Examination and Evaluation of the Persistence of Virulent *Aeromonas hydrophila*, *Edwardsiella ictaluri* and *Flavobacterium covae* Within the Sediments of West Alabama Commercial Catfish Ponds

by

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Abstract

The three most prevalent bacterial pathogens responsible for channel (*Ictalurus punctatus*) and hybrid [♀ channel catfish (*I. punctatus*) × ♂ blue catfish (*I. furcatus*)] catfish losses are virulent *Aeromonas hydrophila* (vAh), *Edwardsiella ictaluri* and *Flavobacterium covae*. Substantial progress has been made regarding management and treatment practices for these bacterial diseases. However, recurring and chronic infections caused by these pathogens continue to cause significant economic losses annually. One aspect of bacterial pathogenesis that has yet to be studied in detail is determining if virulent *A. hydrophila*, *E ictaluri*, and *F. covae* can persist within the bottom sediments of commercial catfish ponds. In a laboratory setting, three separate persistence trials were conducted, each using three 37 L glass aquaria divided into four separate chambers containing sterile sediment from commercial catfish ponds, disinfected water, and known vAh, *E. ictaluri* and *F. covae* inoculum concentrations of 1.64×10^8, 8.33×10^7, and 1.78×10^7 colony forming units (CFU) per mL, respectively. One gram of sediment was extracted on specified sampling days, and serial dilutions of suspended homogenized sediments were plated onto selective media to enumerate bacterial colonies and monitor CFU g^{-1} of bacteria in sediments. After all persistence trials were completed, it was determined that vAh and *E. ictaluri* persisted in this environment, whereas *F. covae* did not survive. Interestingly, vAh and *E ictaluri* populations experience initial growth across all sediments, then plateau after 14- and 5-days post-inoculation, respectively. Future research projects are necessary to determine which environmental factors can influence population changes in these pathogenic bacteria over time and allow them to persist within the sediments of commercial catfish ponds.

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ADA	Ampicillin-dextrin agar
BHI	Brain-heart infusion
BLAST	Basic local alignment search tool
BPC	Bacterial persistence curve
CD	Columnaris disease
CEC	Cation Exchange Capacity
CFU	Colony forming units
CI	Confidence interval
DNA	Deoxyribonucleic acid
EI_{FPT}	*Edwardsiella ictaluri* full persistence trial
EIM	*Edwardsiella ictaluri* medium
ESC	Enteric septicemia of catfish
FC_{FPT}	*Flavobacterium covae* full persistence trial
FPT	Full persistence trial
gDNA	Genomic deoxyribonucleic acid
HGT	Horizontal genetic transfer
MEGA	Molecular evolutionary genetics analysis
MS	Modified Shieh
MST	Modified Shieh with tobramycin
NCBI	National center for biotechnology information
PBS	Phosphate-buffer saline
PCR	Polymerase chain reaction

REML	Restricted maximum likelihood
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SE	Standard error
SS	Smoothing spline
tRNA	Transfer ribonucleic acid
TSB	Tryptic soy broth
USA	United States of America
USD	United States dollar
vAh	Virulent *Aeromonas hydrophila*
VBNC	Viable but not culturable

Literature Review

Aquaculture continues to be a growing and vital sector globally (Pradeepkiran, 2019), as the total production of freshwater and marine aquatic species has increased from 82.5 to 87.5 million tons between 2018 and 2020 (FAO, 2022). However in the United States, outputs from aquatic animal aquaculture operations have slightly declined from 659,000 tons produced in 2010 to 619,200 tons produced in 2020, and annual growth rates of nationwide aquaculture production fluctuated from as low as -2.4 % to as high a 12.7 % within that same time period (Garlock et al., 2020; FAO, 2022). Of all the fish species produced nationwide, the combination of channel catfish (*Ictalurus punctatus*) and hybrid catfish [♀ channel catfish (*I. punctatus*) × ♂ blue catfish (*I. furcatus*)] are the largest contributors to the total United States aquaculture outputs (Cai and Arias 2017), accounting for 51% of total gross sales of finfish food production in 2018 (USDA-NASS, 2019.) Due to issues such as increased production costs, variability in fish prices, and competition from foreign siluriform imports, the recent trend has shown a decrease in overall production area (Hanson et al., 2017). In Mississippi, catfish production area has decreased from 79,557 hectares in 2002 to 13,921 hectares in 2017 (Peterman and Posadas, 2019). West Alabama has displayed a similar trend, with the number of food fish producing farms decreasing from 147 in 2013 to 104 in 2018 (USDA-NASS, 2019), and only 77 farms totaling 7061 surface water hectares by 2020 (Hanson et al., 2020). Economically, national catfish aquaculture sales have been turbulent over the past 34 years, peaking at $501 million during the 2000 production year and dropping down to $380 million in 2012 (USDA-NASS, 2023). Recent sales trends have shown a drastic 20.5 % total sales increase of $371 million to $447 million from 2020 to 2022. (USDA-NASS, 2023). Despite the recent economic rebound, the water surface hetares dedicated to commercial catfish operations has continued to decline

since 2001 (USDA-NASS, 2023), the annual fluctuations in production costs, and irregular sales returns (Hanson et al., 2020) can be detrimental for local catfish farmers. Annual differences in commercial catfish aquaculture yields and sales should be studied to explain and prevent future negative trends in the most important domestic aquaculture industry.

Outside of a reported 7% decrease in water surface acres used from 2020 to 2022 (USDA-NASS, 2023), many more destructive factors explain annual losses in catfish production. In a survey of catfish producers in Alabama, Arkansas, Louisiana, and Mississippi in 2009, losses of food-sized catfish (up to 25.4 cm and 1.36 kg) due to predation, low dissolved oxygen, winter kill (*Saprolegnia fungus*) and other causes were reported on 53.9%, 28.1%, 20.6% and 9.3% of catfish operations, respectively (USDA-NAHMS, 2010). During that same year, losses due to enteric septicemia of catfish (ESC) and columnaris disease (CD) were reported on 36.6 and 39% of catfish farms, respectively. Historically, ESC and CD were the most pressing bacterial disease issues catfish producers had to deal with; however, the statistics representing the other causes revealed something startling. When examining the average losses in catfish per event, 53.2% of farmers reported severe losses due to other causes, meaning more than 900 kg were lost per event, and two-thirds of those reported losses were attributed to the bacteria *Aeromonas* (USDA-NAHMS, 2010). This data indicated the beginning of an epidemic among catfish on farms throughout the southeastern United States.

Hyper virulent strains of *Aeromonas hydrophila* (vAh) were identified as the causative agent from motile *Aeromonas* septicemia (MAS) outbreaks on commercial catfish operations, with the first case reported in Mississippi in 2004 (Hossian et al., 2014), and the first massive outbreak reported in western Alabama in 2009 (Hemstreet, 2010). Severe MAS outbreaks later spread across the southeastern United States, resulting in annual high mortality events and severe

economic losses (Bebak et al., 2011; Pridgeon and Klesius, 2011; Griffen et al., 2013; Hossian et al., 2013; Gresham et al., 2014). For example, in west Alabama, fish losses due to foregone sales, disease treatments, and lost feeding days totaled over $63 million USD between 2015 and 2021 (Abdelrahmen et al., 2023). Nationwide, total economic losses due to MAS, ESC, and CD have been estimated to be $125 million USD annually (Zhou et al., 2018). The emergence of vAh and consistent issues with *Edwardsiella ictaluri* and *Flavobacterium covae* (the causative agents of ESC and CD, respectively) have prompted researchers to analyze as many aspects of the causative pathogenic bacteria as possible.

There are still aspects of bacterial pathogenesis in commercial catfish aquaculture that have yet to be researched, namely how bottom sediment compositions may potentially influence pathogenic bacteria and how bacterial presence impacts the fitness of ponds for catfish aquaculture (Silapajarn et al., 2004). However, some studies have evaluated the ability of biofilms and sediments to harbor bacteria (Cai et al., 2019) and the presence and persistence of *Aeromonas* spp., *Edwardsiella* spp., and *Flavobacterium* spp. in natural (Hazen et al., 1978; Brandi et al., 1996; Kirchman et al., 2002; Madetoja et al., 2002; Olivares-Fuster et al., 2007; Leung et al., 2019) and artificial environments (Hazen et al., 1979; Plumb and Quinlan, 1986; Pridgeon et al., 2014; Leung et al., 2022). One unique aspect of the commercial catfish industry in west Alabama is the renovation frequency of production ponds.

Most ponds in west Alabama are originally constructed as watershed ponds that rely on topwater sources to fill them. After ponds are constructed and stocked, erosion of the embankments naturally occurs over time, and renovation of these earthen ponds is required (Steeby et al., 1998; Hawke and Khoo, 2004; Steeby et al., 2004). The purpose of pond renovation is to make embankments erosion resistant by adequate compaction of dry soils or

sediment on the preexisting embankment (Steeby et al., 1998). Pond embankment erosion results from wave action in larger ponds, water runoff from rainfall, and multi-batch culture of catfish that requires regular seining (Steeby et al., 2004). As the levees erode, sediments accumulate on the pond bottom, decreasing the water depth and increasing the time necessary to seine (Steeby and Lovshin, 1993; Steeby et al., 2004). Pond renovation can be expensive, sometimes as much as one-third of the original pond construction cost. Draining the pond is necessary to properly renovate when sediment erosion drastically impacts production capacity (Steeby et al., 1998; Steeby et al., 2004). The traditional pond renovation method is to drain the pond completely, allow the bottom to dry enough for heavy machinery to enter the pond, and then redistribute and compact the bottom sediment along the levees. However, draining all the water is not feasible in larger ponds filled almost exclusively via water runoff (Steeby et al., 1998). Therefore, west Alabama catfish producers renovate ponds by redistributing and compacting the bottom sediment along the levees or by completely removing the bottom sediment in watershed ponds and bringing in new dirt to rebuild the levees. In speaking with catfish producers, pond renovation can cost an estimated $2023-3240 per ha (A.M. Kelly and L.A. Roy, personal communication 2021).

With long, infrequent periods between renovations and previous studies reporting the ability of pathogenic bacteria to survive under harsh environmental conditions, the bottoms of commercial catfish ponds may have the potential to impact bacteria concentrations in the ponds and influence bacterial persistence. Therefore, the following studies hypothesize that vAh, *F. covae*, and *E. ictaluri* persist in commercial catfish pond sediments.

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Chapter 1

Persistence of a Wild-Type Virulent *Aeromonas hydrophila* Isolate in Pond Sediments from Commercial Catfish Ponds: A Laboratory Study

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1.1 Abstract

Virulent *Aeromonas hydrophila* (vAh) is a major bacterial pathogen in the U.S. catfish industry and is responsible for large-scale losses within commercial ponds. Administering antibiotic feeds can effectively treat vAh infections, but it is imperative to discern new approaches and better understand the mechanics of infection for this bacterium. As such, the persistence of vAh in pond sediments was determined by conducting laboratory trials using sediment from four commercial catfish ponds. Twelve chambers contained sterilized sediment, vAh isolate ML-09-119, and 8 L of water maintained at 28 °C and were aerated daily. At 1, 2, 4, 6, 8 days, and every 7th day post-inoculation for 28 days, 1 g of sediment was removed, and vAh colony forming units (CFU) were enumerated on ampicillin dextrin agar. Viable vAh colonies were present in all sediments at all sampling periods. The vAh growth curve peaked ($1.33 \pm 0.26 \times 10^9$ CFU g^{-1}) at 96 h post-inoculation. The population plateaued between days 14 and 28. No correlations were found between CFU g^{-1} and physicochemical sediment variables. This study validated the ability of vAh to persist within pond sediments in a laboratory setting. Further research on environmental factors influencing vAh survivability and population dynamics in ponds is needed.

1.2 Introduction

Aquaculture is a rapidly expanding agriculture sector, and the production of farm-raised aquatic organisms is essential to a rapidly growing global population. The commercial production of catfish, which includes channel catfish (*Ictalurus punctatus*) and hybrid catfish [♀ channel catfish (*I. punctatus*) × ♂ blue catfish (*I. furcatus*)], exceeds all other finfish species production in the U.S. In 2021, catfish industry sales were nearly $421 million, a 12 percent

increase from the previous year [1]. However, these numbers are negatively affected by fish losses due to diseases.

In 2009, a virulent strain of *Aeromonas hydrophila* (vAh) discovered on catfish farms in Alabama and Mississippi became known as a primary pathogen of motile *Aeromonas* septicemia (MAS) outbreaks in the United States [2]. These outbreaks resulted in high mortalities of farm-raised market-sized catfish and millions of dollars in financial damages [3-7]. *Aeromonas hydrophila* is a gram-negative, facultative, oligotrophic, ubiquitous anaerobe that causes severe hemorrhaging, exophthalmia, and organ failure in numerous species [8-10]. Fish mortalities due to vAh infections can progress rapidly in a pond from a few individuals (5–15%) to the entire pond (up to 100%) in a few days [11], depending on the virulence of the *A. hydrophila* strain [7]. From 2009 to 2021 in Alabama, USA, an estimated 17,064,462 kg of catfish were lost to MAS caused by vAh [12]. From 2015 to 2021, more than 9,500,000 kg of catfish were lost due to vAh, equating to approximately $3.4 million annually in foregone sales [13].

Research on vAh has identified how it enters a fish host and affects specific organ systems and what environmental factors influence pathogenesis [14-15]. For example, *A. hydrophila* is efficient in using siderophores to thrive in iron-limited conditions, and this aspect allows for enhanced virulence, as observed in laboratory settings [16]. In addition, the virulence of attenuated vAh isolates can be reduced by removing certain O-antigens [17], and vAh colonies can produce varying concentrations of proteolytic enzymes, adhesins, and toxins depending on their culture status [18]. Thus, components of biofilm formation and secretion systems are also integral to the virulence of vAh and its ability to evade fish defenses [10].

Most importantly, there have been multiple studies on how vAh can enter and spread across numerous ponds. Many fish-eating aquatic birds prey on alive, dead, or moribund catfish at commercial catfish facilities [19] and serve as vectors for bacterial pathogens, including vAh [20]. Aquatic birds have high vAh recovery rates, and the primary isolation site is the intestines [21]. Multiple studies demonstrated that vAh is still viable when it passes through the digestive tract of predatory birds. Consequently, the bird feces contain substantial concentrations of vAh capable of infecting fish in numerous ponds, causing severe mortalities on farms that can be miles apart [20-22]. Bivalves, aquatic arthropods, and gastropods living in ponds can also harbor vAh, allowing the bacterium to accumulate within aquatic invertebrates [9]. Seining nets can harbor vAh and are most commonly responsible for export to other ponds and farms [2, 23]. These studies have furthered our understanding of the intricacies of vAh, but information on the persistence of this bacterium in pond sediments is lacking.

The bottoms of catfish ponds contain many microorganisms [24] and an accumulation of organic and inorganic materials [25] that may allow pathogenic vAh to persist within this unique environment. The soil, bottom sediments, and biofilms found within catfish production ponds have been known to sequester vAh colonies when water temperatures cool [26]. Notably, Barria et al. [27] reported that cold-response mechanisms are absent in *A. hydrophila*, which would explain the bacterium's ability to enter a viable but not culturable (VBNC) state at colder temperatures. This VBNC state may be responsible for the appearance of multiple distinct strains of *A. hydrophila*, thereby increasing the genetic heterogeneity of the species [28]. Entering a VBNC state would allow a pathogen such as vAh to decrease the rate of cellular processes and then resume normal functions when environmental conditions improve [29]. Bacterial persistence may also explain the phenomenon of vAh causing chronic and recurring MAS

infections [30-35]. Understanding the ability of vAh to persist within commercial catfish pond bottoms, and survive over long periods, will improve our knowledge of this harmful bacterial pathogen. The primary goals of this study were to determine if vAh can persist within pond sediments while simultaneously observing how vAh populations change over time and if any physicochemical components of the sediments were correlated with observed vAh population trends. We hypothesized that the vAh populations would exhibit a typical microbial growth curve and that differences in growth curve values would occur between the four sediment types.

1.3 Methods

1.3.1 Pilot trial

Before initiating the full persistence trial (FPT), a pilot-scale study was conducted to confirm the feasibility of the experimental design and to determine if colonies of vAh could be successfully enumerated from an aqueous environment over time. The pilot and FPT sediment samples, water, bacterial inoculum, and aquaria systems were prepared using the methods described below.

1.3.2 Experimental design and system preparation

Approximately 3–4 kg of top layer sediment was collected from six points within four separate production ponds on two farms in Hale County, Alabama, USA. Two ponds from one of the farms (Farm B) had been recently drained. The third pond had just been completely renovated, and was to be refilled with water shortly after sample collection. From the fourth pond, which was in production at the sampling time, sediment was collected from the embankments 1 m below the water surface. Both the third and fourth ponds were sourced from

Farm A. Sediment samples from each pond were thoroughly mixed to form a single composite sample [36]. Composite samples were then autoclaved at 121 °C, 15 psi, for three 1-h intervals [37-38] using a Market Forge STM-E Sterilmatic Analog Sterilizer (Booth Medical Equipment, Alexander, Arkansas, USA). Once each composite sample was thoroughly autoclaved, each sediment type was quality tested to ensure sterility. The sterilization of soils and seiments can increase the extractability of nitrogen, sulfur, phosphorus, organic matter, and notable metal cations, while sediment ans soil pH, cation exchange capacity (CEC), and surface area typically remain unaffected [38]. Composite samples (1 g each) were vigorously mixed in 15-mL conical tubes (VWR International, Radnor, Pennsylvania, USA) with sterile deionized water, mixed, aseptically plated onto tryptic soy agar, and incubated at 28 °C for 120 h. If no microbial colonies formed, then sediment sterilization was considered successful. If microbial colonies did grow, then the composite sample would be autoclaved for a fourth 1-h interval and re-tested until sterilization was confirmed.

Dechlorinated city water (96 L) was divided among five containers and disinfected using a 5% chlorine bleach solution [39], with a contact time of 18 h. The remaining chlorine was blown off with filtered air for a minimum of 36 h. The containers were then topped off with autoclaved city water containing sodium thiosulfate to neutralize any remaining free chlorine. Water from each container was tested using a Hydrion CH-300 test strip to ensure all chlorine was neutralized and that all microbial activity had ceased. Quality tests on the water were performed by aseptically adding 5 mL of test water to 5 mL of tryptic soy broth (TSB) and incubating at 28 °C for a total of 120 h. If the solution remained translucent, then water solutions were deemed sterile. If the broth appeared cloudy, then the water disinfection process would be repeated and retested until the city water was free of chlorine and microbial activity ceased.

The systems consisted of three 37-L glass aquaria divided into four chambers. The chambers were separated by glass panes held in place with aquarium-safe silicone (Sili-cone 1 All Purpose, General Electric, Waterford, New York, USA). Once the silicone had cured, leak tests on all chambers were conducted to ensure each test chamber was isolated. Before the start of this trial, all tanks were cleaned first with 70% ethanol, followed by 10% Virkon™ S (Antec International, Pittsburgh, Pennsylvania, USA), and 70% ethanol for a second time. Therefore, these systems would only contain the prepared sediment, water, and vAh culture. Once the aquaria chambers had sediment, vAh, and water, they were covered in two layers of plastic wrap (GLAD® Cling'n Seal, Oakland, California, USA) and one layer of styrofoam insulation board (DOW®, Midland, Michigan, USA). This was done to limit potential airborne contaminants from entering the system and better maintain temperatures within the chambers. The preparations for the sediments, water, and aquaria systems were not intended to maintain sterility indefinitely but to create an environment in which the bacterial pathogen of interest would be able to propagate initially without competition from other background microorganisms or external factors. The aquaria were kept in a room with an average temperature of 28.0 ± 0.5 °C maintained throughout the trial.

1.3.3 Bacterial culture and trial preparation

The bacterial culture and inoculum were prepared following the procedure described by Brandi et al. (1996). The wild-type *A. hydrophila* ML-09-119 was isolated from infected catfish during a MAS pond outbreak in west Alabama [40]. ML-09-119 colonies were revived from cryostock by plating on tryptic soy agar and incubated at 28 °C for a minimum of 24 h. Next, a pure colony of vAh was picked and placed in 1 L of TSB and incubated at 28 °C and 115

revolutions per minute for a minimum of 24 h. Next, the bacterial broth culture was centrifuged at 4000 *x g* for 10 min in a 5810 R benchtop centrifuge (Eppendorf North America Inc., Enfield, Connecticut, USA), washed in cold 1X phosphate-buffered saline solution (PBS) adjusted pH of 7.4. Bacterial cells were resuspended and adjusted to an optical density of 0.200 ± 0.005 at 550 nm using an Eppendorf Biospectrometer® Basic (Eppendorf North America). The resulting inoculum had an average concentration of 1.64×10^8 colony forming units (CFU) per mL. A randomized block design was used to assign chambers to the sediment types. In each chamber, 20 mL of bacteria inoculum was added to 200 g of sterilized sediment and 500 mL of disinfected dechlorinated city water. The amalgam was vigorously mixed with a sterile stainless-steel spatula for 1 min durations every 5 mins for 1 h. This would ensure adequate contact time, be conducive to keeping the bacteria primarily in the sediment, and provide bacteria with a nutrient-rich substrate to vivify and reasonably maintain the population. After the 1 h mixing period, water volume within each chamber was increased to a total of 8 L. To simulate the mechanical aeration that takes place within a production pond, a 3.5 cm × 1 cm × 1 cm cuboid Pawfly air stone (at a fixed location within each chamber) would expel air supplied via a Whitewater Silent Air Pump™ v201 (Pentair Aquatic Eco-Systems™, Apopka, Florida, USA) for 12 h beginning at 1800 h and stopping at 0600 h the following morning. Sediments were left untouched until the first sampling.

1.3.4. Sampling and bacterial enumeration

Sediment in each chamber was collected and bacterial populations were evaluated, with sampling times as follows: 24 h post-inoculation (designated as day 0), 48 h post (day 1), 4 d post (day 3), 6 d post (day 5) and 8 d post (day 7), then every seven days following the fifth

sampling. Methods described by Cai et al. (26) were used to extract sediment and enumerate live colonies of ML-09-119 for each sample. Approximately 1 g of sediment was collected from each chamber using a sterile 10-mL serological pipette, placed in a sterile 15-mL centrifuge tube, and centrifuged for 10 min at 667 *x g*. Liquid supernatant was removed and the remaining sediment pellet (~1 g) was resuspended entirely in 0.1X PBS, creating a 1:10 mixture, and vortexed until the pellet was homogenized. Next, 250 µL of homogenized sediment solution was placed into six wells of the leftmost column of a 96-well plate and serially diluted (10-fold) as described by Chen et al. (2003). Four serial dilutions of six 10 µL replicates were each plated onto ampicillin dextrin agar (ADA) for *Aeromonas* spp. selectivity [42]. Plates were dried and then placed in an incubator at 28 °C. The plates required 16 h of incubation at this temperature, and final counts were recorded utilizing the necessary correction factors to determine CFU g^{-1} of sediment accurately. On each sampling day, viable colonies of vAh were picked and either cryopreserved in a 50% glycerol stock at -80 °C for a separate study or had genomic DNA (gDNA) extracted for PCR confirmation. Any bacteria not confirmed to be vAh were designated as "unknown" and labeled as such, followed by their respective chamber name and sampling day.

1.3.5 Pilot and full persistence trial differences

For the pilot trial, only one glass aquarium, divided into four separate chambers, was used. Once sediment samples were inoculated and water volume was increased to the final 8 L per chamber, the system was kept in a room with an average temperature of 21.0 ± 1.2 °C. During the extraction and enumeration process, serial dilutions of sediment samples were plated on ADA via the spread plate method [43] and incubated at 28 °C for 24 h. Two ADA plates were used for each of the four targeted serial dilutions.

1.3.6 DNA extraction and PCR confirmation

Once ML-09-119 colonies formed on selective agar, the viable isolates were confirmed via polymerase chain reaction (PCR). Bacterial colonies were streaked for isolation, and gDNA from all isolated bacterial colonies was extracted using the EZNA® Bacterial DNA Kit (Omega Bio-tek Inc., Norcross, Georgia, USA). Concentration and purity of gDNA were measured using a NanoDrop™ OneC spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The PCR and thermocycling parameters for vAh typing were conducted using methods described by Rasmussen-Ivey et al. (2016). A 25 μL PCR reaction was constructed using 12.5 μL of Hot-Start Taq Master Mix 2X (Amresco LLC, Solon, Ohio, USA), 0.5 μL of ML-09-119F and ML-09-119R primers (initial 10 μM stock solution), and 75 ng of template gDNA. Thermal cycling runs were conducted using an Eppendorf Mastercycler® X50s (Eppendorf North America) with an initial denaturation of 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Positive and negative controls were run in a thermal cycler with test isolates. Then, 5 μL of PCR product was visualized on a 2.0% agarose gel, stained with GelRed (Biotium Inc., Fremont, California, USA), in a 1.0X Tris-acetate-EDTA running buffer using electrophoresis. PCR product bands were visualized via ultraviolet transillumination using a Gel-Doc-Go imaging system (BioRad Inc., Hercules, California, USA). To accurately identify unknown bacterial colonies, PCR products of four unknown isolates and primers 63F and 1387R [44] were sent to Eurofins Genomics LLC, for genetic sequencing of the 16S rRNA gene. After nucleotide base-pair results were trimmed and aligned in the Molecular Evolutionary Genetics Analysis (MEGA) software version 11 [45], base-pair sequences were inputted in the National Center for

Biotechnological Information (NCBI) Basic Local Alignment Search Tool (BLAST) database [46].

1.3.7 Sediment and water chemical analysis

Sediment and water parameters from the chambers were measured to be used in later correlation analyses in conjunction with potential trends in CFU g^{-1}. After composite samples were autoclaved, a portion of each sediment type was sent to the Soil Forage and Water Testing Laboratory (Auburn, Alabama, USA) for alkalinity, organic matter, and Mehlich 1 extractable micronutrient concentrations. Alkalinity was measured in mg L^{-1} as the equivalent percentage of calcium carbonate (% $CaCO_3$ ppm). Total organic matter in mg L^{-1} was determined via loss of ignition. Calcium, potassium, magnesium, phosphorus, copper, iron, manganese, zinc, boron, sodium, and aluminum concentrations in mg L^{-1} were measured via inductively coupled argon plasma spectroscopy. Sediment pH was measured using a SensION+ PH3® pH and ORP meter equipped with a 5021T electrode (HACH, Loveland, Colorado, USA). Cation exchange capacity (CEC) was determined using the Visual MINTEQ 3.1 software [47]. To calculate CEC, values of cation concentrations of each sediment type and measured pH were used as inputs to determine the sum of exchangeable cations each sample can adsorb at their respective pH [48]. All CEC values were reported as milliequivalents per 100 g (meq 100 g^{-1}) sample.

From each test chamber, 10 mL water samples were collected on days 0, 7, 14, 21, and 28. Alkalinity, hardness, total ammonia-nitrogen, nitrite, nitrate, and phosphorus concentrations (mg L^{-1}) were measured using a DR3900 visible spectrophotometer (HACH), and pH as previously described. Water quality parameters were measured to assess any potential effects of water on bacterial colonies.

1.3.8 Statistical analyses

Variances in sediment physicochemical parameters between farms were assessed using a *t*-test. We compared vAh populations (log_{10} CFU g^{-1}) changes over time among four sediment types using a two-way repeated measures analysis of variance test, with sediment type used as a random blocking factor. Differences in overall log_{10} CFU g^{-1} between farms were determined using a paired *t*-test. If there were significant differences, post-hoc analyses were performed using Tukey's Studentized Range — HSD. To test correlations between sediment parameters and vAh population (log_{10} CFU g^{-1}), data from each sediment variable were analyzed for normality. When bivariate normality was verified, data were analyzed through a Pearson correlation. Results not following this assumption were analyzed through a Spearman's rank correlation. All multiple testing *P*-values for correlation analyses have been adjusted to control the false discovery rate using the Benjamini-Hochberg procedure [49]. The Shapiro–Wilk test was utilized for normality analysis of the variables. Statistical significance was set at $P<.05$.

For each farm and the overall study, a bacterial persistence curve (BPC) was created by fitting a smoothing spline (SS) model to vAh population data (log_{10} CFU g^{-1}; y-axis) at sampling days (x-axis) as previously described by Hussain et al. [50]. To ensure the com-promise between the smoothness of the function and the lack of fit, the selection of the smoothing parameter (λ) was based on the restricted maximum likelihood (REML) method [51]. The fitted SS models were used to predict the vAh population using an x-axis scale from 0–28 d with an interval of 0.001. For each BPC, 95% confidence intervals (95% CI) of predicted vAh population curves were created via bootstrapping [52] implemented in the boot package (version 1.3-28) [53]. Data were resampled with replacement 1,000 times, with the SS model re-fitted to these data each

time. The 95% CIs was deter-mined from the 2.5th and 97.5th percentiles. For BPC estimates, we considered descriptors to differ significantly between farms if their 95% CIs did not overlap. The G*Power 3.1.9.4 was used for sample size calculations [54]. All BPC analyses were performed using R soft-ware (version 4.1.1) [55]. All other statistical analyses were performed with SAS® version 9.4 [56]. All figures were plotted using SigmaPlot version 14.5 (Systat Software Inc., San Jose, California, USA). All data were presented as the mean ± standard error of the mean (SE).

1.4 Results

1.4.1 Pilot trial

The duration of the pilot trial encompassed a total of 113 days, with colonies of vAh present from day 0 to the final sampling day. Across all four sediment types, there is a significant relationship between the population (CFU g^{-1}) of vAh and time (Figure 1.1). After data were log-transformed, there was no difference in population among sampling days from day 58 to day 113. All pairwise comparisons among sampling days from day 58 to day 113 were not statistically different ($P > 0.05$). The population of vAh increased during the first seven days, followed by a moderate decline, then a plateauing event. Along with colonies of vAh being produced, colonies of unknown bacteria began appearing on the selective ADA 13 days post-inoculation. These novel bacterial colonies were phenotypically and structurally different from the ML-09-119 colonies, which had previously been solitary on the selective media (Figure 1.2). After following the DNA isolation and thermal cycling procedures described above, PCR product banding displayed distinct differences between the presumed ML-09-119 isolates and these new unknown bacterial colonies (Figure 1.3).

The NCBI BLAST database indicated that the four unknown bacterial isolates were revealed to be *Pseudomonas tohonis, P. alcaligenes, P. taiwanensis,* and *Pseudomonas* spp., with percent identifications of 98.34, 98.97, 97.77, and 99.52, respectively. The results of the pilot persistence trial validated the experimental design as a method for enumerating vAh from aquatic sediments. For the FPT, water parameters and specific sediment physicochemical properties were measured and used as environmental descriptors and components for later correlation analyses.

1.4.2. Full Persistence Trial

All representative vAh colonies counted, from all sampling days when such colonies were present, had their respective DNA extracted and were confirmed via PCR methods. In one of the sediment types from farm A, the unknown bacterial colonies began to appear on the selective ADA media 48 h post-inoculation. By the third sampling day (96 h post-inoculation), unknown colonies were present in all test chambers. The exact identities of most unknown isolates remain unconfirmed at this time. However, they are most likely *Pseudomonas* spp. or a closely related bacterial species based on the previous 16S rRNA sequencing results of the four unknown colonies.

Across all 12 test chambers, populations of vAh initially experienced a rapid increase, followed by a moderate decline and plateauing pattern (Figure 1.4). Collectively from chambers containing samples from farm A and farm B, population numbers on day seven were higher than those on day zero (t_{71} = 7.54, P <.0001). On day 14, vAh populations decreased and were not different from the population on day zero (t_{72} = 0.99, P = 0.9739). On days 21 and 28 post-inoculation, average vAh populations were not different (t_{71} = 2.39, P = 0.2622).

When comparing the sediment types from the two farms, the number of colonies of vAh in farm B sediment was higher than the vAh colonies in farm A sediments (Figure 1.5). On day 14, one of the sediment types from farm A had data points missing due to a much more drastic reduction in the vAh population in that specific sediment than was anticipated ($< 10^6$ CFU g^{-1}). To avoid any further instances of missing data, serial dilutions from days 21 and 28 were decreased by a power of 10. When visualizing the raw values of CFU g^{-1} compared to the $\log_{10}$ transformed CFU g^{-1} values on the smoothing splines encompassed by 95% CIs (Figure 1.6), there is a difference in breadths at 90% of peak raw CFU g^{-1} values between farm A and farm B sediments (Table 1.1). Once data were transformed, there were no significant differences in any population peaks, 90% breadths, or 80% breadths.

Water quality parameters did not noticeably fluctuate throughout the FPT (Table 1.2). After each composite sediment sample was autoclaved, there were no physical and chemical differences in sediment parameters between the sterilized and non-sterilized samples (Data not shown). The water in test chambers containing farm A sediments was not significantly different from that in test chambers containing farm B sediments. There was no difference in CEC and calcium (Ca^{2+}) concentrations between farm A and farm B sediments (Figure 1.7). The boron concentration (B) in all sediment types was below 0.1 mg L^{-1}. In addition to CEC and Ca^{2+} concentrations, there were no differences in any physio-chemical sediment parameters between farm A and farm B sediments (Table 1.3). Due to the small sample size of sediment measurements (2 ponds per farm, two farms), a power analysis was conducted to determine the number of values (*n*) per physicochemical parameter required to reveal statistically significant differences between farms (Table 1.3). The small sample size of sediment chemical and physical properties also resulted in all measured sediment parameters exhibiting no correlation to CFU g^{-1}

of vAh present (Table 1.4). The power analysis revealed the sample size required to determine the statistically significant correlations between vAh populations and each of the sediment physicochemical parameters (Table 1.4).

1.5 Discussion

Persistence trials carried out in this study demonstrated that vAh can survive in a submerged sediment environment in a laboratory setting. The population curve formed in the pilot and FPT followed similar trajectories with an initial rapid growth phase, followed by a steady population decline, concluding in the population of vAh seemingly plateauing within the sediments. Interestingly, the observed population trajectories in CFU g^{-1} be-tween the two vAh trials are worth noting, considering there were only four sediment chambers sampled in the pilot trial and 12 total sediment chambers sampled in the FPT. The final $\log_{10}$ population average on day 113 of the pilot trial was approximately 5.3 CFU g^{-1}. In the FPT, vAh populations in farms A and B sediments reached average $\log_{10}$ values of 5.2 CFU g^{-1} and 6.4 CFU g^{-1}, respectively, after 28 days. These trends in bacterial growth curves are consistent with the findings of other studies on the effect of temperature on the growth dynamics of *A. hydrophila* isolates. While the optimum growth temperature of *Aeromonas* spp. is 20–35 °C, certain strains of *A. hydrophila* can experience positive growth rates from as cold as 0 °C to as warm as 55 °C [57]. Park and Ha [58] also reported that *A. hydrophila* is psychrotrophic due to its ability to continue population increases on squid (*Sepioteuthis sepioidea*) even at 5 °C. Similarly, evaluating the growth rates of *A. hydrophila* on raw tuna (*Thunnus orientalis*), Kim et al. [59] reported an increase in CFU of the target bacterial species over a 168 hour period between 8–15 °C. Storage temperatures are critical environmental factors when developing predictive bacterial growth models and reporting

the effects of temperature on specific growth rates of *A. hydrophila* [57-59]. Future persistence trials at temperatures other than 28 °C and 21 °C would need to be conducted to determine if the population trends of vAh observed in this study are similar or if they die off. Additionally, studies examining cyclical water temperature regimes would more readily reflect natural water temperature fluctuations in commercial aquaculture ponds. Other environmental factors that did not correlate with bacterial growth in this study should also be considered for future studies. For example, adding one physicochemical component of sediment may reveal correlations under controlled conditions where sediment is absent. On the other hand, adding two or more of the physicochemical components of sediment may demonstrate synergism, antagonism, or no effect. Replicating these trials in a commercial pond would be unrealistic.

In this study, the wild-type vAh isolate ML-09-119 was selected primarily due to the extensive research already conducted on this strain. Still, numerous other strains of vAh have been documented after the first pathotype was identified multiple decades ago [60]. Isolates originating from Alabama, Mississippi, and Chinese provinces share a common ancestor [6]; however, there is a higher degree of genetic heterogeneity among Mississippi vAh isolates, and there are distinct subclades among USA and Chinese strains [10]. Due to the strain diversity of vAh, isolates from different geographical regions may exhibit different persistence behaviors.

During the pilot and FPT, once the unknown bacterial colonies began appearing on the ampicillin agar plates, they were detected in all chambers until the trials were concluded. Population trends of the background, putative *Pseudomonas* spp. during the pilot and FPT were not within the scope of this study; however, these bacterial species did not ultimately outcompete the vAh population. The versatility of vAh to withstand environ-mental and ecological

difficulties could explain this. A diverse array of bacteria can persist in the soils or sediments over long periods [37]. In addition, there are adaptations specific to *A. hydrophila* that improve persistence and survivability in harsh aquatic environments. The adaptations include alternative sigma factors, two-component regulatory systems, chaperones, DNA-damage repair pathways, acid resistance systems, and starvation and antibiotic response mechanisms [28, 61-62]. Additionally, *A. hydrophila* can also metabolize a wide variety of carbohydrates [63], specifically chitin, a major component in the aquatic ecosystem [64-66]. Through experimental trials, Zhang et al. [66] reported that vAh isolate ML-10-51K could rapidly proliferate using colloidal chitin and chitin flakes as a sole carbon source at the same rate as if it were supplied glucose. Additionally, virulence factors ex-pressed by establishing biofilm colonies of vAh [18] may allow for more specific niche partitioning [67] between vAh and closely related bacteria like *Pseudomonas* and non-virulent *Aeromonas* spp.

All populations of microorganisms cultured in a closed or batch system exhibit a consistently shaped growth curve consisting of a lag phase, exponential or logarithmic growth phase, stationary phase, and finally, a death phase [29]. However, in any system, complete cell death of a bacterial population is not likely to occur within a short period, as microbial populations exhibit dynamic patterns of ecological succession when environ-mental changes occur [29, 68-69]. In comprehensive studies, researchers noted that successional trends in bacterial populations are dynamic and challenging to predict. However, the taxonomic and functional bacterial community diversities are highest in the initial years of development and then gradually decrease as an ecosystem becomes more developed [68-69]. Understanding ecological interactions of bacteria, such as interspecific and intraspecific competition, and

succession between bacterial communities in commercial catfish pond bottoms would improve the understanding of the mechanisms influencing vAh populations.

This study failed to identify which environmental factors affected the duration of growth, decline, and plateau periods of vAh. The power analysis conducted estimated a total of between 269 and 7,023 samples would be required for testing to determine differences at a high-power level. Unfortunately, that sample number is cost-prohibitive. Although the correlation analyses were weak in this study, increasing sample sizes could reduce variation and perhaps establish better correlative values. Soil microbiome populations are influenced and controlled by multiple abiotic and environmental factors, and these interrelating abiotic factors complicate analyses of specific influences on individual microbial species [70]. Few studies have analyzed relationships between bacteria and specific soil chemical properties in *Aeromonas* spp.

For example, *A. veronii* can volatilize selenium (Se^{4+}) and produce hazardous chemical products such as dimethyl disulfide, methyl selenol, dimethyl selenosulfide, and dimethyl diselenide; however, the rate of volatilization is dependent on pH and salinity of the environment [71]. Awan et al. [28] noted environmental factors, including temperature, pH, surface hydrophobicity, magnesium transport, flagella expression, chemotaxis nutrient limitation, oxygen deprivation, and quorum sensing (QS) could influence the attachment and establishment of colony-forming biofilms of *A. hydrophila*. Isolates of *A. sobria* and another *Aeromonas* spp. collected from mining site soil in Nigeria displayed strong tolerance to lead (Pb^{+}), cadmium (Cd^{2+}), copper (Cu^{2+}), and chromium (Cr^{3+}) at concentrations ≥ 6 mg L^{-1} [72]. Cai et al. [26] noted that aeromonad populations positively correlate with temperature, nitrogen concentration, organic carbon load, and primary productivity. Further research is necessary to determine the

relationships between the physicochemical parameters of catfish pond bottoms and pathogenic bacteria such as vAh.

1.6 Conclusion

Virulent *A. hydrophila* can persist within pond sediments of commercial catfish ponds. This ability to survive within pond sediments may allow vAh populations the opportunity for horizontal genetic transfer [HGT; 73], which can result in more virulent and robust strains of vAh. In addition, with vAh populations persisting in the sediments over long periods, these long-lasting bacterial populations may be more capable of developing antimicrobial resistance via HGT [74]. Therefore, future research projects focusing on understanding the mechanisms and virulence factors that enable vAh to persist are paramount. In addition, further research is needed to determine which sediment physicochemical parameters influence vAh persistence and if other bacterial strains present in pond bottom sediment can outcompete vAh over time.

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Tables

Table 1.1. Mean, standard error (*SE*), and 95% confidence intervals (C.I.) for descriptors of smoothing spline models presented in Figure 1.5. Statistical differences in endpoints between farm sediment are denoted with an asterisk (*).

	Curve descriptor	Overall		Farm A		Farm B	
		Mean ± *SE*	95% C.I.	Mean ± *SE*	95% C.I.	Mean ± *SE*	95% C.I.
CFU g^{-1}	Peak CFU g^{-1} (× 10^{6})	1,329.8 ± 260.9	818.4–1,841.1	449.6 ± 222.0	14.5–884.7	2,046.6 ± 1,266.6	-435.9–4,529.1
	Time (day) at peak CFU g^{-1}	3.16 ± 0.19	2.78–3.53	5.58 ± 1.92	1.82–9.34	3.15 ± 0.79	1.61–4.69
	Time (day) at 90% of peak CFU g^{-1} – lower	2.64 ± 0.12	2.41–2.88	3.52 ± 1.43	0.72–6.32	2.66 ± 0.79	1.10–4.21
	Time (day) at 90% of Peak CFU g^{-1} – upper	3.73 ± 0.32	3.11–4.35	7.97 ± 2.45	3.16–12.77	3.69 ± 0.77	2.18–5.20
	Breadth at 90% of peak CFU g^{-1}*	1.09 ± 0.24	0.61–1.56	4.45 ± 1.47	1.55–7.34	1.03 ± 0.04	0.95–1.11
	Time (day) at 80% of peak CFU g^{-1} – lower	2.41 ± 0.10	2.21–2.60	2.82 ± 1.25	0.37–5.27	2.43 ± 0.79	0.88–3.99
	Time (day) at 80% of peak CFU g^{-1} – upper	4.00 ± 0.47	3.09–4.92	9.07 ± 2.70	3.77–14.36	3.94 ± 0.76	2.45–5.44
	Breadth at 80% of peak CFU g^{-1}*	1.60 ± 0.44	0.74–2.45	6.25 ± 2.06	2.22–10.28	1.51 ± 0.06	1.40–1.62
	Time (day) at 5% of peak CFU g^{-1} – min	0.90 ± 0.19	0.52–1.27	0.00 ± 0.32	-0.62–0.62	1.04 ± 0.71	-0.36–2.43
	Time (day) at 5% of peak CFU g^{-1} – max	12.89 ± 1.89	9.17–16.60	17.32 ± 4.46	8.57–26.07	11.22 ± 1.95	7.39–15.05
	Range at 5% of peak CFU g^{-1}	11.99 ± 1.89	8.28–15.70	17.32 ± 4.59	8.31–26.32	10.18 ± 1.88	6.50–13.86
Log$_{10}$ CFU g^{-1}	Peak log$_{10}$ CFU g^{-1}	8.90 ± 0.12	8.67–9.13	8.62 ± 0.12	8.38–8.85	9.18 ± 0.38	8.43–9.93
	Time (day) at peak log$_{10}$ CFU g^{-1}	3.80 ± 0.23	3.35–4.25	4.59 ± 0.69	3.24–5.95	3.53 ± 0.79	1.98–5.07
	Time (day) at 90% of peak log$_{10}$ CFU g^{-1} – lower	1.71 ± 0.16	1.40–2.01	1.94 ± 0.17	1.61–2.26	1.54 ± 0.84	-0.11–3.19
	Time (day) at 90% of peak log$_{10}$ CFU g^{-1} – upper	7.79 ± 0.78	6.26–9.31	9.28 ± 1.30	6.73–11.83	6.63 ± 0.84	4.98–8.28
	Breadth at 90% of peak log$_{10}$ CFU g^{-1}	6.08 ± 0.84	4.42–7.73	7.34 ± 1.27	4.85–9.84	5.09 ± 0.61	3.88–6.29
	Time (day) at 80% of peak log$_{10}$ CFU g^{-1} – lower	0.54 ± 0.26	0.03–1.04	0.74 ± 0.26	0.23–1.26	0.26 ± 0.88	-1.47–1.98
	Time (day) at 80% of peak log$_{10}$ CFU g^{-1} – upper	11.75 ± 1.61	8.59–14.91	12.68 ± 4.09	4.66–20.70	10.23 ± 1.85	6.61–13.85
	Breadth at 80% of peak log$_{10}$ CFU g^{-1}	11.22 ± 1.71	7.87–14.56	11.94 ± 4.10	3.89–19.98	9.97 ± 1.83	6.38–13.57

Table 1.2. Water quality parameters [mean, standard error (*SE*), minimum measurement (min), and maximum measurement (max)] measured in 12 study tanks containing sediment samples collected from two farms (2 ponds per farm; 3 replicate tanks per pond) for 28 d.

Water quality parameter	**Overall**		**Farm A**		**Farm B**	
	mean ± *SE*	min–max	mean ± *SE*	min–max	mean ± *SE*	min–max
Total alkalinity (ppm)	120.27 ± 3.53	87–206	111.97 ± 3.65	87–167	128.57 ± 5.71	90–206
Total hardness (ppm)	118.93 ± 4.26	67–199	115.60 ± 5.25	67–190	122.27 ± 6.75	73–199
pH	7.59 ± 0.02	7.3–7.9	7.59 ± 0.03	7.4–7.9	7.59 ± 0.03	7.3–7.9
Phosphate (ppm)	1.18 ± 0.15	0.0–4.0	1.50 ± 0.25	0.0–4.0	0.85 ± 0.15	0.0–2.8
Total ammonia nitrogen (ppm)	0.60 ± 0.10	0.0–3.7	0.33 ± 0.05	0.0–1.3	0.88 ± 0.19	0.0–3.7
Nitrite (ppm)	0.07 ± 0.02	0.0–1.0	0.04 ± 0.02	0.0–0.5	0.11 ± 0.04	0.0–1.0
Nitrate (ppm)	0.25 ± 0.06	0.0–1.0	0.20 ± 0.07	0.0–1.0	0.30 ± 0.09	0.0–1.0

Table 1.3. Sediment chemistry parameters [mean, standard error (*SE*), minimum measurement (min), and maximum measurement (max)] measured in sediment samples collected from two farms (2 ponds per farm), test statistics (*t*) and *P*-values of statistical comparison between farms, and the sample size (*n*) required to reveal statistically significant differences between farms.

Sediment parameter	Overall		Farm A		Farm B		Farm A versus Farm B		
	mean ± *SE*	min–max	mean ± *SE*	min–max	mean ± *SE*	min–max	$t_{(2)}$	*P*-value	*n*/farm
Alkalinity (% $CaCO_3$ Equivalence)	13.23 ± 9.36	0.70–41.00	20.85 ± 20.15	0.70–41.00	5.60 ± 1.70	3.90–7.30	0.75	.5295	29
Aluminum (ppm)	246.80 ± 141.25	8.20–648.00	328.10 ± 319.90	8.20–648.00	165.50 ± 64.50	101.00–230.00	0.50	.6677	65
Calcium (× 1,000 ppm)	12.49 ± 2.50	5.32–16.91	9.67 ± 4.36	5.32–14.03	15.31 ± 1.60	13.71–16.91	1.21	.3486	12
CEC (meq/100 g)	0.65 ± 0.13	0.29–0.86	0.50 ± 0.22	0.29–0.72	0.79 ± 0.08	0.71–0.86	1.25	.3363	12
Copper (ppm)	5.90 ± 4.71	0.60–20.00	0.90 ± 0.30	0.60–1.20	10.90 ± 9.10	1.80–20.00	1.10	.4699	15
Iron (ppm)	78.00 ± 36.40	32.00–186.00	111.50 ± 74.50	37.00–186.00	44.50 ± 12.50	32.00–57.00	0.89	.4687	21
Magnesium (ppm)	75.00 ± 24.82	30.00–143.00	54.50 ± 24.50	30.00–79.00	95.50 ± 47.50	48.00–143.00	0.77	.5232	28
Manganese (ppm)	67.25 ± 33.30	10.00–149.00	13.00 ± 3.00	10.00–16.00	121.50 ± 27.50	94.00–149.00	3.92	.0593	3
Organic Matter (%)	3.03 ± 0.99	1.20–5.70	1.55 ± 0.35	1.20–1.90	4.50 ± 1.20	3.30–5.70	2.36	.1422	5
pH	7.03 ± 0.69	5.03–7.94	6.13 ± 1.10	5.03–7.23	7.93 ± 0.01	7.92–7.94	1.64	.3492	7
Phosphorus (ppm)	6.98 ± 5.72	0.10–24.00	0.25 ± 0.15	0.10–0.40	13.70 ± 10.30	3.40–24.00	1.31	.4160	11
Potassium (ppm)	157.25 ± 64.12	50.00–343.00	89.50 ± 39.50	50.00–129.00	225.00 ± 118.00	107.00–343.00	1.09	.3899	15
Sodium (ppm)	189.50 ± 22.63	151.00–250.00	174.50 ± 23.50	151.00–198.00	204.50 ± 45.50	159.00–250.00	0.59	.6173	47
Zinc (ppm)	2.85 ± 1.61	0.70–7.50	1.60 ± 0.90	0.70–2.50	4.10 ± 3.40	0.70–7.50	0.71	.5509	33

Table 1.4. Results from correlation analysis tests between $\log_{10}$ CFU g^{-1} of vAh and sediment chemistry variables. Based on bivariate normality testing, test used was Spearman's rank correlation (coefficient = ρ). All raw *P*-values were adjusted to control the false discovery rate (FDR) using the Benjamini-Hochberg procedure. Significant results at $P<.05$. The sample size (*n*) required to reveal statistically significant correlations.

Variable	***ρ***	***P*-value**		***n*/farm**
		Raw	**FDR**	
Alkalinity (% $CaCO_3$ Equivalence)	0.13	0.2318	0.3327	487
Aluminum (ppm)	-0.13	0.2318	0.3327	487
Calcium (ppm)	0.12	0.2570	0.3327	542
CEC (meq 100 g^{-1})	0.12	0.2570	0.3327	542
Copper (ppm)	0.19	0.0740	0.3327	219
Iron (ppm)	-0.12	0.2570	0.3327	542
Magnesium (ppm)	0.17	0.1075	0.3327	269
Manganese (ppm)	0.11	0.3089	0.3327	672
Organic Matter (%)	0.11	0.3089	0.3327	672
pH	0.16	0.1219	0.3327	291
Phosphorus (ppm)	0.11	0.3089	0.3327	672
Potassium (ppm)	0.17	0.1075	0.3327	269
Sodium (ppm)	-0.03	0.7657	0.7657	7,823
Zinc (ppm)	0.13	0.2272	0.3327	478

Figures

Figure 1.1. Persistence of virulent *Aeromonas hydrophilia* population (log_{10} CFU g^{-1}) over a period of 113 days in sediment samples collected from two farms for the pilot trial. Each symbol indicates the mean of four sediment samples (2 ponds per farm), and error bars around the symbol represent the standard error of the mean. Symbols with different lowercase letters are significantly different at $P < 0.05$.

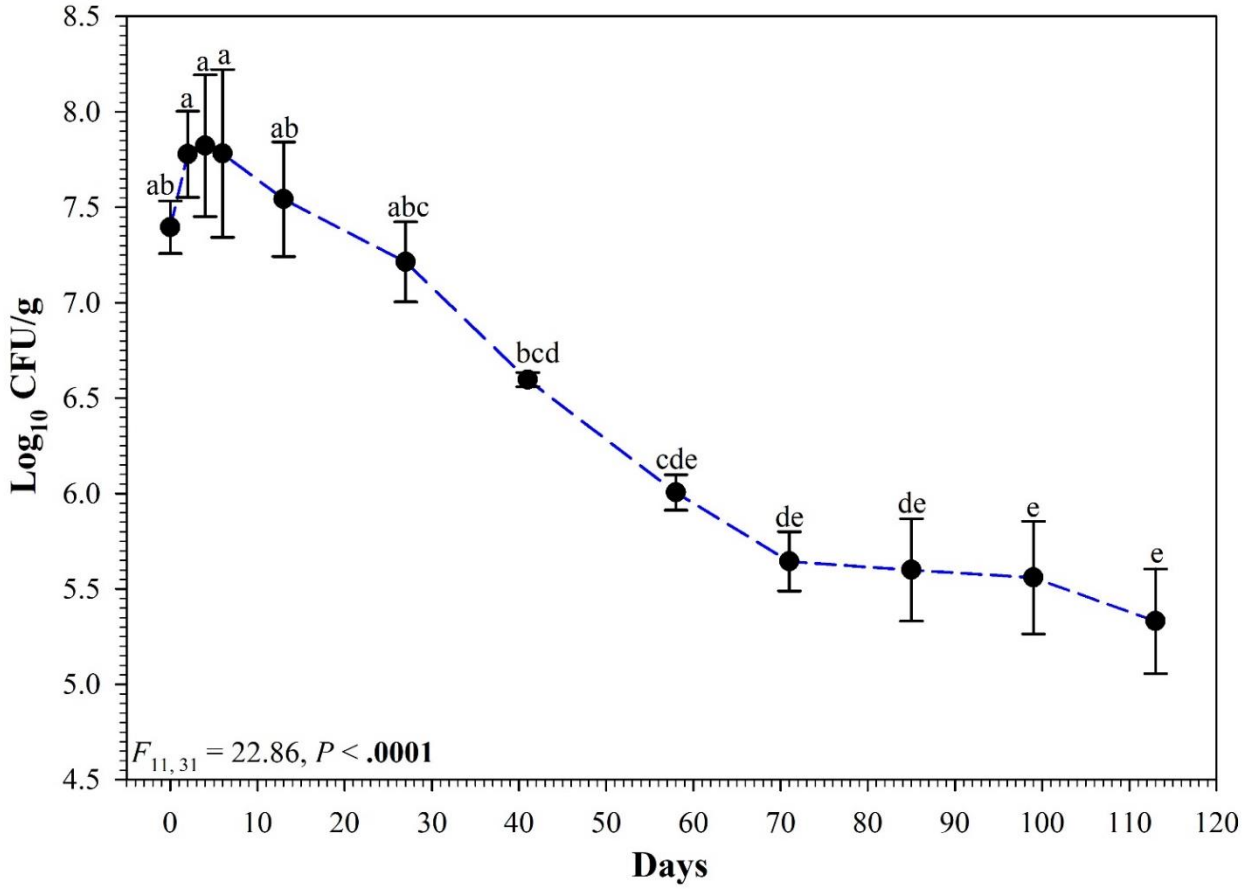


Figure 1.2. Colony growth of virulent *Aeromonas hydrophilia* isolate ML-09-119 (A), and unknown bacterial isolate (B) on ampicillin dextrin agar. Sampling day 13 during pilot trial.

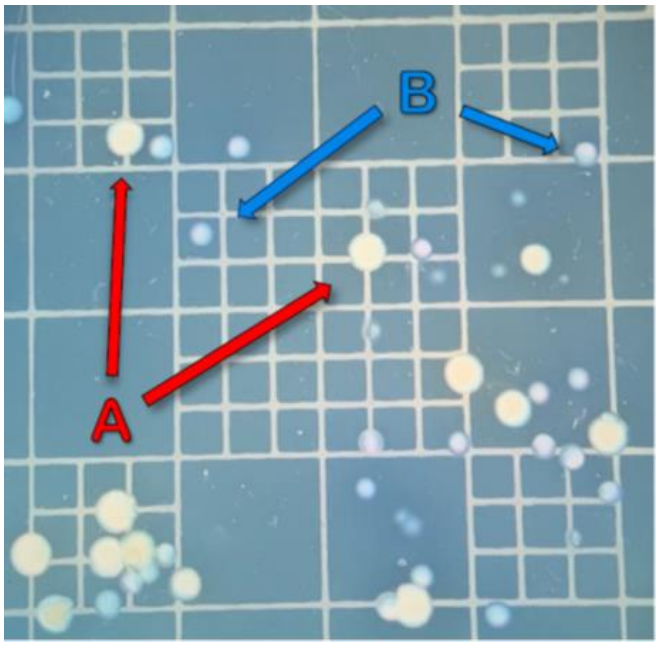


Figure 1.3. PCR product banding of virulent *Aeromonas hydrophilia* and unknown bacterial isolates collected from pilot trial day 41. Positive and negative controls, and DNA ladder were labeled as P, N, and LAD, respectively. Agarose gel (2%) image visualized on an ultraviolet transillumination after. Isolates of vAh formed product bands at 246 bp.

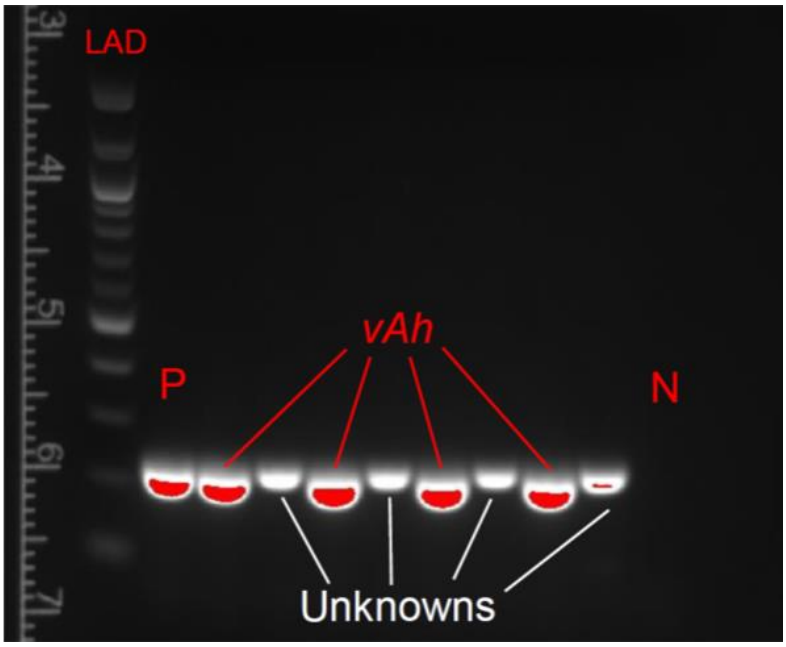


Figure 1.4. Persistence of virulent *Aeromonas hydrophilia* ML-09-119 population (log_{10} CFU g^{-1}) in sediment samples collected from 12 study chambers (2 farms × 2 ponds/farm × 3 replicate tanks per pond). Within each box plot, solid black horizontal line indicates the median, dashed red horizontal line indicates the mean. Box plots with different lowercase letters are significantly different at $P < 0.05$.

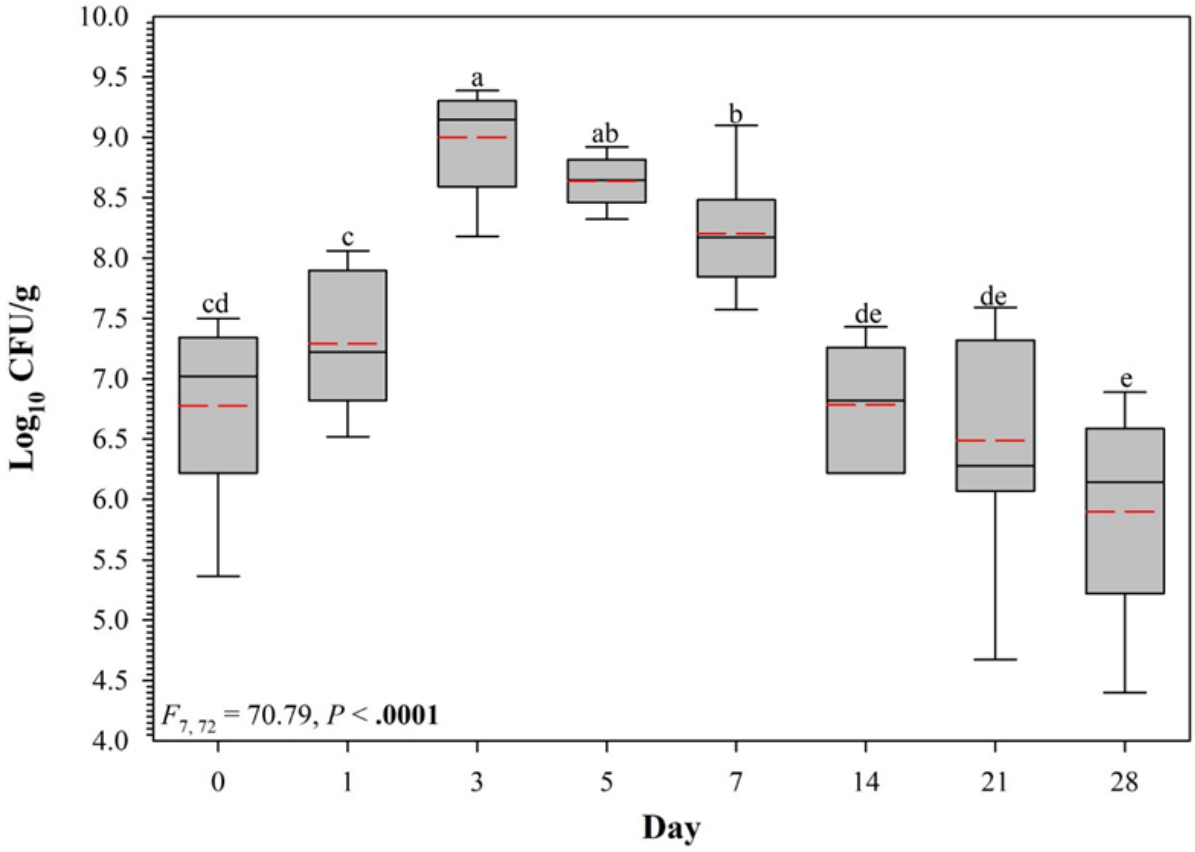


Figure 1.5. Comparison of virulent *Aeromonas hydrophilia* population (log_{10} CFU g^{-1}) in sediment samples collected from two farms (2 ponds/farm × 3 replicate tanks per pond). Within each box plot, solid black horizontal line indicates the median, dashed red horizontal line indicates the mean. Box plots with different lowercase letters are significantly different at $P < 0.05$.

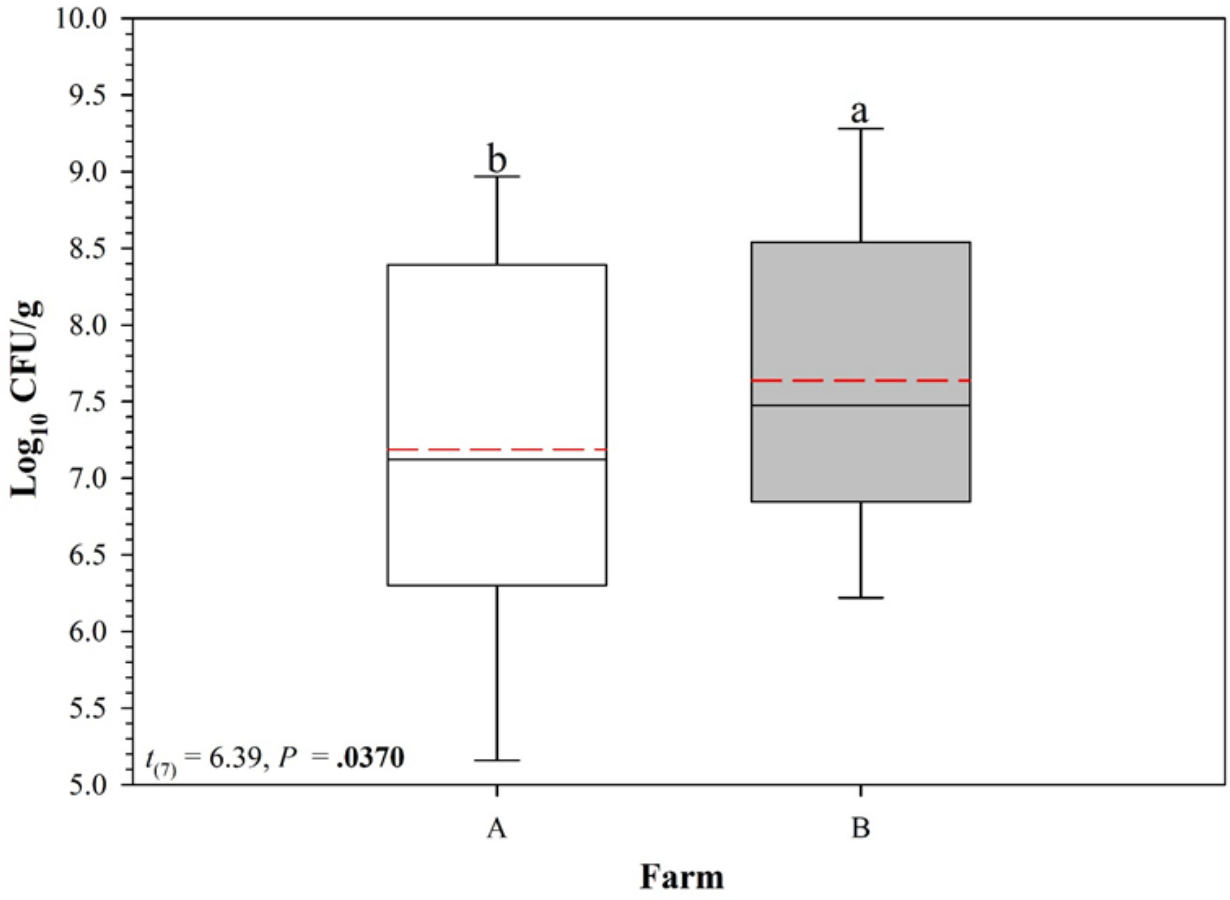


Figure 1.6. Relationship between virulent *Aeromonas hydrophilia* population in sediment (CFU g^{-1}: **A**, **B**, and **C**; log_{10} CFU g^{-1}: **D**, **E**, and **F**) and time (days) using a smoothing spline (SS) model and 95% confidence intervals (gray shadow). Left figures (**A** and **D**) represent all samples; middle figures (**B** and **E**) represent farm A; right figures (**C** and **F**) represent farm B. Estimates of SS model descriptors are summarized in Table 1.

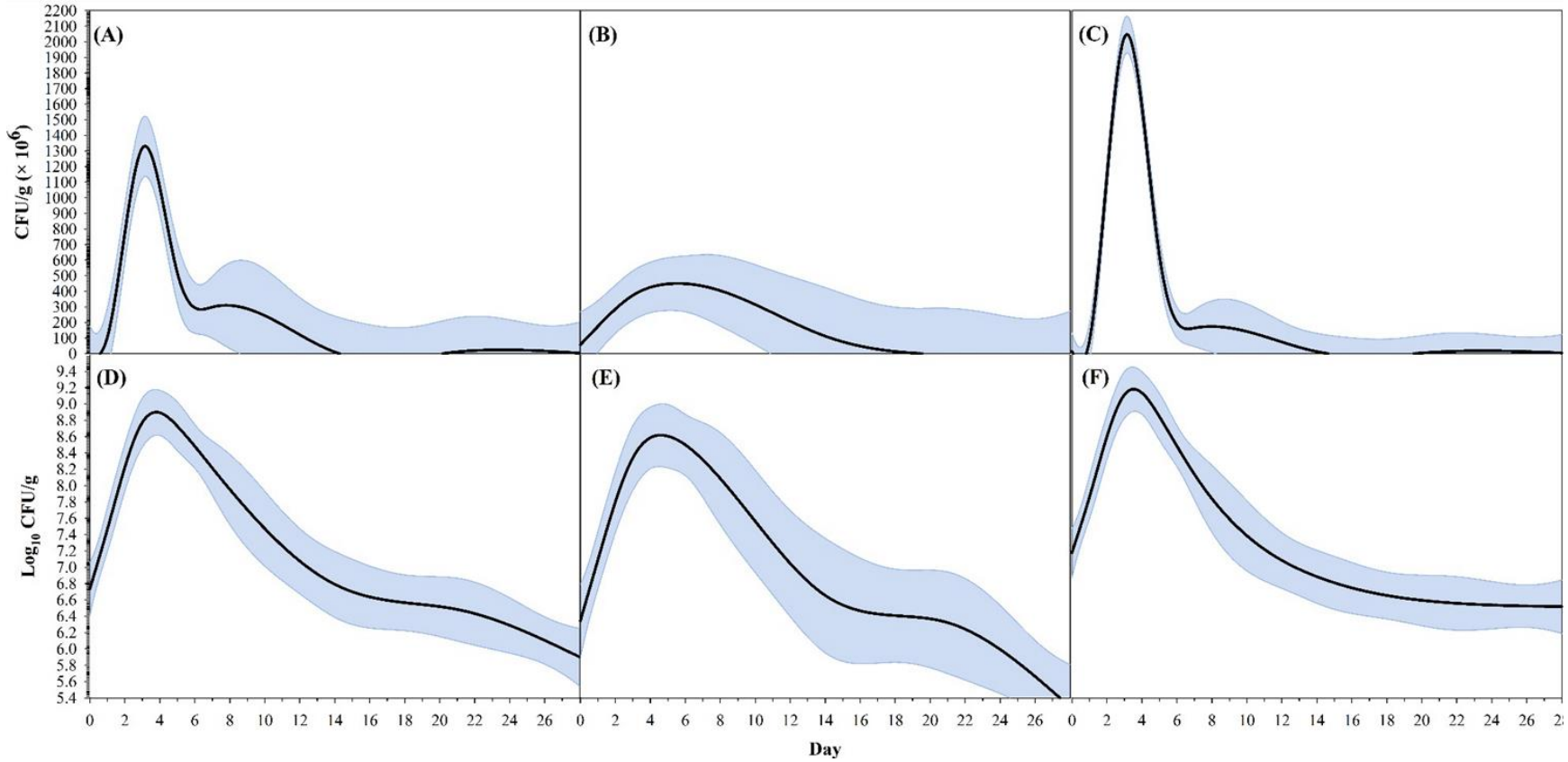
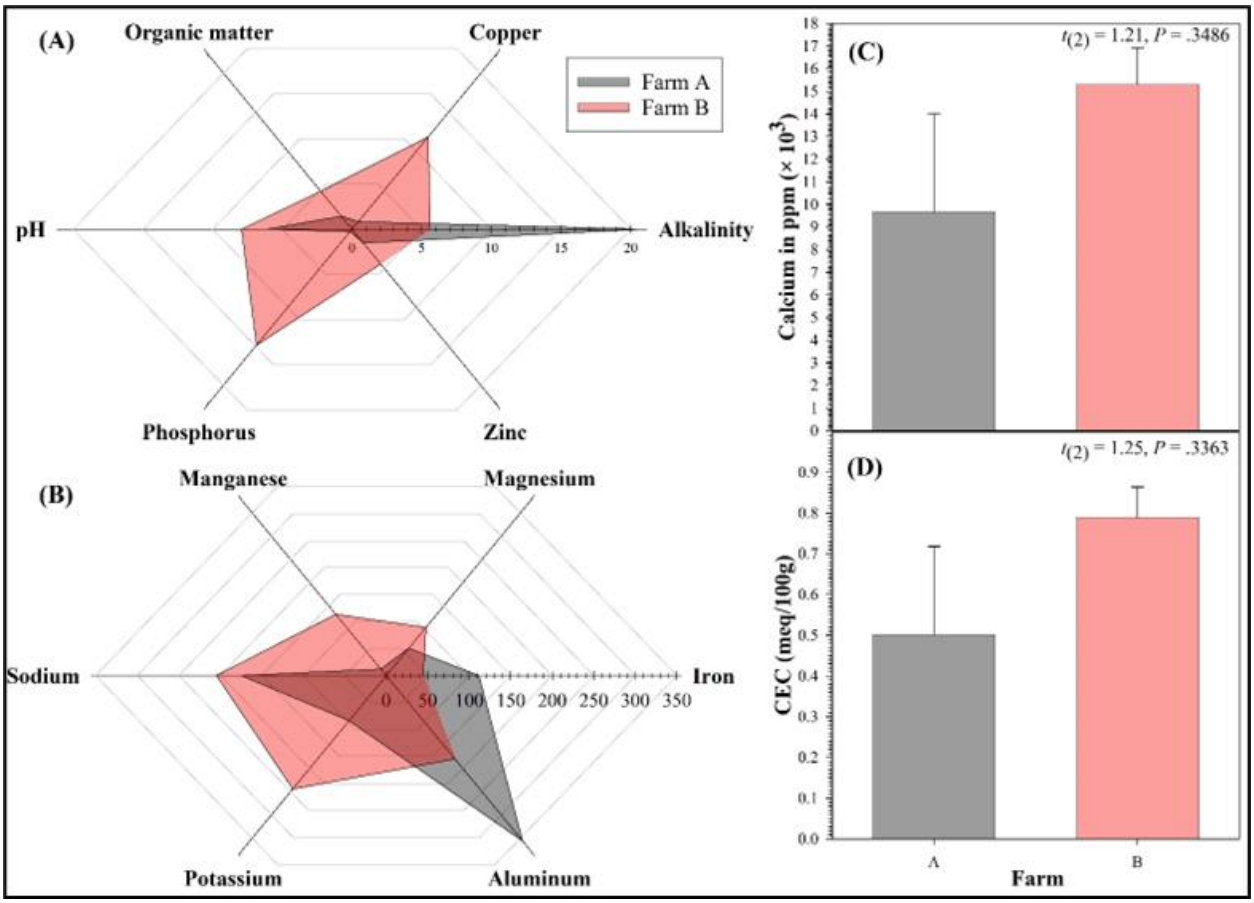


Figure 1.7. Radar plots (A and B) and bar charts (C and D) of sediment chemistry parameters measured in samples collected from two farms (2 ponds per farm). Error bars in bar charts represent the standard error of the mean. Bars with different lowercase letters are significantly different at $P < 0.05$.



Chapter 2

Investigating the Ability of *Edwardsiella ictaluri* and *Flavobacterium covae* to Persist Within Commercial Catfish Pond Sediments Under Laboratory Conditions

This manuscript has been submitted to the Pathogens

Tuttle, J.T.; Bruce, T.J.; Butts, I.A.E.; Roy, L.A.; Abdelrahman, H.A.; Beck, B.H.; Kelly, A.M. Investigating the Ability of *Edwardsiella ictaluri* and *Flavobacterium covae* to Persist Within Commercial Catfish Pond Sediments Under Laboratory Conditions. Manuscript under review for publication.

2.1 Abstract

Two prevalent bacterial diseases in commercial catfish aquaculture are enteric septicemia of catfish and columnaris disease caused by *Edwardsiella ictaluri* and *Flavobacterium covae,* respectively. While disease management options are available, chronic and recurring outbreaks of these bacterial pathogens result in significant economic losses for producers annually. Determining if these pathogens can persist within sediments of commercial ponds is paramount. An experimental full persistence trial (FPT) was conducted to evaluate the persistence of *E. ictaluri* and *F. covae* in pond sediments. Twelve test chambers containing 120 g of sterilized sediment from four commercial catfish ponds, 8 L of disinfected water, and either *E. ictaluri* (EI_{FPT}) or *F. covae* colonies (FC_{FPT}) were inoculated, and at 1, 2, 4, 6, 8, and 15 days post-inoculation, 1 g of sediment was removed, and colony forming units (CFU) were enumerated on appropriate selective media. *E. ictaluri* population peaked on Day 3 at 6.4 ± 0.5 $\log_{10}$ CFU g^{-1}. Correlation analysis revealed no correlation between the sediment physicochemical parameters and CFU g^{-1}. In both FC_{FPT} attempts, no viable *F. covae* colonies recovered. Future studies are needed to evaluate which environmental factors can influence *E. ictaluri* persistence and potential and *F. covae* persistence in pond bottom sediments.

2.2 Introduction

The commercial catfish industry is one of the largest aquaculture industries in the United States and has consistently led all aquatic species in sales from 1988 to 2018 [1]. The professionals responsible for channel catfish (*Ictalurus punctatus*) and hybrid catfish [♀ channel catfish (*I. punctatus*) × ♂ blue catfish (*I. furcatus*)] production have experienced exceptional progress in the industry but have also had to deal with numerous and recurring challenges. One

of the most costly and consistent issues that commercial catfish producers face are bacterial diseases [2], namely enteric septicemia of catfish (ESC) caused by *Edwardsiella ictaluri* [3] and columnaris disease (CD) caused by *Flavobacterium covae* [4].

2.2.1 Edwardsiella ictaluri

Edwardsiella ictaluri is a gram-negative, facultative, rod-shaped, weakly motile, peritrichous bacterium (2, 5-6) and has been one of the causative agents for ESC outbreaks in the commercial catfish industry for nearly 50 years (7-9). In the United States, light (< 90.7 kg), medium (90.7–907 kg), or severe (> 907 kg) losses of catfish to ESC outbreaks caused by *E. ictaluri* in 2002, were reported to be 50.5%, 39.5% and 10.0%, respectively [10]. In East Mississippi, Peterman and Posadas [11] reported that 1.2 million catfish and $0.7 million USD were lost due to *E. ictaluri* outbreaks during the 2016 production season alone. Across western Alabama catfish operations from 2015–2021, Abdelrahman et al. [2] reported that foregone sales from losses due to *Edwardsiella* infections totaled $3.6 million USD. One reason losses due to *E. ictaluri* are not as devastating as other bacterial infections like motile *Aeromonas* septicemia (MAS) and CD [2] could be because fingerlings more often than market-size channel fish succumb to ESC [12]. Fish that have been exposed to and recovered from an *Edwarsiella* infection will have a greater immunological response and become more resistant to later infections [13]. The development and implementation of a live attenuated oral vaccine in 2015 [14] has dramatically reduced losses of channel and hybrid catfish fingerlings in production settings [15-16]. It has been reported that blue catfish and genetically selective strains of channel catfish can exhibit resistance to ESC infections [17-19). Hybrid catfish are moderately susceptible to ESC [13, 19] but are more susceptible than channel catfish when *Edwardsiella piscicida* is the causative agent [15, 20].

Although notable progress has been made in managing and mitigating losses due to ESC, the disease remains to be an annual issue for catfish producers in west Alabama.

Chronic or recurring ESC outbreaks in commercial catfish ponds are commonplace [9, 21-23] and can occur due to numerous factors. For example, the pathogenesis of *E. ictaluri* is distinct compared to other warm-water bacterial infections because it is considered an intracellular pathogen and can replicate within channel catfish macrophages [24]. Pathogenesis typically occurs horizontally when an uninfected fish cannibalizes an infected fish, as it has been known to survive the head kidney and forebrain of channel catfish [24-27]. Mqolomba and Plumb [25] reported that the head kidney, brain, blood, liver, trunk kidney, spleen, gonad, gall bladder, and muscle of fish still contained $>10^4$ bacterial cells g^{-1} 65 days post-exposure to *E. ictaluri.* Surviving fish can remain carriers for *E. ictaluri* even after antibiotic treatment [28].

Another explanation for these recurring infections could be the ability of *E. ictaluri* to persist within commercial catfish ponds. In addition to *E. ictaluri,* bacterial species *E. tarda, F. columnare, Streptococcus iniae,* and *Yersinia ruckeri,* and many strains of *A. hydrophila* have been found in aquaculture pond waters and soils [29-32]. Genetic research has revealed adaptations that would allow the bacterium to survive in stressful environments. Biofilm formation by *E. ictaluri* has been reported on multiple substrates commonly found in aquaculture operations [31]. The genome of this pathogen contains sequences for six different heat shock proteins and 13 universal stress proteins that can be upregulated when exposed to oxidative stress, thermal stress, acid stress, and catfish serum stress [33]. The TonB energy transducing system and TonB-dependent transporters within *E. ictaluri* allow the pathogen to compete for and actively transport essential scarce nutrients [22]. Due to the pathogen's ability to infect diverse fish species, *E. ictaluri* has been reported to express a high level of biochemical

heterogeneity, mainly resulting in differing activities from ornithine decarboxylase, cytochrome oxidase, H_2S production, and production of gas and acid from glucose metabolism [34-36]. Plumb and Quinlan [37] reported direct evidence of *E. ictaluri* surviving within the pond water for a short period and within the mud of commercial catfish pond bottom for several days. While numerous professional and academic contemporaries have widely accepted this, it remains unclear how long *E. ictaluri* would persist in a production environment more analogous to a commercial catfish pond.

2.2.2 Flavobacterium covae

Historically, the causative agent of CD in catfish aquaculture has been turbulent. In 1917, the bacterial pathogen was first named *Bacillus columnaris* due to its tendency to form haystack-like masses when sourced from external catfish lesions [38]. After successful culture conditions were determined, the bacterium was renamed *Chondrococcus columnaris* [39]. The pathogen was reclassified again in 1945 as *Cytophaga columnaris* [40], then *Flexibacter columnaris* [41], then *Flavobacterium columnare* in 1996 [42]. Recent studies have revealed genetic heterogeneity of *F. columnare* isolates worldwide [43-44], which warranted further differentiation into four distinct genomovars [45] and finally, four different species (LaFrentz et al. 2022). Today, and throughout this study, the primary bacterial pathogen responsible for CD outbreaks in channel and hybrid catfish aquaculture is *F. covae* [4, 45].

Explanatorily, *F. covae* is a gram-negative, aerobic, long filamentous rod-shaped, gliding, non-halophilic, yellow-pigmented, opportunistic pathogenic bacterium [4, 46-49]. Outbreaks of CD in commercial catfish species can occur via direct fish-to-fish transmission or when a carrier sheds the bacterium, or through the water column [50]. Pathogenesis of CD occurs during periods

of high fish stress, when temperatures and organic loads in ponds are high, fish are overstocked, and exposed to excessive handling [51]. While the specifics of pathogenesis are not fully understood, generally, the pathogenic bacterium first colonizes the host via attraction, adhesion, and aggregation mechanisms. This is followed by the production of endotoxins, exotoxins, and bacteriocins, which eventually lead to the pathogen overwhelming the host fish's immune system and causing to the disease [52]. In addition, the mucus that naturally covers the gills and skin of catfish causes a more robust chemotactic response in *F. covae* (formerly *F. columnare* genomovar II) compared to *F. columnare* (formerly *F. columnare* genomovar I), indicating a potential relationship involving adhesion [53].

Economically, CD has caused severe losses to the commercial catfish industry since 1922 [54]. It has been reported to be the second-highest disease observed on catfish farms in the United States [29]. Losses are estimated to be $30 million USD annually [55]. While average mortality due to CD is between 50–60%, ponds containing channel catfish fingerlings can experience up to 90% mortality [54]. In west Alabama, the highest number of fish losses were due to CD outbreaks, which equaled an estimated $14.6 million USD in foregone sales from 2015 to 2021 [2]. This discrepancy in economic loss and fish number loss is likely because fingerlings and young fish are the most susceptible to CD [56]. Like *E. ictaluri* and virulent *A. hydrophila* (vAh) diseases, CD outbreaks and infections caused by *F. covae* can be chronic and recurring [48, 50-52, 56-57]. Additionally, *F. covae* has several adaptations for the bacterium to survive and potentially persist in harsh environments.

Historically, *F. covae* growth has been most successful by using low-nutrient media [39, 58]. Concerning growth and prevalence, CD can be influenced by increasing temperature, organic matter, and nitrite concentration in the water [52]. Similar to other aquatic pathogens, *F. covae* can

form biofilms in aquaculture systems, with factors such as calcium concentration, temperature, hardness, salinity, and the presence of certain carbohydrates can impact the formation of biofilm and growth [4, 31, 46]. Cai et al. [46] reported that the optimal conditions for *F. covae* biofilm formation are at 28 ^{o}C, 360 ppm hardness, 5 ppt salinity, and when mannose is present. Shoemaker and LaFrentz [59] have reported the capability of *F. covae* to utilize fish mucus as a nutrient source, which may alter virulence and protein expression. Some *Flavobacterium* spp. can grow at temperatures as high as 45 ^{o}C, while most are considered psychrophilic or psychrotolerant [60]. *Flavobacterium* spp. have been found in numerous environments, including bodies of freshwater and seawater, sediments, soils, glaciers, ice, and freshwater shrimp and catfish ponds [60-62]. Adaptations for dealing with environmental stressors such as peroxide resistance, iron metabolism, heat shock proteins, and multiple stress response mechanisms have been found within *F. columnare* and *F. covae* genomes [44, 63]. The bacterium can also cope with oxidative stress and prolonged starvation, and bacterial cells can be revived following starvation while expressing less virulence [64-65].

With the ability of the opportunistic pathogens *E. ictaluri* and *F. covae* to handle harsh environments, it is plausible that they may potentially be able to persist within commercial catfish ponds over extended periods. Sediments accumulate most rapidly in the first years of pond use and, on average, can accumulate as much as 40 cm of sediment over 15 years [66]. Sediments consist of inorganic and organic matter originating from biological sources, primarily phytoplankton, catfish wastes, and uneaten feed [66-67]. Because sediment and organic materials continue to accumulate on the pond bottoms, and the drastic changes within the pond during a production season allow ample opportunity for *E. ictaluri* and *F. covae* to infect stressed fish [30] and, more importantly, provide a viable environment for the pathogens to persist. The primary

focus of this study was to determine if *E. ictaluri* and *F. covae* can persist within submerged pond sediments while simultaneously observing how their populations change over time. Additionally, physiochemical components of the sediments were examined to determine if they correlated with observed population trends. We hypothesized that both *E. ictaluri* and *F. covae* would successfully propagate within this environment and that differences in population growth would occur between different sediment types.

2.3 Methods

2.3.1 Previous study

Full persistence trials (FPT)s using isolates of *E. ictaluri* and *F. covae* were subjected to the same experimental conditions described [68]. Full persistence trials' sediment samples, water, bacterial inoculum, and aquaria systems were prepared using the methods described below.

2.3.2 Experimental design and system preparation

All methods utilized for sediment sample collection, sediment, water disinfection techniques, and FPT system preparation were the same as those described by Tuttle et al. [68]. In addition, the systems were in temperature-controlled lab spaces set to a targeted 27.5 and 27.0 ºC for the *E. ictaluri* FPT (EI_{FPT}) and *F. covae* FPT (FC_{FPT}), respectively.

2.3.3 Bacterial culture and trial preparation

The wild-type *E. ictaluri* isolate S97-773 was utilized for this study. Cryopreserved S97-773 stocks were revived on brain-heart infusion (BHI) agar and incubated for 48 h at 28 °C.

Next, a pure *E. ictaluri* colony was placed in 1 L of BHI broth and incubated at 28 °C and 115 revolutions per minute (RPM) for approximately 48 h. The broth culture was centrifuged at 4000 *x g* for 10 min in a 5810 R benchtop centrifuge (Eppendorf North America Inc., Enfield, Connecticut, USA), washed in cold 1X phosphate-buffered saline solution (PBS) adjusted pH of 7.4. Bacterial cells were resuspended and adjusted to an optical density of 0.200 ± 0.005 at 550 nm using an Eppendorf Biospectrometer® Basic (Eppendorf North America Inc.), resulting in an average inoculum concentration of 8.33×10^7 colony forming units (CFU) mL^{-1}.

Preparation of the *F. covae* inoculum, using isolate ALG-00-530, followed a similar procedure. However, the culture media was modified Shieh (MS) [58] containing the antibiotic tobramycin at a concentration of 1 mg L^{-1} of media (MST) resulting in a more selective media [69]. The *F. covae* was passed over the selective MST agar five times to ensure the bacterium had grown accustomed to the antibiotic. After the fifth pass, a pure colony of *F. covae* was placed into 1 L of MS broth and incubated for 24 h at 28 °C and 115 RPM. Once the broth culture in broth had grown, the bacterial cells were spun down, as mentioned previously, and instead washed with a 0.1X PBS solution with an adjusted pH of 7.0. Bacterial cells were resuspended and adjusted to an optical density of 0.200 ± 0.005 at 550 nm using a DR3900 visible spectrophotometer (Hach Company, Loveland, Colorado, USA). The final *F. covae* inoculum concentration in PBS was 1.78×10^7 CFU ml^{-1}.

A randomized block design was used for each PT to assign the four sediment types to the 12 total chambers. In each chamber, 20 mL of either *E. ictaluri* or *F. covae* optically adjusted bacterial inoculum was added to 200 g of sterilized sediment and 500 mL of disinfected dechlorinated city water. The sediment mixture was vigorously stirred with a sterile stainless-

steel spatula for 1 min durations every 5 min over 1 h. After the mixing period, water volume within each chamber was increased to a total of 8 L. To simulate production pond aeration, a 3.5 cm × 1 cm × 1 cm cuboid Pawfly air stone at a fixed location within each chamber would expel air supplied via a Whitewater Silent Air Pump™ v201 (Pentair Aquatic Eco-Systems™, Apopka, Florida, USA) for 12 h beginning at 1800 h and stopping at 0600 h the following day.

2.3.4 Sampling and bacterial enumeration

Sediment in each chamber was collected and bacterial populations were evaluated, with sampling times as follows: 24 h post-inoculation (designated as Day 0), 48 h post (Day 1), four days post (Day 3), six days post (Day 5) and eight days post (Day 7), then every seven days following the fifth sampling. Cai et al. [32] described the methodology used to extract sediment and enumerate live colonies of S97-773 and ALG-00-530 for their respective trials. Approximately 1 g of sediment was collected from each chamber using a sterile 10-mL serological pipette, placed in a sterile 15-mL centrifuge tube, and centrifuged for 10 min at 667 x g. Liquid supernatant was removed and the remaining sediment pellet (~1 g) was resuspended entirely in 0.1X PBS, creating a 1:10 mixture, and vortexed until the pellet was homogenized. Next, 250 µL of homogenized sediment solution was placed into six wells of the leftmost column of a 96-well plate and serially diluted (10-fold) as Chen et al. [70] described. Four serial dilutions of six 10 µL replicates were each plated onto E. ictaluri Medium (EIM) [71].

To enumerate ALG-00-530 colonies, the spread plate method [72] and MST media were utilized. Two technical replicate MST agar plates were used for each of the four targeted 10-fold serial dilutions. The EIM and MST plates were incubated at 28 °C for 24 h. The plate counts of *E. ictaluri* and *F. covae* were recorded, and the final counts of CFU g^{-1} of sediment were

determined using the appropriate correction factors. On each sampling day, viable *E. ictaluri* and *F. covae* colonies were picked, and both were cryopreserved in a 50% glycerol stock at -80 °C. Additionally, a representative colony underwent genomic DNA extraction for later polymerase chain reaction (PCR) confirmation. Any bacteria not confirmed to be the isolates of interest were designated as "unknown" and labeled as such, followed by their respective chamber name, sampling day, and PT.

2.3.5 DNA extraction and PCR confirmation

After colony enumeration, the colonies of the bacterial species of interest were picked and confirmed via polymerase chain reaction (PCR) protocols. Genomic DNA (gDNA) from all bacterial colonies was extracted using the EZNA^{â} Bacterial DNA Kit (Omega Bio-tek Inc., Norcross, Georgia, USA). Finally, all concentrations and gDNA purity measurements were assessed measured using a NanoDrop™ One^{C} spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

For the *E. ictaluri* colonies, a 25 µL PCR reaction was constructed using 12.5 µL of Hot-Start Taq Master Mix 2X (Amresco LLC, Solon, Ohio, USA), 1 µL of ESCF and ESCR primers (initial 10 µM stock solution) [73], and 25 ng of template gDNA. Thermal cycling was conducted using an Eppendorf Mastercycler® X50s (Eppendorf North America Inc.) and after optimization, thermal cycling parameters consisted of an initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Positive and negative controls were run in a thermal cycler with test isolates. Then, 5 µL of PCR product was separated on a 2.0% agarose gel, stained with SYBR Safe DNA Stain (Edvotek^{â}, Washington, District of Columbia, USA), in a 1.0X Tris-

acetate-EDTA running buffer using electrophoresis. All gels were run containing a positive control (S97-773), negative control (nuclease-free H_2O), and a 50 bp DNA Step Ladder (Promega, Madison, Wisconsin, USA). PCR products were visualized using a VWR® Real-Time Electrophoresis Systems LED transilluminator (VWR International, Radnor, Pennsylvania, USA).

To confirm *F. covae* colonies, 25 µL PCR reactions were constructed using 12.5 µL of Hot-Start Taq Master Mix 2X (Amresco LLC, Solon, Ohio, USA), 1.25 µL of FcFp and FcRp primers (initial 10 µM stock solution) [74], and 25 ng of template gDNA. Optimized thermal cycling runs began with an initial denaturation of 95 °C for 5 min followed by 40 cycles of 94 °C for 30 s, 56 °C for 20 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Gel electrophoresis protocols were followed, as mentioned above.

Any colonies not PCR-confirmed as *E. ictaluri* or *F. covae* in their respective trials were designated as unknowns and labeled with the sampling day, chamber name, and PT. To accurately identify unknown bacterial colonies via the 16s rRNA gene, PCR products, and primers 63F and 1387R [75] were sent to Eurofins Genomics LLC (Louisville Kentucky, USA). After nucleotide base-pair results were trimmed and aligned in the Molecular Evolutionary Genetics Analysis (MEGA) software version 11 [76], base-pair sequences were inputted in the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) database [77].

2.3.6 Sediment, water, and statistical analysis

All procedures for conducting water quality and sediment physicochemical analyses and statistical analyses were the same as those described by Tuttle et al [68].

2.4 Results

2.4.1 Edwardsiella ictaluri full persistence trial

The temperature during this persistence trial remained at 27.5 ± 0.3 ^{o}C throughout the 14-day trial. Colonies of *E. ictaluri* began appearing on the selective EIM media on Day 0 (24 h post-inoculation). However, some *E. ictaluri* growth across different replicates experienced a lag period and did not begin appearing on the EIM agar until Day 1 (48 h post-inoculation). All test chambers were inoculated and mixed within the same period of 60 min, and there was congruent growth in all sediment types but not all replicates. Despite this, populations of *E. ictaluri* initially experienced a steady increase, followed by a moderate decline and plateauing pattern over a period of 14 days (Figure 2.1). Across all 12 test chambers, population numbers on Day 3 were higher than those on all other days except for Day 5 ($t_{60} = 3.55$, $P = 0.0011$). On Days 5, 7, and 14, the *E. ictaluri* total population was not different according to the pairwise comparisons among those respective sampling days ($P > 0.05$). When comparing the sediment types from the two farms, the overall population of *E. ictaluri* in farm B sediment was not different from the total population of *E. ictaluri* in farm A sediments (Figure 2.2). On the smoothing splines encompassed by 95% CIs, containing the raw values of CFU g^{-1} and $\log_{10}$ transformed CFU g^{-1} values (Figure 2.3), there are no differences in any population peak, breadth, or range values between farm A and farm B sediments, or overall sediment counts (Table 2.1).

Unknown bacterial colonies first appeared in the sediment sourced from farm B on Day 1 (48 h post-inoculation) and were present in all sediment types by Day 3. The morphology and

phenotypic expression of unknown bacterial colonies were more varied and diverse (Figure 2.4). The first colony that appeared in the selective EIM (designated as colony type A) was confirmed to be S97-773 via PCR and 16s rRNA sequencing procedures. Colony type A was present on all sampling days throughout the trial (Figure 2.4). On sampling Day 3, other colonies appeared to have the same color and shape but were small punctiform and pulvinate (colony type C) or intermediate-sized (colony type E). Some colonies appeared to have nearly the same morphology as ones designated as colonies A and C but began to exhibit a translucent and erose margin at both large (colony type B) and smaller sizes (colony type D). On sampling Day 7, there were large colonies that expressed white/opaque (colony type G), dark green (colony type I), and yellow (colony type J) color morphologies. Finally, on sampling Day 14, colonies that exhibited a curled and seemingly dehydrated margin (colony type H) and a noticeably larger colony size with a lobate margin (colony type F) began appearing.

Although colonies more phenotypically varied in this PT, PCR product bands using the ESCF and ESCR primers resulted in all isolated colonies producing the same amplicon region (177 bp) consistent with the positive control, indicating no apparent differentiation between the isolated bacterial colonies (Figure 2.5). A more robust confirmation was conducted, and the 16s rRNA sequencing revealed six different species not identified as *E. ictaluri* (Table 2.2). Additionally, it would appear that three distinct bacterial colonies that initially appeared to be different from the species of interest were identified as *E. ictaluri.* The water quality parameters did not noticeably fluctuate throughout the PT (Table 2.3). The sediments used in this PT are the same as the four sediment types used in a previous study [68], and all physical and chemical parameters between the two farms were not different. Due to the small sample size of sediment physiochemical properties, the correlation analysis indicated no correlation between *E. ictaluri*

populations over time and the sediment parameters (Table 2.4). Power analysis revealed the sample size required to determine the statistically significant correlations between *E. ictaluri* populations and each sediment parameter (Table 2.4). After the bacterial enumeration procedure was complete, all sediment samples were frozen.

2.4.2 Flavobacterium covae full persistence trial

This PT was conducted on two separate occasions following all procedures described above. The water temperatures for the first and second attempts were approximately 27.2 ± 1.2 and 27.0 ± 0.4 ^{o}C, respectively. In both instances, no colonies of *F. covae* were recovered from the sediment over seven days. Therefore, incubation times were increased to 72 h to ensure that *F. covae* colonies were given ample media contact and propagation time. In both FC_{PT} attempts, viable colonies of unknown bacteria appeared on sampling Day 1 (48 h post-inoculation), displaying various unique colony morphologies. However, 16s rRNA sequencing outputs revealed that none of the colonies were *F. covae* or any *Flavobacterium* spp., revealing 12 distinct species (Table 2.5). Like the EI_{PT}, all sediment samples were frozen after sampling.

2.5 Discussion

2.5.1 Edwardsiella ictaluri full persistence trial

The results of the EI_{PT} indicate that *E. ictaluri* can persist within the submerged sediments of commercial catfish ponds in a controlled laboratory setting. The bacterial growth curve illustrated by the data indicated that *E. ictaluri* populations began to plateau by Day 5 and did not change throughout the remainder of the trial. The highest average population across all sediments was $\log_{10}$ 6.4 CFU g^{-1}. Due to no overall difference between sediments sourced from

the two different farms, this suggests that sediment has no apparent influence on the growth and maintenance of pathogen populations. These findings are consistent with those Plumb and Quinlan [37] reported and displayed similarities to how vAh behaves under similar experimental conditions [68]. Like vAh, *E. ictaluri* populations experienced a growth period, followed by reaching the stationary phase, and then plateaued to remain at a consistent population. However, unlike vAh, the bacterial populations in the EI_{PT} began plateauing by the fifth sampling day, compared to a vAh persistence trial when bacterial populations begain to plateau by the fourteenth sampling day [68].

Aside from this study, there are very few publications with direct evidence regarding the ability of *Edwardsiella* spp., let alone *E. ictaluri*, to survive or persist within sediments or soils of aquatic environments. *E. ictaluri*-specific phages found in water and sediments in a river in Hiroshima Prefecture, Japan, have been linked to an individual forktail bullhead (*Pelteobagrus nudiceps*) [78]. Viable *E. tarda* colonies have been found in the sediments and water of aquaculture ponds, and genetic differentiation exists between isolates found exclusively in sediments versus isolates collected from other sources [79-80]. In addition, *E. tarda* found in the soils of Owerri, Nigeria, displayed potential as a species for bioremediation of crude oil [81]. Regarding sediment, the correlation analysis could not distinguish significant physical and chemical factors of the sediment that influenced the population of the bacterial pathogen. The power analysis revealed a larger sample size is necessary to determine statistical significance with high power. These sample size numbers, which were in the thousands, would be unrealistic and cost prohibitive within the scope of this study but would be worth future investigation. It is also necessary to determine which cellular mechanisms and virulence factors allow for the persistence of *E. ictaluri* in the sediments of catfish ponds.

It has been established that species of *Edwardsiella* are naturally resistant to colistin [71]; however, it is notable that the bacteria identified in this study exhibited colistin resistance consistent with previous research findings. Genera from the family Enterobacteriaceae, such as *Salmonella* spp., *Klebsiella* spp., *Aeromonas* spp., *Citrobacter* spp., *Shigella* spp., *Enterobacter* spp., and *Escherichia* spp. contain mobilized colistin resistance genes [82]. *Clostridium perfringens, Bacillus subtilus, Neisseria meningitides, Burkholderia* spp., *Proteus mirabilis, Kluyvera* spp., *Cronobacter sakazakii, Raoultella ornithinolytica,* and *Pseudomonas aeruginosa* have all shown some level of resistance to colistin [83-86]. The most interesting finding from this study comes from verifying the identities of diverse colonies that grew on the selective EIM.

Based on the results of the 16s sequencing, it is apparent that four bacterial colonies were expressing different pigmentation and sizes that were all determined to be *E. ictaluri*. One reason *E. ictaluri* was chosen for this study, as opposed to *E. tarda* or *E. piscicida,* is due to the reported high phenotypic and biophysical homogeneity among isolates [87-88]. During the beginning of the persistence trial, *E. ictaluri* colonies produced a green pigment on the EIM; however, some apparent *E. ictaluri* colonies began expressing a yellow pigment by the fifth sampling day. One study reported that *E. tarda* colony pigmentation was black when grown on Salmonella-Shigella agar [89]. Bacterial pigmentation is quite diverse, and all unique pigments have a specific function essential for bacterial survival and ecological success [90]. Different bacterial genera, including *Pseudomonas* spp., *Janthinobacterium* spp., *Streptomyces spp., Nocardia spp., Thermomonospora spp., Microbispora spp., Streptosporangium spp., Rhodococcus spp.,* and *Kitasatospora spp.,* have diverse pigmentation [91]; however, there have been no studies reporting one species or genus of bacteria being capable of expressing two different pigments. Johansen et al. [92] demonstrated through genetic modification of the motility, cell shape,

stringent response, and tRNA modification genes of a *Flavobacterium* spp. strain Iridescent 1, that they could alter the nanostructure, which resulted in multiple colors observed among the same bacterial colonies. They also suggested that the structural color of bacterial colonies may be linked to cellular functions and gene activity, which may have significant implications for natural populations of pathogenic bacteria. To date, no studies have determined the natural pigments produced by *E. ictaluri* or if there is a linkage between cellular functions and pigmentation. Whole genome sequencing of the isolates collected during the EI_{PT} would reveal what pigments these bacteria can produce and add another level of confirmation to the identity of these bacteria.

2.5.2 Flavobacterium covae Full Persistence Trial

Even though both attempts to propagate *F. covae* colonies within this experimental design were unsuccessful, these findings are intriguing. Multiple factors may have influenced the lack of *F. covae* in these laboratory persistence trials. Environmental conditions such as water hardness, high temperature, organic matter, and nitrite concentration can increase the adhesion and virulence of the bacterial pathogen [93-95]. The biofilm formation is most effective between 25–28 ^{o}C, and can be inhibited when salinity is as low as 3 ppt and significantly reduced at salinities over 7.5 ppt (46, 95). Another factor could be due to ecological interactions and interspecific competition. Bacterial species including *Bacillus subtilis, Luteimonas aestuarii, Rhodococcus qingshengii, Leucobacter luti, Dietzia maris* were antagonistic towards *F. covae* and *F. psychrophilum* [30, 96-97]. Additionally, tannic acid can act as an effective bactericide for *F. columnare* and *E. ictaluri* [98]. It could be possible that some of the other bacteria that

appeared in the FC_{FPT} and some natural compounds or ions in the sediment prevented the establishment of *F. covae*.

The culture conditions necessary for successful *F. covae* growth can be sensitive. Although previous studies have reported that the best growth of *F. covae* is on low nutrient media [58, 69, 99], and the bacteria are slow growing [100-101]. While Shieh media has typically allowed for fast and effective growth of *Flavobacertium* spp. [102], a recent study indicated that G media provides effective and uniform distribution of *F. covae* colonies within 24 h [101]. Other media, such as tryptone yeast extract salt media (TYES) [100] and antibiotics such as polymyxin-neomycin have been utilized to create selective media [69] for successful *F. covae* growth. Media type and culture considerations for future persistence studies may yield more favorable results.

Since we know the pathogen *F. covae* and other members of the *Flavobacterium* genus have been found in aquatic environments outside of a host [50, 103-106], it is plausible that *F. covae* may have the ability to persist within the environment. In addition to biofilm formation, a recent study by Abdelhamed et al. [107] revealed that *F. covae* could grow under anaerobic conditions via denitrification genes and nitrite reduction. However, at this time, we were unable to verify the ability of the pathogen *F. covae* to persist within the sediments of commercial catfish ponds under the conditions outlined in this study.

2.6 Conclusion

Understanding the mechanisms that allow these pathogenic bacteria to persist within sediments is vital for effective disease management strategies for commercial catfish producers. *E. ictaluri* has been confirmed to be able to persist within aquatic sediments based on the results

of this study, however, this may have profound implications. Further gene expression analyses similar to those those conducted on vAh [108] may reveal that cell origins of *E. ictaluri* may result in different virulence factor expressions. Additionally, *E. ictaluri* persisting within sediments may be more susceptible to developing anti-microbial resistance [109], as has been reported in previous studies [36, 110-112]. Conversely, since *F. covae* propagation was unsuccessful, modifications to this experimental design will be necessary for future studies. While *F. covae* can form biofilms, other environmental and experimental factors within the aquatic environment may contribute to them not being recovered in this study.

2.7 References

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Tables

Table 2.1. Mean, standard error (SE), and 95% confidence intervals (95% C.I.) for descriptors of smoothing spline models presented in Figure 2.3.

	Curve descriptor	Overall		Farm A		Farm B	
		Mean ± *SE*	95% C.I.	Mean ± *SE*	95% C.I.	Mean ± *SE*	95% C.I.
CFU g^{-1}	Peak CFU g^{-1} ($\times 10^6$)	3.63 ± 1.31	1.08–6.19	1.29 ± 1.78	-2.20–4.78	2.85 ± 1.97	-1.01–6.71
	Time (day) at peak CFU g^{-1}	2.93 ± 0.90	1.17–4.69	0.00 ± 2.21	-4.32–4.32	0.00 ± 1.63	-3.19–3.19
	Time (day) at 90% of peak CFU g^{-1} – lower	2.17 ± 0.72	0.76–3.57	0.00 ± 1.38	-2.70–2.70	0.00 ± 1.18	-2.32–2.32
	Time (day) at 90% of Peak CFU g^{-1} – upper	3.68 ± 0.72	2.27–5.08	2.41 ± 2.42	-2.33–7.15	1.71 ± 1.5	-1.23–4.64
	Breadth at 90% of peak CFU g^{-1}	1.51 ± 0.55	0.44–2.58	2.41 ± 2.36	-2.21–7.03	1.71 ± 0.96	-0.17–3.59
	Time (day) at 80% of peak CFU g^{-1} – lower	1.81 ± 0.64	0.55–3.07	0.00 ± 1.16	-2.28–2.28	0.00 ± 0.98	-1.93–1.93
	Time (day) at 80% of peak CFU g^{-1} – upper	4.04 ± 0.65	2.76–5.32	4.83 ± 2.71	-0.48–10.13	3.42 ± 1.39	0.70–6.14
	Breadth at 80% of peak CFU g^{-1}	2.23 ± 0.87	0.53–3.93	4.83 ± 3.08	-1.22–10.87	3.42 ± 1.44	0.60–6.24
	Time (day) at 5% of peak CFU g^{-1} – min	0.00 ± 0.20	-0.39–0.39	0.00 ± 0.22	-0.43–0.43	0.00 ± 0.20	-0.39–0.39
	Time (day) at 5% of peak CFU g^{-1} – max	14.00 ± 0.47	13.08–14.92	14.00 ± 0.80	12.43–15.57	14.00 ± 1.77	10.52–17.48
	Range at 5% of peak CFU g^{-1}	14.00 ± 0.51	13.00–15.00	14.00 ± 0.83	12.37–15.63	14.00 ± 1.78	10.51–17.49
Log_{10} CFU g^{-1}	Peak log_{10} CFU g^{-1}	6.32 ± 0.19	5.95–6.68	5.89 ± 1.46	3.04–8.75	6.23 ± 0.32	5.59–6.86
	Time (day) at peak log_{10} CFU g^{-1}	3.27 ± 0.68	1.93–4.61	3.77 ± 3.11	-2.32–9.87	3.31 ± 2.62	-1.81–8.44
	Time (day) at 90% of peak log_{10} CFU g^{-1} – lower	1.85 ± 0.50	0.86–2.83	1.32 ± 0.97	-0.58–3.21	1.20 ± 0.78	-0.33–2.73
	Time (day) at 90% of peak log_{10} CFU g^{-1} – upper	5.37 ± 3.8	-2.08–12.81	14.00 ± 3.63	6.88–21.12	6.66 ± 4.12	-1.41–14.73
	Breadth at 90% of peak log_{10} CFU g^{-1}	3.52 ± 4.03	-4.37–11.42	12.68 ± 4.08	4.69–20.67	5.46 ± 4.61	-3.58–14.50
	Time (day) at 80% of peak log_{10} CFU g^{-1} – lower	0.61 ± 0.65	-0.66–1.87	0.00 ± 0.92	-1.81–1.81	0.00 ± 0.55	-1.09–1.09
	Time (day) at 80% of peak log_{10} CFU g^{-1} – upper	14.00 ± 0.00	14.00–14.00	14.00 ± 0.31	13.39–14.61	14.00 ± 0.89	12.26–15.74
	Breadth at 80% of peak log_{10} CFU g^{-1}	13.40 ± 0.65	12.13–14.66	14.00 ± 1.00	12.04–15.96	14.00 ± 1.11	11.83–16.17

Table 2.2. Results from the NCBI BLAST database for nucleotide 16s rRNA sequences from isolates collected during EI_{PT} and the sampling day durations the unique colonies were present. Bacterial species were determined to have the highest probability under percent maximum identity (Max Ident.), highest total score and highest maximum query cover to show the percentage of query DNA covered.

Colony Morphology	**Sampling Day(s)**	**Confirmation**			**Species ID**
		Total Score[a]	**Query Cover[b]**	**Max Ident.[c]**	
D	5, 7	1762	100	99.37	*Burkholderia contaminans*
F	14	639	98	97.62	Uncultured bacterium
H	14	1954	100	99.53	*Bacillus spp.*
I	14	1599	100	98.84	*Pseudomonas aeruginosa*
C	1–14	1882	94	81.48	*Clostridium hydrogeniformans*
G	7, 14	1792	100	99.59	*Stenotrophomonas pavanii*
A	0–14	1677	100	99.61	*Edwardsiella ictaluri*
B	5–14	1628	100	98.13	*Edwardsiella ictaluri*
E	5, 7	1988	100	99.27	*Edwardsiella ictaluri*
J	7, 14	1831	100	98.90	*Edwardsiella ictaluri*

[a] Sum of alignment scores of all segments from the same subject sequence
[b] Percent of the query length that is included in the aligned segments
[c] Highest percent identity for a set of aligned segments to the same subject sequence

Table 2.3. Water quality parameters [mean, standard error (*SE*), minimum measurement (min), and maximum measurement (max)] measured in 12 study tanks containing sediment samples collected from two farms (2 ponds per farm; 3 replicate tanks per pond) for 14 d EI_{PT}.

Water quality parameter	**Overall**		**Farm A**		**Farm B**	
	mean ± *SE*	min–max	mean ± *SE*	min–max	mean ± *SE*	min–max
Total alkalinity (ppm)	116.72 ± 3.60	87–174	109.50 ± 4.30	87–157	123.94 ± 5.37	90–174
Total hardness (ppm)	123.78 ± 5.03	67–191	105.44 ± 4.92	67–138	142.11 ± 6.36	99–191
pH	7.62 ± 0.02	7.3–7.9	7.57 ± 0.03	7.3–7.8	7.67 ± 0.03	7.4–7.9
Phosphate (ppm)	1.28 ± 0.20	0.0–4.0	1.62 ± 0.32	0.0–4.0	0.94 ± 0.21	0.0–2.8
Total ammonia nitrogen (ppm)	0.60 ± 0.11	0.0–2.2	0.32 ± 0.08	0.0–1.3	0.88 ± 0.18	0.1–2.2
Nitrite (ppm)	0.11 ± 0.04	0.0–1.0	0.16 ± 0.08	0.0–1.0	0.06 ± 0.02	0.0–0.3
Nitrate (ppm)	0.15 ± 0.06	0.0–1.0	0.0 ± 0.0	0.0–0.0	0.31 ± 0.11	0.0–1.0

Table 2.4. Results from correlation analysis tests between $\log_{10}$ CFU g^{-1} of *Edwardsiella ictaluri* and sediment physicochemical variables. Based on bivariate normality testing, Spearman's rank correlation (coefficient = ρ) was used. All raw *P*-values were adjusted using the Benjamini-Hochberg procedure to control the false discovery rate (FDR). Significant results at $P < 0.05$. The sample size (*n*) required to reveal statistically significant correlations.

Variable	ρ	***P*-value**		***n*/farm**
		Raw	**FDR**	
Alkalinity (% $CaCO_3$ Equivalence)	0.05	0.6740	0.2800	2,728
Aluminum (ppm)	-0.04	0.7617	0.9694	5,249
Calcium (ppm)	-0.04	0.7317	0.9694	5,249
CEC (meq 100 g^{-1})	-0.04	0.7414	0.9694	5,249
Copper (ppm)	0.08	0.5447	0.9694	1,224
Iron (ppm)	0.02	0.8857	0.9694	19,260
Magnesium (ppm)	0.09	0.4627	0.9694	896
Manganese (ppm)	<0.01	0.9730	0.9694	422,523
Organic Matter (%)	0.03	0.8450	0.9694	12,627
pH	-0.02	0.9080	0.9694	36,124
Phosphorus (ppm)	0.07	0.6006	0.9694	1,763
Potassium (ppm)	0.08	0.5315	0.9730	1,234
Sodium (ppm)	-0.13	0.3089	0.9730	467
Zinc (ppm)	0.10	0.4373	0.9730	801

Table 2.5. Results from the NCBI Blast database for nucleotide 16s rRNA sequences from isolates collected during FC_{PT} and the sampling day durations the unique colonies were present. Bacterial species were determined to have the highest probability under percent max identity, highest total score, and highest max query cover to show the percentage of query DNA covered.

Colony Morphology	**Sampling Day(s) (FPT attempt)**	**Confirmation**			**Species ID**
		Total Score[a]	**Query Cover**[b]	**Max Ident.**[c]	
A	3–7 (1, 2)	1783	100	99.90	*Brevibacterium sediminis*
B	1–5 (1, 2)	1670	100	100.00	*Micrococcus luteus*
C	1–7 (1, 2)	2021	100	99.91	*Micrococcus sp.*
D	3–7 (1, 2)	1599	100	98.74	*Sphingobium yanoikuyae*
E	3, 5 (1)	1916	99	97.1	*Acinetobacter schindleri*
F	7 (2)	1286	99	89.88	Uncultured Bacterium
G	3–7 (1)	1982	100	98.57	*Massilia neuiana*
H	3–7 (1, 2)	1857	100	100.00	*Stutzerimonas stutzeri*
I	5, 7 (2)	1988	100	99.81	*Bacillus pseudomucoides*
J	7 (2)	1607	100	99.41	*Azospirillum brasilense*
K	7 (1,2)	1700	100	100.00	*Achromobacter marplate*
L	7 (1, 2)	1825	100	99.5	*Cytiolbacillus sp.*
M	7 (1, 2)	1858	98	95.99	*Bacillus firmus*

[a] Sum of alignment scores of all segments from the same subject sequence
[b] Percent of the query length that is included in the aligned segments
[c] Highest percent identity for a set of aligned segments to the same subject sequence

Figure 2.1 Persistence of *Edwardsiella ictaluri* S97-773 population (log_{10} CFU g^{-1}) in sediment samples collected from 12 study chambers (2 farms × 2 ponds/farm × 3 replicate tanks per pond). Within each box plot, the horizontal line indicates the median, symbols indicate the mean, and error bars around the symbol represent the standard error of the mean. Box plots with different lowercase letters are significantly different at $P < 0.05$.

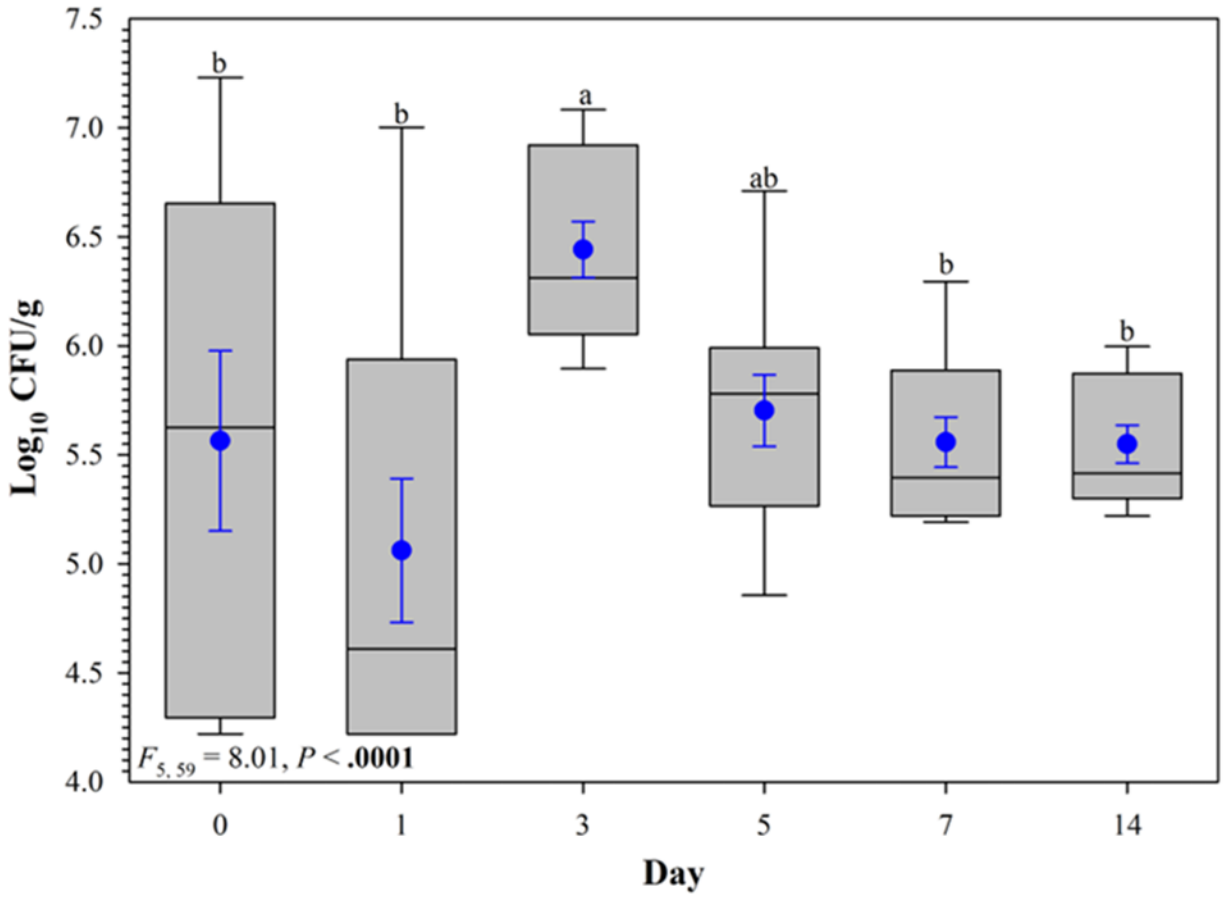

Figure 2.2 Comparison of *Edwardsiella ictaluri* population (log_{10} CFU g^{-1}) in sediment samples collected from two farms (2 ponds/farm × 3 replicate tanks per pond). Within each box plot, the horizontal line indicates the median, symbols indicate the mean, and error bars around the symbol represent the standard error of the mean.

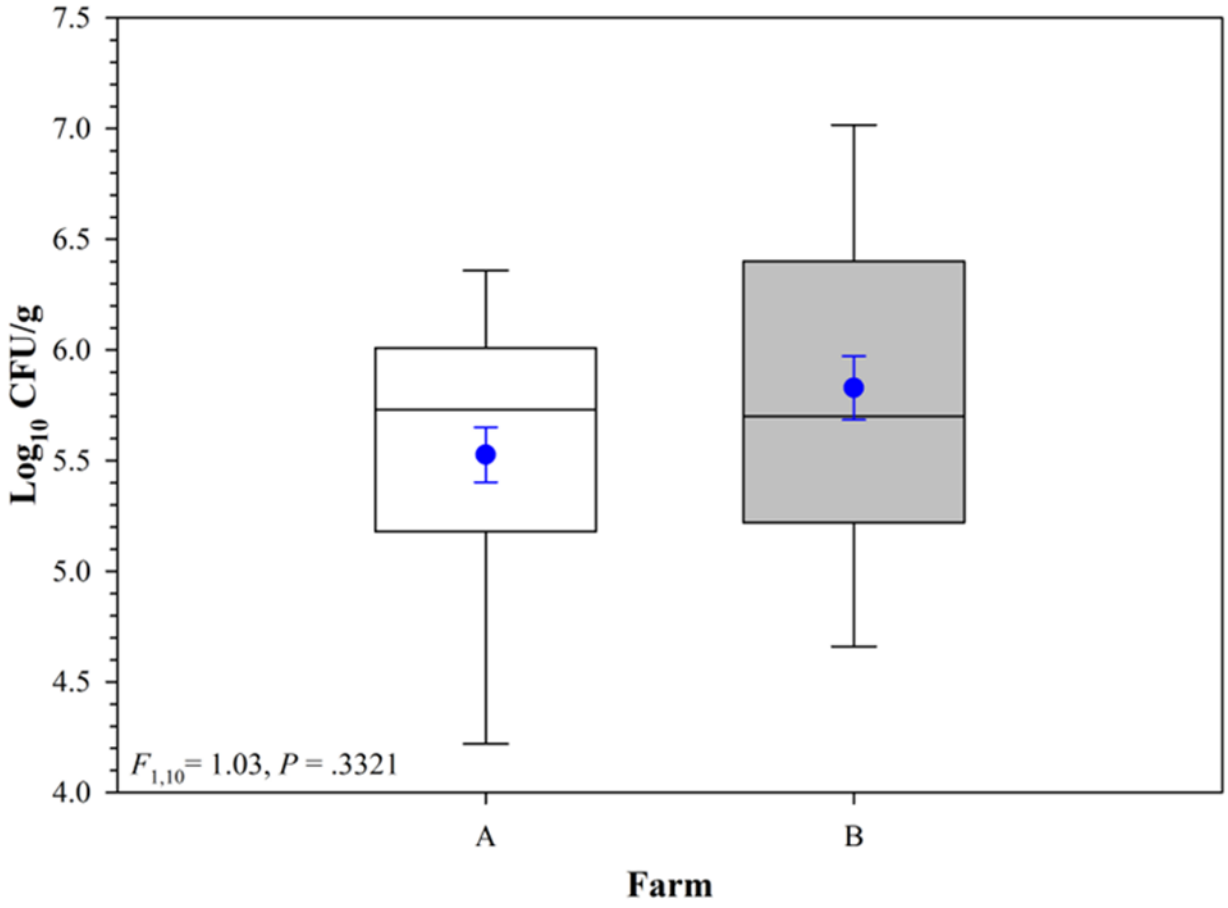

Figure 2.3 Relationship between *Edwardsiella ictaluri* population in sediment (CFU g^{-1}: A, B, and C; log_{10} CFU g^{-1}: D, E, and F) and time (Days) using a smoothing spline (SS) model and 95% confidence intervals (green shadow). Figures A and D represent all samples; B and E represent farm A; C and F represent farm B. Estimates of SS model descriptors are summarized in Table 1.

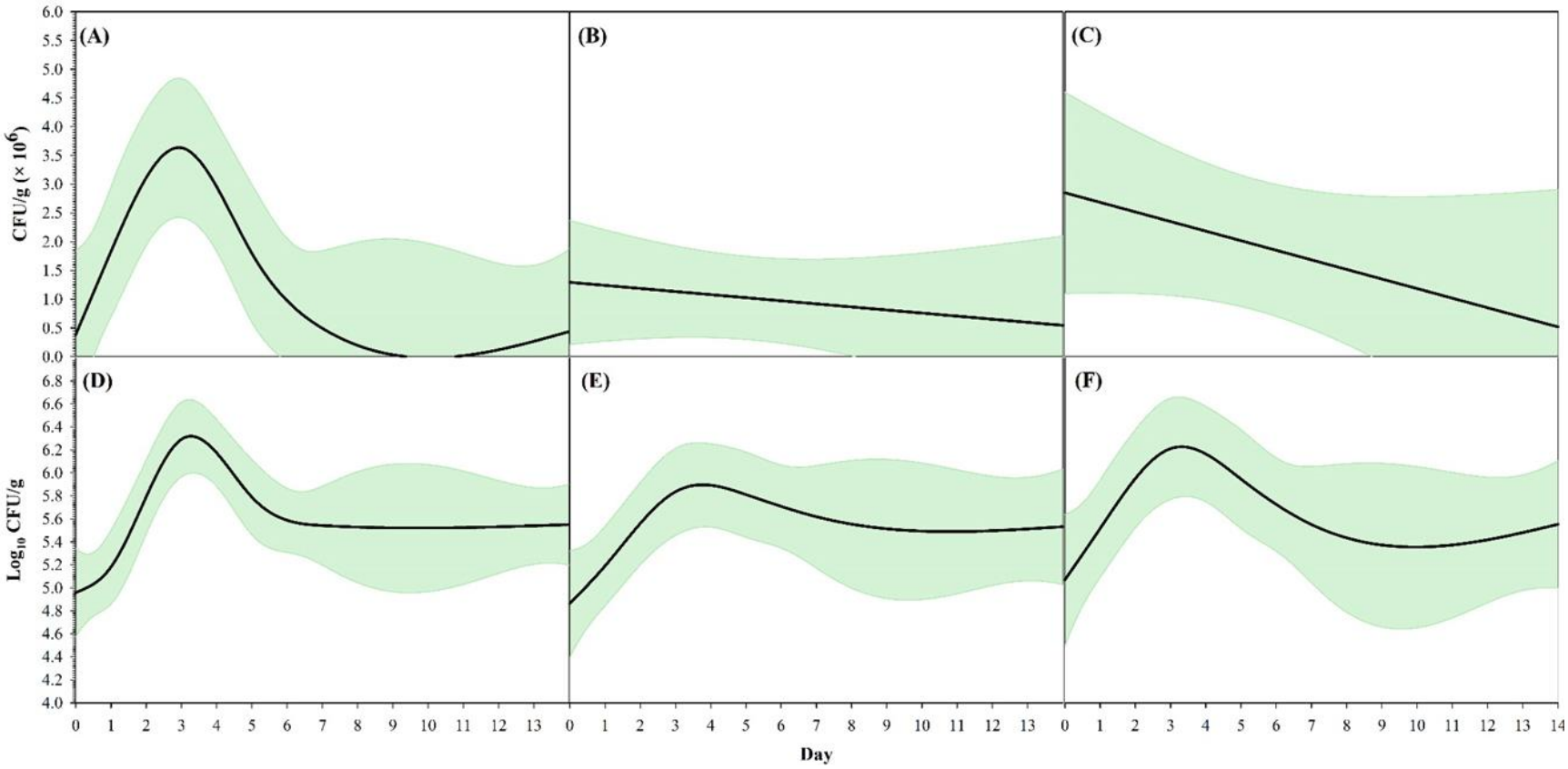

Figure 2.4 Unique bacterial colonies were visually identified on selective EIM during the EI_{PT}. All colonies expressing different sizes, morphologies, and colors were accounted for. All blue lines next to each distinct colony represent 1000 μm. Sampling days of first appearance and identities of colony types A (A), B (B), C (C), D (D), E (E), F (F), G (G), H (H), I (I), and J (J) are listed in Table 2.

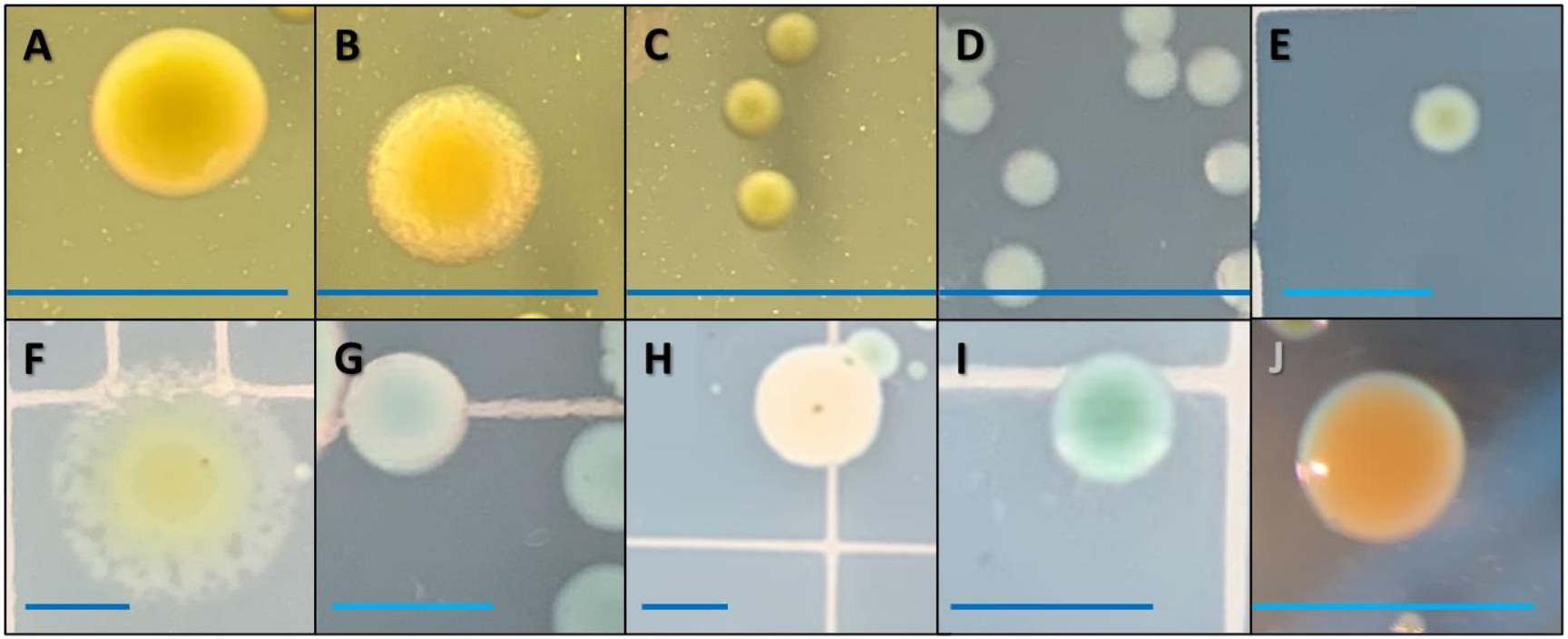

Figure 2.5 Gel electrophoresis image with visualized products of conventional polymerase chain reaction using ESCF and ESCR primers specific to *Edwardsiella ictaluri*. Bacterial isolates (arranged in order in lanes 1-19); 1, positive control (S97-773); 2-4, colony type A (Days 0, 7, and 14); 5–7, colony type B (Days 5, 7, and 14); 8-9, colony type C (Days 1 and 7); 10–11, colony type D (Days 5 and 7); 12–13, colony type E (Days 5 and 7); 14, colony type F (Day 14); 15, colony type G (Day 7); 16, colony type H (Day 14); 17, colony type I (Day 14); 18-19, colony type J (Days 5 and 7); 20, no template, negative control; M = 50 bp DNA ladder.