction

With global populations predicted to rise to 9.7 billion by 2050, attention has been shifted to determine how to meet the needs of a growing global population (UN, 2022). In addition, many people have migrated from rural to urban areas due to better labor markets and access to better amenities such as social and educational services and infrastructure (Selod and Shilpi, 2021). With increased urban population densities, arable land is lost to expand urban development to meet the needs of the growing population (Liu et al., 2010; Radwan et al., 2019). As such, the surrounding environment is unable to meet the resource demands of the cities and provide proper nutrient cycling that previously supported the ecosystem (Rees and Wackernagel, 1996). As a result, decreased farmland due to urban expansion poses a threat to food security (Radwan et al., 2019). In addition, there are food production increases on currently available farmland to meet the increased population demands, which contributes to additional greenhouse gas emissions (Weber and Matthews, 2008).

Aquaponics provides an alternative food production method to address concerns associated with urban population growth, farmland shortage, greenhouse gas emissions associated with food production, and food insecurity (Al-Kodamy, 2018; Greenfeld et al., 2019). Aquaponics, a soilless production system, integrates aquaculture and hydroponics to provide local fresh produce (Delaide et al., 2017). Plant nutrients are supplied through bacterial transformation and metabolism of fish excreta (Figure 1.1) (Wongkiew et al., 2017). The three primary nitrifying bacteria in aquaponic systems are *Nitrosomonas*, *Nitrobacter*, and *Nitrospira*. *Nitrosomonas*

converts ammonium (NH_4^+) to nitrite (NO_2^-) and *Nitrobacter* converts nitrite to nitrate (NO_3^-) (Wongkiew et al., 2017). Uptake of nitrogen by plants in the system occurs predominantly in the form of nitrate (Engels & Marschner, 1995).

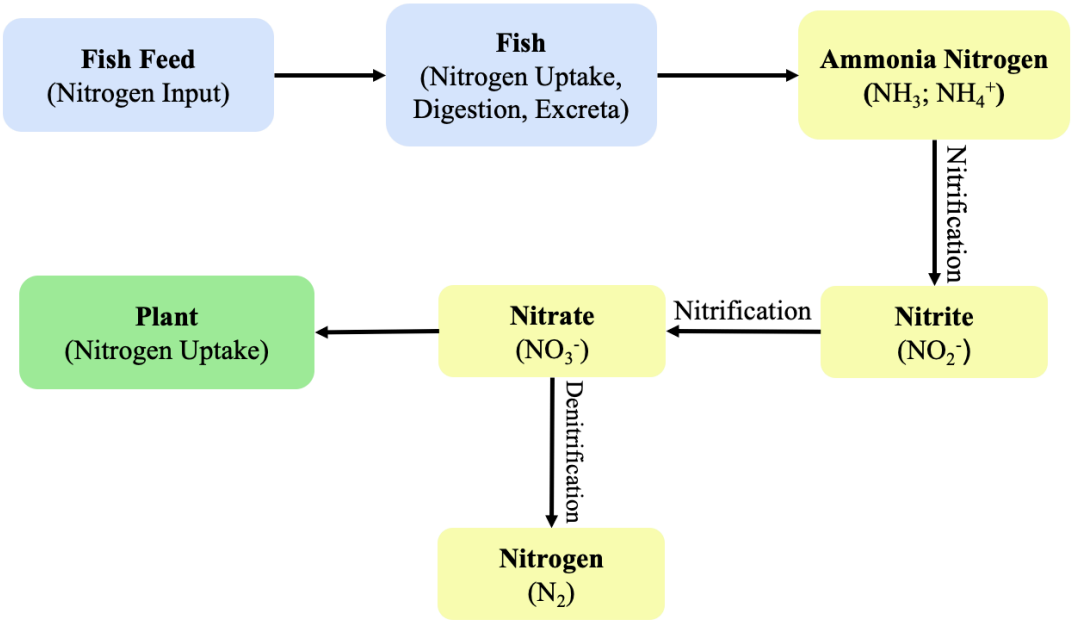


Figure 1.1. Diagram displaying the nitrogen cycle in aquaponics, showing the nitrogen input, uptake, and bacterial transformations.

While the use of aquaculture for plant production has been around for thousands of years, this technology has garnered new attention in the past 30 years (Greenfeld et al., 2019; Palm et al., 2018). As the need for sustainable food production increases and freshwater and phosphorus reserves decrease (Alewell et al., 2020), new methods of food production are being developed to meet that demand (Palm et al., 2018). In addition to food security, aquaponics addresses sustainability issues such as water availability, pollution, increased fertilizer cost, and soil health and quality decline (Yep et al. 2019). The aquaponic food production method has been reported to be more efficient in water usage with no pesticide and antibiotic use

and minimal reliance on synthetic fertilizer than conventional agriculture(Yep et al. 2019).

Due to lack of literature comparing aquaponic, hydroponic, and conventional agriculture crop yield together, an indirect comparison between conventional agriculture and aquaponics was made through their comparisons to hydroponics. Compared to conventional agriculture, aquaponics has higher capital and operational expenditure costs making it less profitable in the short term. In the long term, however, aquaponics is more profitable and uses 85% less water than conventional agriculture (El-Essawy et al., 2019). In terms of crop yield, hydroponics and aquaponics have a higher crop yield than conventional agriculture, but higher energy use is a major drawback due to mechanisms that keep water continuously moving through the system (Barbosa et al., 2015). Hydroponics is a food production method similar to aquaponics, but there is no aquaculture component. Rather, liquid fertilizer is used in place of fish feed and waste as the nutrient input. When comparing yield between aquaponics and hydroponics, the yield was similar, but yield varies based on nutrient supplementation (Ayipio et al., 2019).

With the expansion of aquaponics enterprises, gaps in handling and operational practices, such as those associated with the potential presence of heavy metals, pests, plastics, chemical residues, and pathogens, as well as other unknown food safety risks, need to be addressed support this approach to sustainable food production (Wu et al., 2019). Relatively few research studies report on the presence and potential spread of pathogens and pests in aquaponic systems. Pathogens present on food grown in open-field soil systems that comes in contact with manure or

agricultural water (irrigation or sprays) are common concerns in the human food chain. Pathogens may persist for one month or more after contact with a contaminated source (water, manure, tool/surface) (Nicholson et al., 2005). Pathogens such as *Escherichia coli* (*E. coli*) are active in the rhizosphere of produce plants, can persist in soils for weeks, and can be found inside and outside of contaminated plants (Barnhart et al., 2015; Habteselassie et al., 2010). Fruits and vegetables produced in controlled environments have been regarded as having fewer food safety risks than soil-grown produce due to the lack of contact with natural field soils and isolation from animals (Shaw et al., 2016). The absence of soil is expected to reduce exposure of soilborne pathogens, but there is risk of inadvertent contamination by pathogens spread through seeds, fish, fish waste, tools, equipment, inadequately treated water, substrates, and human activity (Wu et al., 2019; Shaw et al., 2016).

Nutrient solutions often are recirculated in hydroponic systems, which allow pathogens to be introduced and spread throughout the crop (Shaw et al., 2016). In hydroponic systems, *E. coli* was reported to internalize in the edible parts of lettuce plants by transfer from water into the root system through spaces resulting from lateral branch development or physical breaks in the root epidermis (Moriarty et al., 2019; Solomon et al., 2002; Wang et al., 2021; Bernstein et al., 2007; Erickson et al., 2010). Contamination can be traced from various sources, such as contaminated or untreated water and human contact. Roots provide a large surface area to enable bacterial internalization, but analysis of roots has been minimal (Barnhart et al. 2015). However, plants sold with intact roots increase the opportunity for inadvertent

transfer of waterborne food safety bacterial contaminants to consumable foliage at various points during product packaging, distribution, and handling.

As a result, the U.S. Food and Drug Administration (FDA), Food Safety Modernization Act (FSMA) final rule for produce includes microbial water quality criteria for water that likely will contact the edible portions of fruits and vegetables (U.S. Food and Drug Administration, 2015). In 2022, FDA proposed a revision to Subpart E of the FSMA Produce Safety Rule (PSR) that would change the pre-harvest agricultural water requirements for covered produce (other than sprouts) (U.S. Food and Drug Administration, n.d.). The requirements, if finalized, would replace pre-harvest microbial quality criteria and testing requirements in the PSR with requirements for systems-based pre-harvest agricultural water assessments. Such assessments would identify conditions likely to introduce known or reasonably foreseeable hazards into or onto produce or food contact surfaces, and to determine whether corrective or mitigation measures are needed to minimize the risks associated with pre-harvest agricultural water. Currently, FSMA also applies to aquaponic and hydroponic produce, although no regulations are specifically stipulated for aquaponics (U.S. Food and Drug Administration, 2015).

While pathogens can survive in the water of hydroponic and aquaponic systems (Wang et al., 2020), further research is needed to address gaps in key aspects of food safety bacterial pathogen growth and survival responses to conditions and practices associated with aquaponic systems. Handling of plants during and after production involves conditions that can pose a risk for contamination. The two

studies for this thesis conducted and reported as part of this project focused on changes in the generic *E. coli,* specifically *E. coli* TVS 354, concentrations and locations within key compartments of a replicated, bench-scale, aquaponic research system. With no prior data on the microbiological quality of the water in the replicated bench-scale systems, a short-term preliminary study was conducted to determine the response *E. coli* survival in aquaponic system water without inoculating each of the 12-replicate bench-scale aquaponic units. Therefore, a generic strain of *E. coli* obtained from the Salinas, CA leafy greens growing region was selected as the inoculant. The source water was collected from a lab-scale aquaponic unit (1040 L) operating for 3 years. This source water was distributed to sterile bottles (250 ml) according to the inoculation and short-term treatment plan; treatments were aerated by a rotary shaking platform. Concentrations of *E. coli* TVS 354 was determined to understand the different population changes that might occur prior to inoculation of a recirculating aquaponic system (RAS) by inoculating aquaponic effluent in a bench-top lab scale study.

The second study involved inoculating twelve RAS with *E. coli*. Results contribute to a better understanding of *E. coli* harborage sites and survival within a RAS. This knowledge also could lead to development of options for controlling food safety pathogens like *E. coli,* further research on other bacteria that cause food-borne illnesses such as *Salmonella* and *Listeria*, and written guidance and recommendations to improve growth conditions for plants, fish, and essential nitrification bacteria. The objective of this research was to determine survival of *E. coli TVS354* on dose-

inoculated aquaponic effluent with different inoculum concentrations to evaluate survivability over long periods of time.

Overview

This research involved two studies. The first study is referred as Preliminary Experiment or Preliminary. This was a benchtop bottle study that examined *E. coli* TVS 354 population changes in aquaponic effluent in two trials (Trial #1 and #2). This was a short study (72hrs and 48hrs) conducted to provide preliminary information on possible *E. coli* behaviors and what to expect in a functioning aquaponic system since there was no literature available to reference. The second study is referred as Main Experiment or Main. This was lab-scale study that involved inoculating *E. coli* TVS 354 on functioning recirculating aquaponic systems with goldfish. The effluent and plants grown (basil and lettuce) were examined to determine *E. coli* survival in aquaponic systems. The study has two phases (Phase 1 and 2). Phases 1 and 2 involved using the same systems, water, plants, and fish. In each phase, the systems were reinoculated with *E. coli* using different inoculum concentrations and changing the sample collection site from the fish tank water to the biofilter. This study focused on investigating *E. coli* survival over a longer period of time (60 days) and its interaction with plants from a food safety perspective.

Chapter 2: Preliminary Assessment of *Escherichia coli* Survival in Inoculated Challenge Dosing of Aquaponic Effluent

Introduction

Aquaponics is a soilless food production method that integrates aquaculture and hydroponics in a controlled environment (Delaide et al., 2017). Fish feed, fish digestion, and bacterial transformation contribute to plants with nutrients, primarily nitrogen (Wongkiew et al., 2017). Around 48% of foodborne outbreaks worldwide are associated with fruit and vegetable consumption creating the need for improved preventative controls and methods for pathogen detection and elimination (Wang et al., 2020). Fruit and vegetable production in controlled environments have been regarded as having fewer food safety risks than soil-grown produce due to the lack of contact with natural field soils and potential contamination by wild animals, manure, pests, irrigation water, tools/equipment, and personnel (Shaw et al., 2016).

While pathogens are known to survive in water from hydroponic and aquaponic systems (Wang et al., 2020), specific dynamics of *E. coli* TVS345 survival in the existing 2-year continuously operating AP system available for this study was poorly understood. The U.S. Food and Drug Administration (2015), FSMA Produce Safety Rule for produce has microbial quality criteria that requires the following must be met for produce production:

> (1) A geometric mean of your agricultural water samples of 126 or less colony forming units of generic *E. coli* per 100 ml of water; and (2) A

statistical threshold value of your agricultural water samples of 410 or less CFU of generic *E. coli* per 100 ml of water. (Section F)

These regulations also apply to aquaponic production despite experts questioning its ability to meet the regulations (U.S. Food and Drug Administration, 2015). These regulations rely on water testing as an accurate method of monitoring pathogens in AP systems, but the interaction between pathogens and effluents is poorly understood. It also doesn't take into consideration other parts of AP systems that can be used for testing and monitoring such as fish and plants. To gain insight on the population responses of *E. coli* TVS354 to AP effluent, and to avoid inoculation and subsequent decontamination of our replicated AP systems, a preliminary study was conducted to obtain baseline information on the population changes that may be expected in the upcoming replicated full-system study. The objective of this preliminary study was to determine population dynamics of *E. coli* TVS354 in aquaponic effluent and distilled water over 72 h using aerated bottles under controlled conditions.

Material and Methods

Bacterial culture preparation

The non-pathogenic strain of *E. coli* with rifampin resistance (TVS354) used for this study was isolated from Romaine lettuce and provided by Dr. Trevor Suslow from University of California (Davis, CA). A loop was used to transfer the frozen culture (-80 °C) to MacConkey agar (Becton, Dickinson and Company, Franklin Lakes, NJ) and activated at 37°C for 18 hours (h) then enriched with 80 mg/l rifampin

(Thermo Fisher Scientific, Waltham, MA). A single bacterial colony for the strain was inoculated into 30 ml of Trypticase Soy Broth (Becton Dickinson, Sparks, MD) with 80 mg/l of rifampin and vortex mixed then incubated at 37°C for 18 h. Cells were harvested by centrifugation at 4,300 x g for 5 min, then washed once in sterile phosphate-buffered saline (PBS, pH 7.2, Corning, Corning, NY) before re-suspension in 10 ml sterile PBS. Equal volumes of cell suspensions were mixed and diluted in sterile distilled water to an optical density of 0.3 in a cuvette with 1 cm path length at 600 nm (~8 log CFU/ml), before diluting 100-fold with sterile distilled water for use as inoculum. Initial bacterial concentrations were estimated by measuring the absorbance of the bacterial suspension at 600 nm with a spectrophotometer (Genesys 20, Thermo Fisher Scientific, Waltham, MA) and confirmed by plating 0.1 ml aliquots of appropriately diluted culture onto MacConkey Agar (Becton Dickenson, Sparks, MD) supplemented with 80 mg/l of rifampin. After incubation at 37°C for 24 h, colony counts of appropriate spread plated dilutions were recorded and concentration calculated.

Aquaponic effluent conditions and measurements

To examine the changes in *E. coli* TVS354 population in aquaponic effluent, 15 sterile bottles (250 ml) with loosely screwed lids were secured to an orbital shaker (VWR International, Radnor, PA) with Styrofoam and kept at 150 rpm for 3 days at room temperature (20°C) (Figure 2.1). The orbital shaker was used to aerate the bottle to simulate aeration that would be found in an aquaponic system, and 150 rpm was chosen as it provided the highest aeration possible while keeping the contents in the liquid. Dissolved oxygen (DO) was not measured because the DO sensor was not able

to come in contact with the water. While room temperature was set at 20℃, temperature varied based on the weather, and no additional features were installed to strictly maintain the room temperature.

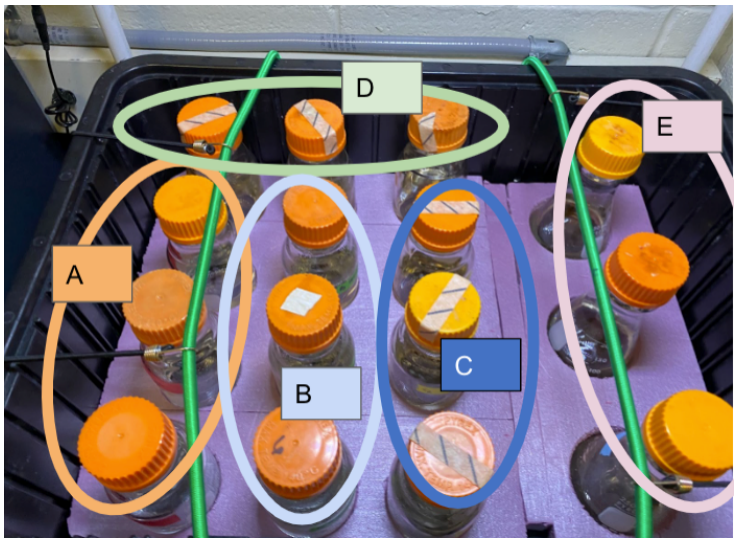


Figure 2.1. Bottle configuration for preliminary experiment Trial #1. Each letter representing one treatment group or control. A = distilled water inoculated with *E. coli* TVS354 (DW); B = aquaponic effluent mixed with distilled water inoculated with *E. coli* TVS354 (IBCW); C= aquaponic effluent inoculated with *E. coli* TVS354 (IBC); D = uninoculated aquaponic effluent (control) (UN); E = replenished aquaponic effluent inoculated with *E. coli* TVS354 (RIBC).

Physicochemical parameters measured included temperature, pH, electrical conductivity (EC), ammonia, nitrite, and nitrate. These were only conducted in Trial #1. Temperature was analyzed with a dissolved oxygen meter (Pro 20, YSI Inc., Yellow Springs, OH) and Salt/TDS/Conductivity/Temp meter was used to collect EC data (Tracer PockeTester, La Motte Company, Chestertown, MD). Ammonia, nitrite, and nitrate was analyzed using water quality test strips (Hach Company, Loveland, CO). A portable pH meter was used for pH (Accumet Portable AP 110, Fisher Scientific, Hampton, NH). All meters were inserted directly into bottles to collect measurements on each sampling day. Water samples (10 mL) were collected at 0, 24, 48, and 72 h post-inoculation and water quality parameters were measured. These

parameters were chosen because they're common water quality parameters measured to ensured ideal fish health. While no fish was used in this study, the values collected in this study would be used understand the possible ranges in the aquaponic systems in the Main experiment.

AP effluent inoculation

Each sterile 250 ml bottle received 150 ml of AP effluent. In Trial #1 of this preliminary study, three bottles contained 150 ml of aquaponic effluent collected from a 1040 L parent aquaponic system (IBC) that had been continuously operating for 3 years and had no detectable generic *E. coli* as determined by standard Colilert 18, IDEXX assay (IDEXX was not inoculated with *E. coli* TVS 354 as the control (UN)) (Figure 2.2). *E. coli* cells were added to the remaining bottles to obtain a concentration of 4 Log CFU/ml and were filled with 150 ml of distilled water (DW) (three bottles), 100 ml of distilled water and 50 ml of aquaponic effluent (IBCW) (three bottles), 150 ml of aquaponic effluent (IBC) (three bottles), or 150 ml of aquaponic effluent with 100 ml removed every 24 h and replenished with 100 ml of fresh aquaponic effluent (RIBC) (three bottles) (Appendix A). All aquaponic effluent was collected from the parent IBC system located at University of Maryland (College Park, MD). The concentration of aquaponic effluent was determined to examine how the lack of nutrients in aquaponic effluent could impact bacterial growth since there was no studies conducted of a similar nature.

In Trial #2 of the preliminary study, six bottles of 150 ml of aquaponic effluent were inoculated at 3 Log *E. coli* TVS 354 CFU/ml (Low Dose, LD), and three more bottles were inoculated at 4 Log CFU/ml *E. coli* TVS 354 (High Dose,

HD) using the inoculum preparation procedure described above (Appendix B). Trial #2 effluent concentration more similarly aligned with the Main experiment; it did not have diluted aquaponic effluent, but the water microbial community was different since the parent IBC system was not used in that study. The inoculum concentration used in Trial #1 and #2 was chosen as there was no information on inoculum size in previous aquaponic food safety studies. With no prior information available, the inoculum concentrations were selected to simulate a large and small contamination event, and there were no expectations for how the *E. coli* population would change overtime.

Figure 2.2. The 1040 L parent aquaponic system (IBC) that had been continuously operating for 3 years. All aquaponic effluent used in preliminary experiment Trial #1 and #2 were collected from this system due to its established microbial community.

Microbiological enumeration

Samples (10 ml) of inoculated and uninoculated AP effluent were collected separately from each replicate of each treatment at 0, 24, 48, and 72 h post-inoculation in Trial #1, and samples were collected at 0, 24, and 48 h post-inoculation in Trial #2 (Appendices A and B). Samples collected from each bottle were serially diluted using sterile PBS in 1:10 dilution, vortex mixed (Thermo Fisher Scientific, Waltham, MA) and spread plated (0.1 ml) onto MacConkey Agar-Rifampicin (MACR) plates and incubated at 37 ℃ for 24 hr before counting colonies of *E. coli* TVS 354 (Figure 2.3). Three 1:10 serial dilutions (10^{-3}) were conducted for MACR. Aerobic plate counts (APC) of each water treatment sample in the Trial #1 preliminary study were determined using 1 ml aliquots of serially diluted water samples plated in duplicate onto APC Petrifilm (3M, Maplewood, MN) and incubated at 37 ℃ for 48 hr. Three 1:10 serial dilutions (10^{-3}) were conducted for APC. APC was used to monitor the change in the native bacteria with the introduction of *E. coli*. Since *E. coli* is aerobic, its counts are included in APC Petrifilm.

Figure 2.3. Experimental set-up for Trial #1 and #2. The bottles were placed on the orbital shaker (right) with a light place on top with the incubator (left) used for microbial enumeration analysis.

Sterilization

All glass bottles used in the preliminary study were sterilized for 20 min in a dry-cycle autoclave process prior to the start of the study. All meters, equipment, and surfaces that came in contact with the water samples were disinfected with 70% ethanol, or 10% bleach and sterile water rinse, before, or in between each measurement, and after all sampling for each day. The bleach contains 7.55% NaClO that is diluted with sterile water to attain a 10% bleach solution (0.755% NaClO treatment).

Statistical analysis

The data collected from plate counts and water quality were analyzed using SAS version 9.4 software (SAS Institute Inc., Cary, NC). A two-way analysis of variance (ANOVA) using the PROC GLM statement was used to assess the

significant difference between treatments or days within the same treatment. Tukey-Kramer test was the post hoc test conducted to identify specific pairs of groups that caused a significant difference after the ANOVA Test with a statistically significant result. This was used to identify which treatments specifically had a significant impact on the bacterial counts throughout the study. Pearson Correlation was used to assess the correlation between variables. The levels of statistical significance were $P < 0.05$ in all cases.

Results and Discussion

Aerobic bacterial population: preliminary study

Aerobic bacterial population counts were only measured in Preliminary study Trial #1 because they were used to provide an estimate of the supplies that would be needed for APC counts in the subsequent multi-replicate low dose, high dose, and control dose treatments. With three 1:10 serial dilutions (10^{-3}), the limit of detection was 1 cell/ml for APC Petrifilm. There was significant difference between each treatment throughout the trial ($P < 0.0001$; $F= 41.16$; $DF = 4$) (Appendix D). A post hoc Tukey test indicated that DW differed significant to IBC and IBCW ($P < 0.0001$), but IBC and IBCW were not different from each other ($P = 0.3073$). RIBC and UN were not compared because data was not collected on those treatments on day 0 and 1 of the trial due to their similarity to IBC in the beginning of Trial #1. During the 24 hrs following inoculation of AP effluent by *E. coli* TVS354, the aerobic bacterial population in the AP water samples changed significantly with a difference in population between each day by treatment following inoculation ($P = 0.0004$; $F =$

4.90; DF = 9) (Appendix D). A Tukey test showed DW was significantly different from all of the treatments and control ($P < 0.05$) throughout the entire trial, but IBC only significantly differed from IBCW on day 1 ($P = 0.0374$). There were no other treatments that had significantly different comparisons on the similar days throughout Trial #1 (Appendix D). The inoculated treatments consisting of 100% aquaponic effluent (IBC) and 33% concentration of aquaponic effluent (IBCW) had mean APC-populations of 2.20 and 2.51 log CFU/ml, respectively.

While both treatments had an increase in population 24 h post-inoculation, the 33% AP treatment (IBCW) had a relatively small increase to 2.34 log CFU/ml, and the IBC treatment had an increase in mean population of 4.07 log CFU/ml. There was no difference in aerobic bacterial populations between all treatments and the uninoculated control. The uninoculated control (UN) samples were collected starting at 24 h (day 1) post-inoculation with a mean population of 3.98 log CFU/ml, and the population decreased to 2.65 log CFU/ml on day 2. The inoculated treatment with aquaponic effluent replenished (RIBC) samples were collected starting at day 2 with a mean population of 3.15 log CFU/ml and decreased to undetectable levels (0 log CFU/ml) on day 3 (Figure 2.4). Due to the limit of detection in MACR plates, counts reaching 0 log CFU/ml indicates the counts are not zero, but below the level of detection needed to quantify the data. The IBCW treatment had a peak mean population of 2.73 log CFU/ml on day 3, the IBC treatment had a peak mean population of 4.07 log CFU/ml on day 1; no other treatment had an increase in population during the trial (Figure 2.4). There is no explanation as to why IBCW

increased throughout the trial and would need to be replicated to see if this is a reoccurring pattern.

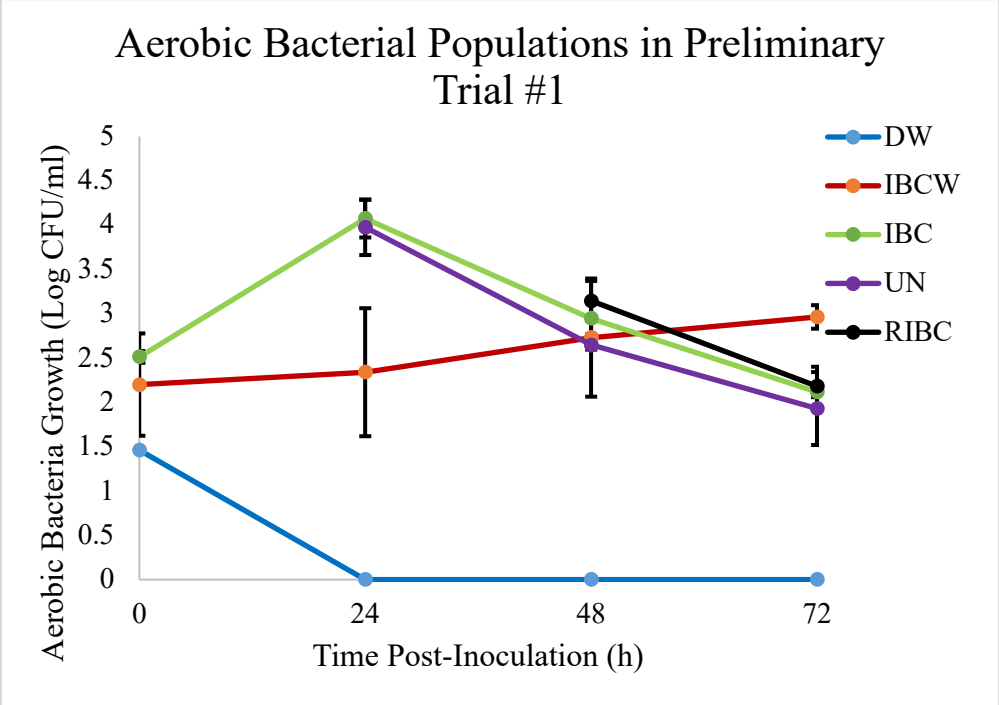


Figure 2.4. Trial #1 water samples were collected from a mature aquaponic system, aliquoted into bottles corresponding to different treatments. Values are means ± standard errors of n=3 per treatment. DW= distilled water inoculated with *E. coli* TVS354; IBCW= aquaponic effluent mixed with distilled water inoculated with *E. coli* TVS354; IBC= aquaponic effluent inoculated with *E. coli* TVS354; UN= uninoculated aquaponic effluent (control); RIBC= replenished aquaponic effluent inoculated with *E. coli* TVS354.

E. coli TVS354 population: preliminary study

In Trial #1, there was a significant reduction in *E. coli* TVS354 24 h post-inoculation in all treatments ($P = 0.0008$; $F = 6.31$; $DF = 4$) (Appendix E). In addition, counts decreased to undetectable numbers after 72 h post-inoculation. The Tukey test showed a significant difference between DW and IBC and a difference between IBC and IBCW ($P < 0.05$) (Appendix E). The ANOVA test showed a significant difference between treatments and days ($P = 0.0153$; $F = 2.82$; $DF = 9$). Despite this, a post hoc Tukey test indicated there was a significant difference in DW

and IBCW day 0 and 1 of Trial #1 ($P < 0.05$). In addition, IBC and IBCW had significantly different values on day 1 when comparing the two treatments (Appendix E).

Distilled water inoculated with *E. coli* TVS354 (DW) had an initial mean population of 2.04 log CFU/ml and decreased to undetectable numbers 24 h post-inoculation. The IBCW had undetectable numbers 48 h post-inoculation with a starting count of 2.38 log CFU/ml. Both IBC and RIBC had undetectable counts at 72 hours post-inoculation with similar counts 48 h post- inoculation (0.51 and 0.57 log CFU/ml, respectively) (Figure 2.5). To confirm that treatments had undetectable numbers, a higher volume of sample was used in spread plating (0.3 and 0.4 ml instead of 0.1 ml). With three 1:10 serial dilutions (10^{-3}), the limit of detection was 10 cell/ml. RIBC did not have data collected the first 24 h post-inoculation because replenishment did not occur until collected samples had been incubated 24 h because IBC and RIBC would have been replicates for the first 24 h post-inoculation.

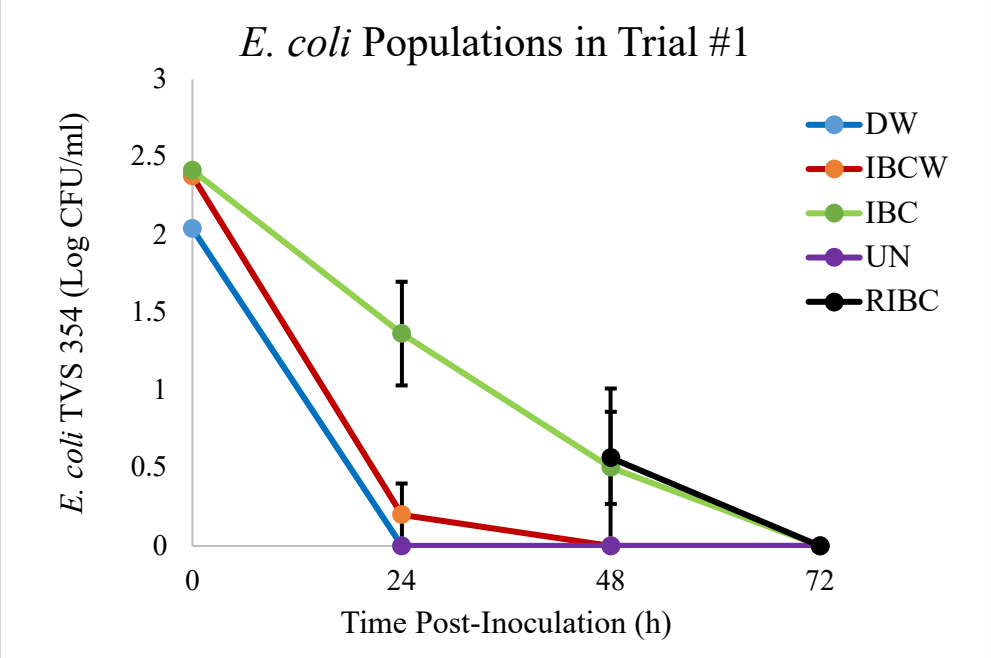


Figure 2.5. Trial #1 water samples were collected from a mature aquaponic system, aliquoted into bottles corresponding to different treatments. Values are means ± standard errors of n=3 per treatment. DW= distilled water inoculated with *E. coli* TVS354; IBCW= aquaponic effluent mixed with distilled water inoculated with *E. coli* TVS354; IBC= aquaponic effluent inoculated with *E. coli* TVS354; UN= uninoculated aquaponic effluent (control); RIBC= replenished aquaponic effluent inoculated with *E. coli* TVS354. All points at zero are considered not detected instead of zero.

For Trial #2, both treatments contained 100% aquaponic effluent, but the treatments were low-dose (LD) and high-dose (HD) inoculum. There was no significant difference between HD and LD treatments ($P = 0.0588$; $F= 4.36$; $DF = 1$), there were no significant differences between treatment and day ($P = 0.9766$; $F= 0.02$; $DF = 2$) in this trial as the *E. coli* counts dropped slower than in Trial #1 (Appendix F). In addition, there was no difference between each other across 48 h since both treatments decrease at the same rate and points in time. HD treatment had a slight reduction after 48 h (3.64 to 2.53 log CFU/ml; $P > 0.05$), and LD treatment had no significant reduction for the entire duration of Trial #2 (2.99 to 1.97 log CFU/ml; $P > 0.05$) as shown in the Tukey tests (Appendix F). Both treatments in Trial #2 had higher counts of *E. coli* compared to Trial #1 with no statistical significance (Figure 2.6).

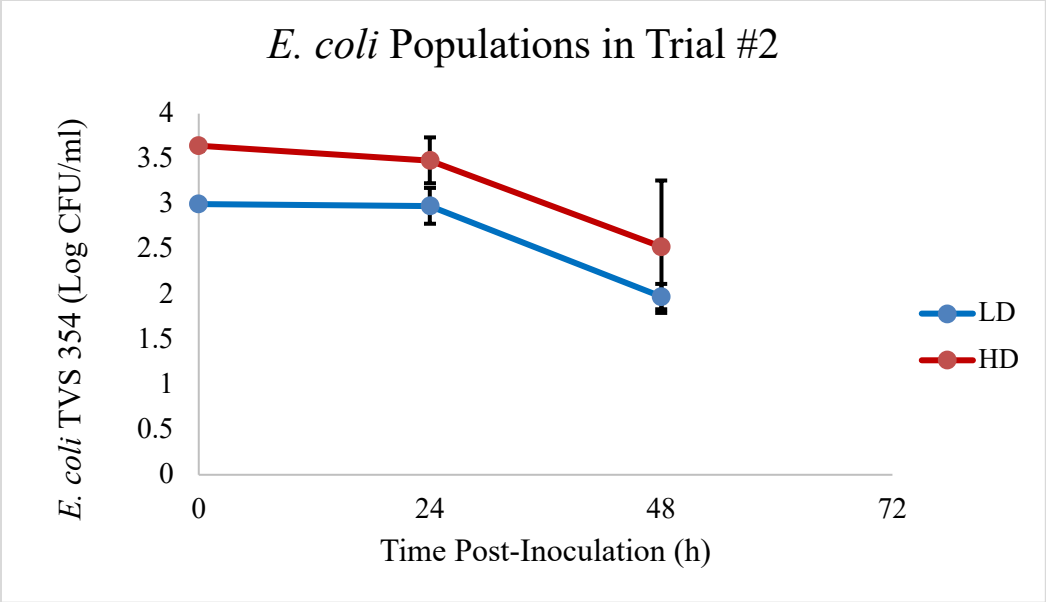


Figure 2.6. Trial #2 water samples were collected from a mature aquaponic system, aliquoted into bottles corresponding to different treatments of n=3 per treatment. Values are means ± standard errors: LD = low dose treatment; HD = high dose treatment.

Physicochemical parameters: preliminary study

Physicochemical parameter data was only collected in Trial #1. Aerobic bacteria had a weak positive correlation with electrical conductivity (EC) and nitrate. Aerobic bacteria had a moderate positive correlation with pH, and a strong positive correlation with nitrite (Table 2.1). Despite a weak and moderate positive correlation coefficient, the correlation between physicochemical parameters and aerobic bacteria is negligible with no noticeable trends or relationships between the two.

Table 2.1. Pearson's correlation between physicochemical parameters and aerobic bacterial populations

Parameter	Correlation Coefficient	P value
Electrical Conductivity	0.3712	0.0195
Nitrite (mg/l)	0.6583	<.0001
Nitrate (mg/l)	0.2808	0.0837
pH	0.4123	0.0086

pH continuously increased and reached a peak for DW, IBCW, IBC, and UN 48 h post-inoculation (10.96, 10.84, 8.89, and 10.95, respectively) (Figure 2.7). There was minimal variability in all treatment groups and control. High pH (8-11) followed by a sudden drop to pH 6 was uncharacteristic and likely inaccurate, possibly resulting from calibration issues displaying higher pH values as calibration was not conducted frequently.

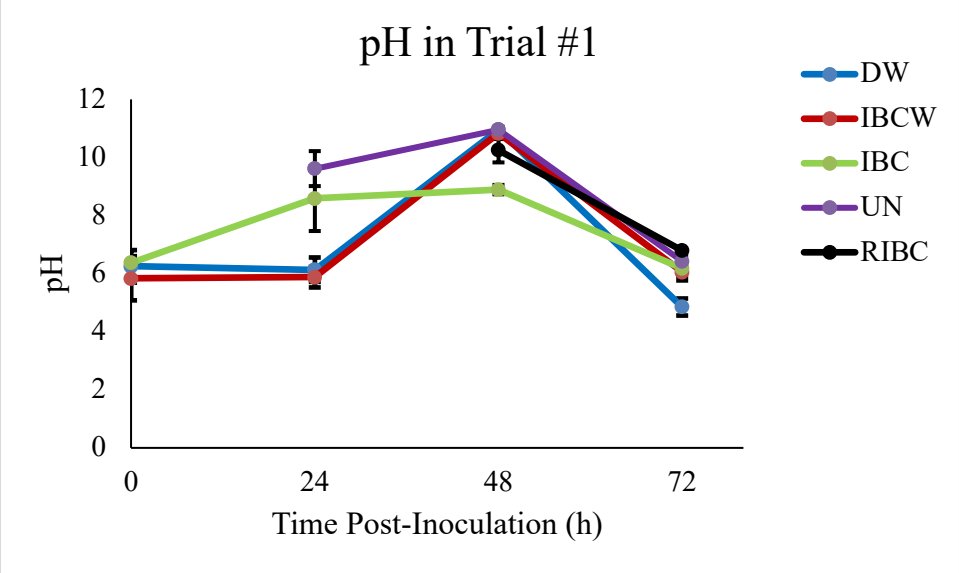


Figure 2.7. pH in all treatments and control with water samples collected from mature aquaponic system during Trial #1. Values are means ± standard errors of n=3 per treatment. DW= distilled water inoculated with *E. coli*; IBCW= aquaponic effluent mixed with distilled water inoculated with *E. coli*; IBC= aquaponic effluent inoculated with *E. coli*; UN= uninoculated aquaponic effluent (control); RIBC= replenished aquaponic effluent inoculated with *E. coli*.

Each treatment had no significant change in EC throughout the entire trial (Figure 2.8). IBC, UN, RIBC had higher EC as expected since they contain 100% aquaponic effluent. There was also minimal variability in all treatment groups and control, indicating there was no drastic differences between replicates. All treatments and control follow a similar pattern of deceasing EC from 48 to 72 h post-inoculation. DW and IBCW have similar patterns throughout the entirety of Trial #1 with the only

difference of IBCW having higher EC due to the aquaponic effluent component in the mixture. In addition to DW and IBCW, UN similarly increases at 24 h before decreasing at 48 to 72 h post-inoculation.

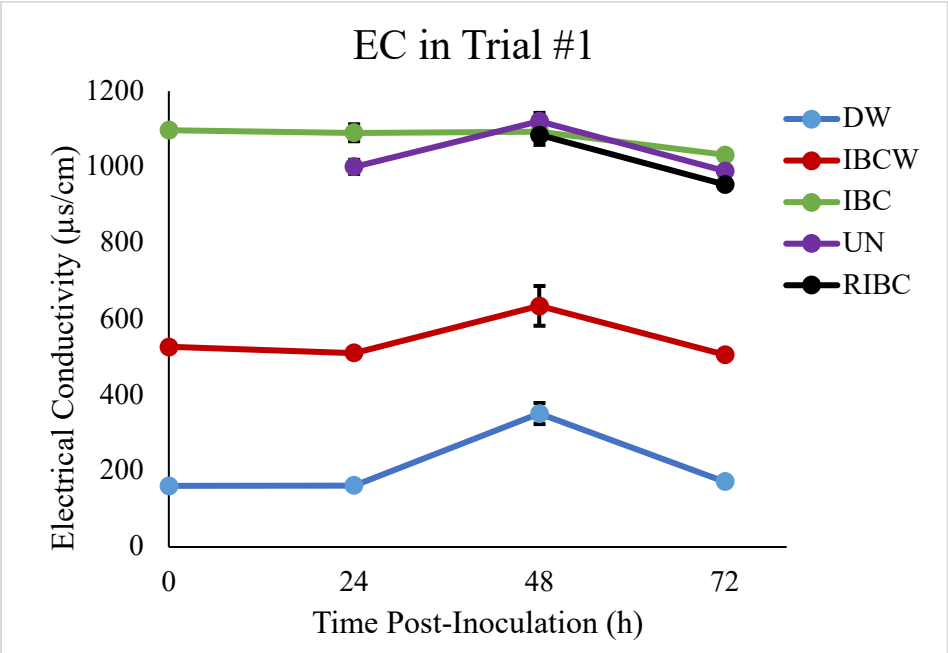


Figure 2.8. Electrical conductivity (EC) in all treatments and control with water samples collected from mature aquaponic system during Trial #1. Values are means ± standard errors of n=3 per treatment. DW= distilled water inoculated with *E. coli*; IBCW= aquaponic effluent mixed with distilled water inoculated with *E. coli*; IBC= aquaponic effluent inoculated with *E. coli*; UN= uninoculated aquaponic effluent (control); RIBC= replenished aquaponic effluent inoculated with *E. coli*.

IBC reached peak nitrite concentration 24 h post inoculation (1.16 mg/l) before decreasing along with all other treatments and control by 72 h; only IBCW remained constant (Figure 2.9). DW was expected to contain no nitrite as it was only comprised of distilled water. UN had high variability, particularly at the 48 h post-inoculation (standard error (SE) = 0.233), but no other parameters displayed the same amount of variability in the control. In addition, nitrite data was collected from color changing nitrite strips. It doesn't provide accurate values as a range of nitrite

concentrations fall under one value, and it is left up to the individual to discern the colors to determine the singular value the sample falls under.

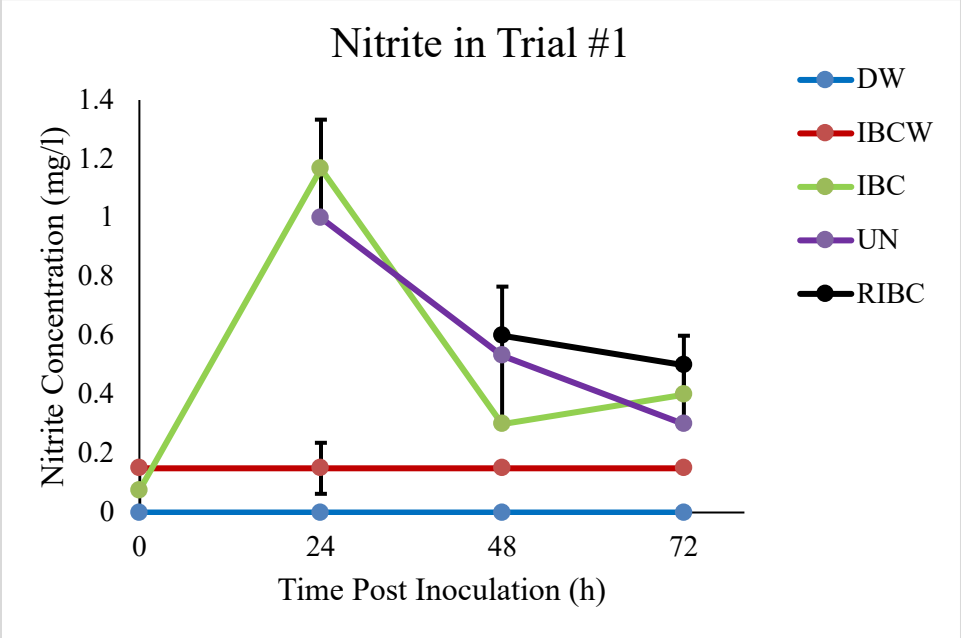


Figure 2.9. Nitrite concentration in all treatments and control with water samples collected from mature aquaponic system during Trial #1. Values are means ± standard errors of n=3 per treatment. DW= distilled water inoculated with *E. coli*; IBCW= aquaponic effluent mixed with distilled water inoculated with *E. coli*; IBC= aquaponic effluent inoculated with *E. coli*; UN= uninoculated aquaponic effluent (control); RIBC= replenished aquaponic effluent inoculated with *E. coli*.

With the exception of IBCW, all treatments and control had a nitrate concentration of 50 mg/l. IBCW had an initial nitrate concentration of 50 mg/l before decreasing to 20 mg/l 24 h post-inoculation (Figure 2.10). There was no variability in all treatments and control. Despite IBCW containing diluted aquaponic effluent, its initial nitrate concentration is the same as IBC, UN, and RIBC that are comprised of 100% aquaponic effluent before decreasing to 20 mg/l. Similar to nitrite, nitrate concertation values were collected using a nitrate strips, so values are not as accurate as using a spectrophotometer. This also leads to no variability as the test strips categorize the water quality in broad ranges.

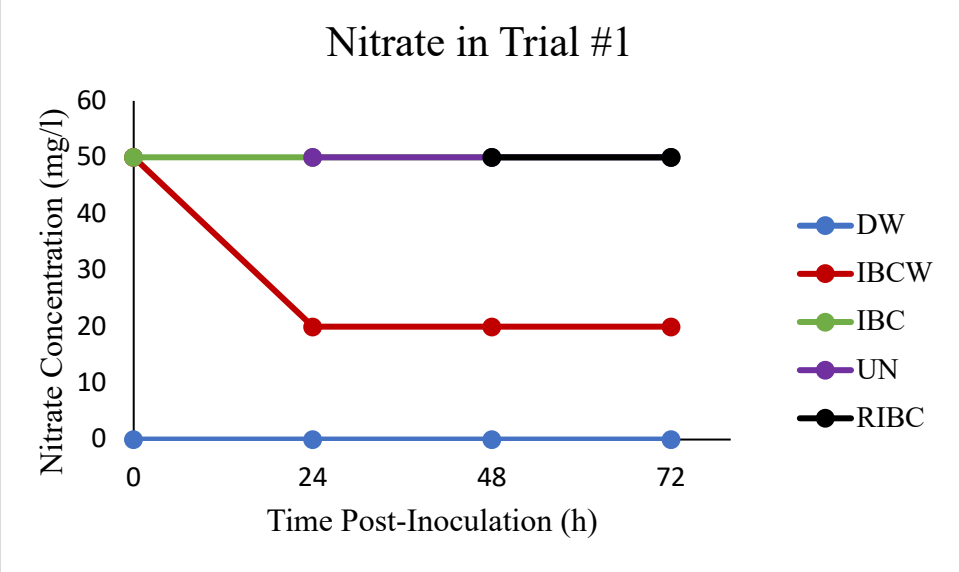


Figure 2.10. Nitrate concentration in all treatments and control with water samples collected from mature aquaponic system during Trial #1. Values are means of n=3 per treatment. DW= distilled water inoculated with *E. coli*; IBCW= aquaponic effluent mixed with distilled water inoculated with *E. coli*; IBC= aquaponic effluent inoculated with *E. coli*; UN= uninoculated aquaponic effluent (control); RIBC= replenished aquaponic effluent inoculated with *E. coli*.

Our study results provided insights on *E. coli TVS354* growth changes in aquaponic effluent and possibly what to expect in an event of contamination during production. The results of Trial #1 did not align with previous studies as counts decreased to undetectable numbers by 72 h post-inoculation. However, the results of Trial #2 more closely align with the results of previous studies that suggest that bacteria are able to quickly adapt to their environment and outcompete native bacteria (Shaw, et al., 2016; Elsas et al., 2011; Lejeune et al., 2001). It is unknown why IBC in Trial #1 had a faster decrease in *E. coli* population than HD in Trial #2. Both treatments were identical in inoculum and aquaponic effluent concentration. In Trial #1, IBC had 1.04 log CFU/ml decrease 24 h post-inoculation, whereas HD only had a 0.16 log CFU/ml decrease over the course of the same time period. Further trials

should be conducted under the same conditions to investigate the variability in results. Results from this study provided evidence that *E. coli TVS354* can survive over a short period of time. While the counts did not increase, the results of Trial #2 showed that *E. coli* populations can decrease gradually and have the possibility to persist over longer periods of time, raising the risk of a food borne outbreak. Studies should be conducted over a longer period of time in the future that mimics the growing season of different plants to examine the growth patterns and how they may differ.

Trial #1 and #2 provided information on the range of *E. coli* and APC growth in populations from inoculated levels. In the preliminary study, *E. coli* population decreased to undetectable levels in short span of time. With consistent input of nutrients through fish feed, *E. coli* population was expected to decrease to undetectable levels in the main experiment similar to the preliminary. However, the population was expected to decrease more gradually over a longer period with the addition of compartments that provide greater surface area, such as the biofilter. Microbial enumeration analysis procedures were identical, and the preliminary experiment was used to identify the amount of the 1:10 serial dilutions. The preliminary experiment was not the focus of this research, rather the results were used to provide insight on the influence of aquaponic effluent composition on *E. coli* population changes to plan for preparations in the main experiment. Since there haven't been any studies conducted of a similar nature to reference, the preliminary study was executed with no expectations.

Chapter 3: Survival of *Escherichia coli* in Aquaponic Systems During Basil and Lettuce Production

Introduction

Aquaponics provides an alternative food production method to address concerns associated with urban population growth, farmland shortage, greenhouse gas emissions associated with food production, and food insecurity (Al-Kodamy, 2018; Greenfeld et al., 2019). Aquaponics, a soilless production system, integrates aquaculture and hydroponics to provide local fresh produce (Delaide et al., 2017). Plant nutrients are supplied through bacterial transformation and metabolism of fish excreta (Wongkiew et al., 2017). Uptake of nitrogen by plants in the system occurs predominantly in the form of nitrate (Engels & Marschner, 1995).

In the United States, there is an estimated 9.4 million cases of foodborne illness caused by major pathogens annually (Scallan et al., 2011). About 51% (4.9 million) of annual outbreak associated illnesses were from plant commodities with the majority of those illnesses specifically associated with leafy vegetables (2.2 million) (Painter et al., 2013). From 2017-2022, there have been 19 *Escherichia coli* O157:H7 outbreaks, of which 8 were from leafy greens, baby spinach, and salad mixes (Centers for Disease Control and Prevention, 2022).

Irrigation water can be a source for foodborne pathogens (Allende and Monaghan, 2015; Critzer and Doyle, 2010; Steele and Odumeru; 2004). If contaminated water is used for aquaculture, pathogens can be introduced into aquaponic systems and contaminate the produce that comes in contact with the water

(Erickson et al., 2010; Macrisin et al., 2013; Lopez-Galvez et al, 2014). The absence of soil is expected to reduce exposure to soilborne pathogens, but there is risk of inadvertent contamination by pathogens spread through seeds, fish, fish waste, tools, equipment, inadequately treated circulated water remains, substrates, and human activity (Wu et al., 2019; Shaw et al., 2016). Pathogens may persist for one month or more after contact with a contaminated source (water, manure, tool/surface) (Nicholson et al., 2005).

Pathogens such as *Escherichia coli* (*E. coli*) are active in the rhizosphere of produce plants, can persist in soils for weeks, and can be found both within and on the surfaces of contaminated plants (Barnhart et al., 2015; Habteselassie et al., 2010). In hydroponic systems, *E. coli* was reported to internalize in the edible parts of lettuce plants by transfer from water into the root system through spaces resulting from lateral branch development or physical breaks in the root epidermis (Moriarty et al., 2019; Solomon et al., 2002; Wang et al., 2021; Bernstein et al., 2007; Erickson et al., 2010). Pathogens like Salmonella can also survive, and avoid removal by washing, or inactivation by sanitizing rinse, when they colonize plant stomata (REF, Barack). While pathogens can survive in the water (Wang et al., 2020), their survival and growth in bulk, and water samples from commercial aquaponic facilities has not been reported. The objective of this research was to determine survival of *E. coli* TVS354 in aquaponic effluent as well as key compartments of the AP research system, especially harvested leaves and roots of basil and lettuce. These replicated AP systems provided a means to compare the survival of low and high dose-inoculated *E.*

coli, relative to key compartments of the recirculating aquaponic research units under controlled conditions during a 60-day period.

Materials and Methods

Overview

The University of Maryland College Park IACUC approved this Animal Study Protocol as this study involves vertebrates (goldfish).
IACUC Reference #: R-FEB-21-10.

Bacterial strains and culture conditions

A non-pathogenic strain of *E. coli* with rifampin resistance (TVS354 isolated from Romaine lettuce) was used for inoculations in this study. The strain, originally provided by Dr. Trevor Suslow from University of California, Davis, CA, has been used in other environmental survival studies (Tomás-Callejas et al., 2011; Gutiérrez-Rodríguez et al., 2012; Zhao et al., 2021). A loop of frozen culture (-80 °C) was activated at 37°C for 18 hours in MacConkey agar (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 80 mg/l rifampin (Thermo Fisher Scientific, Waltham, MA). Then, a single colony for the strain was inoculated into Trypticase Soy Broth (30 mL) (Becton Dickinson, Sparks, MD) containing rifampin (80 mg/l) (Thermo Fisher Scientific, Waltham, MA) and incubated at 37°C for 18 hours with shaking. Cells were harvested by centrifugation at 4,300 x *g* for 5 min, washed once in sterile phosphate-buffered saline (PBS), and re-suspended in 10 ml of PBS. Equal volumes of cell suspensions were mixed and diluted in sterile distilled water to an

optical density of 0.3 (in a cuvette with 1 cm pathlength) at 600 nm (~8 log CFU/ml), which then was diluted 100-fold with sterile distilled water for use as inoculum.

Plant growing conditions

To examine the changes in the inoculated population in controlled environmental conditions, the study was conducted using 12 individual recirculating aquaponic systems placed on two metal racks, each 1.8 m long; each rack contained six aquaponic systems. Each system contained four plants, two Italian basil plants (*Ocimum basilicum* var. Elidia) and two little gem lettuce plants (*Lactuca sativa*), and four 1 year old goldfish (*Carassius auratus*). All seeds were purchased from Johnny's Selected Seeds (Winslow, ME), and all goldfish were purchased from Eaton's Goldfish Hatchery (Thurmont, MD). Each system, spaced 5 cm from an adjacent system, was composed of a 37.8 L fish tank and a 15.1 L deep-water plant grow bed and separated with plastic boards on both shelves (Figure 3.1).

These deep-water systems utilized a Styrofoam raft to elevate the stems and shoots/leaves above water while allowing roots to remain submerged in the continuously recirculating water, that contained the plant nutrients. Plants were elevated above water using 2.5 cm insulation foam boards (Owens Corning, Toledo, OH) with four 7.8 cm holes to hold plastic net pots (Bywater Shop, Pomona, NY) with a top diameter of 7.8 cm, bottom diameter of 5 cm, and height of 7.5 cm filled with hydroton (clay pellets) as the growth media (Sunlight Supply, Vancouver, WA). A 3-stage biofiltration system was used to filter solids in water pumped from the fish tank and convert the ammonia by microbial nitrification from the fish waste into nitrates suitable for plant uptake as fertilizer.

The biofiltration system contained three acrylic containers (7.62 cm wide, 7.62 cm long, and 7.62 cm high) vertically stacked with holes drilled to allow for water flow through each. The bottom layer had a spigot attached to allow water to drain into the 15.1 L grow tank. The top layer was layered with one layer of aquarium filter foam sponge (CLL Pet Supplies, Madison, WI) and two layers of cotton cheesecloth (10.5 cm wide and 14 cm long, weight 1.34 g) (SCENG, Shenzhen, CN). The cheesecloth was later changed to an aquarium filter media (sponge) with the same dimensions (2.5 cm thickness) due to the cheesecloth's lack of structural integrity (Aqua-Flo Industries, La Porte, IN). The second layer was composed of hollow ceramic rings (12.7 mm diameter) as filter media (CLL Pet Supplies, Madison, WI). The bottom layer was filled with K1 filter media (Evolution Aqua, Wigan, UK), hollow plastic cylinder (12 mm x 9 mm) with ridges along the exterior. This not only filters out solid waste, but it also provides ample surface area for bacteria to adhere to and grow.

All 12 systems were located in a constant temperature room, 20°C, for the entire duration of the study (62 days) (Figure 3.2). Each grow bed and fish tank were separated by plastic boards to prevent water splashing from adjacent systems. Grow lights (75W UV IR LED) (Osunby, Shenzhen, CN) on a 12 h on/off cycle were located 20.3 cm above the grow bed in the beginning of the study to 43.1 cm above the grow bed at the end of the study as the lights were raised on day 16, 23, 30, 37, 44, and 51 as plant height increased. Light intensity ranged from 95 to 175 µmol/m²/s and 60 to 115 µmol/m²/s based on the rack. The rack is covered in mylar to contain the LED lights. Plant height was measured weekly. Seeds were germinated in

rockwool (Hydroponic City Inc., La Puente, CA) for 14 days in a bench-top growth chamber (EarlyGrow, North Yorkshire, UK) before they were transferred into net pots filled with hydroton as the grow media. The growth chamber was placed on a heating mat (VIVOSUN, Ontario, CA) set at 22 ℃ with the rockwool kept damp with distilled sterile water. Basil was germinated and transferred into the APs with two to three true leaves one week earlier than the lettuce was with one to two true leaves. All plants survived by the end of the Main Experiment with no plants showing signs of distress, root rot, disease, or needing to be replaced after transplant from growth chamber.

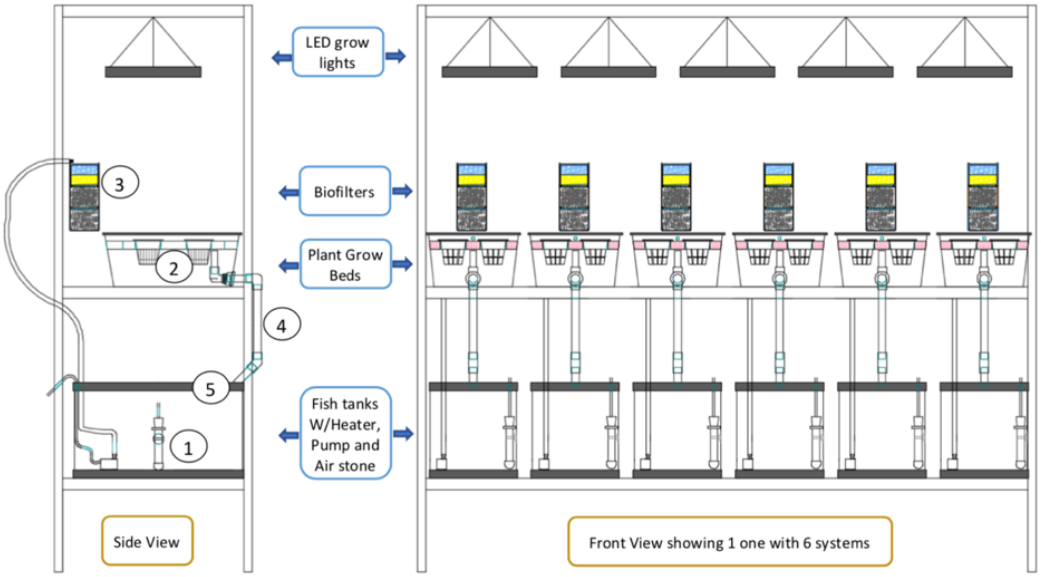


Figure 3.1. Schematic of lab scale recirculating aquaponic systems. 1. Fish Tank; 2. Plant Grow Bed; 3. Water Pump and Biofiltration System; 4. Bell Siphon; 5. Water Discharge

Figure 3.2. Picture of the 12 aquaponic systems used in Phase 1 and 2. Each system contained a fish tank with 4 goldfish, grow bed with 4 plants (2 basil and 2 lettuce), 3-tier biofiltration system and LED lights.

Fish growing conditions

Each system contained four 1-year old goldfish (*Carassius auratus*) and were purchased from Eaton's Goldfish Hatchery (Thurmont, MD). The average weight of all 48 goldfish (32g) was used to determine the amount of fish feed (Table 3.1). All fish was fed 0.8g of goldfish flakes (Tetra, Melle, DE) once a day (2.5% of the average weight).

Table 3.1. Average weight of four goldfish per aquaponic system

Tank	Average Weight (g)
1	28.93
2	38.91
3	35.36
4	34.56
5	24.65
6	33.93

7	36.35
8	24.72
9	32.23
10	33.78
11	31.56
12	25.01

Each system, spaced 5 cm from an adjacent system, was composed of a 37.8 L fish tank and a 15.1 L deep-water plant grow bed with a plastic board covering the top of the tank (Figure 3.1). Each water tank bottom had a 4 cm layer of gravel; water was circulated by a 900 L/h water pump (Kedsum, Shenzhen, CN). Each set of six fish tanks was connected to a 1.5 m^3/h air pump (Hydrofarm, Fairless Hills, PA) with 4 cm air stones (Pawfly, Guangzhou, CN) dispersing ambient air throughout each tank, and 36W LED aquarium lights (NICREW, Shenzhen, CN) were hung above 6 tank set (on rack) on a 24 h light cycle (12 h on, 12 h off). Water quality parameters were collected on a weekly basis to ensure fish heath (Table 3.2). If measured parameters were outside of expected ranges, adjustments would be made. Potassium hydroxide (10 ml) was used to raise system pH to return to expected range. No changes were made to adjust ammonia, nitrite, and nitrate levels. Ambient room temperature was set at 20°C, but temperature varied due to external weather and foot traffic from outside to the lab where the systems were located. With no heater installed in the fish tanks, temperature fluctuations were dependent on external factors. Fish displayed no adverse behaviors for the duration of the main experiment with all 48 fish surviving by the end of the study.

Table 3.2. Aquatic parameter range in aquaponic systems compared to expected range for goldfish based on IACUC Protocols

Parameter	System Range	Expected Range
Temperature (°C)	18.9 – 23.6	17.8 – 23.3

Dissolved Oxygen (mg/l)	5.8 – 11.4	6 – 8
Total Ammonia (mg/l)	0.06 – 2.81	0
Nitrite (mg/l)	0.02 – 0.55	0
Nitrate (mg/l)	11.8 – 88.1	<20
pH	4.6 – 7.3	7.2 – 7.6

Water inoculation

Basil and lettuce were grown in deep-water recirculating aquaponic systems in this two-phase experiment with a randomize block design. In Phase #1, four systems were inoculated with a low dose inoculum (3 Log CFU/ml), a high dose inoculum (4 Log CFU/ml), or no inoculation each. Prepared inoculum of *E. coli* TVS354 was added into the 37.8 L fish tank and left to circulate in the system by a 900 L/h water pump for 1 hour prior to microbiological analysis. Water in the fish tank was sampled and analyzed for *E. coli* TVS354 on 0, 1, 2, 3, 7, 8, 9, 10 days post-inoculation (dpi). Water in the fish tank was sampled and analyzed for aerobic bacteria on 0, 1, 2, 3, 7, 8, 9, 10, 14, 21, 23 dpi.

A second inoculation experiment (Phase 2) was conducted using *E. coli* TVS354 at 2 Log CFU/ml (low dose) and 4 Log CFU/ml (high dose) with no controls. Lettuce and basil transplanted to the recirculating aquaponic systems from the previous experiment was kept in the system for this experiment and was harvested at the end of the second experiment. Water was sampled for *E. coli* TVS354 and aerobic bacteria from the fish tank on 0, 1 and 2 dpi. Water was also sampled for *E. coli* TVS354 and aerobic bacteria from the biofiltration system on 2, 9, 14, 21, and 28 dpi. Basil and lettuce leaves and roots were sampled for *E. coli* TVS354 on 30 and 32 dpi (day 58 and 60 of the entire study), respectively. The protocol for the remainder of the experiment was conducted in the same manner as Phase 1 as described above,

with the exception of microbiological analysis for basil and lettuce only conducted after completing Phase 2. The inoculum size for Phase 1 was based on Trial #2 of the preliminary experiment. Based on the results of the preliminary experiment, it was expected that the *E. coli* population would reach undetectable number after 48 hrs. In Phase 2, the LD treatment was lowered to still simulate a low contamination event, but it was intended to show a larger contrast between the HD treatment. The Phase 2 LD treatment was sought to examine possible differences in *E. coli* population changes, such as the rate at which population decrease.

Microbiological analysis

Water samples (10 ml) were collected from the fish tanks in Phase 1 and from the biofilters in the Phase 2 of the study. Serial 1:10 dilutions (4 dilutions per sample) of water samples prepared in sterile phosphate-buffered saline (PBS) were spread plated (0.1 ml) onto MacConkey Agar-Rifampicin (MACR) to detect *E. coli* TVS354. Colonies were counted after incubation at 37 ℃ for 24 hours. Three 1:10 serial dilutions (10^{-3}) were conducted for MACR. were conducted for MACR. Aerobic mesophilic heterotrophic bacteria were enumerated using Aerobic Plate Count (APC) Petrifilm (3M, Maplewood, MN) in duplicate for each water sample dilution after incubation at by 37 ℃ for 48 hours according to the manufacturer's instructions. Three 1:10 serial dilutions (10^{-3}) were conducted for APC. APC was used monitor the change in the native bacteria populations and how it would change with the introduction of *E. coli*. Since *E. coli* is an aerobic bacterium, its counts are included in APC. Of the 10 ml water samples that were collected, 1 ml was used for direct plating in 1:10 serial dilutions (MACR and APC) with the remaining 9 ml samples used for

enrichment. For filter samples, 1 ml of water was squeezed out of the cheesecloth/sponge for serial dilution with filter used for enrichment. Enrichment was only conducted on filter samples in Phase 2 and plants samples collected at the end of the Main Experiment.

Leaves and roots from each plant were separated from each whole plant with 70% EtOH sterilized, air-dried scissors following removal from aquaponic systems. For *E. coli* TVS354 enumeration from leaf surfaces, 25 g of leaves collected from the two plants of the same species were suspended in 100 ml sterile PBS in a Whirl-Pak filter bag (Nasco, Fort Atkinson, WI) and pummeled for 2 mins at 200 rpm in a Stomacher 400 Circulator (Steward, London, UK). For root surfaces, all roots from both plants (same species) were suspended in a Whirl-Pak filter bag with 200 ml sterile PBS and pummeled as described above. Prepared, stomached tissue suspensions were assayed for *E. coli* TVS354 by enumeration using spread plating and aerobic plate count Petrifilm as described above.

Filter samples and plant samples were also assayed using Tryptic Soy Broth with Rifampicin (TSB-r) for enrichment to verify a positive or negative presence of *E. coli* TVS354. Enrichment is able to detectable lower counts of *E. coli* in order to verify the presence of the pathogen when MACR plates indicate undetectable counts. All samples, including UN control, was enriched to verify positive or negative counts. Samples were collected and separated for immediate plating (APC and MACR) and enrichment each sampling day (Appendix C). Enrichment for filter sample was conducted starting on day 9 (day 37) of Phase 2 and continued to the conclusion of Phase 2 (day 14, 21, 28, 32 dpi). For filter samples, liquid samples (5 ml) were added

into a Whirl-Pak filter bag containing 100 ml of TSB-R and massaged for 1 min. before incubation at 37℃ for 24 h. Samples (2µl aliquots) were streak plated onto MACR then incubated at 37℃ for 24 h before counting *E. coli* colonies. For root samples, 10 g root homogenate samples were added to (90 ml) TSB-R incubated at 37℃ for 24 h. For leaf samples, 5 g leaf homogenate samples were added to (45 ml) TSB-R and incubated at 37℃ for 24 h. Samples (2µl aliquots) were streak plated onto MACR then incubated at 37℃ for 24 h before counting *E. coli* colonies.

Physicochemical water analysis

Physicochemical parameters were measured on fish tank samples and included: dissolved oxygen (DO), pH, temperature using a Hanna HI98196 portable meter (Hanna Instruments, Woonsocket, RI); electrical conductivity (EC) using a tracer PockeTester (LaMotte Company, Chestertown, MD); ammonia, nitrite, and nitrate using a Hach DR 3900 laboratory spectrophotometer (Hach Company, Loveland, CO). For DO, pH and temperature, the sensor was directly inserted into the tanks and removed when values stabilized, whereas 30 ml of water was removed to measure EC due to the short length of the sensor. For ammonia, 20 ml of water was removed, and the ammonia reagent set was used according to manufacture instructions. For nitrite and nitrate, 20 ml of water was removed for each test, and the nitrite and nitrate reagent were used according to manufacture instructions, respectively.

Physicochemical parameters were measured on 1, 3, 8, 9, 15, 17, 22, and 24 dpi for the Phase 1 period, and on 1, 3, 4, 8, 10, 15, 17, 22, 24, 29, and 31 dpi in the Phase 2 period (day 29, 31, 32, 36, 38, 43, 45, 50, 52, 57, and 59). Parameters were

measured to ensure water quality was maintained with a predetermined range to ensure optimal fish health (Table 3.2). Adjustments were made to water quality when certain parameters fell outside the expected range.

Sterilization

All meters, tools, equipment, and surfaces that came in contact with *E. coli* TVS354 were disinfected with 70% EtOH or 10% bleach before, in between each measurement, and after all sampling for each day. The bleach contains 7.55% NaClO that is diluted with sterile water to attain a 10% bleach solution (0.755% NaClO treatment).

Statistical analysis

Data collected were input into SAS 9.4 software (SAS Institute Inc., Cary, NC) to conduct a two-way analysis of variance (ANOVA) using the PROC GLM statement. A post hoc Tukey-Kramer test was conducted to identify specific pairs of groups that caused the difference after an ANOVA Test and used to gauge the significant difference between treatments and days for both water and plants. Pearson Correlation was used to evaluate the correlation between physicochemical parameters and aerobic bacteria. The levels of statistical significance were $P < 0.05$ in all cases.

Results

E. coli TVS354 population

In Phase 1, there was a significant decrease in *E. coli* TVS354 populations in low-dose (LD) and high-dose (HD) treatments during the initial 24 h post-inoculation

and throughout Phase 1 ($P < 0.0001$; $F = 4691.19$; $DF = 16$). The LD and HD had mean population reductions of 2.57 and 3.54 log CFU/ml, respectively. The *E. coli* TVS354 populations remained undetectable after the initial 24-h post-inoculation time period for the remaining duration of Phase 1 (Figure 3.3). With three 1:10 serial dilutions (10^{-3}), the limit of detection was 10 cell/ml for samples collected from fish tank water. There was also a significant difference between treatments ($P < 0.0001$; $F = 4500.89$; $DF = 2$) throughout Phase 1 (Appendix G). A post hoc Tukey test showed the LD and HD differed significantly with each other on day 0 and 1 post inoculation ($P < 0.0001$).

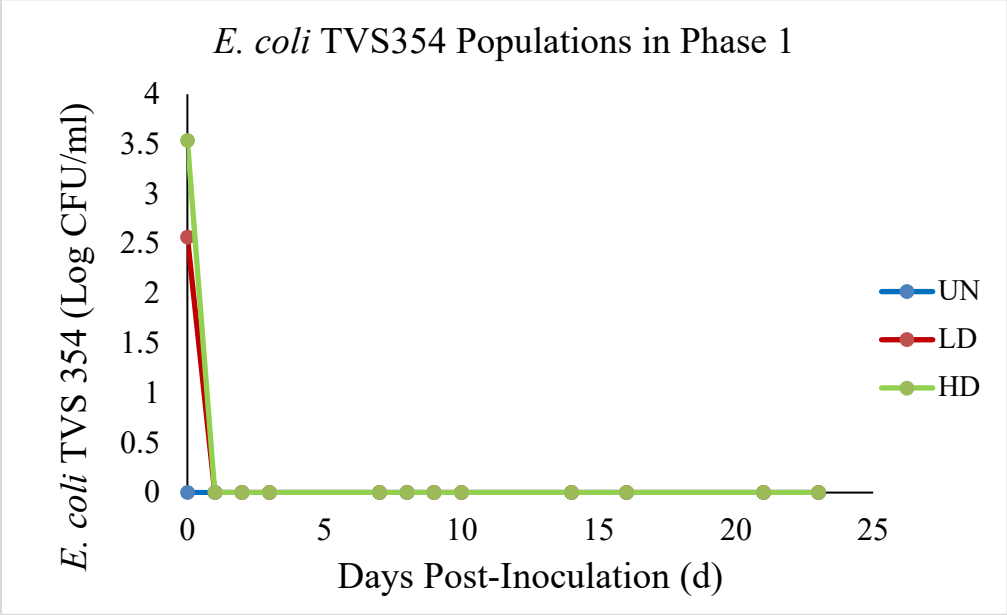


Figure 3.3. Phase 1 water samples were collected from the fish tanks to measure *E. coli* TVS 354 counts. Values are means ± standard errors of n=4 per treatment: UN = uninoculated (control); LD = low dose treatment; HD = high dose treatment. All points at zero are considered not detected instead of zero.

In Phase 2, the *E. coli* TVS354 populations for low-dose (LD) and high-dose (HD) treatments also exhibited a significant decrease during the 24-h post-inoculation period ($P < 0.0001$; $F = 33.21$; $DF = 2$); water samples were collected from the fish

tanks. Water samples also had significant difference between the treatments ($P < 0.0001$; $F = 185.95$; $DF = 2$). A post hoc Tukey test showed a significant difference in HD and LD between the first day 0 and 1 post inoculation of Phase 2 (Day 28 and 29) ($P < 0.0001$ and $P = 0.0008$, respectively). In addition, Tukey test showed a significant difference in treatments between HD, LD, and UN ($P < 0.0001$) (Appendix H). Samples collected from the biofiltration system showed a significant decrease in mean populations from 2 days (48 h) to 7 days post-inoculation ($P < 0.0001$; $F = 200.34$; $DF = 8$). In addition, treatments displayed significant difference throughout the remainder of Phase 2 ($P < 0.0001$; $F = 214.52$; $DF = 2$). A post hoc Tukey test showed a significant difference between treatments and between days within the same treatments ($P < 0.0001$) (Appendix H). The LD and HD treatments exhibited mean population reductions of 2.05 and 3.63 log CFU/ml (Figure 3.4 and 3.5), respectively. Enrichment indicated all treatments had positive counts of *E. coli,* and UN had no positive counts throughout the entire duration of Phase 2.

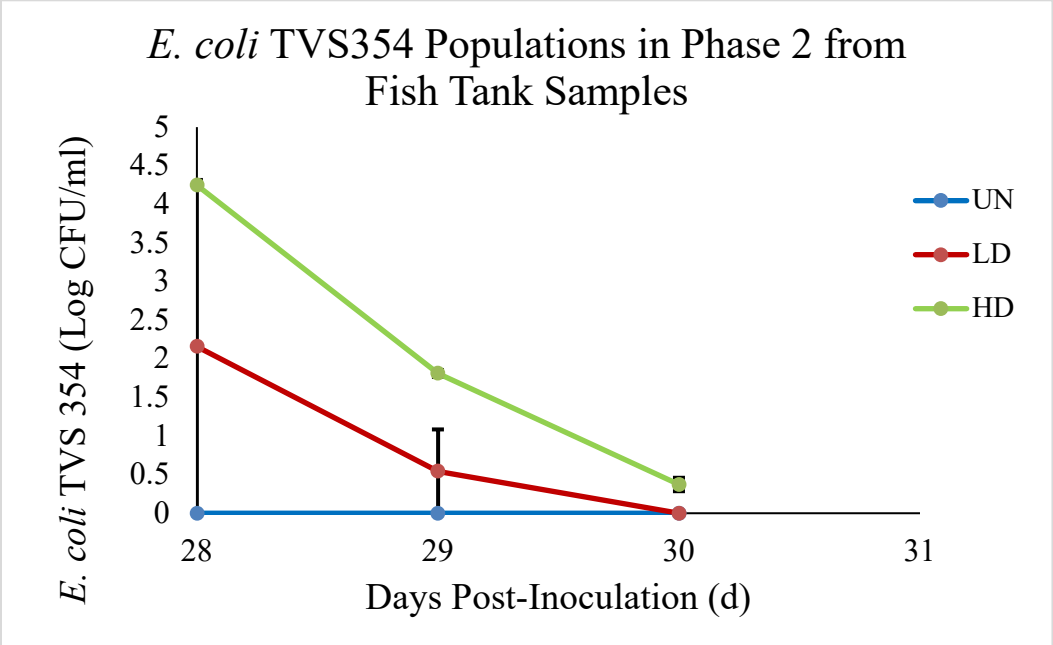


Figure 3.4. Phase 2 water samples in the first three days (day 28, 29, 30) following the second inoculation event were collected from the fish tanks. Values are means ± standard errors of n=4 per treatment: UN = uninoculated (control); LD = low dose treatment; HD = high dose treatment. All points at zero are considered not detected instead of zero.

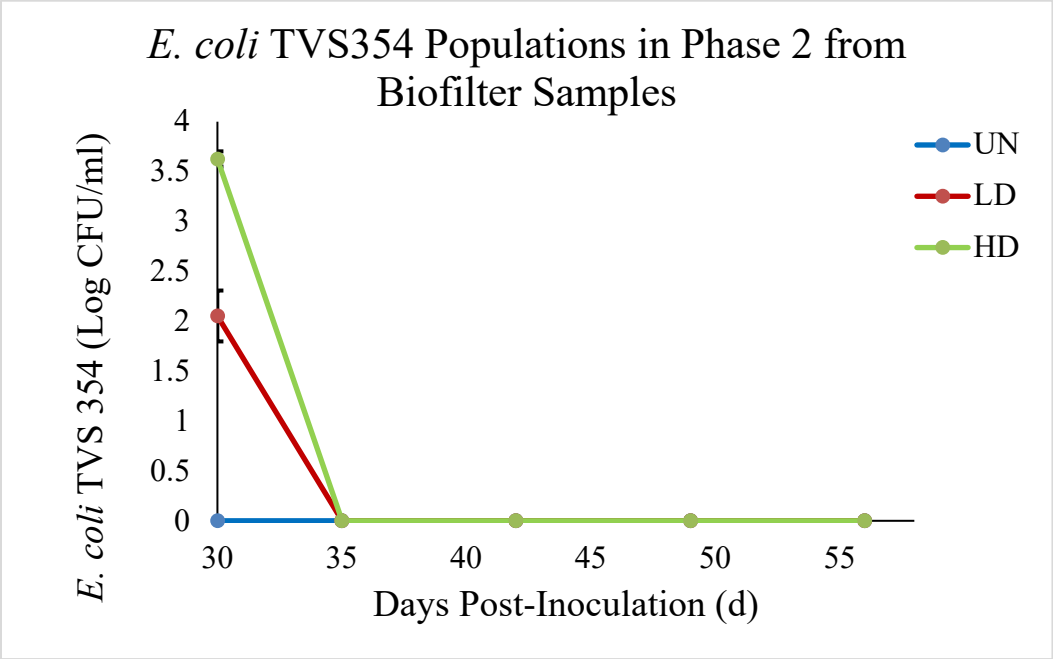


Figure 3.5. Phase 2 water samples in the second inoculation event were collected from the biofilters starting on day 30. Values are means ± standard errors of n=4 per treatment: UN = uninoculated (control); LD = low dose treatment; HD = high dose treatment. All points at zero are considered not detected instead of zero.

Aerobic bacterial populations

After inoculating *E. coli* TVS354 into the aquaponic systems in Phase 1, the aerobic bacterial population in fish tank water declined significantly during the initial 24 h, and with a significant daily reduction in the populations within the same treatment ($P < 0.0001$; $F = 3.92$; $DF = 16$) (Appendix I). The uninoculated control, low-dose treatment, and high-dose treatment all started with mean aerobic bacterial populations of approximately 3 log CFU/ml with population decreases to 2.60, 2.66, and 2.83 log CFU/ml (Figure 3.6), respectively. According to post hoc testing (Tukey test), there was only a statistically significant decrease in the first 24 h of the HD

treatment ($P < 0.0001$), and no significant decrease in the LD treatment ($P = 0.3783$) and UN control ($P = 0.0660$) across the same span of time (Appendix I).

However, there was no significant difference in aerobic bacterial populations between the HD and LD treatments and the uninoculated control ($P = 0.1753$; $F = 1.78$; $DF = 2$). Following the 24 h post-inoculation population decrease, aerobic bacterial populations increased starting 48 h (day 2) and continued to increase until day 16, at which all three treatments (uninoculated control, LD, and HD) had peak mean populations of 7.93, 6.74, and 6.84 log CFU/ml, respectively. The limit of detection was 1 cell/ml for samples collected from fish tank water, and 1 CFU/filter for samples collected from the cheesecloth/sponge in the biofilter.

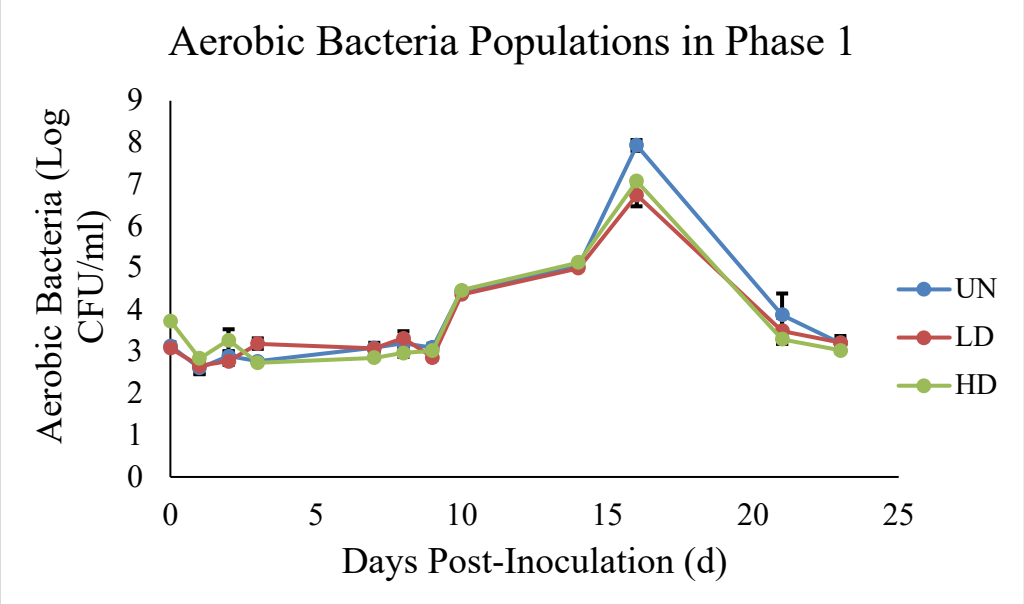


Figure 3.6. Phase 1 water samples were collected from the fish tanks. Values are means ± standard errors of n=4 per treatment: UN = uninoculated (control); LD = low dose treatment; HD = high dose treatment.

In Phase 2, water samples were collected from fish tanks 24 h post inoculation, then collected from aquaponic biofiltration systems for the remainder of the study. In the fish tank, there was no significant difference between treatments ($P =$

0.4646; F = 0.81; DF = 2), but there was significant difference within the same treatments (P = 0.0213; F = 5.14; DF = 2) 24 h post inoculation (Appendix J). Despite this, the Tukey test showed there was no significant difference in mean populations for the low-dose treatment (3.63 to 3.44 log CFU/ml; P = 0.9947) and high-dose treatment (4.57 to 3.09 log CFU/ml; P = 0.0728) 24 h post-inoculation (Figure 3.7 and 3.8). Samples collected from the biofiltration systems had significant differences between treatments (P = 0.0002; F = 11.05; DF = 2), but there was no significant difference within the same treatment across multiple days (P = 0.6254; F = 0.78; DF = 8). A post hoc Tukey test also supported this, but there was only a significant difference between HD and UN (P = 0.0064) and LD and UN (P = 0.0001). There was no significant difference between the treatments (P = 0.2996) (Appendix J).

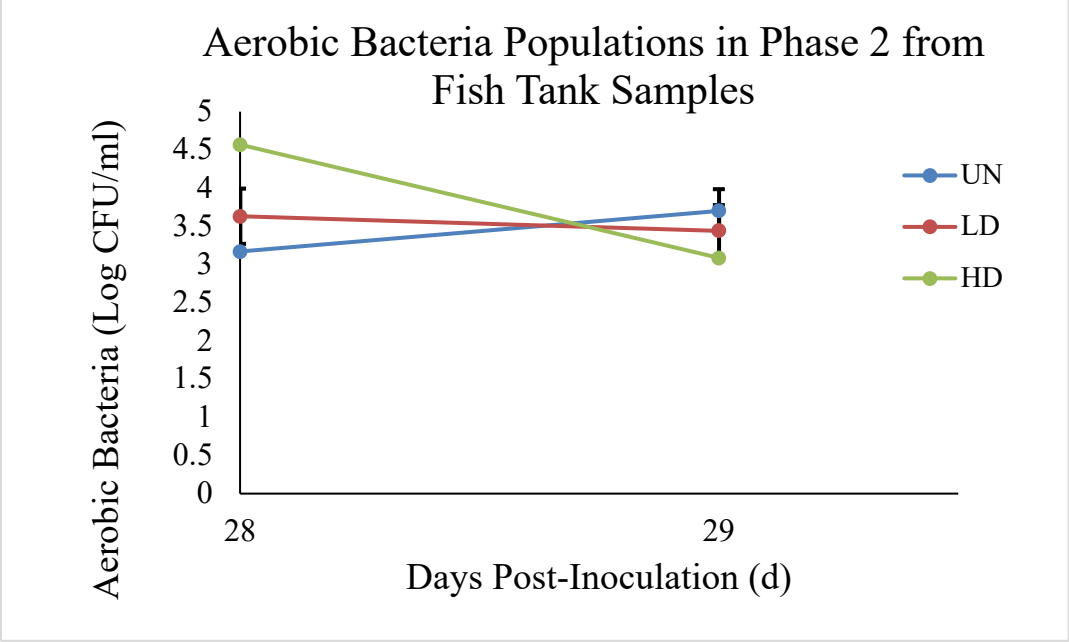


Figure 3.7. Phase 2 water samples in the first two days (day 28, 29) following the second inoculation event were collected from the fish tanks. Values are means ± standard errors of n=4 per treatment: UN = uninoculated (control); LD = low dose treatment; HD = high dose treatment.

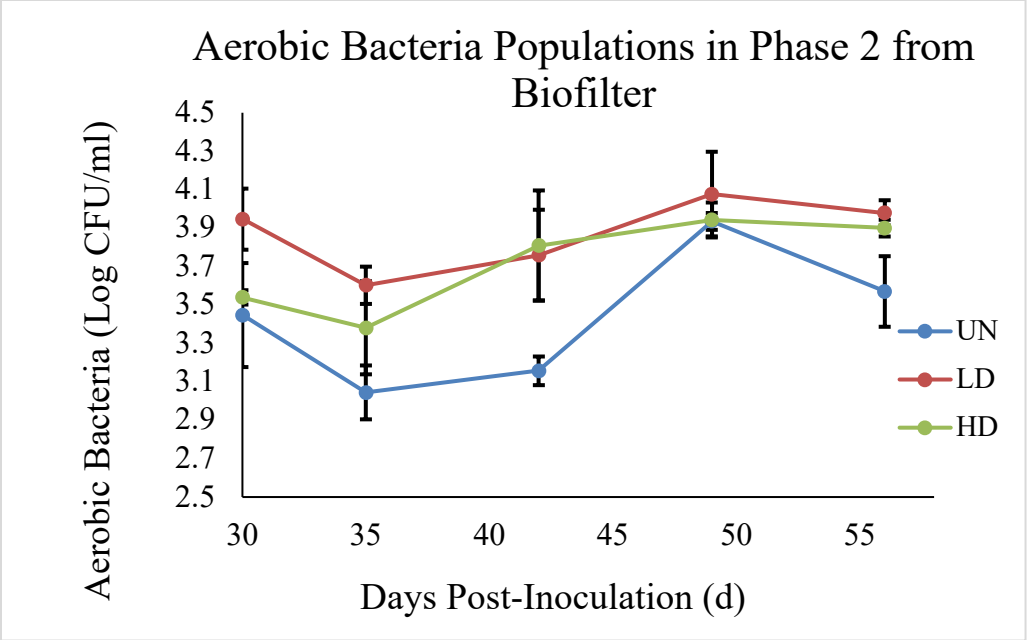


Figure 3.8. Phase 2 water samples in the second inoculation event were collected from the biofilters starting on day 30 Values are means ± standard errors of n=4 per treatment: UN = uninoculated (control); LD = low dose treatment; HD = high dose treatment.

Physicochemical parameters

For physicochemical parameters, aerobic bacteria had a strong positive correlation with nitrate and ammonia. Whereas aerobic bacteria had a moderate positive correlation with electrical conductivity (EC) and nitrite (Table 3.3). Despite as moderate and strong positive correlation coefficient, there were no patterns or trends visually seen that indicated any relationship with aerobic bacteria.

Table 3.3. Pearson's correlation between physicochemical parameters and aerobic bacterial populations

Parameter	Correlation Coefficient	P value
Electrical Conductivity	0.4801	0.0008
Ammonia	0.6148	<.0001
Nitrite	0.5967	<.0001
Nitrate	0.7069	<.0001

The EC continuously increased until reaching peak values for all three treatments, UN, LD, and HD: 743.33, 731.5, and 723 µs/cm, respectively, before

decreasing (Figure 3.9). There was moderate variability in all treatment groups EC continuously increased throughout both trials. In Phase 1, EC increase up until day 24, before increasing again on day 28 (reinoculation date). On day 52, all treatments decrease up until the end of Phase 2 (day 59) with EC still being higher than Phase 1.

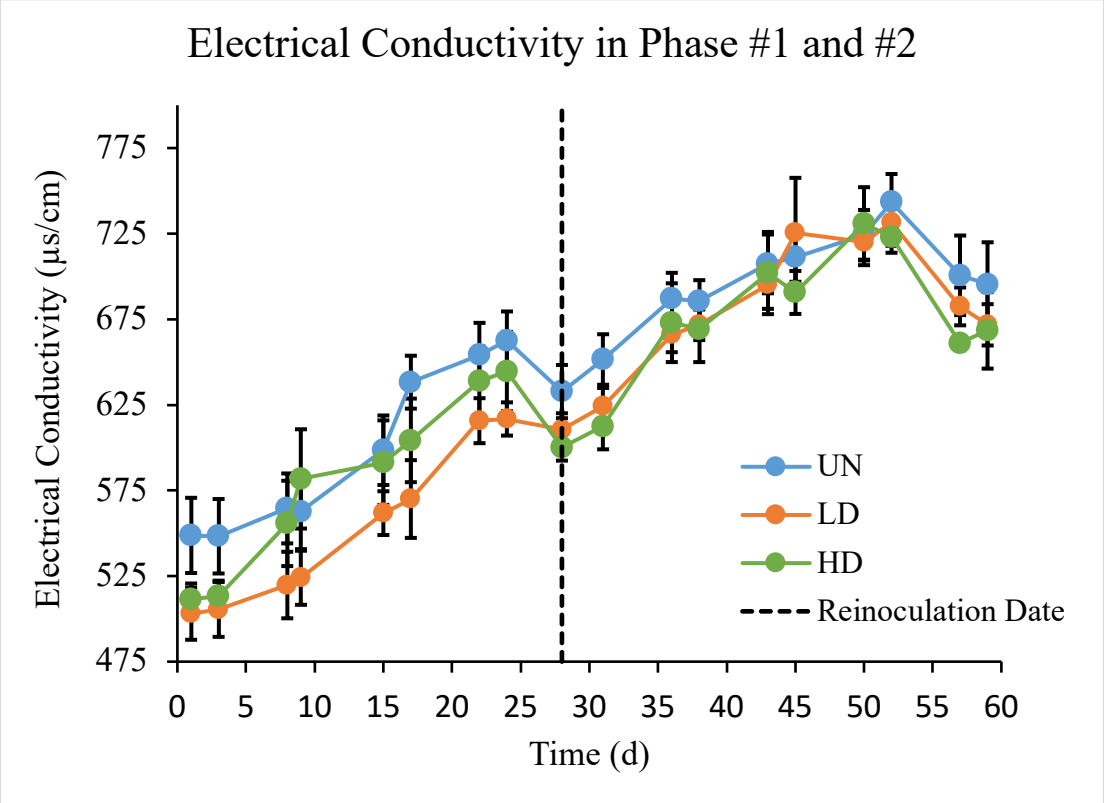


Figure 3.9. Electrical conductivity (EC) in control, low and high dose treatment samples collected from fish tank water during Main experiment Phase 1 and 2. Dotted line indicates the start of Phase 2, where fish tanks were reinoculated with *E. coli* TVS 354. Values are means ± standard errors of n=4 per treatment: UN = uninoculated (control); LD = low dose treatment; HD = high dose treatment.

The pH in Phase 1 began decreasing drastically approximately 10 days post-inoculation up until day 28, with pH reaching 4-5, at which point potassium hydroxide was added to raise and maintain a desirable water quality pH of 6 to 7 to maintain optimal fish health. The increase in ammonia levels was likely due to the limitations in handling fish waste in our small-scale system. Because pH levels were

maintained intentionally in the range of 6 to 7 for fish and plant health, no correlation analysis was conducted reflecting aerobic bacterial changes. Following the spike, ammonia levels decreased on day 30 and reached levels similar to those present at the beginning of the study (Figure 3.10). LD exhibited higher variability on all days in both phases with start of Phase 2 (day 28) having the highest variability with UN and HD following behind (SE = 0.476, 0.307, 0.137, respectively). LD also had higher ammonia concentrations from day 15 to day 45. The largest difference between LD with HD and UN occurring on day 28 (0.878 and 0.8 mg/l, respectively).

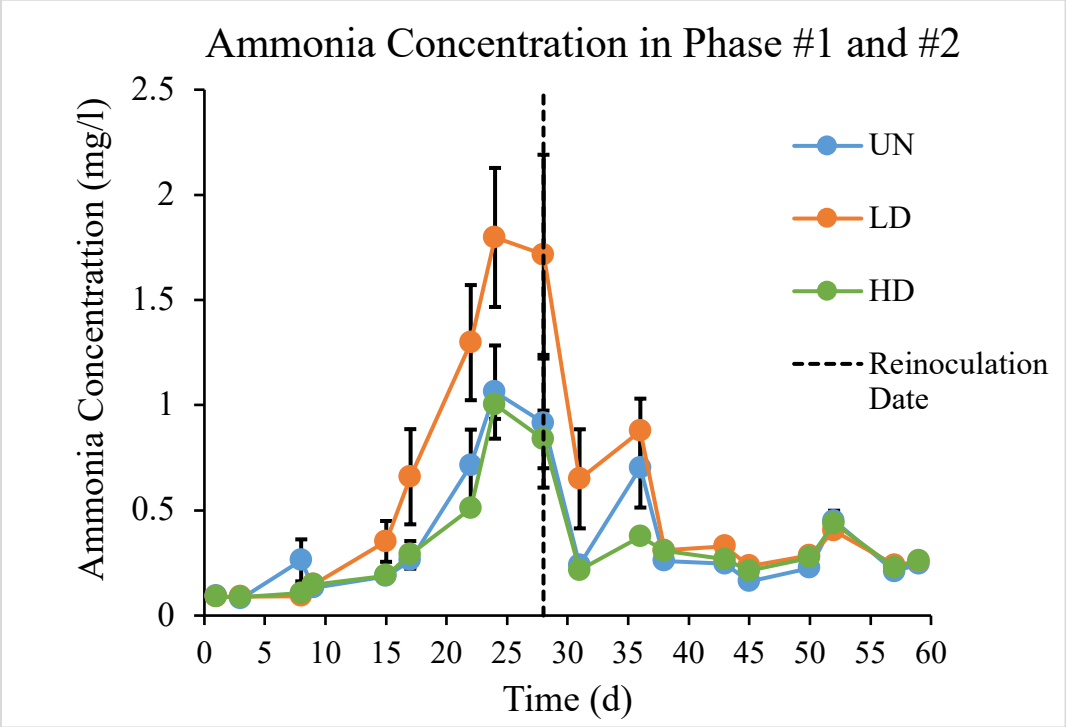
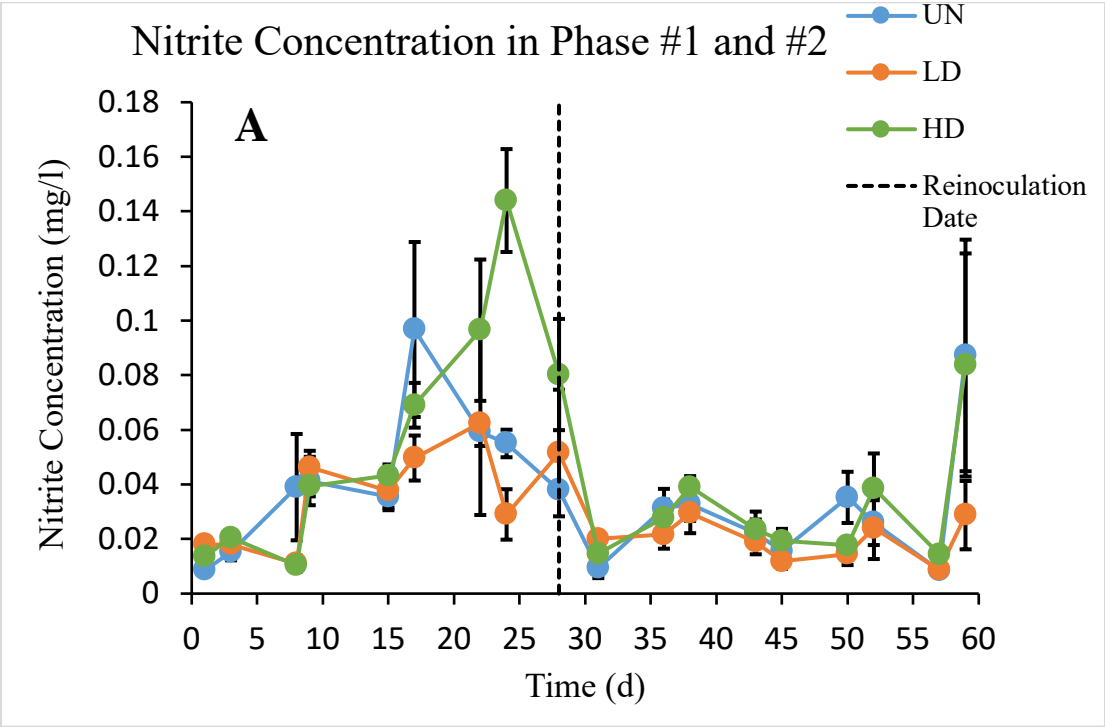


Figure 3.10. Ammonia concentration in control, low and high dose treatment samples collected from fish tank water during Main experiment Phase 1 and 2. Dotted line indicates the start of Phase 2, where fish tanks were reinoculated with *E. coli* TVS 354. Values are means ± standard errors of n=4 per treatment: UN = uninoculated (control); LD = low dose treatment; HD = high dose treatment.

Nitrite increased and decreased similarly to ammonia (Figure 3.10 and 3.11). Specifically, HD followed a similar rise and fall pattern to ammonia on similar days,

with its peak also at day 24 (0.144 mg/l). UN had a smaller peak at day 17 (0.097 mg/l) before decreasing to levels similar to initial dpi. Both UN and HD had a major spike on day 59 (0.087 and 0.084 mg/l, respectively) with high variability (SE = 0.042 and 0.041, respectively), but LD only increased to 0.029 mg/l with low variability (SE = 0.013). No noticeable trends were found in nitrate concentration throughout the study with the moderate variability in majority of sampling days in all treatment (Figure 3.11). In Phase 1, nitrate levels steadily increase. On day 22, LD decreases to 39.15 mg/l, but there is high variability (SE = 8.65) compared to UN and HD (SE = 1.58 and 0.15, respectively). However, Phase 2 began with nitrate levels increasing up until day 36, where nitrate levels sharply decrease and increase every sampling day. UN had slightly lower average nitrate concentrations throughout the majority of Phase 2 with the highest variability on day 45 (SE = 13.31).



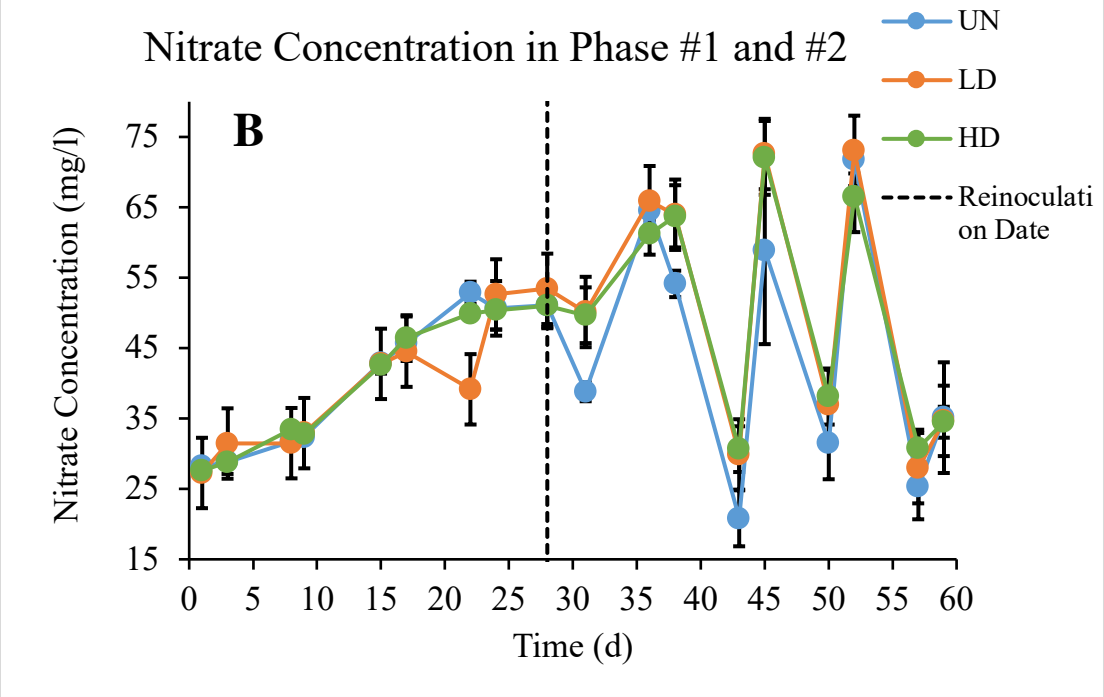


Figure 3.11. Nitrite (A) and nitrate (B) concentration in control, low and high dose treatment samples collected from fish tank water during Main experiment Phase 1 and 2. Dotted line indicates the start of Phase 2, where fish tanks were reinoculated with *E. coli* TVS 354. Values are means ± standard errors of n=4 per treatment: UN = uninoculated (control); LD = low dose treatment; HD = high dose treatment.

Plants

Basil was harvested on day 30 of Phase 2 (day 58 of Main Experiment), and lettuce were harvested on day 32 of Phase 2 (day 60 of Main Experiment). There was a significant difference in aerobic bacterial populations between basil and lettuce ($P < 0.0001$; $F= 289.09$, $DF = 1$), and there was a significant difference in aerobic bacterial populations between leaves and roots ($P < 0.0001$; $F= 412.15$, $DF = 1$). There was also a significant difference between the treatments ($P < 0.0001$; $F= 23.51$, $DF = 2$), their corresponding parts (leaves and roots) of the plant ($P < 0.0001$; $F= 31.48$, $DF = 2$), and all three factors together (plant, parts, and treatment) ($P < 0.0001$; $F= 22.98$, $DF = 5$). A post hoc Tukey test confirmed the significant difference of the treatments

(P < 0.0001) (Appendix K). When comparing the plant parts with their treatments, there was as significant difference between the treatments with the leaves (P < 0.0001), but not with the roots (P > 0.9798) because the aerobic bacteria count for basil roots were similar in all treatments and uninoculated control (Figure 3.12). While post hoc Tukey test showed a significant difference in the leaves in basil and lettuce plants in all treatments and control (P < 0.05), there was no significant difference between the basil and lettuce roots for all treatments and control (P > 0.05) (Appendix K).

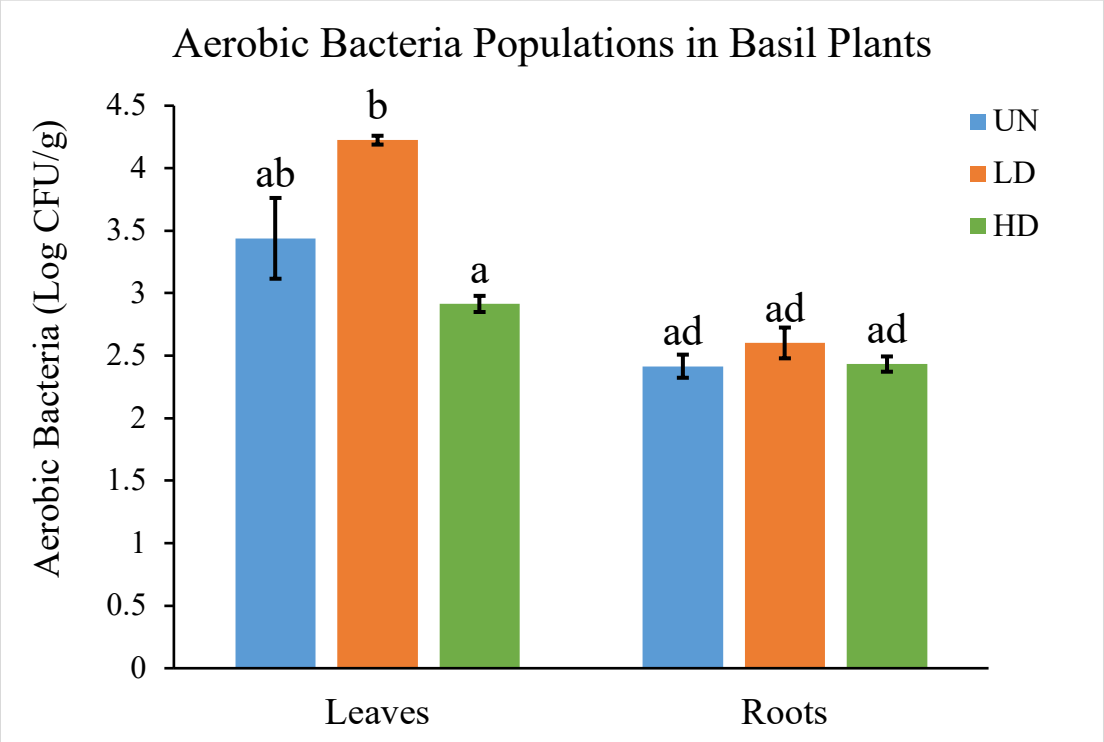


Figure 3.12. Aerobic bacterial populations from basil leaves and roots collected at harvest following Phase 2 (Day 60). Values are means ± standard errors of n=4 per treatment: UN = uninoculated (control); LD = low dose treatment; HD = high dose treatment. Different lowercase letters on top of the bar graphs specify significant difference between treatment and plant parts.

Basil leaves had higher aerobic bacteria populations on average than the roots (Figure 3.12). The limit of detection for APC Petrifilm was 5 cell/g for leaves and 3

cell/g for roots. The highest variability found in UN leaves (SE = 0.32). All remaining treatment groups in leaves and roots had minimal variability (SE < 0.12). Lettuce leaves followed a similar pattern with higher aerobic bacterial counts from leaves than from roots, but lettuce leaves and roots had higher bacterial counts than basil (Figure 3.13). Lettuce plants had a similar variability pattern with UN leaves being the highest (SE = 0.38) and the remaining treatment groups in leaves and roots having minimal variability (SE < 0.2).

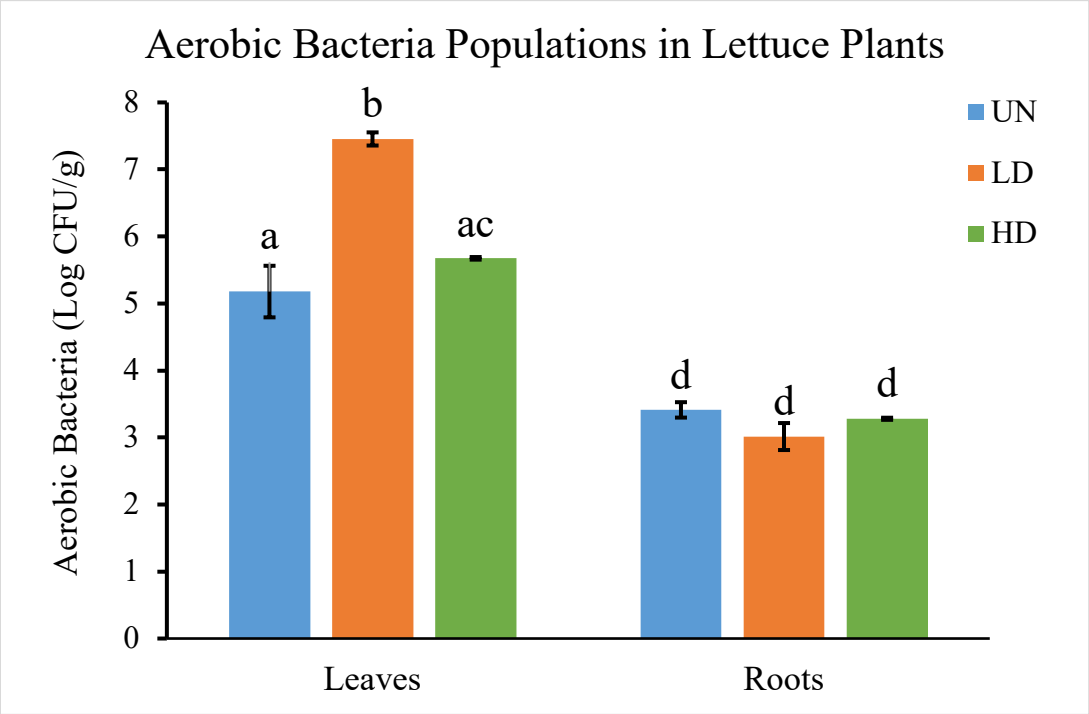


Figure 3.13. Aerobic bacteria population in lettuce leaves and roots collected at harvest following Phase 2 (Day 62). Values are means ± standard errors of n=4 per treatment: UN = uninoculated (control); LD = low dose treatment; HD = high dose treatment. Different lowercase letters on top of the bar graphs specify significant difference between treatment and plant parts.

E. coli TVS354 population was undetectable from basil leaves and roots. Enrichment revealed detectable *E. coli* TVS354 in HD basil roots that were undetectable on MACR spread plates. However, *E. coli* TVS354 was not detected from basil leaves even by enrichment. There was significant difference in treatments

(P = 0.0287; F = 4.72; DF = 2), but a post hoc Tukey test revealed that only HD and UN were significantly different (P = 0.0229) with no significant difference between the two treatments (Appendix L). However, there was a significant difference between the treatments and the plant parts (P = 0.0002; F = 18.38; DF = 2), and the Tukey test indicated there was a significance between the leaves and roots of the HD (P = 0.0160) and LD (P = 0.0107) treatments (Appendix L). It also revealed there was a difference between HD and LD leaves (P = 0.0049) in addition to HD and LD roots (P = 0.0392). Only HD treated lettuce leaves showed detectable *E. coli* TVS35*4* populations (5.71 log CFU/g) (Figure 3.14). Both LD and HD treatments of lettuce roots revealed detectable *E. coli* TVS354 populations for (4.89 and 1.39 log CFU/g). The limit of detection for MACR was 50 cell/g for leaves and 30 cell/g for roots. The highest variability was found in UN roots due to two replicates having positive counts of *E. coli* (SE = 1.39); a result of cross-contamination occurring during the production or harvesting process. The remaining treatment groups had minimal or no variability (SE < 0.18).

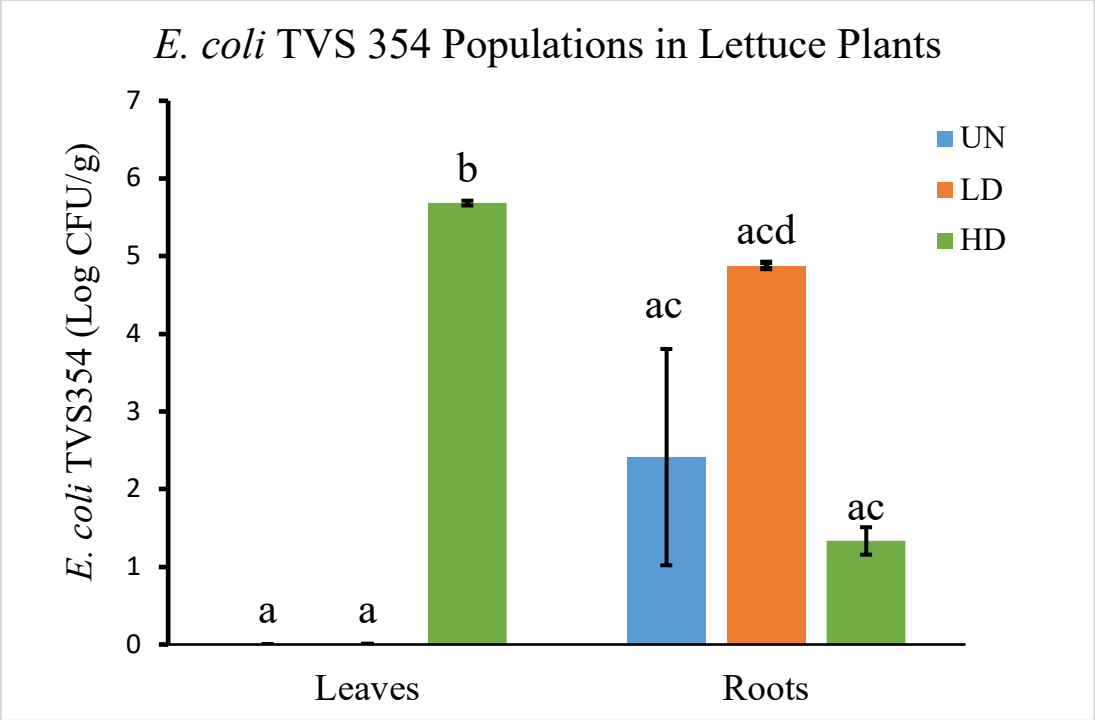


Figure 3.14. *E. coli* TVS 354 population in lettuce leaves and roots collected at harvest following Phase 2 (Day 62). Values are means ± standard errors of n=4 per treatment: UN = uninoculated (control); LD = low dose treatment; HD = high dose treatment. Different lowercase letters on top of the bar graphs specify significant difference between treatment and plant parts.

Discussion

E. coli TVS354 population

E. coli TVS354 was detected in low quantities through enrichment in both LD and HD treatments throughout the entire duration of Phase 2 despite being undetectable by spread plating. In both Phase 1 and 2, a higher volume of sample was used in direct spread plating (0.3 ml instead of 0.1 ml) to confirm undetectable counts of *E. coli* in the fish tank water and filter. The limit of detection was 10 cell/ml for samples collected from fish tank water and 10 ml/filter for samples collected from cheesecloth/sponge in the biofilter. Based on the Tukey test conducted on the *E. coli* results for LD and HD treatments, there was a significant difference between each

other 24 h post inoculation in both phases. This was to be expected as HD had a higher concentration inoculum added to the AP systems. The difference between the two indicated that HD treatments took a longer period to reach undetectable levels compared to LD. If bacterial competition is at play, then this would explain why LD, with fewer counts of *E. coli* initially, would decrease quicker than its HD counterpart (Figure 3.4) (Shaw, et al., 2016).

Aerobic bacterial populations

There was no significant difference between the treatments, and their counts were similar throughout the entirety of Phase 1 (Figure 3.6). The only difference occurs the first day post inoculation since *E. coli* counts decreased to the undetectable levels for the remainder of Phase 1 (Figure 3.3 and 3.6). In Phase 2, there was only a significant difference in HD between Day 0 and 1 post inoculation in the fish tank results (Figure 3.7). It was expected for LD to have no significant decrease due to its low initial counts. The *E. coli* population decreased to no detectable counts, so it was minimally reflected in the aerobic bacterial counts (Figure 3.4 and 3.7). In the biofilter results, treatments differed more, but HD and LD were only significantly different than UN. HD and LD had similar populations throughout Phase 2 with some variability, and this study revealed that initial inoculum concentration had less influence on results than initially anticipated.

Physicochemical parameters

Pearson's correlation was conducted on the physicochemical parameters with the aerobic bacteria populations. The results indicated moderate to strong position

correlation among all parameters (Table 2.1). Despite the results indicating statistically positive correlations, there was no visual relationships in the graphs that would have enabled me to pinpoint days throughout the study that would have indicated the positive correlation. Therefore, the correlation coefficients were not used to draw conclusions on their results.

Plants

Since plant roots are able to foster a microbial community in the roots, competition was likely a factor in managing the populations regardless of the treatments (Shaw, et al., 2016). The aerobic bacteria in plant roots were similar regardless of treatment, but plants leaves indicated that LD had the highest counts of aerobic bacteria in both basil and lettuce (Figure 3.12 and 3.13). This means the scale at which the contamination event occurred doesn't greatly increase the pathogenic risk factor in plant roots when given time as seen in the basil plants as there were no detectable counts of *E. coli* following the conclusion of Phase 2. This is not the case in lettuce as there were indeed high counts of *E. coli* in the leaves and roots. This suggests plant structure can be a used to assess the level of risk in terms of food safety prior to germination.

It is in these results that we can see competition at play. In the leaves, only HD had detectable levels of *E. coli*, whereas roots had detectable levels in all treatments (Figure 3.14). Since the LD leaves had no counts, *E. coli* mostly was out competed by native bacteria due to its initial low counts compared to HD. The LD roots had higher counts than HD indicating the impact microbial communities in the roots have on bacteria. However, this would need to be explored more in future

research to understand the reason how the native microbial communities interact with pathogens. This revealed that not only are some treatments undetectable, but it also showed that the plants provided ideal conditions for *E. coli* to persist for extended periods of time increasing the food safety risk of AP systems. In addition, cross contamination occurred where 2 replicates of the UN (control) displayed positive counts of *E. coli* despite practicing aseptic techniques. In addition, enrichment was conducted on all plant samples. All treatments in basil and lettuce displayed positive counts for *E. coli*. Except for UN lettuce roots, no other UN plant parts had no positive counts.

Food safety studies involving *E. coli* and hydroponic components were important guides for this study. Previous studies have concluded that bacteria are able to adapt to their environment quickly in the presence of native bacteria and can out compete them (Shaw, et al., 2016). However, our results don't fully align with their results due to differences in conditions and experimental set-up. Despite the water reaching undetectable levels throughout the majority of the study (Phase 1 and 2), lettuce plants had notably high detectable counts of *E. coli* by the end of Phase 2. The presence of *E. coli* TVS354 from the plants showed that it could compete with and successfully adapt to the indigenous bacterial community present on biofilter surfaces (sponge filter) and plant roots (Elsas et al., 2011; Lejeune et al., 2001). Plant exudates could be impacting pathogen survival by promoting other microbial growth, which could explain why basil had undetectable levels of *E. coli* TVS354 (Shaw, et al., 2016). Enrichment showed that *E. coli* TVS354 was present from the basil roots.

The persistence of *E. coli* TVS354 in the root zone could have indicated adaptation to natural environmental background or a continuous decrease in population due to competition. This could also explain the presence of *E. coli* TVS354 in both LD and HD treatments in the fish tank water through enrichment. Despite the lack of *E. coli* TVS354 presence in the basil leaves, internalization can occur, and *E. coli* can enter plants through leaves and roots as previous studies have shown (Solomon et al., 2002; Wang et al., 2021; Bernstein et al., 2007; Erickson et al., 2010). A potential explanation as to why lettuce has a higher aerobic bacteria population is the shape and height of the lettuce plants compared to the basil. At harvest, the leaves closest to the surface of the water were collected and assayed. Lettuce leaves were situated right above the water surface and in constant contact with water droplets, increasing exposure to *E. coli* TVS354 over a longer period of time, compared to basil leaves located farther from the water surface.

Chapter 4: Conclusion

In this study, we examined the changes in *E. coli* TVS354 population in 12 aquaponic systems. With significant decrease in *E. coli* TVS354 populations in water, it gave the impression aquaponics was a safer food production method compared to conventional agriculture. However, the plant results revealed a different result. Despite having undetectable levels of *E. coli* in the water for the majority of the study, the plant results indicated that it could survive long periods of time at high counts, increasing the risk of an outbreak. Cross-contamination was also difficult to prevent as shown in the lettuce counts.

High counts of *E. coli* were found in the uninoculated lettuce plants despite our best efforts to prevent this. In a commercial setting, this would most certainly occur since system maintenance is needed to address issues in the systems or adjust/test water quality parameters for fish and plant health. This also indicated that certain plants have higher risks of contamination than others based on their structure. In this case, the basil had lower *E. coli* counts present compared to lettuce with the leaves having less consistent exposure to contaminated water as a result of height. Our research suggests that future research should be focused on pathogen populations in plants to better explain the high *E. coli* counts in plants found following the conclusion of this study with an emphasis on using plants as an effective monitoring tool. In addition, future research on the relative food safety risk based on plant structure should be explored to design horticulture management guidelines tailored to different species.

Challenges of the study

Some challenges we encountered during the main study pertained to fish management and system constraints. In terms of fish and plant care, there was no official guidelines to reference when determining management procedures in aquaponic systems. Instead, guidelines were determines based on recommended conventional agriculture growing and aquarium conditions. In addition, we applied aseptic techniques to reduce the risk of contamination between systems. The lack of standardized horticulture guidelines means all farmers are left to their own devise to determine how to process produce, which can increase the risk of a food outbreak.

System constraints were observed throughout the main study. Our biofilters were physically too small to handle the amount of waste the goldfish produced, leading to high ammonia concentrations in the systems. To combat this, the volume of fish feed was reduced prior to the start of the study to manage the waste. We discovered halfway through Phase 2 of the study that cheesecloth was not strong enough to hold the fish waste and started to fray to the point that we were unable to remove the cheesecloth from the biofilter, so we changed to an aquarium filter media that held up much better. For future reference, a biofiltration system needs to be larger than 7.62 x 7.62 cm for a 37.8 L fish tank and thick aquarium filter media is needed to hold one week's worth of fish waste for easy cleaning and collection for analysis.

Recommendations for food safety testing

Based on the *E. coli* counts in the water throughout both phases, it indicated that water testing was not a sufficient method of pathogen monitoring and therefore,

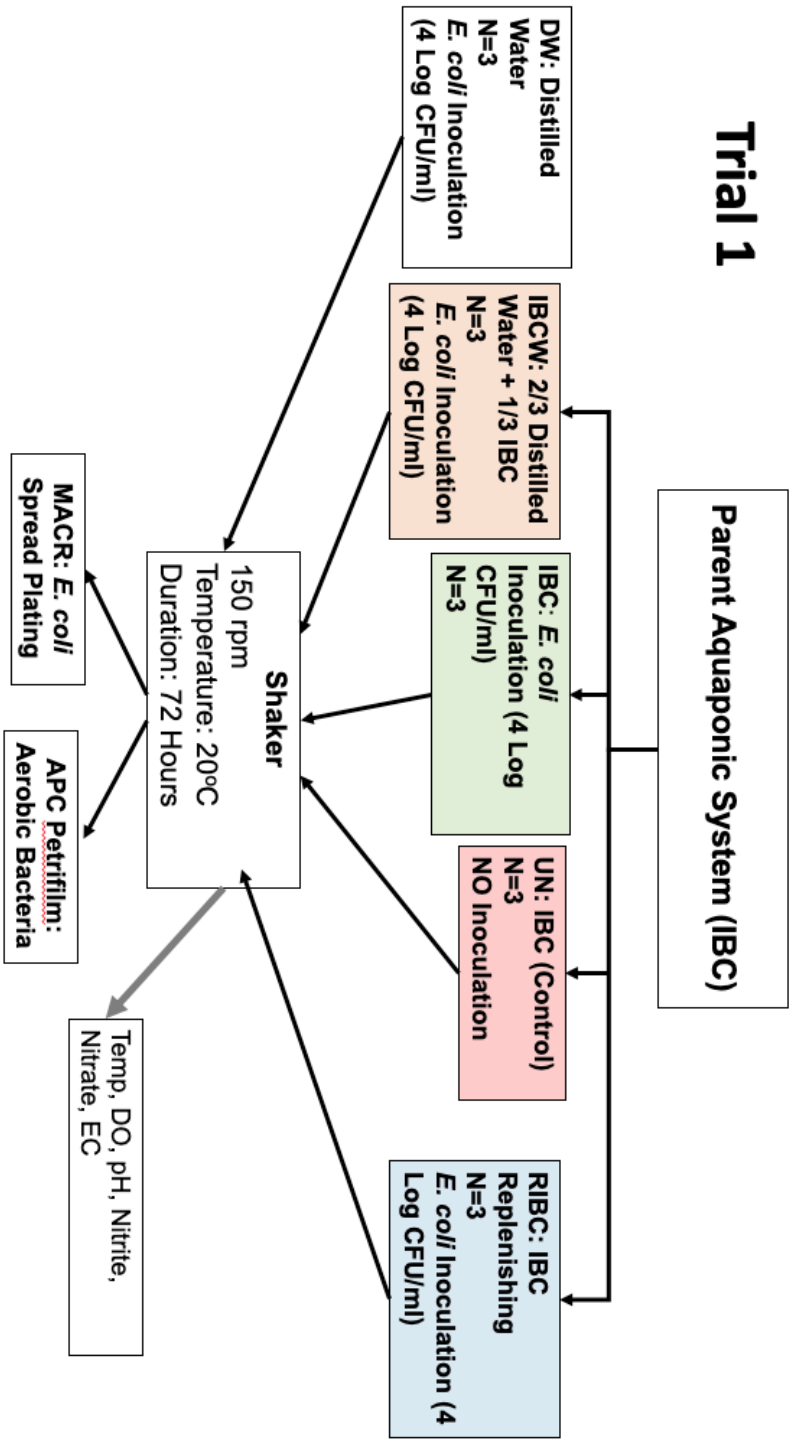
should not be used as such. Results suggest plant testing is needed as *E. coli* counts were still countable through spread plating by the end of Phase 2. Instead, plant testing should be used as an indicator tool for food risks and should be conducted frequently to intervene and remediate the issue as early as possible. Increase access to plant testing resources where farmers can send plant samples to labs for microbial enumeration analysis will need to be made more readily available for this to be an effective monitoring tool. While this study did not analyze the aquaculture component of food safety, it shouldn't be overlooked. Fish intestine and muscle tissue should be evaluated in future research to assess the food safety risk as they can be found in aquaculture settings (Dang and Dalsgaard, 2012). Research would determine if it can be used as a tool for food safety monitoring.

Based on these results, standardized horticulture and aquaculture management guidelines specifically applicable to the diversity of AP and HP systems need to be developed to aid farmers in proper aseptic handling and processing to prevent cross-contamination as was seen in the lettuce samples following Phase 2. Guidelines should also focus on prioritizing aquatic and plant health by creating ideal conditions. In addition, guidelines will also need to include procedures to best address contamination events such as proper plant and fish disposal and equipment sanitization. Further research is needed to better understand the pathogen population changes, the role of native bacteria competition, and interactions within aquaponic system components. Studies should also be conducted for other bacteria commonly responsible for foodborne disease outbreaks, such as *Listeria monocytogenes* and *Salmonella*, to compare the differences and similarities in population changes. In

addition, studies on common contamination locations in production and harvesting can help identify and develop food safety measures needed to reduce risk.

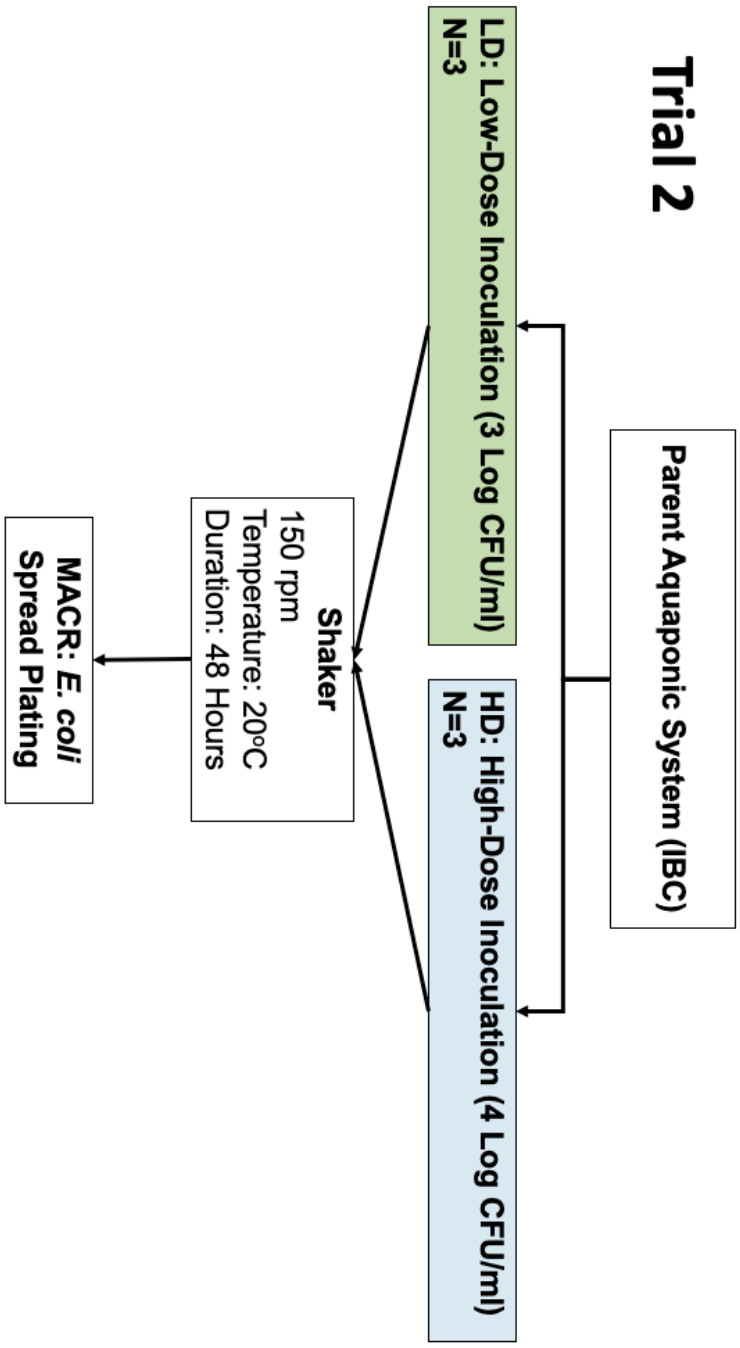
Appendix A

Diagrams displaying the methodology for Trial #1 of the Preliminary Experiment.



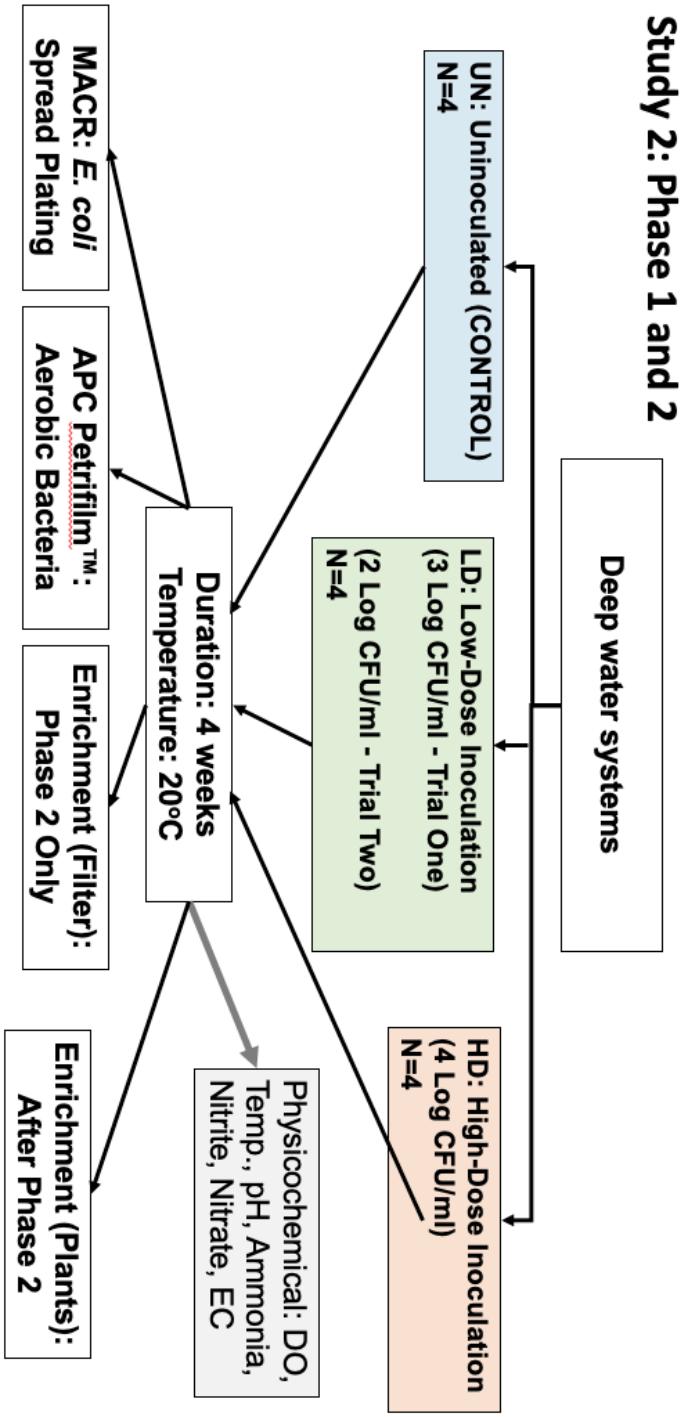
Appendix B

Diagrams displaying the methodology for Trial #2 of the Preliminary Experiment.



Appendix C

Diagrams displaying the methodology for Phase 1 and 2 of the Main Experiment.



Appendix D

SAS results of Trial #1 of the Preliminary experiment for APC counts. PROC GLM statement was used to conduct ANOVA (A). The table encased in the black box contains P values, F value, and degrees of freedom (DF) values used in the study. Tukey-Kramer test was used to examine comparisons between treatment and day. The table displays the comparisons between treatments (B). The table (C and D) are similar except the comparison is between the treatment and day.

A

The GLM Procedure

Dependent Variable: APC APC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	16	72.71103096	4.54443943	15.17	<.0001
Error	31	9.28496490	0.29951500		
Corrected Total	47	81.99599586			

R-Square	Coeff Var	Root MSE	APC Mean
0.886763	24.89431	0.547280	2.198413

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	4	54.13588716	13.53397179	45.19	<.0001
Day	3	5.37104170	1.79034723	5.98	0.0024
Treatment*Day	9	13.20410210	1.46712246	4.90	0.0004

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	4	49.31791665	12.32947916	41.16	<.0001
Day	3	5.37104170	1.79034723	5.98	0.0024
Treatment*Day	9	13.20410210	1.46712246	4.90	0.0004

B

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	APC LSMEAN	LSMEAN Number
DW	0.36534068	1
IBC	2.91334109	2
IBCW	2.55915835	3
RIBC	Non-est	4
UN	Non-est	5

Least Squares Means for effect Treatment
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: APC

i/j	1	2	3	4	5
1		<.0001	<.0001	.	.
2	<.0001		0.3073	.	.
3	<.0001	0.3073		.	.
4	.	.	.		.
5	.	.	.	.	

C

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	Day	APC LSMEAN	LSMEAN Number
DW	0	1.46136273	1
DW	1	0.00000000	2
DW	2	-0.00000000	3
DW	3	-0.00000000	4
IBC	0	2.51347081	5
IBC	1	4.07413338	6
IBC	2	2.94836601	7
IBC	3	2.11739417	8
IBCW	0	2.20070027	9
IBCW	1	2.34039643	10
IBCW	2	2.73127334	11
IBCW	3	2.96426334	12
RIBC	2	3.14644423	13
RIBC	3	2.18161429	14
UN	1	3.97585855	15
UN	2	2.64733602	16
UN	3	1.93050507	17

D

Least Squares Means for effect Treatment*Day
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: APC

i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		0.2768	0.2768	0.2768	0.8655	0.0011	0.2531	0.9943	0.9925	0.9262	0.4949	0.2392	0.1172	0.9859	0.0019	0.6047	0.9999
2	0.2768		1.0000	1.0000	0.0019	<.0001	<.0001	0.0042	0.0101	0.0011	<.0001	<.0001	<.0001	0.0029	<.0001	0.0002	0.0125
3	0.2768	1.0000		1.0000	0.0019	<.0001	<.0001	0.0042	0.0101	0.0011	<.0001	<.0001	<.0001	0.0029	<.0001	0.0002	0.0125
4	0.2768	1.0000	1.0000		0.0019	<.0001	<.0001	0.0042	0.0101	0.0011	<.0001	<.0001	<.0001	0.0029	<.0001	0.0002	0.0125
5	0.8655	0.0019	0.0019	0.0019		0.1931	1.0000	1.0000	1.0000	1.0000	1.0000	0.9999	0.9961	1.0000	0.2759	1.0000	0.9984
6	0.0011	<.0001	<.0001	<.0001	0.1931		0.5094	0.0108	0.0508	0.0374	0.2405	0.5325	0.7890	0.0156	1.0000	0.1689	0.0036
7	0.2531	<.0001	<.0001	<.0001	1.0000	0.5094		0.8915	0.9802	0.9919	1.0000	1.0000	1.0000	0.9387	0.6533	1.0000	0.6672
8	0.9943	0.0042	0.0042	0.0042	1.0000	0.0108	0.8915		1.0000	1.0000	0.9911	0.8772	0.6511	1.0000	0.0189	0.9981	1.0000
9	0.9925	0.0101	0.0101	0.0101	1.0000	0.0508	0.9802	1.0000		1.0000	0.9995	0.9761	0.8781	1.0000	0.0794	0.9999	1.0000
10	0.9262	0.0011	0.0011	0.0011	1.0000	0.0374	0.9919	1.0000	1.0000		1.0000	0.9896	0.9119	1.0000	0.0625	1.0000	0.9999
11	0.4949	<.0001	<.0001	<.0001	1.0000	0.2405	1.0000	0.9911	0.9995	1.0000		1.0000	0.9999	0.9971	0.3486	1.0000	0.9159
12	0.2392	<.0001	<.0001	<.0001	0.9999	0.5325	1.0000	0.8772	0.9761	0.9896	1.0000		1.0000	0.9286	0.6762	1.0000	0.6442
13	0.1172	<.0001	<.0001	<.0001	0.9961	0.7890	1.0000	0.6511	0.8781	0.9119	0.9999	1.0000		0.7411	0.8929	0.9990	0.3848
14	0.9859	0.0029	0.0029	0.0029	1.0000	0.0156	0.9387	1.0000	1.0000	1.0000	0.9971	0.9286	0.7411		0.0269	0.9996	1.0000
15	0.0019	<.0001	<.0001	<.0001	0.2759	1.0000	0.6533	0.0189	0.0794	0.0625	0.3486	0.6762	0.8929	0.0269		0.2546	0.0064
16	0.6047	0.0002	0.0002	0.0002	1.0000	0.1689	1.0000	0.9981	0.9999	1.0000	1.0000	1.0000	0.9990	0.9996	0.2546		0.9640
17	0.9999	0.0125	0.0125	0.0125	0.9984	0.0036	0.6672	1.0000	1.0000	0.9999	0.9159	0.6442	0.3848	1.0000	0.0064	0.9640	

Appendix E

SAS results of Trial #1 of the Preliminary experiment for *E. coli* counts. PROC GLM statement was used to conduct ANOVA (A). The table encased in the black box contains P values, F value, and degrees of freedom (DF) values used in the study. Tukey-Kramer test was used to examine comparisons between treatment and day. The table (B) displays the comparisons between treatments. The table (C and D) are similar except the comparison is between the treatment and day.

A

The GLM Procedure

Dependent Variable: EC EC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	16	29.03975829	1.81498489	18.89	<.0001
Error	31	2.97829180	0.09607393		
Corrected Total	47	32.01805009			

R-Square	Coeff Var	Root MSE	EC Mean
0.906981	68.93592	0.309958	0.449632

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	4	4.81841388	1.20460347	12.54	<.0001
Day	3	21.78491693	7.26163898	75.58	<.0001
Treatment*Day	9	2.43642747	0.27071416	2.82	0.0153

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	4	2.42422550	0.60605638	6.31	0.0008
Day	3	21.78491693	7.26163898	75.58	<.0001
Treatment*Day	9	2.43642747	0.27071416	2.82	0.0153

B

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	EC LSMEAN	LSMEAN Number
DW	0.50989766	1
IBC	1.07161497	2
IBCW	0.64484617	3
RIBC	Non-est	4
UN	Non-est	5

Least Squares Means for effect Treatment
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: EC

i/j	1	2	3	4	5
1		0.0006	0.5790	.	.
2	0.0006		0.0091	.	.
3	0.5790	0.0091		.	.
4	.	.	.		.
5	.	.	.	.	

C

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	Day	EC LSMEAN	LSMEAN Number
DW	0	2.03959062	1
DW	1	0.00000000	2
DW	2	0.00000000	3
DW	3	-0.00000000	4
IBC	0	2.41465189	5
IBC	1	1.36563667	6
IBC	2	0.50617131	7
IBC	3	-0.00000000	8
IBCW	0	2.37869801	9
IBCW	1	0.20068666	10
IBCW	2	0.00000000	11
IBCW	3	0.00000000	12
RIBC	2	0.56632333	13
RIBC	3	-0.00000000	14
UN	1	-0.00000000	15
UN	2	-0.00000000	16
UN	3	0.00000000	17

D

Least Squares Means for effect Treatment*Day
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: EC

i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		<.0001	<.0001	<.0001	0.9976	0.5994	0.0007	<.0001	0.9992	<.0001	<.0001	<.0001	0.0012	<.0001	<.0001	<.0001	<.0001
2	<.0001		1.0000	1.0000	<.0001	0.0007	0.8290	1.0000	<.0001	1.0000	1.0000	1.0000	0.6928	1.0000	1.0000	1.0000	1.0000
3	<.0001	1.0000		1.0000	<.0001	0.0007	0.8290	1.0000	<.0001	1.0000	1.0000	1.0000	0.6928	1.0000	1.0000	1.0000	1.0000
4	<.0001	1.0000	1.0000		<.0001	0.0007	0.8290	1.0000	<.0001	1.0000	1.0000	1.0000	0.6928	1.0000	1.0000	1.0000	1.0000
5	0.9976	<.0001	<.0001	<.0001		0.0560	<.0001	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
6	0.5994	0.0007	0.0007	0.0007	0.0560		0.1117	0.0007	0.0748	0.0060	0.0007	0.0007	0.1807	0.0007	0.0007	0.0007	0.0007
7	0.0007	0.8290	0.8290	0.8290	<.0001	0.1117		0.8290	<.0001	0.9977	0.8290	0.8290	1.0000	0.8290	0.8290	0.8290	0.8290
8	<.0001	1.0000	1.0000	1.0000	<.0001	0.0007	0.8290		<.0001	1.0000	1.0000	1.0000	0.6928	1.0000	1.0000	1.0000	1.0000
9	0.9992	<.0001	<.0001	<.0001	1.0000	0.0748	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
10	<.0001	1.0000	1.0000	1.0000	<.0001	0.0060	0.9977	1.0000	<.0001		1.0000	1.0000	0.9856	1.0000	1.0000	1.0000	1.0000
11	<.0001	1.0000	1.0000	1.0000	<.0001	0.0007	0.8290	1.0000	<.0001	1.0000		1.0000	0.6928	1.0000	1.0000	1.0000	1.0000
12	<.0001	1.0000	1.0000	1.0000	<.0001	0.0007	0.8290	1.0000	<.0001	1.0000	1.0000		0.6928	1.0000	1.0000	1.0000	1.0000
13	0.0012	0.6928	0.6928	0.6928	<.0001	0.1807	1.0000	0.6928	<.0001	0.9856	0.6928	0.6928		0.6928	0.6928	0.6928	0.6928
14	<.0001	1.0000	1.0000	1.0000	<.0001	0.0007	0.8290	1.0000	<.0001	1.0000	1.0000	1.0000	0.6928		1.0000	1.0000	1.0000
15	<.0001	1.0000	1.0000	1.0000	<.0001	0.0007	0.8290	1.0000	<.0001	1.0000	1.0000	1.0000	0.6928	1.0000		1.0000	1.0000
16	<.0001	1.0000	1.0000	1.0000	<.0001	0.0007	0.8290	1.0000	<.0001	1.0000	1.0000	1.0000	0.6928	1.0000	1.0000		1.0000
17	<.0001	1.0000	1.0000	1.0000	<.0001	0.0007	0.8290	1.0000	<.0001	1.0000	1.0000	1.0000	0.6928	1.0000	1.0000	1.0000	

Appendix F

SAS results of Trial #2 of the Preliminary experiment for *E. coli* counts. PROC GLM statement was used to conduct ANOVA. The table (A) encased in the black box contains P values, F value, and degrees of freedom (DF) values used in the study. Tukey-Kramer test was used to examine comparisons between treatment and day. The table (B) displays the comparisons between treatment and day.

The GLM Procedure

A

Dependent Variable: Ec Ec

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	5.70469631	1.14093926	3.43	0.0372
Error	12	3.99107545	0.33258962		
Corrected Total	17	9.69577176			

R-Square	Coeff Var	Root MSE	Ec Mean
0.588369	19.65451	0.576706	2.934217

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	1	1.45039894	1.45039894	4.36	0.0588
Day	2	4.23852443	2.11926222	6.37	0.0130
Treatment*Day	2	0.01577294	0.00788647	0.02	0.9766

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	1.45039894	1.45039894	4.36	0.0588
Day	2	4.23852443	2.11926222	6.37	0.0130
Treatment*Day	2	0.01577294	0.00788647	0.02	0.9766

B

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Treatment	Day	Ec LSMEAN	LSMEAN Number
HD	0	3.64493696	1
HD	1	3.48306719	2
HD	2	2.52623217	3
LD	0	2.99831787	4
LD	1	2.97906694	5
LD	2	1.97367862	6

Least Squares Means for effect Treatment*Day
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Ec

i/j	1	2	3	4	5	6
1		0.9992	0.2382	0.7413	0.7190	0.0363
2	0.9992		0.3799	0.8991	0.8840	0.0646
3	0.2382	0.3799		0.9084	0.9216	0.8410
4	0.7413	0.8991	0.9084		1.0000	0.3149
5	0.7190	0.8840	0.9216	1.0000		0.3325
6	0.0363	0.0646	0.8410	0.3149	0.3325	

Appendix G

SAS results of Phase 1 of the Main experiment for *E. coli* counts. PROC GLM statement was used to conduct ANOVA (A). The table encased in the black box contains P values, F value, and degrees of freedom (DF) values used in the study. Tukey-Kramer test was used to examine comparisons between treatment and day. The table (B and C) displays the comparisons between treatment and day. The table (D) results show the comparison between treatment groups.

A

The GLM Procedure

Dependent Variable: EC EC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	26	70.70750540	2.71951944	8638.90	<.0001
Error	78	0.02455434	0.00031480		
Corrected Total	104	70.73205975			

R-Square	Coeff Var	Root MSE	EC Mean
0.999653	7.631238	0.017743	0.232499

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	2	3.13828667	1.56914334	4984.58	<.0001
Day	8	43.94068113	5.49258514	17447.9	<.0001
Treatment*Day	16	23.62853760	1.47678360	4691.19	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	2	2.83375374	1.41687687	4500.89	<.0001
Day	8	43.95543082	5.49442885	17453.8	<.0001
Treatment*Day	16	23.62853760	1.47678360	4691.19	<.0001

B

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	Day	EC LSMEAN	LSMEAN Number
HD	0	3.53728753	1
HD	1	-0.00000000	2
HD	2	-0.00000000	3
HD	3	0.00000000	4
HD	7	-0.00000000	5
HD	8	-0.00000000	6
HD	9	-0.00000000	7
HD	10	-0.00000000	8
HD	14	0.00000000	9
LD	0	2.56582203	10
LD	1	-0.00000000	11
LD	2	-0.00000000	12
LD	3	-0.00000000	13
LD	7	-0.00000000	14
LD	8	-0.00000000	15
LD	9	-0.00000000	16
LD	10	-0.00000000	17
LD	14	0.00000000	18
UN	0	0.00000000	19
UN	1	-0.00000000	20
UN	2	-0.00000000	21
UN	3	0.00000000	22
UN	7	0.00000000	23
UN	8	-0.00000000	24
UN	9	0.00000000	25
UN	10	-0.00000000	26
UN	14	-0.00000000	27

C

Least Squares Means for effect Treatment*Day
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: EC

i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
3	<.0001	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
4	<.0001	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
5	<.0001	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
6	<.0001	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
7	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
8	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
9	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
10	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
11	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
12	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
13	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
14	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
15	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
16	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
17	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
18	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
19	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
20	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
21	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
22	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000
23	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000
24	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000
25	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000
26	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000
27	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	

D

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	EC LSMEAN	LSMEAN Number
HD	0.39303195	1
LD	0.28509134	2
UN	0.00000000	3

Least Squares Means for effect Treatment
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: EC

i/j	1	2	3
1		<.0001	<.0001
2	<.0001		<.0001
3	<.0001	<.0001	

Appendix H

SAS results of Phase 2 of the Main experiment for *E. coli* counts. PROC GLM statement was used to conduct ANOVA. The fish tank data (A) was collected for the first two days (day 28 and 29) of Phase 2. The table encased in the black box contains P values, F value, and degrees of freedom (DF) values used in the study. Tukey-Kramer test was used to examine comparisons between treatment and day. The table (B) displays the comparisons between treatments. The table (C) displays the comparisons between treatment and day. The filter data (D) collected the following day (day 30) up until the end of Phase 2. The table (E) displays the comparisons between treatments. The table (F and G) displays the comparisons between treatment and day.

A

The GLM Procedure

Dependent Variable: EC EC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	43.59035606	8.71807121	124.90	<.0001
Error	14	0.97721113	0.06980080		
Corrected Total	19	44.56756719			

R-Square	Coeff Var	Root MSE	EC Mean
0.978073	19.44984	0.264198	1.358358

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	2	31.24425428	15.62212714	223.81	<.0001
Day	1	7.70942747	7.70942747	110.45	<.0001
Treatment*Day	2	4.63667431	2.31833716	33.21	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	2	25.95926524	12.97963262	185.95	<.0001
Day	1	8.56824141	8.56824141	122.75	<.0001
Treatment*Day	2	4.63667431	2.31833716	33.21	<.0001

B

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	EC LSMEAN	LSMEAN Number
HD	3.02920474	1
LD	1.35031821	2
UN	-0.00000000	3

Least Squares Means for effect Treatment
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: EC

i/j	1	2	3
1		<.0001	<.0001
2	<.0001		<.0001
3	<.0001	<.0001	

C

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	Day	EC LSMEAN	LSMEAN Number
HD	28	4.24779247	1
HD	29	1.81061700	2
LD	28	2.15796237	3
LD	29	0.54267406	4
UN	28	-0.00000000	5
UN	29	-0.00000000	6

Least Squares Means for effect Treatment*Day
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: EC

i/j	1	2	3	4	5	6
1		<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001		0.6593	0.0008	<.0001	<.0001
3	<.0001	0.6593		<.0001	<.0001	<.0001
4	<.0001	0.0008	<.0001		0.1392	0.0968
5	<.0001	<.0001	<.0001	0.1392		1.0000
6	<.0001	<.0001	<.0001	0.0968	1.0000	

D

The GLM Procedure

Dependent Variable: EC EC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	57.47806001	4.10557571	356.67	<.0001
Error	40	0.46043493	0.01151087		
Corrected Total	54	57.93849494			

R-Square	Coeff Var	Root MSE	EC Mean
0.992053	28.55722	0.107289	0.375697

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	2	4.98764470	2.49382235	216.65	<.0001
Day	4	34.04160536	8.51040134	739.34	<.0001
Treatment*Day	8	18.44880995	2.30610124	200.34	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	2	4.93864651	2.46932326	214.52	<.0001
Day	4	28.81196711	7.20299178	625.76	<.0001
Treatment*Day	8	18.44880995	2.30610124	200.34	<.0001

E

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	EC LSMEAN	LSMEAN Number
HD	0.72526097	1
LD	0.41054242	2
UN	-0.00000000	3

Least Squares Means for effect Treatment
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: EC

i/j	1	2	3
1		<.0001	<.0001
2	<.0001		<.0001
3	<.0001	<.0001	

F

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	Day	EC LSMEAN	LSMEAN Number
HD	30	3.62630486	1
HD	35	0.00000000	2
HD	42	0.00000000	3
HD	49	0.00000000	4
HD	56	0.00000000	5
LD	30	2.05271208	6
LD	35	0.00000000	7
LD	42	0.00000000	8
LD	49	0.00000000	9
LD	56	-0.00000000	10
UN	30	-0.00000000	11
UN	35	0.00000000	12
UN	42	-0.00000000	13
UN	49	-0.00000000	14
UN	56	0.00000000	15

G

Least Squares Means for effect Treatment*Day
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: EC

i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001		1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
3	<.0001	1.0000		1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
4	<.0001	1.0000	1.0000		1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
5	<.0001	1.0000	1.0000	1.0000		<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
6	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
7	<.0001	1.0000	1.0000	1.0000	1.0000	<.0001		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
8	<.0001	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
9	<.0001	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
10	<.0001	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000
11	<.0001	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000
12	<.0001	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000
13	<.0001	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000
14	<.0001	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000
15	<.0001	1.0000	1.0000	1.0000	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	

Appendix I

SAS results of Phase 1 of the Main experiment for APC counts. PROC GLM statement was used to conduct ANOVA (A). The table encased in the black box contains P values, F value, and degrees of freedom (DF) values used in the study. Tukey-Kramer test was used to examine comparisons between treatment and day. The table (B and C) displays the comparisons between treatment and day.

A

The GLM Procedure

Dependent Variable: APC APC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	26	63.15102996	2.42888577	60.13	<.0001
Error	78	3.15074331	0.04039415		
Corrected Total	104	66.30177327			

R-Square	Coeff Var	Root MSE	APC Mean
0.952479	5.901640	0.200983	3.405544

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	2	0.29993448	0.14996724	3.71	0.0288
Day	8	60.31661275	7.53957659	186.65	<.0001
Treatment*Day	16	2.53448273	0.15840517	3.92	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	2	0.14387229	0.07193614	1.78	0.1753
Day	8	60.48785108	7.56098138	187.18	<.0001
Treatment*Day	16	2.53448273	0.15840517	3.92	<.0001

B

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	Day	APC LSMEAN	LSMEAN Number
HD	0	3.72972374	1
HD	1	2.83259692	2
HD	2	3.27125503	3
HD	3	2.73088415	4
HD	7	2.84932691	5
HD	8	2.96804114	6
HD	9	3.01945677	7
HD	10	4.46751505	8
HD	14	5.12971219	9
LD	0	3.08182109	10
LD	1	2.65834126	11
LD	2	2.76304024	12
LD	3	3.19009561	13
LD	7	3.07032881	14
LD	8	3.32008829	15
LD	9	2.85832514	16
LD	10	4.36781824	17
LD	14	4.99470796	18
UN	0	3.13522358	19
UN	1	2.60409927	20
UN	2	2.89246025	21
UN	3	2.76458121	22
UN	7	3.09016157	23
UN	8	3.19974244	24
UN	9	3.09752483	25
UN	10	4.44737240	26
UN	14	5.03072542	27

C

Least Squares Means for effect Treatment*Day
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: APC

i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1		<.0001	0.2339	<.0001	<.0001	0.0003	0.0010	0.0005	<.0001	0.0049	<.0001	<.0001	0.0558	0.0037	0.4455	<.0001	0.0062	<.0001	0.0173	<.0001	<.0001	<.0001	0.0060	0.0675	0.0213	0.0008	<.0001
2	<.0001		0.3106	1.0000	1.0000	1.0000	0.9999	<.0001	<.0001	0.9908	1.0000	1.0000	0.7139	0.9951	0.1469	1.0000	<.0001	<.0001	0.9202	0.9972	1.0000	1.0000	0.9860	0.6659	0.9925	<.0001	<.0001
3	0.2339	0.3106		0.1175	0.5435	0.9187	0.9895	<.0001	<.0001	0.9999	0.0113	0.1019	1.0000	0.9996	1.0000	0.4291	<.0001	<.0001	1.0000	0.0030	0.6056	0.1048	0.9999	1.0000	1.0000	<.0001	<.0001
4	<.0001	1.0000	0.1175		1.0000	0.9984	0.9783	<.0001	<.0001	0.8532	1.0000	1.0000	0.3705	0.8883	0.0496	1.0000	<.0001	<.0001	0.6288	1.0000	1.0000	1.0000	0.8242	0.3303	0.8782	<.0001	<.0001
5	<.0001	1.0000	0.5435	1.0000		1.0000	1.0000	<.0001	<.0001	0.9988	1.0000	1.0000	0.8845	0.9995	0.3226	1.0000	<.0001	<.0001	0.9805	0.9974	1.0000	1.0000	0.9980	0.8549	0.9989	<.0001	<.0001
6	0.0003	1.0000	0.9187	0.9984	1.0000		1.0000	<.0001	<.0001	1.0000	0.9016	0.9995	0.9981	1.0000	0.7400	1.0000	<.0001	<.0001	1.0000	0.6820	1.0000	0.9995	1.0000	0.9966	1.0000	<.0001	<.0001
7	0.0010	0.9999	0.9895	0.9783	1.0000	1.0000		<.0001	<.0001	1.0000	0.6961	0.9867	1.0000	1.0000	0.9249	1.0000	<.0001	<.0001	1.0000	0.4172	1.0000	0.9877	1.0000	0.9999	1.0000	<.0001	<.0001
8	0.0005	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.0034	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0713	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0343
9	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0034		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0002	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0021	1.0000
10	0.0049	0.9908	0.9999	0.8532	0.9988	1.0000	1.0000	<.0001	<.0001		0.3783	0.8741	1.0000	1.0000	0.9949	0.9980	<.0001	<.0001	1.0000	0.1729	0.9999	0.8791	1.0000	1.0000	1.0000	<.0001	<.0001
11	<.0001	1.0000	0.0113	1.0000	1.0000	0.9016	0.6961	<.0001	<.0001	0.3783		1.0000	0.0652	0.4338	0.0035	0.9996	<.0001	<.0001	0.1753	1.0000	0.9960	1.0000	0.3402	0.0538	0.4608	<.0001	<.0001
12	<.0001	1.0000	0.1019	1.0000	1.0000	0.9995	0.9867	<.0001	<.0001	0.8741	1.0000		0.3617	0.9082	0.0391	1.0000	<.0001	<.0001	0.6400	1.0000	1.0000	1.0000	0.8452	0.3189	0.9017	<.0001	<.0001
13	0.0558	0.7139	1.0000	0.3705	0.8845	0.9981	1.0000	<.0001	<.0001	1.0000	0.0652	0.3617		1.0000	1.0000	0.8276	<.0001	<.0001	1.0000	0.0209	0.9317	0.3688	1.0000	1.0000	1.0000	<.0001	<.0001
14	0.0037	0.9951	0.9996	0.8883	0.9995	1.0000	1.0000	<.0001	<.0001	1.0000	0.4338	0.9082	1.0000		0.9905	0.9991	<.0001	<.0001	1.0000	0.2077	1.0000	0.9123	1.0000	1.0000	1.0000	<.0001	<.0001
15	0.4455	0.1469	1.0000	0.0496	0.3226	0.7400	0.9249	<.0001	<.0001	0.9949	0.0035	0.0391	1.0000	0.9905		0.2225	<.0001	<.0001	0.9999	0.0009	0.3591	0.0403	0.9969	1.0000	0.9994	<.0001	<.0001
16	<.0001	1.0000	0.4291	1.0000	1.0000	1.0000	1.0000	<.0001	<.0001	0.9980	0.9996	1.0000	0.8276	0.9991	0.2225		<.0001	<.0001	0.9675	0.9881	1.0000	1.0000	0.9965	0.7879	0.9982	<.0001	<.0001
17	0.0062	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0002	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.0082	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0034
18	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0713	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0082		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0477	1.0000
19	0.0173	0.9202	1.0000	0.6288	0.9805	1.0000	1.0000	<.0001	<.0001	1.0000	0.1753	0.6400	1.0000	1.0000	0.9999	0.9675	<.0001	<.0001		0.0660	0.9934	0.6480	1.0000	1.0000	1.0000	<.0001	<.0001
20	<.0001	0.9972	0.0030	1.0000	0.9974	0.6820	0.4172	<.0001	<.0001	0.1729	1.0000	1.0000	0.0209	0.2077	0.0009	0.9881	<.0001	<.0001	0.0660		0.9501	1.0000	0.1505	0.0169	0.2398	<.0001	<.0001
21	<.0001	1.0000	0.6056	1.0000	1.0000	1.0000	1.0000	<.0001	<.0001	0.9999	0.9960	1.0000	0.9317	1.0000	0.3591	1.0000	<.0001	<.0001	0.9934	0.9501		1.0000	0.9997	0.9082	0.9999	<.0001	<.0001
22	<.0001	1.0000	0.1048	1.0000	1.0000	0.9995	0.9877	<.0001	<.0001	0.8791	1.0000	1.0000	0.3688	0.9123	0.0403	1.0000	<.0001	<.0001	0.6480	1.0000	1.0000		0.8508	0.3256	0.9056	<.0001	<.0001
23	0.0060	0.9860	0.9999	0.8242	0.9980	1.0000	1.0000	<.0001	<.0001	1.0000	0.3402	0.8452	1.0000	1.0000	0.9969	0.9965	<.0001	<.0001	1.0000	0.1505	0.9997	0.8508		1.0000	1.0000	<.0001	<.0001
24	0.0675	0.6659	1.0000	0.3303	0.8549	0.9966	0.9999	<.0001	<.0001	1.0000	0.0538	0.3189	1.0000	1.0000	1.0000	0.7879	<.0001	<.0001	1.0000	0.0169	0.9082	0.3256	1.0000		1.0000	<.0001	<.0001
25	0.0213	0.9925	1.0000	0.8782	0.9989	1.0000	1.0000	<.0001	<.0001	1.0000	0.4608	0.9017	1.0000	1.0000	0.9994	0.9982	<.0001	<.0001	1.0000	0.2398	0.9999	0.9056	1.0000	1.0000		<.0001	<.0001
26	0.0008	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0021	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0477	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.0222
27	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0343	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0034	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0222	

Appendix J

SAS results of Phase 2 of the Main experiment for APC counts. PROC GLM statement was used to conduct ANOVA. The fish tank data (A) was collected for the first two days (day 28 and 29) of Phase 2. The table encased in the black box contains P values, F value, and degrees of freedom (DF) values used in the study. Tukey-Kramer test was used to examine comparisons between treatment and day. The table (B) displays the comparisons between treatment and day. The filter data (C) collected the following day (day 30) up until the end of Phase 2. The table (D and E) displays the comparisons between treatment and day. The table (F) displays the comparisons between treatments.

A

The GLM Procedure

Dependent Variable: APC APC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	4.03322209	0.80664442	2.88	0.0541
Error	14	3.91797878	0.27985563		
Corrected Total	19	7.95120087			

R-Square	Coeff Var	Root MSE	APC Mean
0.507247	14.60100	0.529014	3.623133

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	2	0.84010206	0.42005103	1.50	0.2567
Day	1	0.31892381	0.31892381	1.14	0.3038
Treatment*Day	2	2.87419622	1.43709811	5.14	0.0213

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	2	0.45204549	0.22602274	0.81	0.4656
Day	1	0.68148831	0.68148831	2.44	0.1410
Treatment*Day	2	2.87419622	1.43709811	5.14	0.0213

B

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	Day	APC LSMEAN	LSMEAN Number
HD	28	4.56786194	1
HD	29	3.08583398	2
LD	28	3.63088874	3
LD	29	3.43885723	4
UN	28	3.16910414	5
UN	29	3.70027870	6

Least Squares Means for effect Treatment*Day
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: APC

i/j	1	2	3	4	5	6
1		0.0728	0.2494	0.1168	0.0539	0.3194
2	0.0728		0.8346	0.9682	1.0000	0.7588
3	0.2494	0.8346		0.9947	0.8555	1.0000
4	0.1168	0.9682	0.9947		0.9828	0.9790
5	0.0539	1.0000	0.8555	0.9828		0.7728
6	0.3194	0.7588	1.0000	0.9790	0.7728	

C

The GLM Procedure

Dependent Variable: APC APC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	5.78033628	0.41288116	4.10	0.0002
Error	40	4.03185590	0.10079640		
Corrected Total	54	9.81219217			

R-Square	Coeff Var	Root MSE	APC Mean
0.589097	8.703801	0.317484	3.647653

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	2	2.51486221	1.25743111	12.47	<.0001
Day	4	2.63920892	0.65980223	6.55	0.0004
Treatment*Day	8	0.62626515	0.07828314	0.78	0.6254

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	2	2.22795364	1.11397682	11.05	0.0002
Day	4	2.68368546	0.67092137	6.66	0.0003
Treatment*Day	8	0.62626515	0.07828314	0.78	0.6254

D

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	Day	APC LSMEAN	LSMEAN Number
HD	30	3.53903205	1
HD	35	3.38077576	2
HD	42	3.80922874	3
HD	49	3.94183041	4
HD	56	3.89943948	5
LD	30	3.94602947	6
LD	35	3.60202464	7
LD	42	3.75874552	8
LD	49	4.07674894	9
LD	56	3.97895097	10
UN	30	3.17642317	11
UN	35	3.04456400	12
UN	42	3.15682129	13
UN	49	3.93436046	14
UN	56	3.56970447	15

E

Least Squares Means for effect Treatment*Day
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: APC

i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		1.0000	0.9957	0.8867	0.9475	0.9276	1.0000	0.9995	0.5320	0.8784	0.9698	0.6613	0.9200	0.9412	1.0000
2	1.0000		0.8351	0.4633	0.5892	0.5752	0.9998	0.9260	0.1612	0.4844	0.9999	0.9694	0.9994	0.6077	0.9999
3	0.9957	0.8351		1.0000	1.0000	1.0000	0.9999	1.0000	0.9961	1.0000	0.3938	0.0819	0.2368	1.0000	0.9987
4	0.8867	0.4633	1.0000		1.0000	1.0000	0.9824	0.9999	1.0000	1.0000	0.1431	0.0183	0.0660	1.0000	0.9337
5	0.9475	0.5892	1.0000	1.0000		1.0000	0.9948	1.0000	1.0000	1.0000	0.2048	0.0302	0.1026	1.0000	0.9739
6	0.9276	0.5752	1.0000	1.0000	1.0000		0.9892	1.0000	1.0000	1.0000	0.2100	0.0381	0.1156	1.0000	0.9594
7	1.0000	0.9998	0.9999	0.9824	0.9948	0.9892		1.0000	0.8097	0.9760	0.9381	0.5969	0.8691	0.9921	1.0000
8	0.9995	0.9260	1.0000	0.9999	1.0000	1.0000	1.0000		0.9807	0.9998	0.5278	0.1357	0.3516	1.0000	0.9999
9	0.5320	0.1612	0.9961	1.0000	1.0000	1.0000	0.8097	0.9807		1.0000	0.0386	0.0033	0.0139	1.0000	0.6241
10	0.8784	0.4844	1.0000	1.0000	1.0000	1.0000	0.9760	0.9998	1.0000		0.1626	0.0268	0.0849	1.0000	0.9247
11	0.9698	0.9999	0.3938	0.1431	0.2048	0.2100	0.9381	0.5278	0.0386	0.1626		1.0000	1.0000	0.2290	0.9434
12	0.6613	0.9694	0.0819	0.0183	0.0302	0.0381	0.5969	0.1357	0.0033	0.0268	1.0000		1.0000	0.0431	0.5698
13	0.9200	0.9994	0.2368	0.0660	0.1026	0.1156	0.8691	0.3516	0.0139	0.0849	1.0000	1.0000		0.1285	0.8677
14	0.9412	0.6077	1.0000	1.0000	1.0000	1.0000	0.9921	1.0000	1.0000	1.0000	0.2290	0.0431	0.1285		0.9684
15	1.0000	0.9999	0.9987	0.9337	0.9739	0.9594	1.0000	0.9999	0.6241	0.9247	0.9434	0.5698	0.8677	0.9684	

F The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	APC LSMEAN	LSMEAN Number
HD	3.71406129	1
LD	3.87249991	2
UN	3.37637468	3

Least Squares Means for effect Treatment
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: APC

i/j	1	2	3
1		0.2996	0.0064
2	0.2996		0.0001
3	0.0064	0.0001	

Appendix K

SAS results of the APC counts for plant data of the Main experiment. PROC GLM statement was used to conduct ANOVA. The table (A) encased in the black box contains P values, F value, and degrees of freedom (DF) values used in the study. The post hoc Tukey-Kramer test was used to examine comparisons between treatment and the plants parts. The table contains the comparison for the treatments (B), the treatment and the plants parts (C); and the plants, plant parts, and treatment (D and E).

A

The GLM Procedure

Dependent Variable: APC APC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	79.34473238	7.21315749	80.38	<.0001
Error	28	2.51270394	0.08973943		
Corrected Total	39	81.85743632			

R-Square	Coeff Var	Root MSE	APC Mean
0.969304	7.953943	0.299565	3.766250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Plants	1	22.69846216	22.69846216	252.94	<.0001
Treatment	2	3.02703501	1.51351751	16.87	<.0001
Part	1	36.53890800	36.53890800	407.17	<.0001
Treatment*Part	2	6.76817715	3.38408857	37.71	<.0001
Plants*Treatmen*Part	5	10.31215006	2.06243001	22.98	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Plants	1	25.94298447	25.94298447	289.09	<.0001
Treatment	2	4.21867366	2.10933683	23.51	<.0001
Part	1	36.98584936	36.98584936	412.15	<.0001
Treatment*Part	2	5.65052298	2.82526149	31.48	<.0001
Plants*Treatmen*Part	5	10.31215006	2.06243001	22.98	<.0001

B

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	APC LSMEAN	LSMEAN Number
HD	3.62661990	1
LD	4.31808467	2
UN	3.61114649	3

Least Squares Means for effect Treatment
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: APC

i/j	1	2	3
1		<.0001	0.9903
2	<.0001		<.0001
3	0.9903	<.0001	

C

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	Part	APC LSMEAN	LSMEAN Number
HD	Leaves	4.33096643	1
HD	Roots	2.92227337	2
LD	Leaves	5.82796493	3
LD	Roots	2.80820442	4
UN	Leaves	4.30826369	5
UN	Roots	2.91402928	6

Least Squares Means for effect Treatment*Part
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: APC

i/j	1	2	3	4	5	6
1		<.0001	<.0001	<.0001	1.0000	<.0001
2	<.0001		<.0001	0.9798	<.0001	1.0000
3	<.0001	<.0001		<.0001	<.0001	<.0001
4	<.0001	0.9798	<.0001		<.0001	0.9855
5	1.0000	<.0001	<.0001	<.0001		<.0001
6	<.0001	1.0000	<.0001	0.9855	<.0001	

D The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Plants	Treatment	Part	APC LSMEAN	LSMEAN Number
Basil	HD	Leaves	2.98841341	1
Basil	HD	Roots	2.56522982	2
Basil	LD	Leaves	4.22427346	3
Basil	LD	Roots	2.60218554	4
Basil	UN	Leaves	3.43889262	5
Basil	UN	Roots	2.41612351	6
Lettuce	HD	Leaves	5.67351945	7
Lettuce	HD	Roots	3.27931693	8
Lettuce	LD	Leaves	7.43165640	9
Lettuce	LD	Roots	3.01422330	10
Lettuce	UN	Leaves	5.17763476	11
Lettuce	UN	Roots	3.41193505	12

E

Least Squares Means for effect Plants*Treatmen*Part
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: APC

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.6911	0.0005	0.8590	0.7088	0.3787	<.0001	0.9764	<.0001	1.0000	<.0001	0.6901
2	0.6911		<.0001	1.0000	0.0272	0.9999	<.0001	0.1269	<.0001	0.6142	<.0001	0.0177
3	0.0005	<.0001		<.0001	0.1057	<.0001	0.0001	0.0245	<.0001	0.0006	0.0225	0.0506
4	0.8590	1.0000	<.0001		0.0675	0.9997	<.0001	0.2463	<.0001	0.8044	<.0001	0.0519
5	0.7088	0.0272	0.1057	0.0675		0.0113	<.0001	0.9999	<.0001	0.7746	<.0001	1.0000
6	0.3787	0.9999	<.0001	0.9997	0.0113		<.0001	0.0531	<.0001	0.3185	<.0001	0.0073
7	<.0001	<.0001	0.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	0.6727	<.0001
8	0.9764	0.1269	0.0245	0.2463	0.9999	0.0531	<.0001		<.0001	0.9882	<.0001	1.0000
9	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001
10	1.0000	0.6142	0.0006	0.8044	0.7746	0.3185	<.0001	0.9882	<.0001		<.0001	0.7626
11	<.0001	<.0001	0.0225	<.0001	<.0001	<.0001	0.6727	<.0001	<.0001	<.0001		<.0001
12	0.6901	0.0177	0.0506	0.0519	1.0000	0.0073	<.0001	1.0000	<.0001	0.7626	<.0001	

Appendix L

SAS results of the *E.* coli counts for plant data of the Main experiment. PROC GLM statement was used to conduct ANOVA. The table (A) encased in the black box contains P values, F value, and degrees of freedom (DF) values used in the study. The post hoc Tukey-Kramer test was used to examine comparisons between treatment and the plants parts. The table contains the comparison for the treatments (B) and the treatment and lettuce parts (C).

A

The GLM Procedure

Dependent Variable: EC EC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	88.0429429	17.6085886	9.75	0.0005
Error	13	23.4806515	1.8062040		
Corrected Total	18	111.5235944			

R-Square	Coeff Var	Root MSE	EC Mean
0.789456	50.84762	1.343951	2.643095

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	2	17.92895012	8.96447506	4.96	0.0250
Part	1	3.72553352	3.72553352	2.06	0.1746
Treatment*Part	2	66.38845922	33.19422961	18.38	0.0002

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	2	17.06187381	8.53093690	4.72	0.0287
Part	1	4.32702462	4.32702462	2.40	0.1457
Treatment*Part	2	66.38845922	33.19422961	18.38	0.0002

B

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	EC LSMEAN	LSMEAN Number
HD	3.50845457	1
LD	2.44011722	2
UN	1.20589230	3

Least Squares Means for effect Treatment
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: EC

i/j	1	2	3
1		0.4014	0.0229
2	0.4014		0.2842
3	0.0229	0.2842	

C

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Treatment	Part	EC LSMEAN	LSMEAN Number
HD	Leaves	5.68357581	1
HD	Roots	1.33333333	2
LD	Leaves	-0.00000000	3
LD	Roots	4.88023443	4
UN	Leaves	-0.00000000	5
UN	Roots	2.41178459	6

Least Squares Means for effect Treatment*Part
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: EC

i/j	1	2	3	4	5	6
1		0.0160	0.0049	0.9658	0.0019	0.0625
2	0.0160		0.8782	0.0392	0.8223	0.8920
3	0.0049	0.8782		0.0107	1.0000	0.3572
4	0.9658	0.0392	0.0107		0.0040	0.1662
5	0.0019	0.8223	1.0000	0.0040		0.2425
6	0.0625	0.8920	0.3572	0.1662	0.2425	

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