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Research Paper

Longitudinal Survey of *Aeromonas hydrophila* and Foodborne Pathogens in a Commercial Aquaponics System

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ABSTRACT

Aquaponic production of fresh produce is a sustainable agricultural method becoming widely adopted, though few studies have investigated potential food safety hazards within commercial systems. A longitudinal study was conducted to isolate and quantify several foodborne pathogens from a commercial, aquaponic farm, and to elucidate their distribution throughout. The survey was conducted over 2 years on a controlled-environment farm containing Nile tilapia (*Oreochromis niloticus*) and lettuce (*Lactuca sativa*). Samples (N = 1,047) were collected bimonthly from three identical, independent systems, and included lettuce leaves, roots, fingerlings (7–126 d old), feces from mature fish (>126 d old), water, and sponge swabs collected from the tank interior surface. Most probable number of generic *Escherichia coli* were determined using IDEXX Colilert Quanti-Tray. Enumeration and enrichment were used to detect Shiga toxin-producing *E. coli* (STEC), *Salmonella enterica*, *Listeria monocytogenes*, *Aeromonas* spp., *Aeromonas hydrophilia*, and *Pseudomonas aeruginosa*. Generic *E. coli*, STEC, *L. monocytogenes,* and *S. enterica* were not detected in collected samples. *P. aeruginosa* was isolated from water (7/351; 1.99%), swabs (3/351; 0.85%), feces (2/108; 1.85%), and lettuce leaves (2/99; 2.02%). *A. hydrophila* was isolated from all sample types (623/1047; 59.50%). The incidence of *A. hydrophila* in water (X^2 = 23.234, p < 0.001) and sponge samples (X^2 = 21.352, p < 0.001) increased over time.

Between 2010 and 2020, there were 245 outbreaks of foodborne illness associated with fresh produce in the United States (US), with 7,140 illnesses, 792 hospitalizations, and 21 deaths (CDC, 2022). Specifically, 66 outbreaks, 2,032 illnesses, 580 hospitalizations, and 15 deaths were associated with leafy greens contaminated by *Salmonella enterica* subspecies *enterica*, *Listeria monocytogenes*, or Shiga toxin-producing *Escherichia coli* (STEC; CDC, 2022). These outbreaks have likely contributed to an increase in the adoption of commercial-scale, soil-less systems throughout the US. To-date aquaponic systems have not been involved in any foodborne illness outbreaks, nevertheless, some recent recalls with hydroponic systems have indicated that hazard analysis and risk mitigation strategies are still necessary in soil-less produce production (McClure et al., 2023). In 2021, the first documented outbreak associated with hydroponically grown lettuce occurred due to contamination by *Salmonella* Typhimurium (FDA, 2022), and in 2023, hydroponically grown lettuce was recalled due to contamination of *L. monocytogenes* on the product (FDA, 2023). Extensive research has identified potential routes of contamination in conventionally grown produce, but few studies have been performed on soil-less systems using aquaculture effluent (Buscaroli et al., 2021; Wang et al., 2020; Weller et al., 2020).

According to the Food and Agriculture Organization of the United Nations (FAO), to continue feeding the world's growing population, the food system must be made sustainable by increasing efficacy, inclusivity, and resiliency (FAO et al., 2022). Aquaponic production is one approach to improve sustainability in agriculture, since it optimizes the use of nitrogen by-products from aquaculture for use as nutrients for fresh produce production (Tyson et al., 2011). In a recirculating aquaponic system, nitrogen enters the system through fish feed; the fish consume the feed and produce ammonia (NH_3), which is transferred to the water when excreted through their gills and feces (Ru et al., 2017). Ammonia (NH_3) is oxidized by nitrifying bacteria to nitrites (NO_2^-) and then to nitrates (NO_3^-; Goddek et al., 2016). Plants absorb the nitrates and other dissolved macro and micronutrients,

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preventing the accumulation in the water. As a result, the water can be returned to the fish tanks with safe levels of potentially toxic nutrients (Eck, Körner, et al., 2019).

In previous microbiome studies, *Aeromonas* and *Pseudomonas* (Eck, Sare, et al., 2019) were identified within the aquaponic microbial community, but several studies have failed to isolate the enteric pathogens *E. coli*, *Salmonella*, and *Listeria* (Day et al., 2021; Schmautz et al., 2017). Concerns regarding pathogen prevalence within the aquaponic microbiome exist because produce comes in close contact with water during production. Prior observational studies briefly evaluated the presence of foodborne pathogens on varying types of small-scale, experimental, or commercial aquaponics systems. Fox et al. (2012) assessed commercial and backyard aquaponic systems in Hawai'i and found no detectable *E. coli* O157:H7 or *Salmonella* in the water or on produce or fish filets. Another study evaluated the presence of foodborne pathogens in three experimental aquaponic and hydroponic systems (Wang et al., 2020). While STEC was detected in fish feces, water, and on the surface of plant roots in the aquaponic systems, and water and plant roots in the hydroponic systems, it was not detected internally or on the surface of the edible portion of the produce, nor internally in the roots in either system (Wang et al., 2020). Alternatively, another study compared five experimental hydroponic and three experimental aquaponic systems, while generic *E. coli* was detected in three hydroponic systems, it was not detected in any of the aquaponic systems (Weller et al., 2020).

A major source of foodborne pathogens in conventional agriculture is from feces transferred through irrigation water (Benjamin et al., 2013). Although fish are intentionally present in the aquaponics system, limited research exists addressing the potential for fish to introduce foodborne pathogens such as STEC, *Salmonella*, or *Listeria* into the water. Nile tilapia (*Oreochromis niloticus*) are the most commonly raised fish in aquaponic systems in the U.S (Love et al., 2015) and generic *E. coli*, *S. enterica*, and *A. hydrophila* have been isolated from these fish (Thaotumpitak et al., 2022). Therefore, Nile tilapia could be a host to these pathogens, which could potentially transfer to the water, thrive within or establish biofilms, and ultimately transfer to the produce through splashing, harvest, or even potentially via internalization through the roots (Macarisin et al., 2014), although internalization may be a greater concern when the plant root structure is damaged (Wang et al., 2021).

While prior studies have examined single small-scale, experimental, or well-established commercial aquaponic systems with mature microbial communities, the lack of consistency among different system types prompted the current evaluation of a commercial system. Pathogen prevalence and distribution throughout a triplicated, commercial farm was determined bimonthly from startup through the first 2 years of production. Changes in physicochemical water quality were also documented to determine the potential impact water quality had on foodborne pathogens. This study adds to a growing body of knowledge intended to assist aquaponic producers assess potential food safety hazards throughout their own systems and implement appropriate risk mitigation strategies.

Materials and methods

Aquaponic system design. The examined commercial farm was comprised of three adjacent, recirculating aquaponic systems in a controlled environment (Fig. 1). This farm represents a large segment of systems in the U.S., as 77% (n = 186) of commercial aquaponic producers use deep water culture beds (DWC) to grow produce and 69% (n = 185) of commercial aquaponic producers raise tilapia (Love et al., 2015). The DWC hydroponic system utilized floating polystyrene resin mats with 2 in × 2 in holes were cut at approximately 1 ft intervals; lettuce transplants (~3–4 weeks) in rockwool were placed within each hole. Each independent system was coupled, meaning water flowed directly from fish tanks through a clarifier and biofilter until it reached the DWC (Palm et al., 2018); all three aquaponic systems functioned independently of each other. The systems were filled with charcoal-filtered municipal water upon system start-up and replenished with as needed. Each system was comprised of five fish tanks containing adult fish and two DWC grow beds. Lettuce was harvested weekly from the DWC mats, and as the mats were removed for harvest, new mats containing seedlings were transferred to the system.

Nile tilapia (*O. niloticus*) fingerlings were obtained at 7–14 d and confirmed negative for Aeromonas salmonicida, Yersinia ruckeri, Piscirickettsia salmonis, and Streptococcus iniae at the Washington Animal Disease Diagnostics Laboratories at Washington State University prior to receipt. Fingerlings were grown outside the system in nursery tanks until 84–126 d, after which they were introduced to the fish tank, where fish age ranged from 84 to 238 d. Approximately, 80 fish were harvested from each system weekly.

Lettuce (*Lactuca sativa*) was grown hydroponically by DWC. Cultivars included red butterhead, green butterhead, romaine, green oakleaf, and red oakleaf. Each system contained roughly 11,400 heads of lettuce with 1,900 heads of lettuce harvested weekly at 42 d from transplant. Light was provided to the lettuce by an artificial light source. The water temperature in each unit was maintained at 25–28°C.

Sample collection. Sample collection was performed once every 2 months for 2 years (November 2020 – September 2022) with an additional preproduction sample collection (October 2020; N = 1,047). Sample types included water, interior surface sponge swabs (3 M, Saint Paul, MN), feces from mature fish (>126 d old), fingerlings (7–126 d old), lettuce leaves, and lettuce roots. Water samples (c. 500 mL) were collected in sterile polyethylene bottles (Thermo Fisher Scientific Inc., Waltham, MA) from the fish tank, beginning of DWC (BDWC), and end of DWC (EDWC; Fig. 1). In addition, dry sponge-sticks (3 M, Saint Paul, MN) were used to swab the interior walls of the fish tank, BDWC, and EDWC (Fig. 1). Each sponge swab was moistened with aquaponic water to assist in the removal of the adhered bacteria on each surface. The swab was used to sample a 0.93 m^2 (30.5 cm × 30.5 cm) surface area as described in the FDA Bacteriological Analytical Manual Chapter 10 (Hitchins et al., 2022).

Lettuce was collected using sterile scissors to remove the root base, and undesired leaves were removed to mimic commercial practice. The entire head of lettuce and the root base (>25 g) were transferred to separate Whirl-Pak bags (Whirl-Pak, Madison, WI). Fingerlings (>10 g) and fish were harvested by the farm on the morning of sample collection and placed in individual Whirl-Pak bags. All samples were immediately placed on ice upon collection and chilled until processing.

Sample preparation. Samples were processed within 10 h of collection. Sponge swabs (0.93 m^2), lettuce leaves (25 g ± 1 g), lettuce roots (25 g ± 1 g), fingerlings (10 g ± 5 g), and fish feces (1 mL) were diluted (1:10) with 0.1% peptone (Life Technologies Corporation, Detroit, MI). Sponge swabs were hand massaged for 60 s. Lettuce leaves, roots, and fingerlings were stomached (Seward Inc, Bohemia, NY) in Whirl-Pak bags at 230 rpm for 150 s. Fish feces were extracted from the fish and placed in a 2 mL microcentrifuge tube (Thermo Fisher Scientific, Waltham, MA) before dilution.

Enumeration of target organisms. All samples were enumerated on agar for total aerobic plate count (APC), STEC, *L. monocytogenes*, *S. enterica*, *P. aeruginosa*, *Aeromonas* spp., and *E. coli* (excluding water samples). Samples were spread plated (100 µL) onto CHROMagar™ STEC (CHROMagar, Paris, France; STEC), CHROMagar™ Listeria (CHROMagar; *L. monocytogenes*), XLT-4 (Neogen, Lansing, MI; *S. enterica*), CRITERION™ Ampicillin Dextrin Agar with 100 ppm ampicillin (ADA; Hardy Diagnostics, Santa Maria, CA; for quantification and enrichment of *Aeromonas* spp.), Difco™ Tryptic Soy Agar (TSA; Becton, Dickinson and Company, Sparks, MD; APC), TSA containing 5% sheep

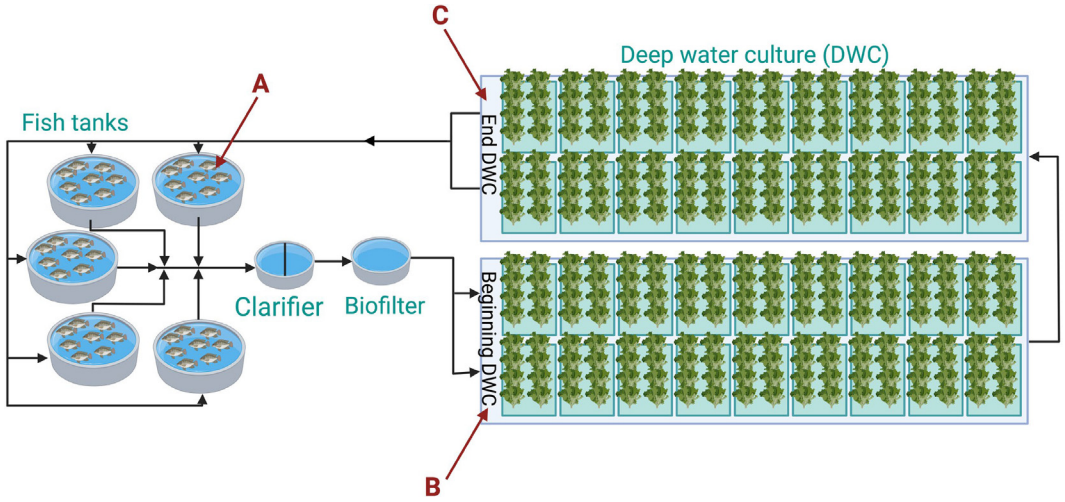

Figure 1. Schematic of one replicate of three recirculating aquaponics system. The red arrows indicate where water and sponge swab samples were collected, including the fish tank (A), the beginning of the deep-water culture (DWC; B), and the end of the DWC (C) grow bed. This figure was created with BioRender.com.

blood (Northeast Lab Services, Waterville, MA) and 100 ppm ampicillin (Hardy Diagnostics; for the enrichment of *Aeromonas* spp.), Difco™ Cetrimide Agar (Becton, Dickinson and Company; *P. aeruginosa*), and Difco™ MacConkey Agar (excluding water samples; Becton, Dickinson and Company; *E. coli*). ADA and 5% Sheep Blood Agar were incubated at 25°C for 24 h. CHROMagar Listeria was incubated at 37°C for 48 h. Cetrimide agar, TSA, and MacConkey Agar were incubated at 37°C for 24 h. CHROMagar STEC was incubated at 44.5°C for 24 h. XLT-4 was incubated at 37°C for 72 h, and plates were observed every 24 h. Positive controls for each corresponding media type included *S.* Newport MDD 689 (clinical isolate from tomato outbreak), *E. coli* O157:H7 ATCC 35150 (American Type Culture Collection, Manassas, Virginia), *L. monocytogenes* F8027 (serotype 4b; celery isolate), *P. aeruginosa* ATCC 27853, and *A. hydrophila* ATCC 49140 obtained from the University of Georgia Department of Food Science and Technology culture collection.

IDEXX Colilert Quanti-tray 2000 (IDEXX Laboratories, Westbrook, ME, USA) was used to quantify generic *E. coli* and coliforms in 100 mL water samples per manufacturers' instructions.

Microbial enrichment and isolation. Samples (sponge swab, fingerling, lettuce leaf, and root) were enriched at a 1:10 dilution in 90 mL Tryptic Soy Broth (TSB; Becton, Dickinson and Company), Universal Preenrichment Broth (UPB; Becton, Dickinson, and Company), and Listeria Enrichment Broth (LEB; Neogen). Due to the small sample size, fish feces samples were enriched at a 1:10 dilution in 9 mL TSB, UPB, and LEB. For water samples, bottles were inverted 25 times and 100 mL were vacuum filtered on 0.45-µm 47 mm mixed cellulose ester membrane filters (Whatman, Maidstone, UK). The filtration apparatus was rinsed twice with approximately 30 mL of sterile deionized water (SDW). The filter paper was transferred to bottles containing 100 mL UPB, TSB, or LEB, then mixed by shaking for 10 s.

UPB dilution bottles were incubated at room temperature for 1 h; then, 10 mL were transferred to a sterile test tube; for fish feces samples, 1 mL was transferred to 9 mL of UPB. Tubes were incubated at 44.5°C for 24 h for selective STEC enrichment. After incubation, a loopful of UPB was streaked on CHROMagar STEC and incubated at 44.5°C for 24 h for STEC isolation.

TSB, LEB, and the remainder of the UPB were incubated at 35°C for 24 h. After 24 h incubation, a loopful (10 µL) of TSB was streaked on Cetrimide agar (for *P. aeruginosa*) and a loopful of LEB was streaked on CHROMagar Listeria (for *L. monocytogenes*) and incubated at 37°C for 48 h. Presumptive *L. monocytogenes* colonies were further confirmed by streaking on RAPID'L.mono (Bio-Rad Laboratories, Inc. Hercules, CA) and incubated at 37°C for 24 h.

Additionally, 1 mL enriched TSB was transferred to 9 mL TSB containing ampicillin (100 ppm) and incubated at 35°C for 24 h. A loopful (10 µL) of overnight enrichment was streaked onto ADA and 5% Sheep Blood Agar and incubated at 25°C for 24 h for *Aeromonas* spp. isolation. Enriched UPB (1 mL) was transferred to 9 mL Difco™ Tetrathionate Broth (Becton, Dickinson and Company) and incubated at 35°C for 24 h. After 24 h, the culture was streaked on XLT-4 and BD CHROMagar Salmonella (Becton, Dickinson and Company) and incubated at 37°C for 72 h. Plates were observed every 24 h for *S. enterica* growth.

Presumptive positives, TSB, and UPB were stored at −80°C in 25% glycerol by volume for further confirmation.

Real-time PCR confirmation. DNA extraction was performed using a heat lysis method described in the FDA BAM Chapter 4A (Feng et al., 2020). Briefly, a 1 mL aliquot of culture was centrifuged at 12,000*g* for 180 s (accuSpin Microcentrifuge 17R, Fisher Scientific, Waltham, MA). The supernatant was removed, and pellets were washed with 1 mL 0.85% NaCl (Thermo Scientific) and centrifuged at 12,000*g* for 180 s. The supernatant was removed, pellets were resuspended in molecular grade water (Thermo Scientific) and heated to 100°C for 10 min. DNA quantification was performed using NanoDrop 2000 spectrophotometer (NanoDrop, Wilmington, DE, USA). DNA extractions were stored at −80°C for real-time PCR analysis.

A real-time PCR was optimized to confirm STEC, *S. enterica*, *L. monocytogenes*, *A. hydrophila*, and *P. aeruginosa* on QuantStudio3 Real-Time PCR System (Applied Biosystems, Waltham, MA) using PowerUp SYBR Green Master Mix (Applied Biosystems). Primer (Thermo Fisher Scientific) sequences can be found in Table 1. The amplification reaction contained 10 µL Powerup SYBR Green Master Mix, 4 µL molecular grade water, forward and reverse primers (Table 1), and 2 µL DNA template. Amplification reactions were carried out with uracil-DNA glycosylases (UDG) activation of 120 s at 50°C, denaturation of 120 s at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C, and melt curve stage of 15 s at 95°C, 60 s at 60°C, and 15 s at 95°C.

Physicochemical water quality. Water samples were analyzed for carbonaceous biochemical oxygen demand (CBOD), according to Delzer & McKenzie (2003). Three water samples were collected from the fish tank, BDWC, and EDWC and slowly transferred into a 300 mL glass biochemical oxygen demand (BOD) bottle. The nitrifying bacteria inhibitor 2-Chloro-6-(trichloromethyl) pyridine (Hach,

Table 1
Primers used in real-time PCR assays for confirmation of presumptive *Aeromonas hydrophila*, *Aeromonas* spp., *Pseudomonas aeruginosa*, *Salmonella enterica*, *Listeria monocytogenes* (LM), and Shiga toxin-producing *Escherichia coli* (STEC)

Target organism	Target gene	Oligonucleotide	Sequence (5′→3′)	Primer Concentration (nM)	Amplicon size (bp)	Tm °C	Ref.
A. hydrophila	*ahh1*	AHH1-F	GCCGAGCGCCCAGAAGGTGAGTT	500	130	87.1 ± 1	(Chowdhury et al., 2019)
		AHH1-R	GAGCGGCTGGATGCGGTTGT	500			
	ast	Ast-F	GACTTCAATCGCTTCCTCAACG	250	536	87.2 ± 1	(Robertson et al., 2014)
		Ast-R	GCATCGAAGTCACTGGTGAAGC	250			
Aeromonas spp.	*gyrB*	gyrB-F	GAAGGCCAAGTCGGCCGCCAG	500	198	89.5 ± 1	(Robertson et al., 2014)
		gyrB-R	ATCTTGGCATCGCCCGGGTTTTC	500			
P. aeruginosa	*oaa*	PA431CF	CTGGGTCGAAAGGTGGTTGTTATC	500	232	92.1 ± 1	(Choi et al., 2013)
		PA431CR	GCGGCTGGTGCGGCTGAGTC	500			
S. enterica	*iroB*	iroB-12-deg-F	GG**R**ACAAAAATGGGG**M**ACTTCT	500	105	80.5–83.0	(Barbau-Piednoir, Bertrand, et al., 2013)
		iroB-12-deg-R	AAGGGGAGG**R**TAGACGATGA	500			
LM	*hlyA*	hlyA-177-F	TGCAAGTCCTAAGACGCCA	500	112	74 ± 1	(Barbau-Piednoir, Botteldoorn, et al., 2013)
		hlyA-177-R	CACTGCATCTCCGTGGTATACTAA	500			
STEC	*stx1*	stx1-185-F	GTCACAGTAACAAACCGTAACA	500	95	77.1 ± 1	(Barbau-Piednoir et al., 2018)
		stx1-185-R	TCGTTGACTACTTCTTATCTGGA	500			
	stx2	stx2-81-deg	GTTTCCATGACRACGGACAGCAG	750	122	78.5–80 ± 1	
		stx2-81-alt-R	CTGAACTCCATTAAMKCCAGATATG	750			

Loveland, CO, US) was immediately added to each sample. Polyseed (InterLab, Woodlands, TX, US), a BOD seed inoculum, was prepared per the manufacturer's instructions and added to each bottle. Dissolved oxygen (DO) was measured from each bottle using a DO meter (Accumet, Waltham, MA, USA) and recorded as initial DO (d_0). The samples were sealed with the cap and placed in an incubator at 20°C for 5 days. After 5 days, DO was measured (d_5) and CBOD was calculated (Delzer & Mckenzie, 2003).

$$\mathrm{CBOD} = \mathrm{DO(mg/L)\ of\ d_0} - \mathrm{DO(mg/L)\ of\ d_5}.$$

A 10 mL sample was removed from the collected water and equilibrated to room temperature. The pH (Accumet), electrical conductivity (EC; Accumet), total dissolved solids (TDS; Accumet), and turbidity (Orion AQ3010, Waltham, MA, USA) were measured for each sample.

Statistical analysis. During the bimonthly samplings ($n = 12$), the following samples were collected from each identical system ($n = 3$): lettuce leaf ($n = 3$), lettuce root ($n = 3$), feces from mature fish ($n = 3$), interior surface sponge swab ($n = 9$), water ($n = 9$) and fingerling ($n = 1$).

The age of the system was divided into four groups, 0–122, 183–305, 366–488, and 549–671 d, with 0 d being the day fish were placed into the system. This grouping was necessary to fulfill the limitations of the statistical analysis. The impact of seasonality was not evaluated since the system was in a controlled environment with no seasonal fluctuation in natural light or other exposure to natural environment factors.

For IDEXX Colilert MPN values, the lower limit of detection (LOD) was 1 MPN/100 mL, while the upper LOD was 5.38 log MPN/100 mL. For microbial enumeration, the lower LOD was 1.00 log CFU/mL for water, 2.00 log CFU/g for lettuce leaf, lettuce root, fish feces, and fingerling, and 2.00 log CFU/cm^2 for sponge swabs.

A repeated measures one-way and two-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test ($P \leq 0.05$) was performed to test the associations among the concentrations of total plate count (TPC), *Aeromonas* spp., coliforms, and physicochemical water quality measurements and age of the system and sample location. Pearson's chi-square goodness of fit test ($P \leq 0.05$) was performed to test the association of *A. hydrophila* and the age of the systems based on each sample type. Pearson's chi-square test of independence ($P \leq 0.05$) was performed to test the association of *A. hydrophila* in water and sponge swab samples by the age of the system and sample collection location. All analyses were performed using RStudio version 4.1717 (R Core Team, 2021). Figures were produced on JMP Pro 16.0.0 (Cary, NC, USA).

Results and discussion

Physicochemical water quality conditions. It has been well-documented that physicochemical water quality parameters, including pH, dissolved oxygen, nutrient levels, turbidity, and temperature, affect microbial communities in aquaponics systems (Joyce et al., 2019; Sun et al., 2021; Wongkiew et al., 2018). Therefore, aquaponic farms monitor these parameters closely to ensure homeostasis among fish, plants, and microorganisms.

The optimal pH for aquaculture and nitrifying bacteria in an aquaponic system is 7.0–8.5 (Ebeling & Timmons, 2012), while plants favor slightly more acidic conditions (5.5–6.5) for optimal nutrient absorption (Hochmuth, 2021). The pH of the commercial system ranged from 6.85 to 7.69 (Fig. 2) and was higher between 549–671 d (7.41 ± 0.12) than 183–305 d (7.10 ± 0.11) and 366–488 d (7.14 ± 0.14; $p < 0.001$; Table 2). The EDWC (7.28 ± 0.17) had a higher pH than the BDWC (7.18 ± 0.18) and fish tank (7.17 ± 0.19; $p = 0.0014$). The pH difference in the EDWC can be attributed to nutrient uptake in the water. The uptake of nitrates can lead to an increase in pH, while potassium has the opposite effect, resulting in acidification in the water (Hochmuth, 2021). Although pH was slightly above the optimal range for lettuce production, studies have found no differences in growth rates or yields in produce grown in pH conditions between 5.0 and 7.0 within an aquaponic system (Blanchard et al., 2020). Maintaining a pH above 6.0, such as the examined system, promotes nitrification and minimizes pH-induced fish stress (Singh & Dunn, 2016; Wongkiew et al., 2018).

Water sample collection location (i.e., fish tank, BDWC, and EDWC) did not impact EC and TDS ($p > 0.05$), with average EC and TDS readings throughout the entire system of 1.50 ± 0.24 mS/cm and 903.7 7 ± 193.34 ppm, respectively (Table 2). However, the TDS and EC increased as the farm matured ($p < 0.001$). EC measures the total soluble salt content, with an optimal range for hydroponically grown lettuce between 1.20 and 1.80 mS/cm (Singh & Dunn, 2016). In conventional agriculture, soil acts as a buffer to maintain pH and EC. In soil-less systems, these parameters must be monitored closely and maintained since water has less buffering capacity than soil (Hochmuth, 2021). Fish are sensitive to changes in EC, with an ideal range for freshwater fish between 0.10 and 2.00 mS/cm. Some salt in the water is necessary for fish cells to maintain an osmotic balance, but an EC outside of the optimal range results in stress, making fish susceptible to disease and minimizing productivity in lettuce production (Hosseini et al., 2021). TDS measures the content of inorganic salts, organic matter, and other dissolved materials in water. As with

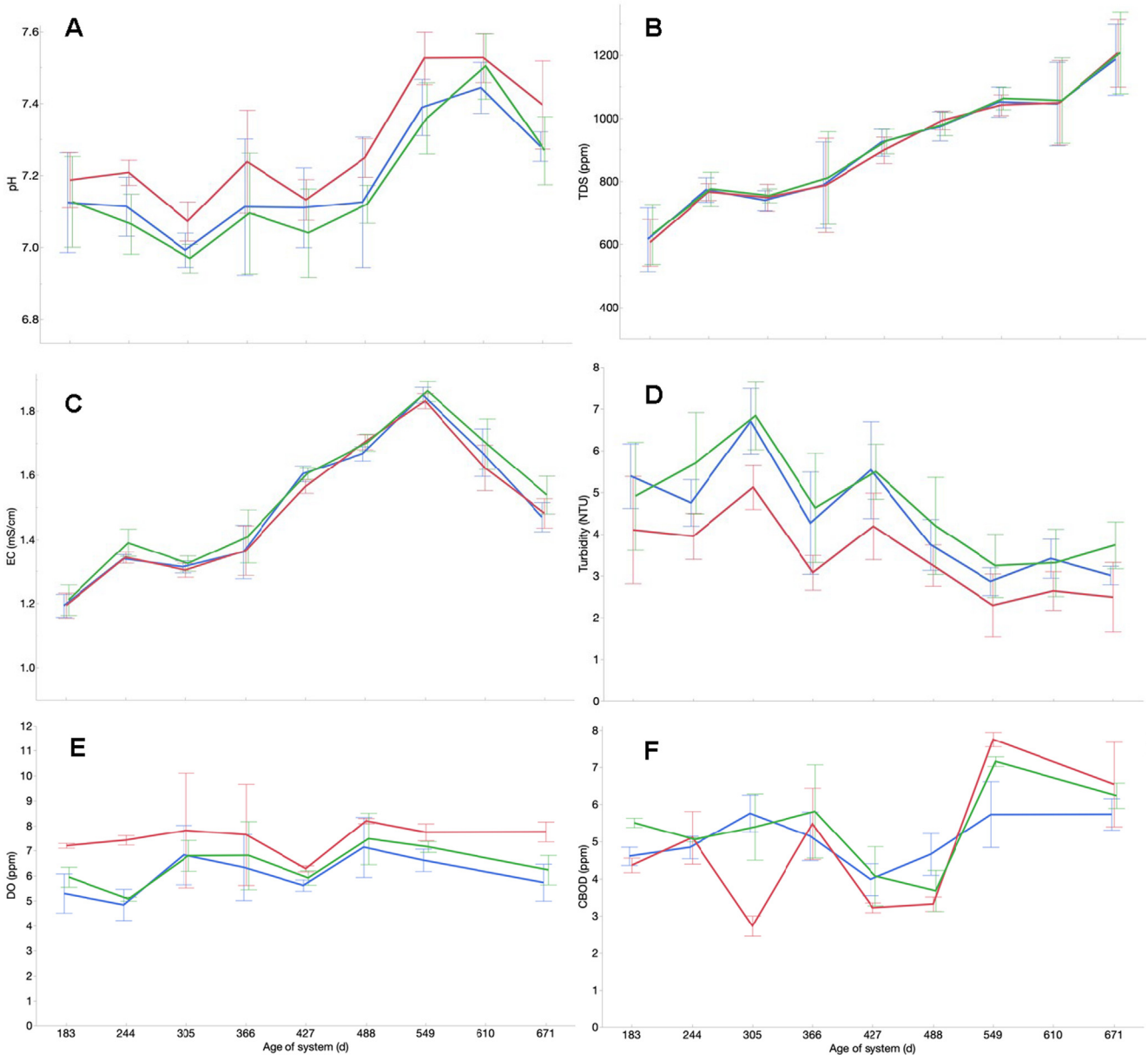

Figure 2. Change of pH (a), total dissolved solids (b), electrical conductivity (c), turbidity (d), dissolved oxygen (e), and carbonaceous biochemical oxygen demand (f) over 2 years in the fish tank (green), beginning deep water culture (blue), and end deep water culture (red). Each point represents the mean of 9 measurements, 3 replicates from 3 farms.

EC, variance in TDS has been shown to cause shifts in biotic communities, limit biodiversity, and can be detrimental to fish health (Duffy & Weber-Scannell, 2007).

The turbidity in aquaponic water is due primarily to suspended solids from uneaten feed, fecal matter, microorganisms, sloughed-off biofilms, and plant matter (Rakocy, 2012). The turbidity of the examined systems, as measured in total suspended solids, ranged from 1.63 to 8.54 NTU. The turbidity was lower in the EDWC (3.47 ± 1.12 NTU) than in the BDWC (4.42 ± 1.43) or fish tank (4.68 ± 1.47; $p = 0.0013$; Table 2), which is likely due to solids having more time to settle out of the water as it moves through the system before reaching the EDW. As the age of the farm increased, the turbidity decreased ($p < 0.001$). This decrease in turbidity over time could be due to the facility optimizing fish feed, flow rate, and production practices with the lettuce since after the first year, the system was considered at equilibrium. Imbalance of feed quantity or stocking density during the first few months of startup can result in uneaten feed or excess waste, increasing turbidity (Sipaúba-Tavares et al., 2010). Flow rate correlates positively with water turbidity, so flow rate reduction by farm management during the startup adjustment period could be a potential explanation for decreased turbidity as the system aged. Elevated suspended solids (>90 NTU) may have deleterious effects to fish by adhering to their gills, potentially lowering oxygen transfer rates and ammonia exchange rates (Lennard & Goddek, 2019). High turbidity can also affect plant health by creating anaerobic zones around the roots that inhibit nutrient uptake or by facilitating colonization by pathogenic organisms on the roots leading to poor root health (Rakocy et al., 2006). Also, because suspended solids are high in organic matter, they can promote the growth of heterotrophic organisms rather than the nitrifying bacteria that are critical for balance within the system (Lennard & Goddek, 2019).

Dissolved oxygen is necessary for fish, plant roots, and microbial health (Rakocy et al., 2006). Most plant roots and microorganisms in an aquaponic system require >3 ppm (Goto et al., 1996), whereas

Table 2
Average physicochemical water quality measurements of each collection location (between days 183–671) in the recirculated aquaponic farm (n = 27; mean ± SD). The means followed by different letters, in the same column, are significantly differences ($p < 0.05$), per Tukey's HSD

Age of system (d)	Collection location	pH	Total dissolved solids (ppm)	Electrical conductivity (mS/cm)	Turbidity (NTU)	Dissolved oxygen (ppm)	Carbonaceous biochemical oxygen demand (ppm)
183–305	Fish tank	7.05 ± 0.11 cd	720.30 ± 89.58cde	1.31 ± 0.14bc	5.82 ± 1.35a	5.95 ± 0.83bc	5.32 ± 0.81abc
	BDWC	7.08 ± 0.11d	709.33 ± 93.37e	1.28 ± 0.10c	5.62 ± 1.08a	5.66 ± 1.19c	5.07 ± 0.76bc
	EDWC	7.16 ± 0.08bcd	706.73 ± 88.74de	1.28 ± 0.10c	4.39 ± 0.98ab	7.48 ± 1.18ab	4.07 ± 1.25c
366–488	Fish tank	7.08 ± 0.12 cd	906.64 ± 113.11b	1.57 ± 0.19ab	4.78 ± 1.17ab	6.74 ± 1.10abc	4.52 ± 1.69c
	BDWC	7.12 ± 0.16bc	895.34 ± 116.11bcd	1.54 ± 0.20abc	4.55 ± 1.27ab	6.37 ± 1.12abc	4.60 ± 0.98c
	EDWC	7.21 ± 0.11bc	892.97 ± 122.62bc	1.54 ± 0.20abc	3.51 ± 0.75bc	7.38 ± 1.33ab	4.01 ± 1.40c
549–671	Fish tank	7.38 ± 0.13abc	1,108.87 ± 22.30a	1.70 ± 0.22a	3.43 ± 0.72bc	6.70 ± 0.64abc	6.69 ± 0.63ab
	BDWC	7.37 ± 0.09ab	1,094.56 ± 120.01a	1.66 ± 0.21ab	3.10 ± 0.42bc	6.18 ± 0.73abc	5.73 ± 1.08abc
	EDWC	7.48 ± 0.11a	1,099.26 ± 124.39a	1.65 ± 0.21ab	2.49 ± 0.69c	7.76 ± 0.33a	7.15 ± 1.43a

most fish require >5 ppm (Timmons et al., 2018). The DO in the fish tank (6.43 ± 0.95 ppm) and BDWC (6.05 ± 1.07 ppm) were lower than in the EDWC (7.51 ± 1.07 ppm; $p < 0.0001$; Table 2). The differences among sample collection locations are likely due to fish in the fish tank and beneficial bacteria depleting the dissolved oxygen in the biofiltration component immediately prior to the water entering the BDWC (Fig. 1). Once water reaches the EDWC, fewer beneficial bacteria are present, therefore using less oxygen, leading to higher DO concentrations.

Carbonaceous biochemical oxygen demand is the amount of dissolved oxygen needed by microorganisms to break down carbonaceous organic matter in the water in an aerobic environment; oxygen demand due to nitrification is inhibited by this method (Delzer & Mckenzie, 2003). Heterotrophic organisms use the carbonaceous organic matter (excess fish feed and fish waste) in the system, including carbohydrates, amino acids, peptides, and lipids as their carbon and energy source (Munguia-Fragozo et al., 2015). The CBOD of the system increased over time, with an average of 4.80 ± 1.97 ppm. The last 6 months of sampling (549–671 d) had a higher CBOD (5.64 ± 1.67 ppm) than 183–488 d (4.30 ± 1.97 ppm). No studies have evaluated CBOD in aquaponics systems; however, a few studies have evaluated biochemical oxygen demand (BOD) in aquaponic systems (Deswati et al., 2020; Su et al., 2020). CBOD and BOD evaluate oxygen needed to break down carbonaceous material; however, CBOD purposefully inhibits nitrifying bacteria. Nitrification can occur within 5 days if there are high amounts of nitrifying bacteria and can skew the results of the oxygen demand assay (Delzer & Mckenzie, 2003). In a 70 d small-scale experimental study, the average BOD was 1.51 ppm with 100% fish survival (Su et al., 2020). In another small-scale experimental system, the BOD over 0–42 d ranged between 0.48 and 4.64 ppm and increased as the age of the system increased (Deswati et al., 2020). These systems were younger in age and smaller in size than our study system, leaving less time for the organic load to accumulate. Additionally, the differences between our study and published data could be due to system maturity, production density, flow rate of each system, and the differences presented by measuring CBOD versus BOD. The evaluation of CBOD in aquaponic systems could assist in optimizing the oxygen demand that is needed to break down the carbonaceous organic matter in the water.

Total aerobic plate count. TPC quantified aerobic organisms that were able to grow on TSA within 24 h at 37°C (Fig. 3). The sample location (fish tank, BDWC, and EDWC) did not influence TPC in sponge samples and water samples ($p > 0.05$). The age of the system affected TPC in lettuce leaves, sponge swabs, fish feces, and water samples ($p < 0.05$), but root and fingerling TPC were not impacted by system age ($p > 0.05$). TPC in lettuce leaves and sponge swabs changed similarly over time. Between 0 and 122 d, the TPC was lower than between 183 and 305 d. After 1 year, the TPC on leaves and sponge swabs continuously decreased up to 671 d. In water samples, the TPC decreased over 0–488 d (5.30–3.08 log CFU/mL). However, between 549 and 671 d, the TPC was higher than between 366 and 488 d. The TPC in feces from mature fish ranged between 2.81 and 7.59 log CFU/mL. During 0–122 d, fish feces had a lower TPC than 183–305 and 549–671 d. However, on 366–488 d, fish feces TPC was similar among all sampling periods. A similar trend occurred in a smaller experimental recirculating aquaponics system analyzing TPC; between 0 and 63 d the TPC increased, followed by a 3-log reduction from 63 d to 118 d (Elumalai et al., 2017), this is likely due to the establishment of the biological filter which contains heterotrophic and autotrophic organisms (Schmautz et al., 2022). Unlike *Aeromonas* spp. and coliforms, TPC decreased over time in lettuce leaf, sponge swab, and water. Since coliforms and *Aeromonas* are heterotrophic organisms, they do not directly compete with nitrifying bacteria for nutrients (Taabodi et al., 2020), and are therefore able to proliferate in the ecosystem. The decreasing TPC may be attributed to increased populations of slower-growing nitrifying bacteria and ammonia-oxidizing archaea in the biological filter, which take longer to form a robust microbial community than most mesophilic, aerobic organisms (Goddek et al., 2019; Kasozi et al., 2021). Nitrifying bacteria can take anywhere from 28 to 244 d to form a robust community and are not quantifiable on TSA (Bartelme et al., 2017; Sallenave, 2016).

Coliforms and generic *E. coli* in aquaponic water. Coliforms and generic *E. coli* were in water samples collected from the fish tank, BDWC, and EDWC from all three systems were analyzed using IDEXX Colilert (n = 351). Generic *E. coli* was below the detectable limit, but coliforms were quantified in all water samples. Coliform MPN were similar among the three systems and collection locations ($p > 0.05$) but differed based on the age of the farm ($p < 0.05$; Fig. 4). Coliform MPN were the lowest when the farm was the youngest (0–122 d; 3.43 ± 0.69 log MPN/100 mL) while 183–305 and 549–671 d had the greatest coliforms 4.62 ± 0.30 and 4.69 ± 0.34 log MPN/100 mL, respectively. These findings were analogous to current research on coupled aquaponics systems. A study performed by Weller et al. (2020) evaluated generic *E. coli* and coliforms in three disparate recirculating experimental aquaponics systems. Generic *E. coli* was below the detectable limit, but coliform MPN within the countable range (9/29) ranged from 3.24 to 3.55 log MPN/100 mL. However, in a decoupled system using surface water, generic *E. coli* levels were as high as 5.32 log CFU/100 mL (Dorick et al., 2021). This variability is likely due to the type of system and water source used.

Prevalence of *E. coli*, *L. monocytogenes*, *S. enterica*, and *P. aeruginosa*. No generic *E. coli*, STEC, *S. enterica*, and *L. monocytogenes* were isolated from the system. These findings were consistent with similar studies. Aquaponic systems have been evaluated for STEC, *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* in varying experimental designs (Elumalai et al., 2017; Fox et al., 2012; Wang et al., 2020; Weller et al., 2020). One study identified STEC in the water, root surface, and fish feces of aquaponic systems, and the pathogen was also identified in the water and root surface of neighboring hydroponic

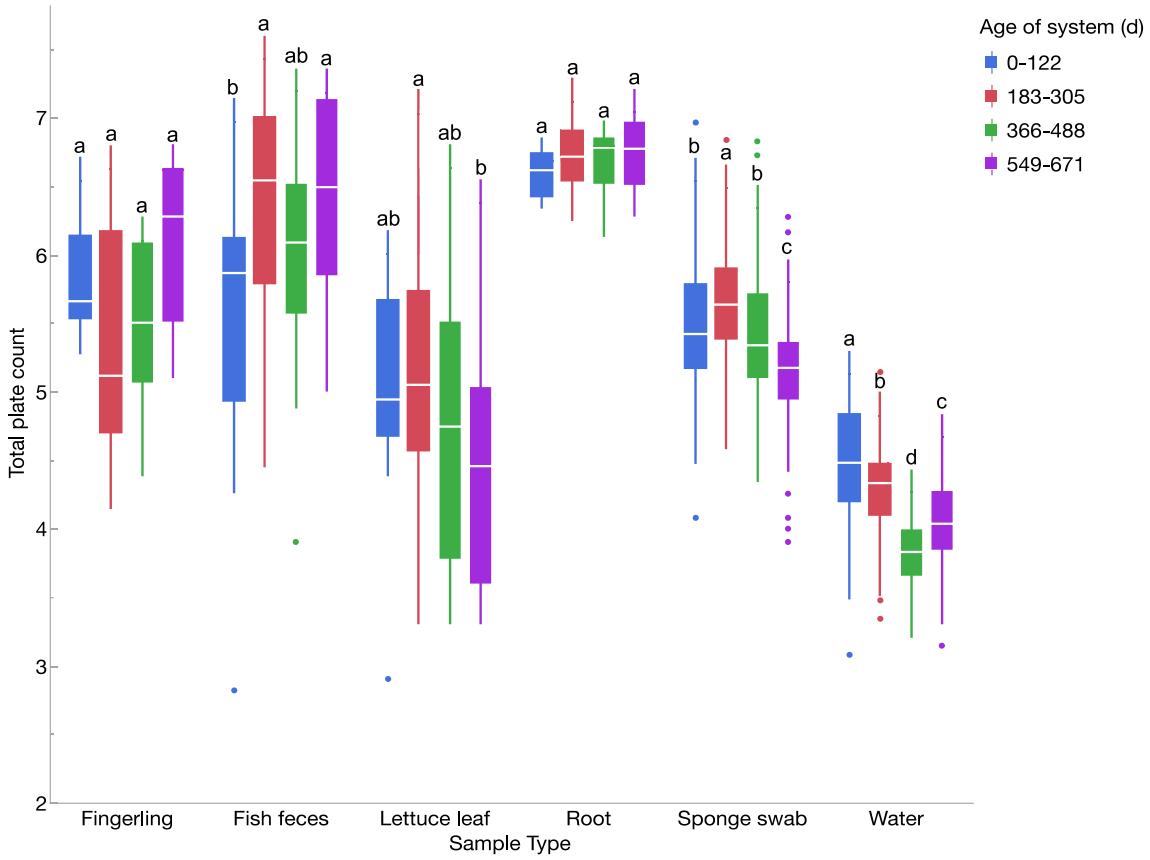

Figure 3. Boxplot of the total aerobic plate count (log CFU/g) in fingerling, fish feces, lettuce leaf, root, sponge swab (log CFU/cm^2), and water samples (log CFU/mL; N = 1,047) based upon the age of the system (0–671 d). The colored boxes represent the interquartile range (25–75 percentile) of the distribution. The middle white bar of each boxplot represents the median, and the whiskers indicate range. Each dot denotes outliers. The different letters, in each sample type, are significant differences ($p < 0.05$) per Tukey's HSD.

systems in the same greenhouse. Contamination in both system types within the same facility suggests contamination from a non–aquaponic-specific source. Although STEC was present in both types of systems, it had not internalized in the roots, lettuce or basil leaves, or fruit of tomatoes grown (Wang et al., 2020). Outside of the mentioned study, there have been no findings of STEC, *S. enterica*, or *L. monocytogenes* in aquaponic systems. This has been attributed to the robust microflora necessary for nitrification, which is hypothesized to outcompete many pathogens (Kasozi et al., 2021).

P. aeruginosa was detected in 1.34% (14/1,047) of the samples but was below the quantifiable limit (Table 3). Of the 14 samples that were positive for *P. aeruginosa*, four were from water and sponge samples collected before placing fish in the system. The age of the system influenced the probability of *P. aeruginosa* being in the system (p = 0.03). Over the first 1.5 years, *P. aeruginosa* prevalence was variable and overall was identified in less than 3% of samples collected: 0–122 d, 5/247 (2.02%); 182–305 d, 2/247 (0.81%); 366–488 d, 7/247 (2.83%). In the last 6 months of the study (549-671d), *P. aeruginosa* was not isolated from any samples collected. *P. aeruginosa* specifically has not been evaluated in current literature, but *Pseudomonas* spp. has been identified in aquaponic microbiome studies. In a mature experimental aquaponic system, Schmautz et al. (2017) found that *Pseudomonas* had a relative abundance of 2.2% in roots from lettuce (n = 2), 0.58% in biochips from the biofilter (n = 20), and 0.17% in a biofilm sample in the fish tank (periphyton).

***Aeromonas* spp. quantification and *A. hydrophila* prevalence.** *Aeromonas* is a ubiquitous aquatic organism in the fish microbiome, but it can cause mortality in fish if they undergo stress or injury (Janda & Abbott, 2010). *A. hydrophila* is considered the most harmful to farm-raised fish, causing hemorrhagic septicemia; however, disease has also been caused by *Aeromonas caviae*, *Aeromonas veronii*, *Aeromonas salmonicida*, and *Aeromonas sobria* (Chen et al., 2019). *Aeromonas* also causes illness in humans, and in more recent studies, *A. hydrophila* was identified in 5–9% of diarrheal illnesses associated with *Aeromonas* infection (Pessoa et al., 2022).

Aeromonas has been reported in tilapia fecal samples from fish raised in an aquaponic system (Schmautz et al., 2017). The pathogen could transfer via feces to the water, integrate into the existing biofilm, and ultimately transfer to produce if biofilm disruption occurs and cells are dislodged. In the current study, *Aeromonas* spp. was quantified in all sample types by enumerating ADA. The age of the system significantly influenced *Aeromonas* spp. levels quantified in roots, sponge swabs, and water ($p < 0.05$; Fig. 5). The root samples contained the lowest *Aeromonas* levels from 0 to 122 days (3.51 ± 1.08 log CFU/g); levels increased between 183 and 305 d (4.67 ± 0.58 log CFU/g) and remained constant throughout 366–671 d (5.61 ± 0.73 log CFU/g). There was a similar trend among sponge and water samples; *Aeromonas* was lowest from 0 to 122 d (3.12 ± 0.81 log CFU/cm^2 and 2.30 ± 0.62 log CFU/mL) and remained consistent between 183 and 671 d (3.77 ± 1.06 log CFU/cm^2 and 2.74 ± 0.91 log CFU/mL). In addition to the age of the system, *Aeromonas* spp. were also affected by collection location (fish tank, BDWC, and EDWC; $p < 0.05$). Water collected from the BDWC contained significantly higher *Aeromonas* spp. (2.96 ± 0.91 log CFU/mL) than EDWC (2.53 ± 0.93 log CFU/mL) and fish tank (2.61 ± 0.75 log CFU/mL). This could be due to changes in nutrient

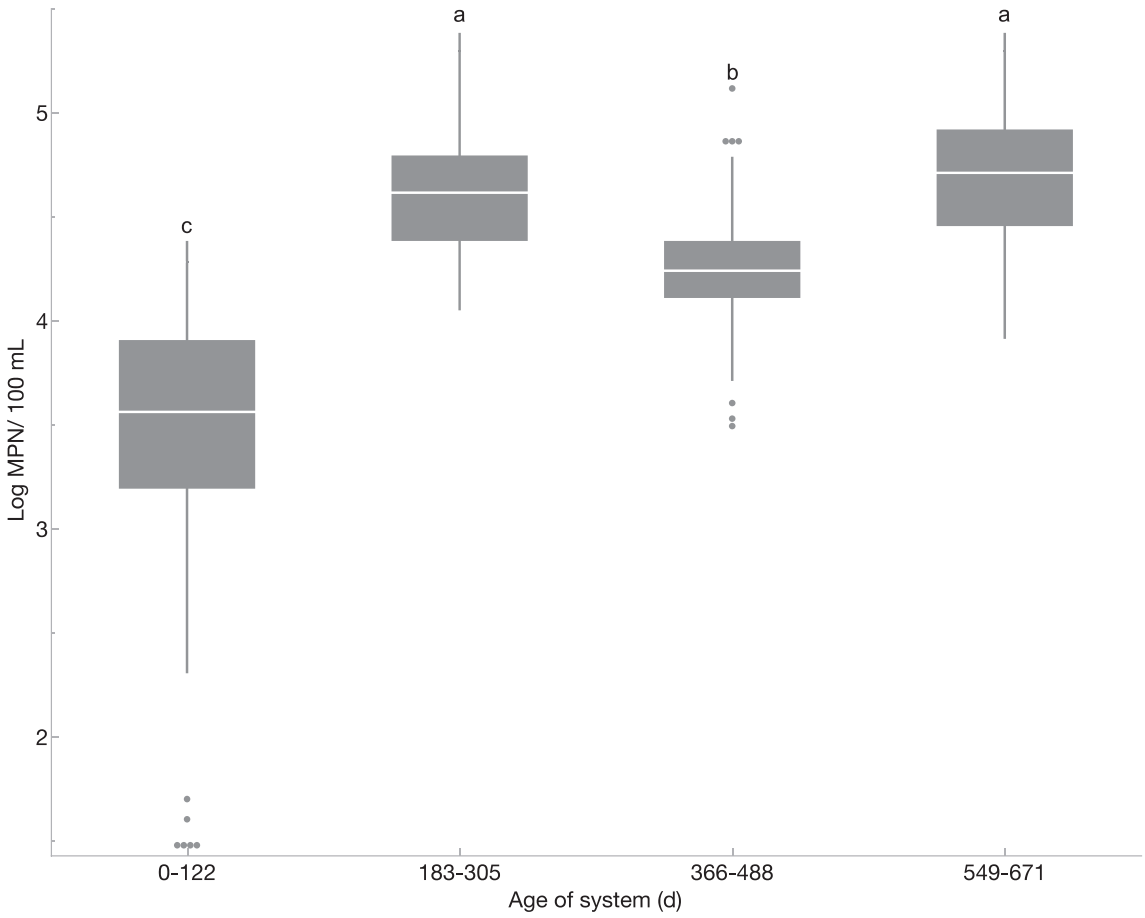

Figure 4. Boxplot of the total coliform count (log MPN/100 mL) in aquaponic water (n = 351) from the fish tank, beginning deep water culture (BDWC), and end deep water culture (EDWC) based upon the age of the system. The boxes represent the interquartile range (25–75 percentile) of the distribution. The middle white bar of each boxplot represents the median, and the whiskers indicate range. Each dot denotes outliers. The different letters are significantly differences ($p < 0.05$), per Tukey's HSD.

Table 3
Distribution of *Aeromonas hydrophila* and *Pseudomonas aeruginosa* according to sample type from a coupled aquaponics system over 2 years

Type of sample (*n*)	No. of positive (%)	
	A. hydrophila	*P. aeruginosa*
Fingerling (39)	16 (41.03)	0 (0.00)
Fish feces (108)	63 (58.33)	2 (1.85)
Lettuce leaf (99)	38 (38.38)	2 (2.02)
Root (99)	82 (82.83)	0 (0.00)
Sponge swab (351)	210 (59.83)	3 (0.85)
Water (351)	214 (60.97)	7 (1.99)
Total (1,047)	623 (59.50)	14 (1.34)

concentrations at each collection point. After the water exits the fish tank, the water flows through a clarifier and a biofiltration system before entering the BDWC to filter out the biosolids (excess fish feed and fish waste) and oxidize ammonia in the water (Fig. 1). Heterotrophic organisms tend to proliferate in this part of the system since there is ample organic matter to break down and use as energy (Joyce et al., 2019). In addition to carbon sources, nitrification occurs in the biofilter, producing high amounts of nitrites and nitrate at this point in the system. *Aeromonas*, in addition to other heterotrophic organisms (Padhi et al., 2013), can reduce nitrites and nitrates through denitrification (Munguia-Fragozo et al., 2015; Taabodi et al., 2020). Fig. 6.Fig. 7..

Aeromonas was quantified in the water from two out of three systems (0.55 ± 0.19 and 1.44 ± 0.28 log CFU/mL) prior to placing the fingerlings, but *A. hydrophila* was not identified in any samples prior to fish placement, including fingerling samples. The system that was *Aeromonas* negative before placing fish into the system, but a fingerling sample contained *Aeromonas* prior to introduction to the system. In the subsequent sampling, *Aeromonas* was identified throughout the system. Therefore, it is possible the fingerlings were a source of *Aeromonas* transferred into the system.

Although *A. hydrophila* was not identified in preproduction samples, it was present in all sample types (59.53%) over the survey period (Table 3). There was an association between the age of the system and the presence of *A. hydrophila* for water samples (X^2 = 23.234, df = 3, $p < 0.001$) and sponge samples (X^2 = 21.352, df = 3, $p < 0.001$). Between 0 and 122 d, *A. hydrophila* was lower in water and sponge samples than between 183 and 671 d ($p < 0.001$). The location where the water and sponge samples were collected (fish tank, BDWC, and EDWC) did not affect the likelihood of *A. hydrophila* presence in the system. The age of the system did not affect the likelihood of *A. hydrophila* contamination in lettuce leaves, roots, fingerlings, and fish feces ($p > 0.05$). *A. hydrophila* was most abundant in roots (82.83%, 82/99), followed by water (60.97%, 214/351), sponge swabs (59.83%, 210/351), and fish feces (58.33%, 63/108).

Over the collection period, 38/99 (38.38%) of the lettuce leaves were positive for *A. hydrophila.* Kasozi et al. (2022), reported a relative abundance of 3.14% from the genus *Aeromonas* on 12 lettuce

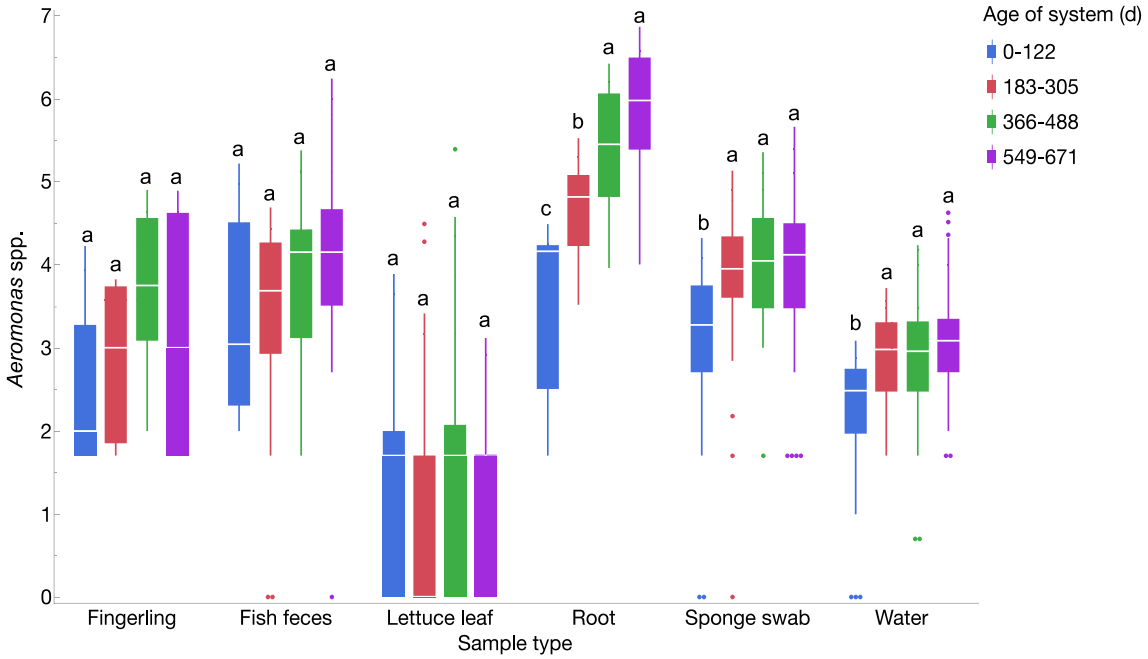

Figure 5. Boxplot of *Aeromonas* spp. (log CFU/g) in a recirculating aquaponics system from fingerling, fish feces, lettuce leaf, root, sponge swab (log CFU/cm^2), and water samples (log CFU/mL; n = 1,047) based upon the age of the system (0–671 d). The different letters, in each sample type, are significantly different ($p < 0.05$), per Tukey's HSD. The colored boxes represent the interquartile range (25–75 percentile) of the distribution. The middle white bar of each boxplot represents the median, and the whiskers indicate range. Each dot denotes outliers.

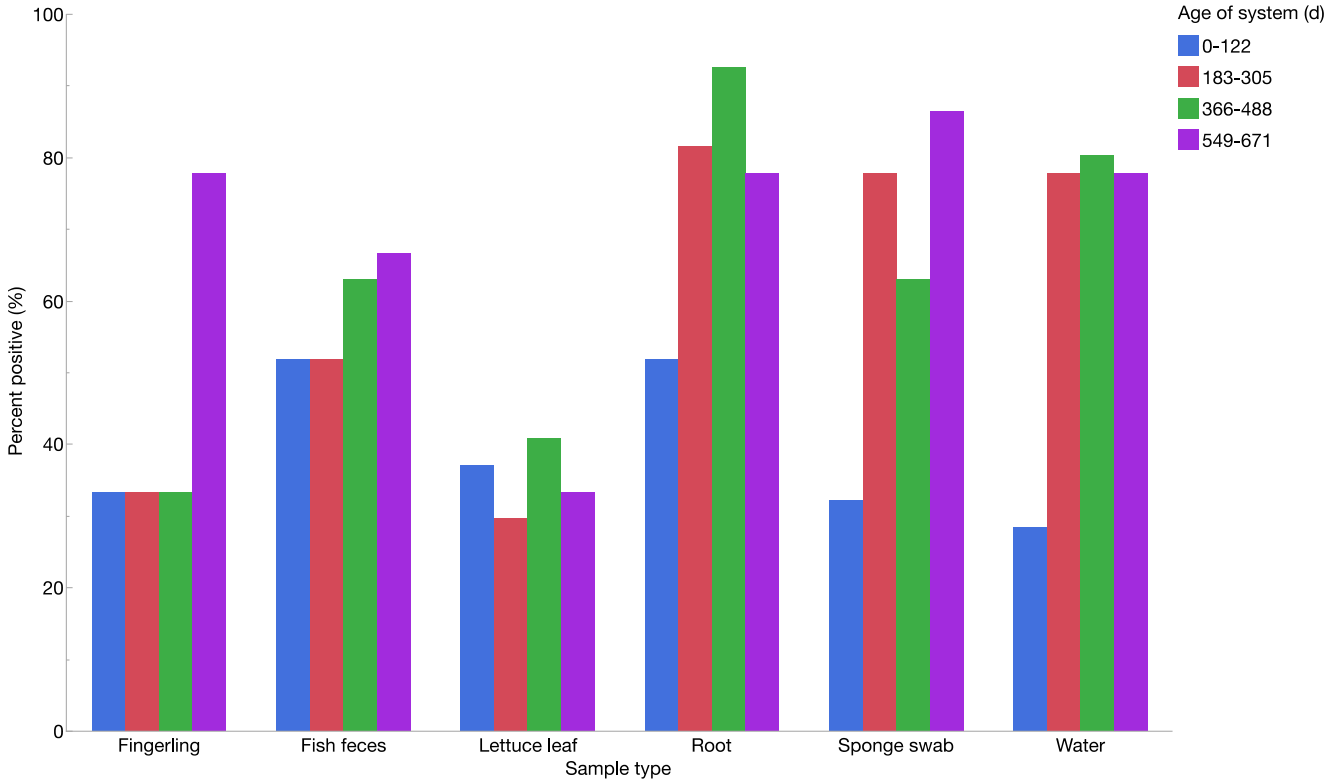

Figure 6. The incidence and distribution of *A. hydrophila* identified in each sample type based on the age of the system (0–671 d).

epiphytes grown in a small-scale experimental aquaponics system. According to the CDC National Outbreak Reporting System, there have not been any foodborne outbreaks associated with *A. hydrophila* from 1970 to 2020 in the U.S., but there were two waterborne outbreaks (1989; 26 individuals and 2013; five individuals) from the consumption of untreated drinking water. It was suspected that *A. hydrophila* was present in the water, but never confirmed (CDC, 2022). Additionally, *A. hydrophila* has been identified in retail

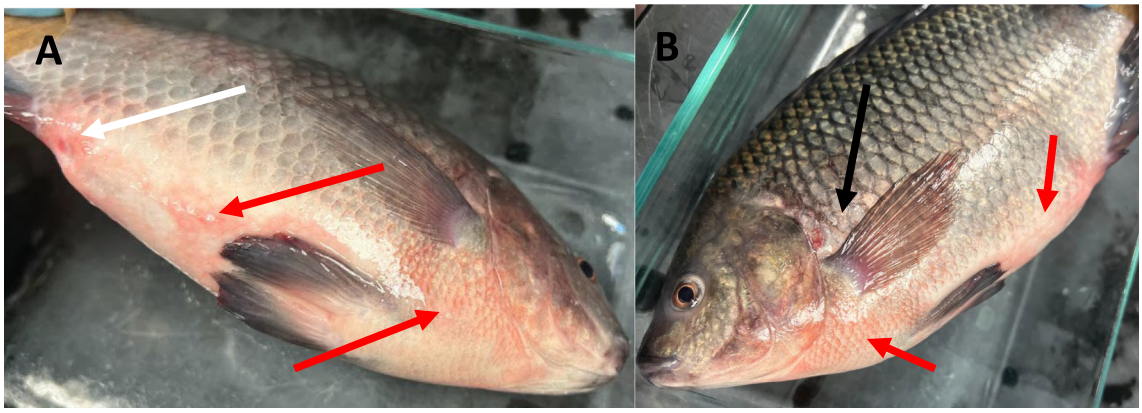

Figure 7. Photograph of Nile tilapia (*Oreochromis niloticus*) with signs of detached scales (black arrow; B) and external hemorrhagic patches on fish and fins (red arrows; A and B) and hemorrhaging around the anus (white arrow; A).

ready-to-eat vegetable salad mixes, although there were no reports of consumers becoming ill (Umutoni et al., 2020; Xanthopoulos et al., 2010). Due to the limited number of outbreaks and understanding of the pathogenicity, *A. hydrophila* is not considered a foodborne pathogen in the United States (FDA, 2020), though it has caused small outbreaks globally (Tsheten et al., 2016; Zhang et al., 2012).

A. hydrophila pathogenicity in fish has been studied more extensively since it can cause high mortality in aquaculture. In our study, before the extraction of fish feces, the exterior of each fish was examined for signs of disease. On day 671, a fish showed signs of hemorrhaging on the underside and around the anus. Additionally, there were early signs of fin rot on the ventral fins and detached scales. It cannot be concluded *A. hydrophila* was the cause for the symptoms, but the feces contained 3.7 log CFU/mL *Aeromonas* spp. and was positive for *A. hydrophila*. *A. hydrophila* was also confirmed in the fish tank water and sponge swab samples. The farm had not reported any prior fish showing similar symptoms.

A. hydrophila is well established as a human pathogen capable of causing wound infections and septicemia in humans, as well as gastroenteritis when consumed by immunosuppressed individuals (Bhunia, 2018; Daskalov, 2006). The pathogen is considered an emerging foodborne pathogen which can cause diarrheal disease in young children, the elderly, and travelers to endemic regions, with some infections resulting in the development of hemolytic uremic syndrome (Bhunia, 2018). *A. hydrophila* has been isolated from water and foods, and cases due to consumption of contaminated fish in Sweden (Krovacek et al., 1995) and Norway (Granum et al., 1998) have been reported. Many strains demonstrate reduced susceptibility to antibiotic therapies, and *Aeromonas* not only can multiply under refrigeration but also produces enterotoxin and hemolysin at these temperatures (Martins et al., 2002). Interest in aquaponic agriculture continues to grow, and marketing for these farms often relies on the perception of the enhanced safety of these foods. This perception of safer, cleaner food may increase the consumption of this produce by immunocompromised individuals who are known to be at an increased risk of infection by *A. hydrophila*. Therefore, as with any fresh produce production system, aquaponic farmers should take measures to reduce the likelihood of produce contamination to ensure the continued safety of the foods they sell.

Understanding the microorganisms endemic to aquaponic systems is critical to successfully conduct comprehensive hazard analyses. Although several foodborne pathogens commonly associated with produce were not detected in this commercial system, it should not be concluded that all such systems are free from these pathogens. Good agricultural practices, regular water quality monitoring, and maintenance of a healthy fish population can help reduce the presence of *A. hydrophila* and foodborne pathogens, thereby reducing the potential contamination risk posed to fresh produce grown in aquaponic systems.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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