Variations in Antibiotic Resistance of *Aeromonas* Observed Across Lubbock Canyon Lake System

by

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ABSTRACT

Aeromonas is an aquatic organism that can be found ubiquitously all over the world. While usually a pathogenic bacterium to fish and other aquatic animals, instances of human infection have been recorded. Although uncommon, *Aeromonas'* ability to acquire new virulence factors, along with the increasing prevalence of antibiotic resistance, poses it as a possible future concern. This study aims to evaluate the prevalence of antibiotic resistance among *Aeromonas* along a connecting lake system. Total numbers of *Aeromonas* as well as ampicillin and ciprofloxacin resistance of the bacterium were evaluated among four connecting canyon lakes in Lubbock, TX. The percent difference of antibiotic resistance was evaluated for each lake and compared, and whereas no ciprofloxacin-resistant *Aeromonas* colonies were observed, the percent difference in ampicillin resistance traveling down the lake system showed a strong negative correlation (R= –0.9346) for a Pearson's Correlation Coefficient. However, this value fell below the overall critical (α=0.05, cv=0.950, df=2, 2-tailed) and cannot be considered significant. While the data show an overall increase in rates of ampicillin antibiotic resistance, decrease in percent difference, additional studies will be needed to confirm this with a larger sample size and more lakes to observe to see if a significant correlation really does exist or not.

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LIST OF ABBREVIATIONS

AIM	Aeromonas Isolation Media
AMP	Ampicillin
ANOVA	Analysis of Variance
CFU	Colony Forming Units
CIP	Ciprofloxacin
CV	Critical Value
°C	Degrees Celsius
°F	Degrees Fahrenheit
df	Degrees of Freedom
g	Grams
L	Liter
µL	Microliter
mg	Microgram
mL	Milliliter
µm	Micrometer
NF	North Fork
SF	South Fork

TX	Texas
TNTC	Too Numerous to Count
TSA	Tryptic Soy A

CHAPTER I

INTRODUCTION

Antibiotic Resistance

Antibiotics are small organic molecules produced by microorganisms that have the ability to kill or prevent the growth of bacteria. They were originally discovered by Sir Alexander Fleming in 1929 (Fleming, 1929) and since then have been the primary method for combatting bacterial infections in humans. In 2014, over 100 different antibiotics, and 10 different classes of antibiotics have been discovered and approved for medical use by the U.S. Food and Drug Administration (Berger, 2014). However, over time bacteria have become increasingly more resistant to various antibiotics, which has made it more difficult to treat certain bacterial diseases. Because of this, the Centers for Disease Control and Prevention has listed antibiotic resistance as one of the world's most immediate public health threats (Smolinski et al. 2003). With bacteria becoming resistant to greater numbers of antibiotics, along with higher resistance to them, the need for combatting and controlling antibiotic resistance is at an all-time high. Seventy percent of nosocomial infections are resistant to at least one antimicrobial drug that previously was effective for the causative pathogen (Carmeli, 2008). Certain bacteria such as *Staphylococcus aureus, Enterococcus spp., Enterobacteriaceae, Pseudomonas aeruginosa,* and *Acinetobacter spp.,* which contain notable virulence factors, have shown resistance patterns to antibiotics which include the problematic methicillin-resistant *S. aureus*, vancomycin-resistant enterococci, and even vancomycin- and methicillin-

resistant *S. aureus* (Carmeli, 2008). Recently more research and technology have been focused on discovering new antibiotics as well as new methods to combat bacterial infections (Clardy et al., 2006). However more research is also being done in order to better understand and prevent or slow down the process of antibiotic resistance for current drugs as well as future potential ones.

The occurrence of antibiotic resistance is due to several various factors, but the main one is the unavoidable process of mutation, natural selection, and evolution (Woodford and Ellington, 2006). This occurs by bacteria being able to produce molecules to directly combat an antibiotic, modify the drug's target site, or even modify their metabolic pathway to bypass the effects of the drug (Tenover, 2006). Antibiotic resistance can be found naturally in the environment through contact with wild antibiotic-producing microorganisms (Ash et al., 2002). However, several human-caused interactions have been driving forces for increased evolutionary pressures for antibiotic resistance; the most predominant of these being the inappropriate use of antibiotics and resulting increase of antibiotics entering the environment (Smolinski et al., 2003). Antibiotic resistance can also spread in the environment by means of antibiotic-resistant bacteria passing genes to the environmental flora via plasmids or transposons (Neu, 1992). These environmental florae can then serve as reservoirs for antibiotic-resistance genes and pass them along to other organisms. Antibiotic-resistant bacteria can also reach humans through agriculture and animals. Antibiotic agents used in humans are also frequently used in agriculture and food animals for promoting growth and disease prevention (Angulo et al., 2004). Antibiotic resistance can develop from these methods

via selective pressures, and these antibiotic-resistance genes can then be transferred to human microflora (Witte, 1998) or into the environmental flora.

Along with the increased problem of antibiotic resistance, the development of new antibiotics and antimicrobial drugs has been decreasing. Over a 20-year period (1998–2002 vs. 1983–1987) approval of new antimicrobial drugs by the U.S. Food and Drug Administration decreased by 56% (Spellberg et al., 2004). When looking at the number of newly approved antimicrobial drugs in 5-year intervals from 1983–2007, the number has steadily decreased, from 16 (1983–1987) to five (2003–2007) (Spellberg et al., 2008). Luckily, it seems as though more research now is being focused on development and discovery of new antimicrobial drugs.

Aeromonas

Bacteria the genus *Aeromonas*, are Gram negative, rod-shaped, non-spore forming, facultative anaerobes that are ubiquitous and primarily inhabit aquatic environments. Sharing many similarities to the family *Enterobacteriaceae*, despite being oxidase positive, the genus *Aeromonas* was originally placed in the family *Vibrionaceae* before later being placed in the family *Aeromonadaceae* (Colwell et al., 1986) along with the genera *Tolumonas*, *Oceanimonas*, *Oceanisphaera*, and *Zobellella* (Huys, 2014). There are currently 36 identified species in the genus *Aeromonas*, which include the mesophilic, motile (e.g., *A. hydrophila*, *A. veronii*, *A. caviae*) and the psychrophilic, non-motile (e.g., *A. salmonicida*) species. *Aeromonas* is ubiquitous to freshwater aquatic systems, such as lakes and rivers, and can be found at moderate variations of salinity, temperature, turbidity, and pH, but not the extremes (Hazen et al., 1978). All *Aeromonas*,

except for *A. trota,* were thought to be naturally resistant to ampicillin; however, studies by Huddleston (2007) showed that around 17% of isolated strains from playa lakes in the Lubbock, TX region were sensitive.

Aeromonas is known for being an opportunistic pathogen especially in aquatic animals, including but not limited to fish, amphibians, bivalves, reptiles, etc. (Parker and Shaw, 2011). *Aeromonas* is also known to infect humans, causing conditions such as gastroenteritis, wound infections, and septicemia (Trust and Chipman, 1979; Gracey et al., 1982). Infections from *Aeromonas* can occur through contaminated food (Kirov, 1993) as well as water-contaminated wounds from accidents such as fish hook accidents, animal bites, boating accidents, etc. (Janda and Abbott, 1996). *Aeromonas* can possess a variety of virulence factors including aerolysin, hemolysins, proteases, glycero-phospholipid:cholesterol acyltransferase, lipase, and cytotoxic enterotoxins (Janda and Abbott, 2010; Chopra et al., 2000). These virulence factors can vary depending on the individual strain, but all strains involve multiple virulence factors to infect the host (Yu et al., 2005). Because of its ability to gain and lose virulence factors, while not a big concern in medicine, the possibilities for it being a larger issue in the future are present as *Aeromonas* becomes an increasing threat to human health in the United States (Lederberg et al., 1992).

Lubbock Canyon Lake System and Playa Lakes

The Brazos River is the 11th longest river in the United States and runs through central East Texas, North Texas, and Northwest Texas and empties into the Gulf of Mexico. The Brazos begins in far North West Texas in two parts: The North Fork Double

Mountain Brazos (NF), which runs through Lubbock TX and begins between north Highway 289 and Highway 84, and the South Fork Double Mountain Brazos (SF), which runs west of Lake Alan Henry. East of Justiceburg TX, the two forks merge into the Double Mountain Fork of the Brazos River. The river runs down to northeast of Rule, TX where it merges with the Salt Fork of the Brazos River and becomes the Brazos River. From there the river runs southeast and empties into the Gulf of Mexico.

Within the city of Lubbock, a series of lakes has been built along the NF which is called the "Canyon Lake System" (Stafford, 1981). The Lake System consists of five lakes which begin at Conquistador Lake, which runs into Llano Estacado Lake (both located in the Buddy Holly Recreation Area), which then goes into Comancheria Lake, then Mackenzie Park Lake, and then Dunbar Historical Lake. From there the water of the NF travels southeast into two large reservoirs: Buffalo Springs Lake and Lake Ransom Canyon (Figure 1.1). Some of the water that feeds into the Canyon Lake System is from urban runoff, but the majority is from reclaimed water used in agriculture that, after percolation into groundwater, is collected by wells and pumped into the head of the Canyon Lake System (Conquistador Lake) (Smith et al., 1979).

Throughout the city of Lubbock, dozens of small runoff lakes called "playa lakes" can be found, and over 20,000 of these lakes are present throughout the High Plains of Texas, New Mexico, Colorado, and Oklahoma (Gustavson, 1994). These playa lakes are fed purely by rainfall and urban runoff water and the water quality is directly related to land surrounding the lake (Hall, 1997). These playa lakes are frequently seen in parks and recreational areas throughout the city of Lubbock (Figure 1.2).

Canyon Lakes and Antibiotic Resistance

Bacteria harboring antibiotic resistance are ubiquitous and able to transfer antibiotic-resistance genes from cell to cell through plasmids and transposons (Neu, 1992; Alonso et al., 2002). Water systems, such as lakes and rivers, are a major environment where this phenomenon can occur. Here, bacteria can acquire antibiotic resistance through human and animal contact and influences (Baquero et al., 2008), runoff, and effluent (Goni-Urriza et al., 2000). This process can be observed worldwide (Zhang et al., 2009). This occurs through bacteria already containing antibiotic-resistance genes being released into the environment and spreading these genes to naturally occurring microflora that can then serve as reservoirs for these genes (Martinez, 2008) including those bacteria that are not yet culturable (Riesenfeld et al., 2004). This is a major issue since water sources such as these can be major sources of consumable water and recreational activity.

The Lubbock, TX Canyon Lakes System is no exception to this. Horizontal gene transfer has been observed in *Aeromonas* isolates from Lubbock, West Texas, and New Mexico geographical area, with all observed strains acting as donors and 73% as acceptors by transformation from other aeromonads (Huddleston et al., 2013). Also, antibiotic-resistant *Aeromonas* has been isolated from Lubbock playa lakes, which includes resistance to antibiotics such as co-trimoxazole, tetracycline, and cefuroxime (Warren et al., 2004; Huddleston et al., 2006). Because all canyon lakes, playa lakes, and reservoirs in Lubbock, TX and the surrounding area are publicly accessible, it is expected that introduction of antibiotic-resistance genes to these lakes should be seen, especially in

late spring and late summer when they are used the most and bacterial numbers are highest (Warren et al., 2004).

Justification

With the increasing issue of antibiotic resistance, research has recently been focusing more on attempts to find new antibiotics and antimicrobial drugs as well as better understanding antibiotic resistance and its causes. This study aims to focus on how antibiotic resistance plays a role in environmental water systems. While there are a good number of studies focusing on antibiotic resistance in aquatic systems such as lakes and rivers, little research has been done on entire lake systems such as the Canyon Lakes System in Lubbock, TX and how antibiotic resistance can differ across the system. The bacterial genus *Aeromonas* is a good subject to observe this since it is ubiquitous to aquatic systems. Because of this, there is the potential that *Aeromonas* can serve as a reservoir for antibiotic-resistance genes in aquatic systems. Moreover, *Aeromonas* can potentially be used as an indicator species to determine the levels of antibiotic resistance that are occurring as a consequence of human manipulation.

This study will focus on the spatial trend of antibiotic resistance among the Lubbock Canyon Lakes system as water moves from its source in Conquistador Lake, down to Buffalo Springs Lake and Lake Ransom Canyon. All these lakes are publicly accessible and recreational activities have been observed at all these locations, fishing, boating, kayaking, etc. As the initial source of the water for the Canyon Lakes is secondary sewage effluent that has been land-farmed, and as all these lakes are connected, one of the major questions in this study is whether levels of antibiotic

resistance decrease as the water interacts with the natural microflora. In addition, aquatic systems such as rivers have been observed to filter out and remove some biochemicals such as hormones out of the system (Barel-Cohen, 2006). Whether or not antibiotics can be filtered out of the system is unknown; however it is likely that it would act the same, and if so there would be less pressure for antibiotic resistance farther down the lake system. To help place the Canyon Lakes antibiotic resistance dynamics in perspective, selected playa lakes in Lubbock, TX will also be assessed for antibiotic resistance. Playa lakes differing in size and amounts of public use will be compared to the results of the Canyon Lakes System for any similarities or trends.

Research Objectives

1. Observe rates of antibiotic resistance in *Aeromonas* across the Lubbock, TX Canyon Lake System from Conquistador Lake, to Mackenzie Park Lake, Dunbar Historical Lake, and to Ransom Canyon Lake.
2. Observe trends of antibiotic resistance in *Aeromonas* from the playa lakes in Maxey Park, Higginbotham Park, and Miller Park in Lubbock, TX.

Research Hypotheses

1. If antibiotic-resistance genes can pass into the environmental microflora because of human and animal exposure and we observe antibiotic resistance across the canyon lakes, then we will see the most antibiotic resistance in *Aeromonas* bacteria at the end of the lake system, Ransom Canyon, due to the cumulative effect of human and animal exposure.

2. If antibiotic-resistance genes can pass into the environmental microflora because of human and animal exposure and we observe antibiotic resistance among the playa lakes, we should see the highest rate of antibiotic resistance in those lakes that have the most human contact, Maxey Park Lake and Higginbotham Park Lake, compared to those that don't have as much, Miller Park.

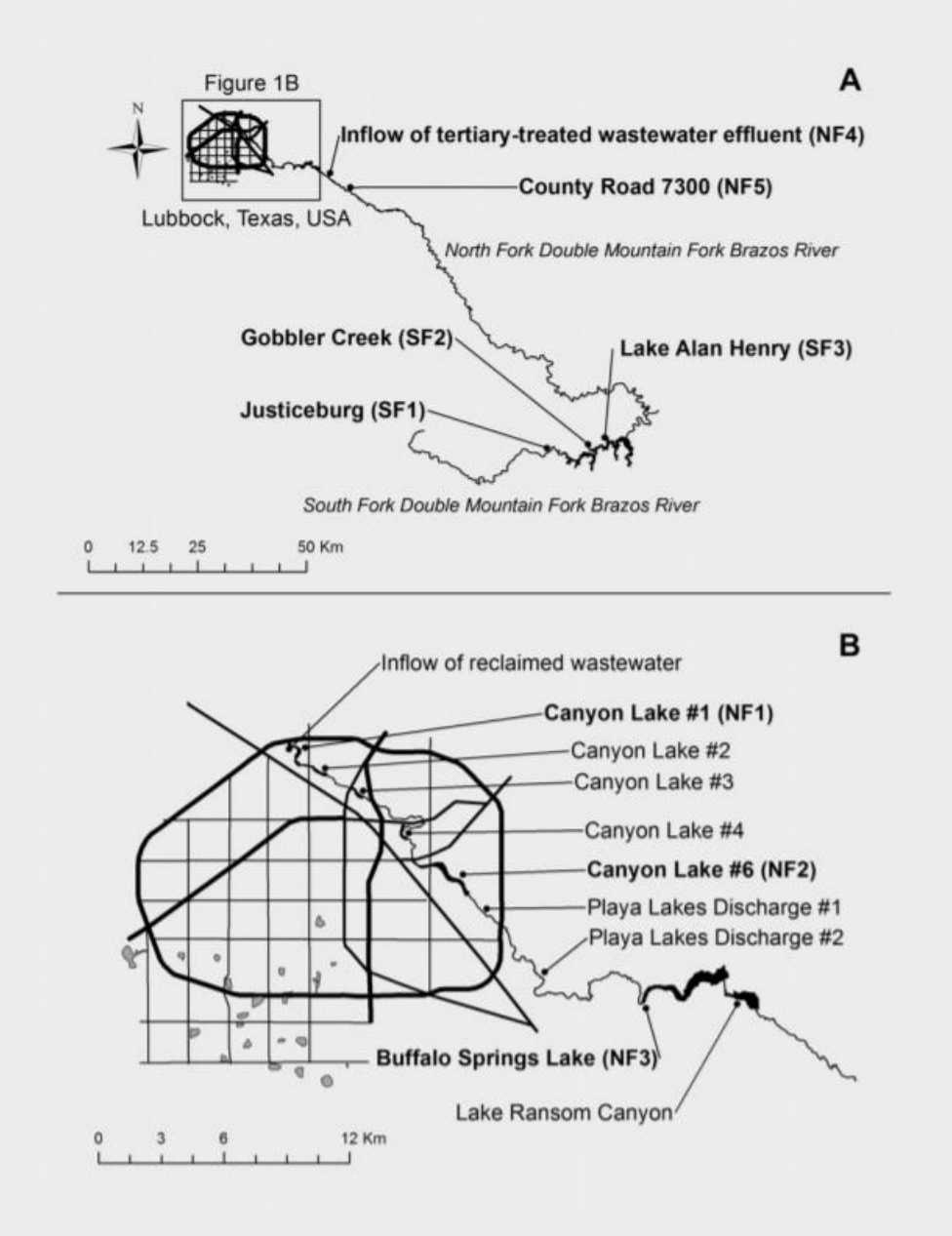

Figure 1.1: (A) Locations of the Canyon Lakes along the Double Mountain Fork Brazos River. (B) Location of Canyon Lakes through Lubbock, TX along the NF of the Double Mountain Fork Brazos River. Canyon Lakes shown here are: #1- Conquistador Lake, #2- Llano Estacado Lake, #3- Comancheria Lake, #4- Mackenzie Park Lake, #6- Dunbar Historical Lake. (Source: VanLandeghem et al., 2012).

Figure 1.2: Locations of Lubbock Playa Lakes. Lakes used in this study are also noted and as follows: a- Higginbotham Park Lake, b- Maxey Park Lake, c- Miller Park Lake.

CHAPTER II

MATERIALS AND METHODS

Materials

Media and Chemicals

Specialized *Aeromonas* isolation media were made based on the recipe from Huddleson et al. (2007): 1.0 L distilled H_2O, 4 g Soluble Starch (Difco, 0178-17), 0.25 g Ammonium Chloride (Fisher Scientific, A-649, 12125-02-9), 1.0 g Tryptone (BD Bacto, 211705, 2022-03-31), 0.5 g Yeast Extract (Fisher Biotech, BP1422-500, 8013-01-2), 40 mg Bromothymol Blue (Fisher Scientific, B-388, FL-01,0784), 15 g Agar (Fisher Scientific, BP1356-500, 987556), 50 mg Sodium Desoxycholate (Difco, 0248-15), 5.0 mL of 0.41% L-Tryptophan (Sigma, T-0254), 5.0 mL of 0.99% L-Phenylalanine (Sigma, P-2126), 1.0 mL of 10% Ampicillin (Sigma, A-9518), 1.0 mL of 10% Ciprofloxacin (TCI, C2510, 85721-33-1). Other Chemicals: Ethyl Alcohol 95% (Fisher Science Education, S73985A, AD-12171-11), NaCl (EM Science, SX0420-3, 7647-14-5). Other Media: Tryptic Soy Agar (TSA) (Difco, 236950, 3056695).

Equipment and supplies

Sterile 200-mL dilution bottles (Pyrex, Kimax), Sterile 250-ml graduated cylinders (Pyrex, Kimax), 1-L Pyrex storage bottles, Sterile Petri Dishes 100 mm x 15 mm (Fisherbrand, FB0875713), Bunsen Burner, Metal Spreader, Sterile 1.0-ml disposable pipettes (Greiner Bio-One, F160534R), Dissecting microscope (Olympus SZ Stereo), Large orifice pipet tips 1–200 µL (Fisherbrand, 21-197-2B, 20103041),

Micropipette 20–200 µL (Gilson), Autoclave (Steris, SV-120 Scientific Prevacuum Sterilizer) , Oxidase Test pads (Sigma-Adrich, BCBW3915).

Bacterial Strains

Aeromonas strains for testing and observation were taken from Lubbock isolates (Huddleston, 2003): *A. encheleia* 86 M2-1, *A. hydrophila* 88 C3-1, *A. media* 98 C2-1, *A. sobria* 103 H2-1, *A. ichthiosmia* 106 YS3-1, *A. caviae* S3-1, *A. veronii* 113 MS3-1, *A. enteropelogenes* 116 HS3-1.

Test strains from Texas Tech University MBIO 3400 teaching lab: *Pseudomonas stutzeri, Pseudomonas fluorescens, Serratia marcescens, Bacillus subtilis, Staphylococcus epidermidis.*

Methods

Collecting Water Samples

Water samples for analysis of *Aeromonas* antibiotic resistance were taken from four of the Lubbock Canyon lakes which included: Conquistador Lake (in the Buddy Holly Recreation Area), Mackenzie Park Lake, Dunbar Historical Lake, and Lake Ransom Canyon (Figure 1.1). Samples from three Lubbock playa lakes at public parks were also taken which differed in size and amounts of recreational activity: Miller Park, Maxey Park, and Higginbotham Park (Figure 1.2). Three samples of 100 mL of lake water were taken from each location and at different spots on the lake using sterilized 250-mL glass graduated cylinders. Samples were taken from shore surface water of each lake and samples were then each placed into individual sterilized 200-mL dilution bottles

and kept on ice. Samples were then stored at 4°C until ready to be used. Another set of samples were taken 2 weeks later at the same lakes and locations for additional replicates.

Preparing *Aeromonas* Isolation Media

Culture media used in this study were based on the medium used by Huddleston et al. (2007) to isolate *Aeromonas.* One change was made to this medium by decreasing the amount of sodium desoxycholate from 100 mg to 50 mg in order to prevent stress of growing *Aeromonas*. For preparing the *Aeromonas* media, 1.0 L of distilled water was mixed with 4 g soluble starch, 0.25 g ammonium chloride, 1.0 g tryptone, 0.5 g yeast extract, 40 mg bromothymol blue, and 15 g agar. The solution was mixed and autoclaved using the Liquid 20 cycle (20 min sterilization) at 121°C and 15 psi. After autoclaving and before pouring, 50 mg of sodium desoxycholate, 5 mL of 0.41% L-tryptophan, and 5 mL of 0.99% L-phenylalanine were added and mixed. Plates contained either ampicillin (AMP) or ciprofloxacin (CIP) at a concentration of 1 mL of a 100 mg/mL solution of the antibiotic was also added before pouring. AMP and CIP were chosen because most *Aeromonas* strains are known to contain ampicillin resistance and ciprofloxacin is used for treatment of *Aeromonas* infections. Medium was poured into sterile 100 mm x 15 mm Petri dishes and allowed to solidify. After the agar had gelled, plates were left at room temperature for 48 hr to dry and any contaminated plates were discarded.

Identification of *Aeromonas* on agar plates

Aeromonas isolation media was tested by growing eight different species of *Aeromonas* strains isolated from Lubbock (Huddleston, 2003) including: *A. encheleia, A. hydrophila, A. media, A. sobria, A. ichthiosmia, A. caviae, A. veronii,* and *A.*

enteropelogenes. Individual colonies of each species were grown on *Aeromonas* isolation media, with and without ampicillin, at 30°C and observed under a dissecting microscope at one day and two days of growth. Colonies were observed and recorded for their varying, shape, margin, form, and other characteristics across different species on the *Aeromonas* isolation media. Colonies were also stained with bromothymol blue and observed under oil immersion for cell size, shape, and other morphological characteristics of *Aeromonas*.

Inoculating Samples onto Agar Plates

Each water sample was tested for *Aeromonas* on the three different types of plates: *Aeromonas* isolation media (AIM), AIM+AMP, and AIM+CIP. Each water sample was inoculated onto each type of plate three times, giving nine plates total for each sample. One-tenth mL of water from the samples was spread onto each AIM and AIM+AMP plate and 1.0 mL onto each AIM+CIP plate. One mL was used for the AIM+CIP plates due to preliminary testing of the media which displayed little to no growth when 0.1 mL was inoculated. Water was spread using a metal spreader rod sterilized with 95% ethyl alcohol and a flame. Water was left to soak into the medium and then the plates were incubated upside down at 30°C for 48 hr. Visual representation of this process is shown in Figure 2.1.

Observing Plates for Growth of *Aeromonas*

After 48 hr of growth, each plate was observed under a dissecting microscope and the number of *Aeromonas* colonies were counted and recorded based on their colony characteristics. Frequently observed colonies were also chosen at random and tested for

an oxidase-positive phenotype to verify they were not *Enterobacteriaceae*, tested for a salt-sensitive phenotype by lack of growth on a TSA plate containing 3% NaCl to verify they were not *Vibrio*, and observed in the Gram stain under the oil immersion objective of a brightfield microscope. Only colonies that were confirmed as *Aeromonas* were recorded and those that had an ambiguous identification were excluded.

Data Analysis

Data from all samples and runs were averaged together for each lake, and overall means were compared. Percent differences between overall averages of total colony counts and averages for colony counts of each antibiotic resistance were calculated and compared (Difference of the two values divided by the average of the two values multiplied by 100). Percent difference was used because neither value, total *Aeromonas* or ampicillin resistant *Aeromonas*, are considered the old or original value. We are only comparing the difference in the values compared to each other, which is why percent difference instead of percent change is used. Also, because recorded total *Aeromonas* and ampicillin resistant Aeromonas were not from the same colonies (recorded ampicillin resistant *Aeromonas* were not evaluated based on those colonies isolated from the total *Aeromonas* plates) it would be inappropriate to compare the two by just a percentage of each other. A Pearson's Correlation Coefficient was used to access the significance of any trends seen between the canyon lakes.

A one-way Analysis of Variance (ANOVA) was run to access differences between total numbers of *Aeromonas* for each canyon lake as well as the percent differences between total *Aeromonas* and antibiotic-resistant *Aeromonas* between each

lake. Percent differences were converted using square-root transformation. Tukey's post-hoc test was also conducted if shown to be statistically significant ($p<0.05$) in ANOVA.

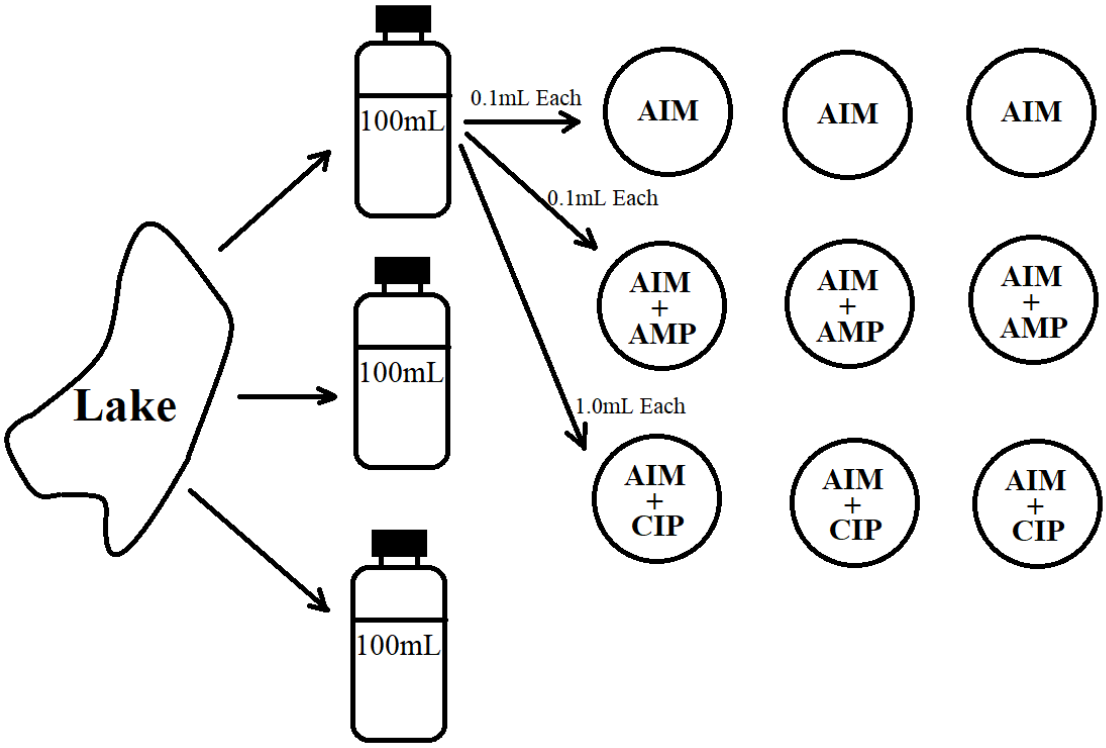

Figure 2. 1: Display of the process by which samples were taken and inoculated onto the three different agar plates: AIM, AIM+AMP, AIM+CIP. Three 100-mL samples were taken from each lake and from each of those samples, water was inoculated onto three replicates of each type of plate. 0.1 mL were inoculated onto AIM and AIM+AMP plates and 1.0 mL was inoculated onto AIM+CIP plates.

CHAPTER III

RESULTS

Identification of *Aeromonas* on Agar Plates

Individual *Aeromonas* colonies for each species were observed growing after 24 hr on AIM. All colonies were yellow in color, ranging from a pale yellow getting darker towards the center, to a deep golden yellow (Figure 2.1 and Figure 2.2). Size of colonies varied between species with *A. hydrophila, A. media,* and *A. ichthiosmia* showing larger-sized colonies, approximately 2 mm in diameter, and with the other five species producing smaller colonies, less than 1 mm in diameter (Figure 2.1 and Figure 2.2). In form, all species displayed roundish, but not perfectly round, colonies with a slightly irregular shape. Most all species showed a convex elevation except for *A. hydrophila* showing a more raised elevation. Almost all species showed an entire margin except for *A. ichthiosmia*, which displayed an undulate margin (Figure 2.3). One of the most distinct features shown for all the *Aeromonas* species was that the insides of the colonies showed a textured/cloudy structure. This ranged from being subtle to very distinct, although all species showed this characteristic.

After 48 hr of growth most species looked the same apart from the colonies being slightly larger in size and darker in color. Some species that had a more textured center became sparkly around the margins as well (Figure 2.5). *Aeromonas hydrophila* took on a darker center with the outer parts of the colony showing a pale-yellow halo, looking a little bit like a fried egg (Figure 2.6). All species were also Gram stained and examined

under the oil immersion objective with all showing thin pink/red rods of about 0.5 µm in diameter and 2.5–4 µm in length (Figure 2.7).

Observing Plates for Growth of *Aeromonas*

After 48 hr of growth all plates were examined under a dissecting microscope for *Aeromonas* colonies and colony counts were recorded. Observations were concentrated on colonies that were yellow, roundish but not perfectly round, and were textured inside the colony. Colonies that were questionable or appeared frequently were also subjected to an oxidase test and growth in the presence of 3% NaCl. All tested colonies were oxidase positive. Only two colonies tested on 3% NaCl grew, which is characteristic of the genus *Vibrio* but not *Aeromonas*, and were not counted in this study.

Aeromonas grown on media with 100 mg sodium desoxycholate grew very slowly, formed small colonies, and seemed to be under stress; some strains would not grow at all. Fifty mg desoxycholate-containing media showed much better overall growth of *Aeromonas* while still inhibiting the growth of Gram-positive bacteria when tested with *S. epidermidis* and *B. subtilis*.

All values for counted *Aeromonas* are shown in Table 1 for the first round of samples and Table 2 for the second round of samples, taken 2 weeks after the first round. All averages on AIM+AMP were less than those on the control AIM plates except for the Dunbar Historical Lake second-round samples, which showed the same average between AIM and AIM+AMP. Averages from each lake for both rounds were also averaged and are shown in Table 3.3. Overall numbers of *Aeromonas* per 0.1 mL of water at each lake

varied, with Mackenzie Park Lake showing the most *Aeromonas* overall at 93.6 cells/0.1 mL and Buddy Holly (Conquistador Lake) the least at 17.7 cells/0.1 mL for the Canyon Lakes System (Table 3.3). Playa lakes also varied in overall *Aeromonas* growth with Maxey Park Playa Lake showing the most at 246.6 cells/0.1 mL and Miller Park Playa Lake showing the least at 37.6 cells/0.1 mL (Table 3.3). The percent differences between overall *Aeromonas* and ampicillin-resistant *Aeromonas* were also recorded and are shown in Table 4. For the canyon lakes, Buddy Holly Lake showed the largest percent difference at 45.5%, which slowly decreased down the canyon lakes, except for Mackenzie Park Lake which increased slightly to 47.1%, with Ransom Canyon Lake having the smallest percent difference at 24.3%. Among playa lakes, Miller Park Playa Lake showed the highest percent difference at 79.9% and Higginbotham Park Playa Lake showed the least at 17.8%.

No *Aeromonas* grew on any of the AIM+CIP plates. While mostly fungal colonies grew on these plates, a few non-*Aeromonas* bacteria were seen growing and were recorded and shown in Table 5. Most lakes showed less than one ciprofloxacin-resistant colony per mL except for Dunbar Historical Lake and Maxey Park Playa Lake, with Maxey being the highest at approximately 1.8 colonies/mL.

Spatial Distribution of Antibiotic Resistance

Data plots for each canyon lake were put into a scatter plot, and linear regression was shown with a best fit line for the points (Figure 3.8). Pearson's Correlation Coefficient from this plot gave an R value of –0.9346. Our critical value in this situation is 0.950 (α-0.05, df-2). The R value is less than the Critical value, and therefore the null

hypothesis cannot be rejected. A third-order polynomial graph was shown for percent difference averages and displayed a sizable decrease between Mackenzie and Dunbar lakes with not much difference between the others (Figure 3.9). Scatterplot showing both samples separate is also shown in Figure 3.10 with a best-fit linear trend line and third-order polynomial for each sample. Percent differences for sample 1 showed near opposite results from sample 2 and both with poor R^2 values of 0.0769 and 0.2083 respectively.

One-way ANOVA for total *Aeromonas* was run for both rounds of samples, and both were shown to be statistically significant ($p=0.01$ & $p<0.001$) for variance between lakes (Table 3.6). Tukey's post-hoc test was run for these data sets to access the location of the variance (Table 3.7). For the first sample, Buddy Holly (Conquistador Lake) was not significant between Dunbar and Ransom Canyon but was shown to be significant between Mackenzie ($p=0.08$). Additionally, Mackenzie was shown to not be significant between Dunbar and Ransom Canyon, although close, and Dunbar was not significant between Ransom Canyon. For sample 2, Mackenzie was shown to be significant between all three lakes ($p<0.001$ for all) while all other lakes were shown to be not significant between each other.

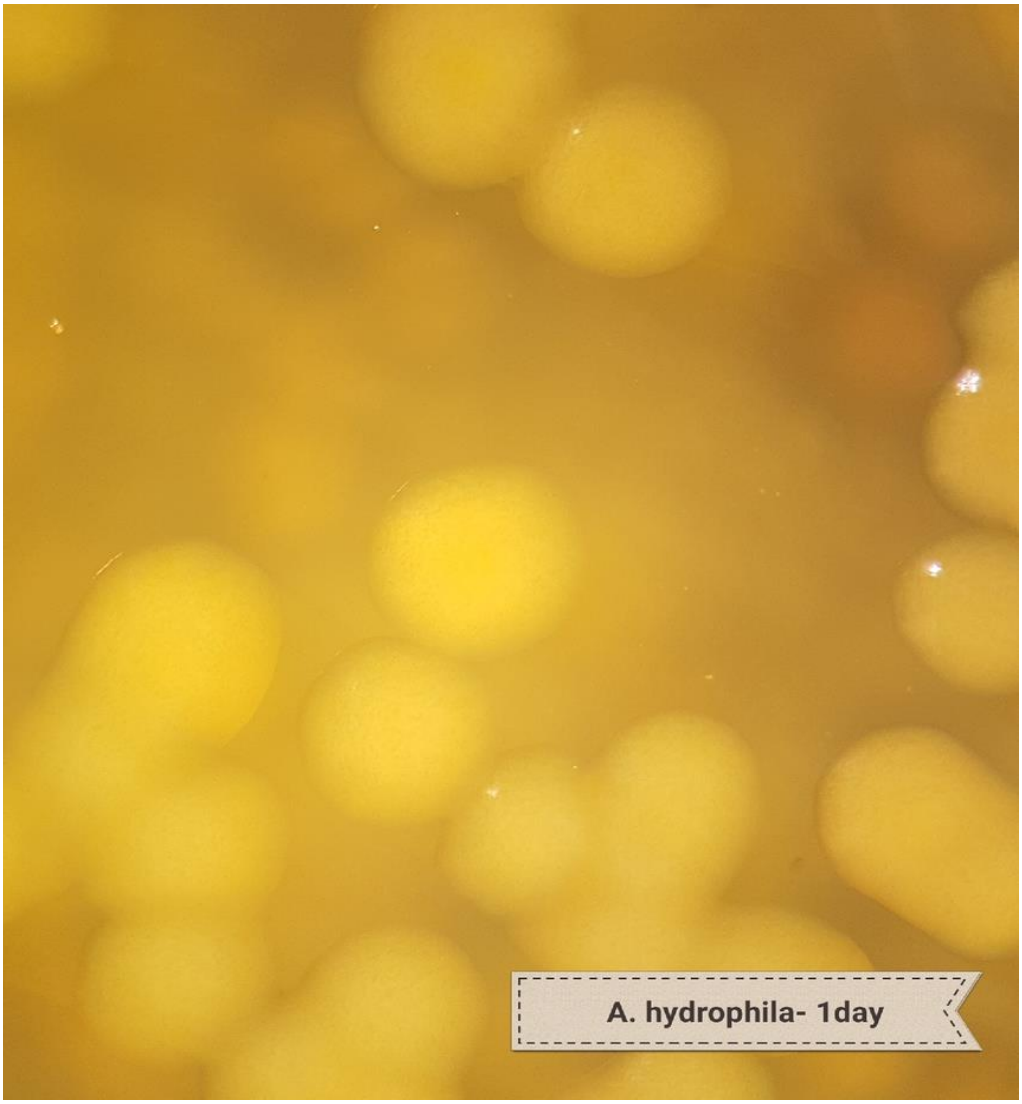

Figure 3.1: *Aeromonas hydrophila* colonies after 24 hr of growth on AIM.

Figure 3.2: *Aeromonas encheleia* colonies after 24 hr of growth on AIM.

Figure 3.3: *Aeromonas ichthiosmia* colonies after 24 hr of growth on AIM.

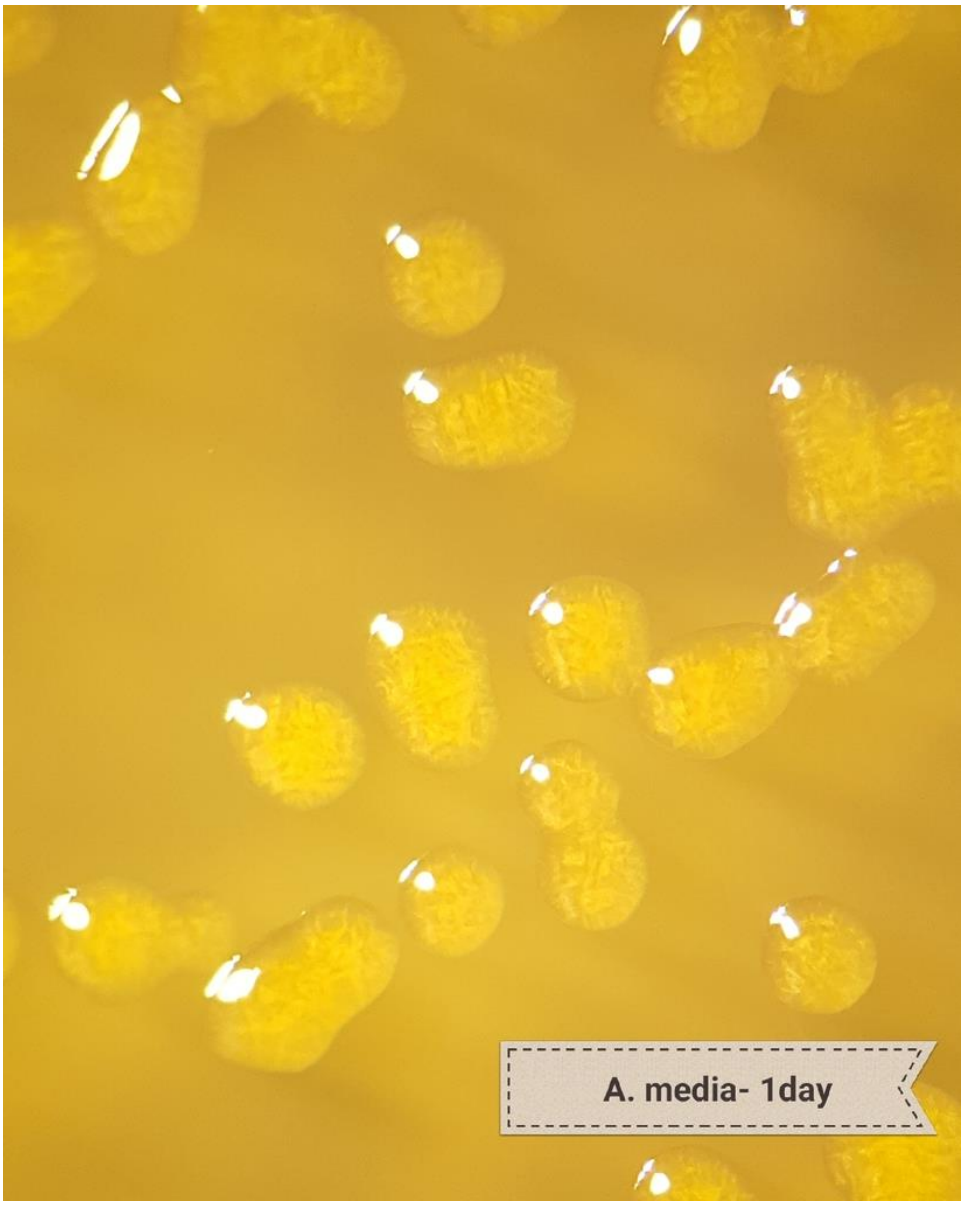

Figure 3.4: *Aeromonas media* colonies after 24 hr of growth on AIM.

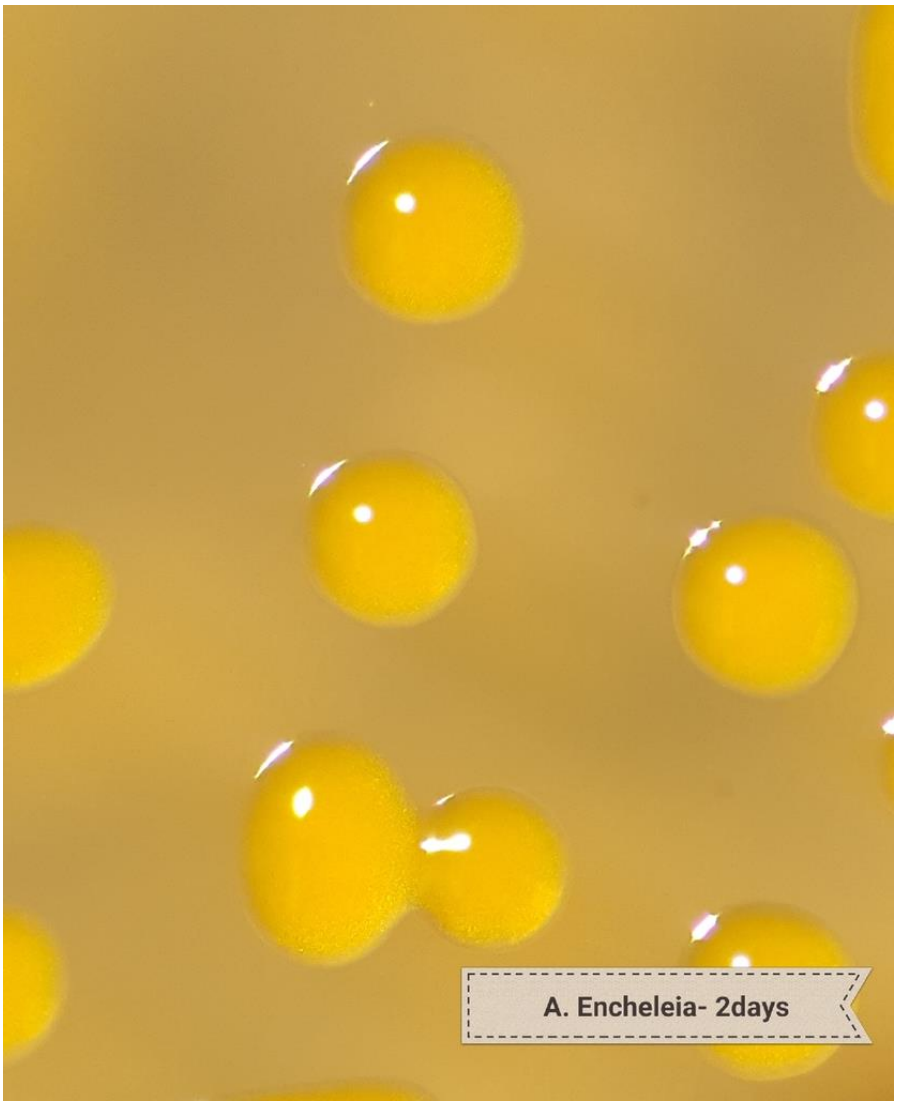

Figure 3.5: *Aeromonas encheleia* colonies after 48 hr of growth on AIM.

Figure 3.6: *Aeromonas hydrophila* colonies after 48 hr of growth on AIM.

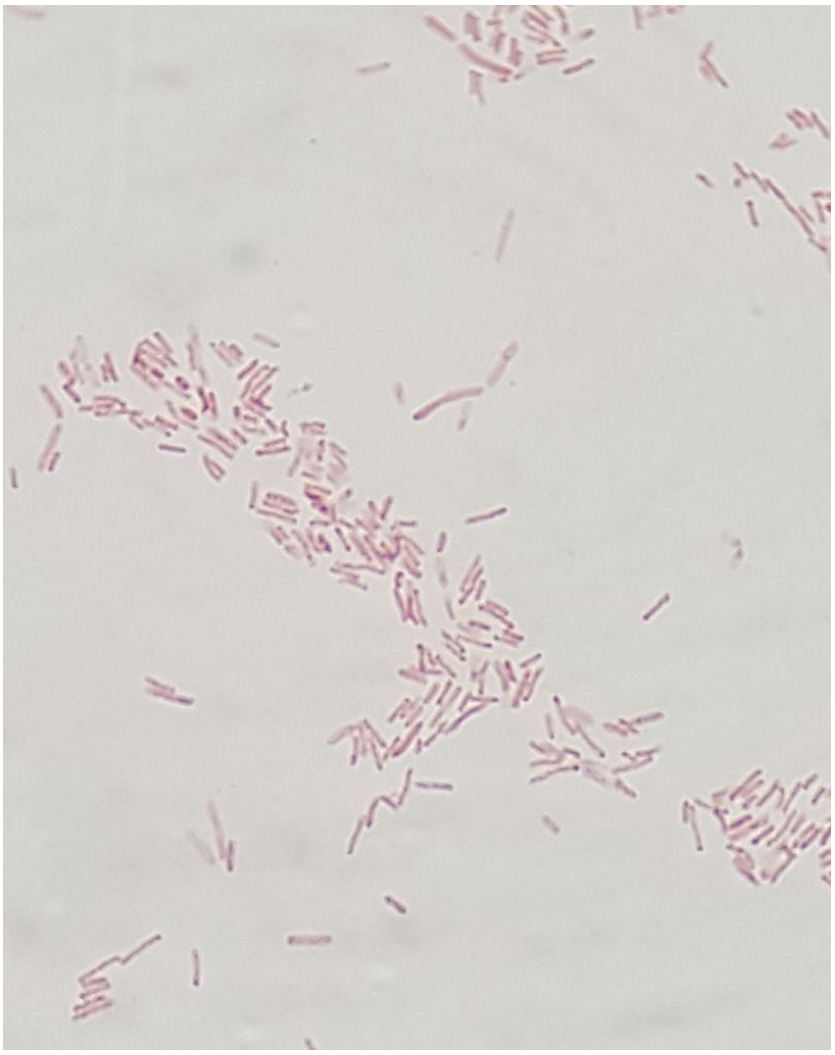

Figure 3.7: Gram stain of *Aeromonas media* under oil immersion showing thin pink rod shaped cells approx. 3 µm in length and approx. 0.5 µm in width.

Table 3.1: *Aeromonas* colony counts on each plate for the first round of samples including those for AIM and AIM+AMP. Three replicates were made for each sample and those values were averaged for each sample. Three samples for each lake were averaged giving an overall average for the lake, shown in bold. Samples were taken at: Buddy Holly Park (Conquistador Lake), Mackenzie Park Lake, Dunbar Historical Lake, Ransom Canyon Lake, Miller Park Playa Lake, Maxey Park Playa Lake, and Higginbotham Park Playa Lake. Numbers indicate the counted number of *Aeromonas* colonies on each plate. TNTC = Too numerous to count.

	AIM1	AIM2	AIM3	AVG	AMP1	AMP2	AMP3	AVG
BH1	31	17	23	23.7	7	10	14	10.3
BH2	14	21	15	16.7	8	2	6	5.3
BH3	19	29	16	21.3	11	24	9	14.7
				20.6				**10.1**
Mack1	16	19	33	22.7	15	21	17	17.7
Mack2	60	32	47	46.3	18	24	30	24.0
Mack3	147	118	166	143.7	150	120	122	130.7
				70.9				**57.4**
Dunbar1	13	22	13	16.0	11	24	19	18.0
Dunbar2	39	60	26	41.7	9	10	5	8.0
Dunbar3	42	33	55	43.3	23	24	28	25.0
				33.7				**17.0**
Ransom1	33	25	33	30.3	21	16	16	17.7
Ransom2	22	32	51	35.0	28	18	15	20.3
Ransom3	39	TNTC	22	30.5	34	28	26	29.3
				31.9				**22.4**
Miller1	63	49	23	45.0	9	9	11	9.7
Miller2	86	146	56	96.0	23	28	31	27.3
Miller3	45	55	43	47.7	36	24	42	34.0
				62.9				**23.7**
Maxey1	440	400	360	400.0	360	310	320	330.0
Maxey2	270	214	251	245.0	269	224	255	249.3
Maxey3	166	196	146	169.3	58	73	63	64.7
				271.4				**214.7**
Higgin1	84	159	142	128.3	164	127	103	131.3
Higgin2	314	320	321	318.3	300	221	293	271.3
Higgin3	78	87	55	73.3	56	67	63	62.0
				173.3				**154.9**

Table 3.2: *Aeromonas* colony counts on each plate for the second round of samples, 2 weeks after the first samples were taken, including those for AIM and AIM+AMP. Three replicates were made for each sample and those values were averaged for each sample. Three samples for each lake were averaged giving an overall average for the lake, shown in bold. Samples taken were: Buddy Holly Park (Conquistador Lake), Mackenzie Park Lake, Dunbar Historical lake, Ransom Canyon Lake, Miller Park Playa Lake, Maxey Park Playa Lake, and Higginbotham Park Playa Lake. Numbers indicate the counter number of *Aermonas* colonies on each plate X = No growth on plate.

	AIM1	AIM2	AIM3	AVG	AMP1	AMP2	AMP3	AVG
BH1	8	9	13	10.0	9	6	11	8.7
BH2	1	8	7	5.3	3	4	2	3.0
BH3	24	40	23	29.0	19	33	22	24.7
				14.8				**12.1**
Mack1	191	202	184	192.3	88	105	112	101.7
Mack2	97	103	66	88.7	41	44	33	39.3
Mack3	54	56	X	55.0	37	42	23	34.0
				112.0				**58.3**
Dunbar1	97	64	63	74.7	113	93	76	94.0
Dunbar2	21	28	22	23.7	6	11	8	8.3
Dunbar3	14	9	12	11.7	8	9	6	7.7
				36.7				**36.7**
Ransom1	18	15	11	14.7	12	17	13	14.0
Ransom2	33	25	19	25.7	30	24	24	26.0
Ransom3	9	15	17	13.7	8	9	13	10.0
				18.0				**16.7**
Miller1	7	8	4	6.3	6	3	2	3.7
Miller2	14	6	11	10.3	9	10	4	7.7
Miller3	10	17	33	20.0	15	12	16	14.3
				12.2				**8.6**
Maxey1	253	199	88	180.0	98	115	78	97.0
Maxey2	212	X	89	150.5	102	60	82	81.3
Maxey3	415	310	183	302.7	303	159	197	219.7
				211.1				**132.7**
Higgin1	37	30	38	35.0	10	31	17	19.3
Higgin2	80	81	79	80.0	45	55	41	47.0
Higgin3	71	50	43	54.7	46	47	44	45.7
				56.6				**37.3**

Table 3.3: Average number of *Aeromonas* colonies counted per 0.1 mL on AIM and AIM+AMP for each canyone lake and playa lake. values are means ± standard error from each lake for both sample times combined.

Lake	Average number of *Aeromonas*/0.1 mL	Average number of ampicillin-resistant *Aeromonas*/0.1 mL
Buddy Holly	17.7 ± 9.7	11.1 ± 8.4
Mackenzie	93.6 ± 63.4	57.9 ± 44.7
Dunbar	35.2 ± 24.1	26.8 ± 32.3
Ransom Canyon	24.6 ± 11.0	19.6 ± 7.6
Miller	37.6 ± 36.2	16.1 ± 11.5
Maxey	246.6 ± 107.4	173.7 ± 106.0
Higginbotham	114.9 ± 99.3	96.1 ± 90.1

Table 3.4: Percent difference between average overall *Aeromonas* colonies and ampicillin-resistant *Aeromonas* colonies (Table 3.3) for each canyon lake and playa lake tested.

Lake	% Difference
Buddy Holly	45.6
Mackenzie	47.1
Dunbar	26.9
Ransom Canyon	24.3
Miller	79.9
Maxey	34.7
Higginbotham	17.8

Table 3.5: Overall number of isolated Ciprofloxacin-resistant bacterial colonies from each canyon lake and playa lake. Middle column shows total bacterial colonies isolated from all plates for each lake. Right column shows calculated number of resistant bacteria for each lake per mL of lake water.

Lake	Total number of bacterial ciprofloxacin-resistant colonies	Ciprofloxacin-resistant bacteria per mL
Buddy Holly	4	0.7
Mackenzie	2	0.3
Dunbar	7	1.2
Ransom Canyon	2	0.3
Miller	4	0.7
Maxey	11	1.8
Higginbotham	0	0

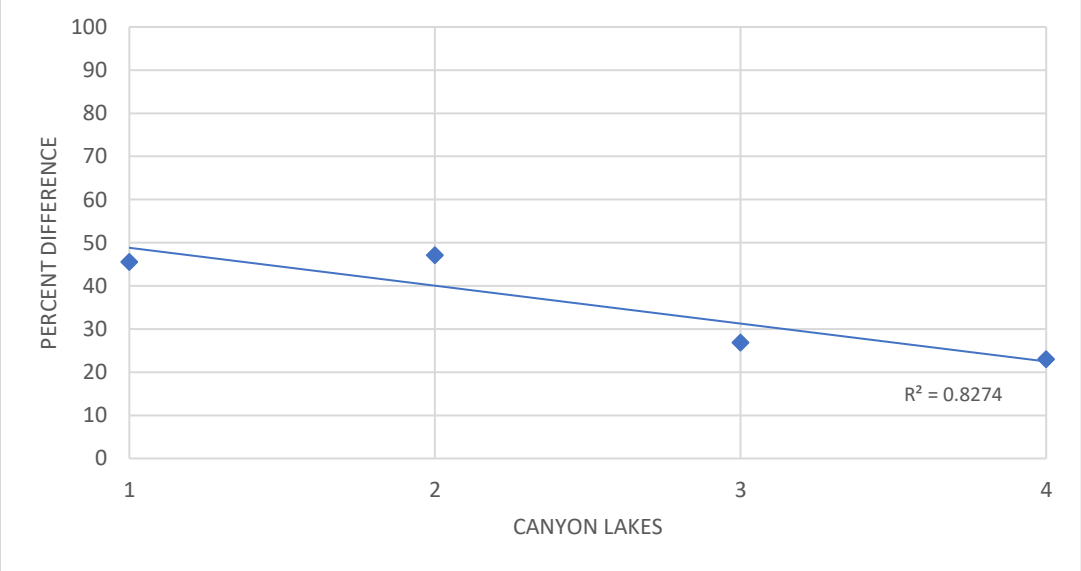

Figure 3.8: Scatter plot for the percent difference values (Table 3.4) of the Canyon Lakes and a best-fit trend line and R^2 Value was added. X-axis for lakes are placed in order from left to right based on water flow through the canyon lakes from start to end. Numbers associated with the following lakes: 1-Buddy Holly (Conquistador), 2-Mackenzie, 3-Dunbar, 4-Ransom Canyon.

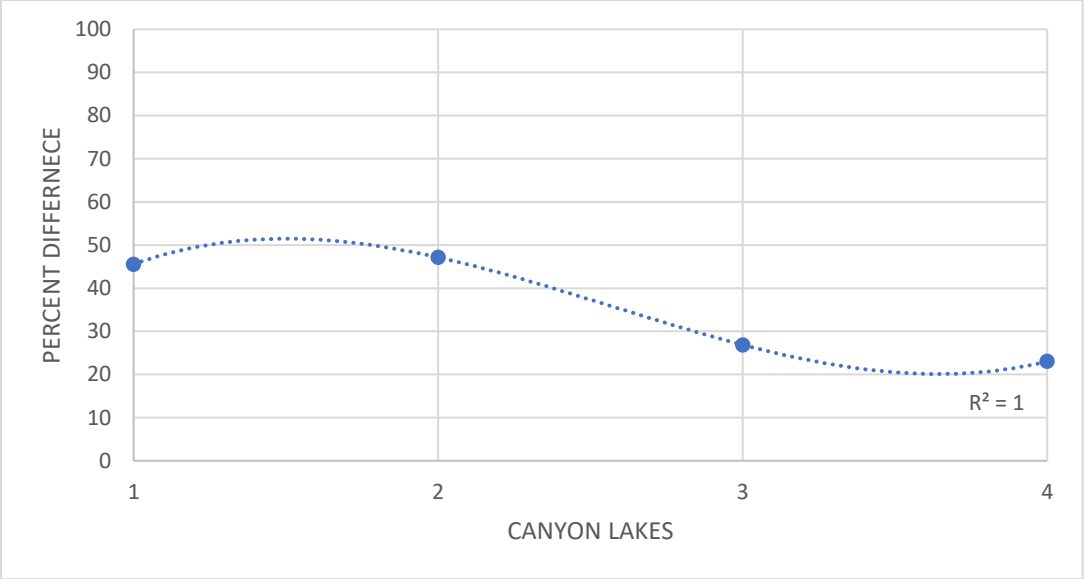

Figure 3. 9: Third-order polynomial graph for the percent difference values between total *Aeromonas* and antibiotic resistant *Aeromonas* of the Canyon Lakes (Table 3.4) with best fit trend line and R^2 value added. X-axis for lakes are placed in order from left to right based on water flow through the canyon lakes from start to end Numbers are associated with the following lakes: 1-Buddy Holly (Conquistador), 2-Mackenzie, 3-Dunbar, 4-Ransom Canyon.

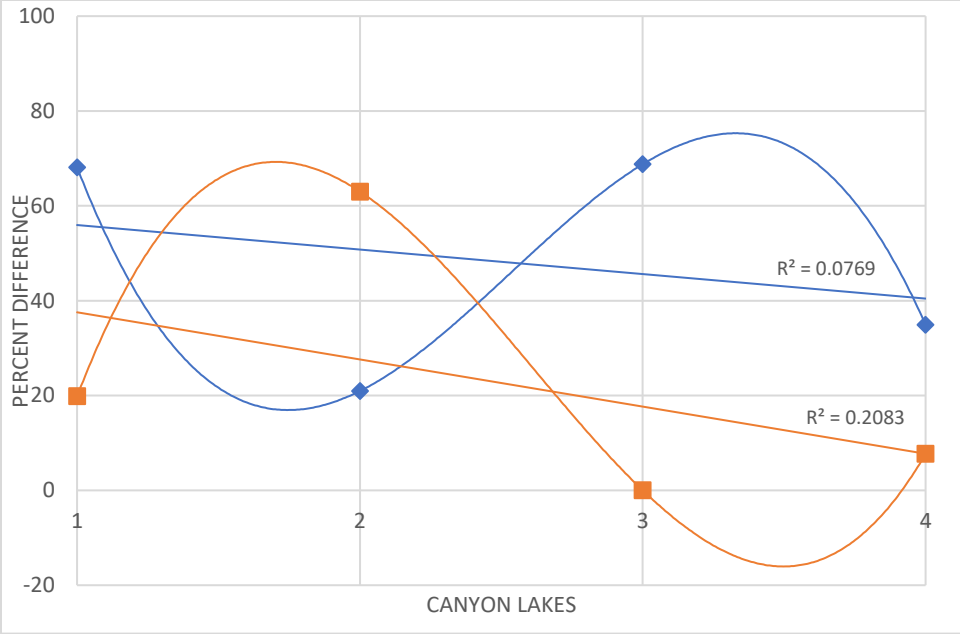

Figure 3.10: Scatter plot showing percent difference values between total *Aeromonas* and antibiotic resistant *Aeromonas* of the Canyon Lakes for both samples separate: Blue- Sample 1, Orange- Sample 2. Best-fit linear and third-order polynomial trend lines weres added for each as well as R^2 values for both linear trend lines. X-axis for lakes are placed in order from left to right based on water flow through the canyon lakes from start to end Numbers are associated with the following lakes: 1-Buddy Holly (Conquistador), 2-Mackenzie, 3-Dunbar, 4-Ransom Canyon.

Table 3.6: One-way ANOVA for both samples, sample 1 and sample 2, of total *Aeromonas* between canyon lakes using IBM SPSS 24. Shows values between lake groups, within lake groups, and total values for the sum of squares, df, mean square, F value, and Signifigance.

		Sum of Squares	df	Mean Square	F	Sig.
Total *Aeromonas* Isolated from Canyon Lakes, Sample 1	Between Groups	12838.757	3	4279.586	4.474	.010
	Within Groups	29653.986	31	956.580		
	Total	42492.743	34			
Total *Aeromonas* Isolated from Canyon Lakes, Sample 2	Between Groups	59362.312	3	19787.437	16.518	.000
	Within Groups	37136.431	31	1197.949		
	Total	96498.743	34			

Table 3.7: Tukey's post-hoc test for both samples, sample 1 and sample 2, of total *Aeromonas* between canyon lakes using IBM SPSS 24. Shows the mean difference values, standard error, and significance for each canyon lake compared to the others.

Dependent Variable		(I) Canyon Lakes	(J) Canyon Lakes	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
Total *Aeromonas* Isolated from Canyon Lakes, Sample 1	Tukey HSD	Buddy Holly	Mackenzie	-50.33333*	14.57990	.008	-89.9042	-10.7624
			Dunbar	-13.11111	14.57990	.805	-52.6820	26.4598
			Ransom_Canyon	-11.56944	15.02861	.867	-52.3582	29.2193
		Mackenzie	Buddy Holly	50.33333*	14.57990	.008	10.7624	89.9042
			Dunbar	37.22222	14.57990	.071	-2.3487	76.7931
			Ransom_Canyon	38.76389	15.02861	.067	-2.0248	79.5526
		Dunbar	Buddy Holly	13.11111	14.57990	.805	-26.4598	52.6820
			Mackenzie	-37.22222	14.57990	.071	-76.7931	2.3487
			Ransom_Canyon	1.54167	15.02861	1.000	-39.2471	42.3304
		Ransom_Canyon	Buddy Holly	11.56944	15.02861	.867	-29.2193	52.3582
			Mackenzie	-38.76389	15.02861	.067	-79.5526	2.0248
			Dunbar	-1.54167	15.02861	1.000	-42.3304	39.2471
Total Aeromonas Isolated from Canyon Lakes, Sample 2	Tukey HSD	Buddy Holly	Mackenzie	-104.34722*	16.81812	.000	-149.9928	-58.7016
			Dunbar	-21.88889	16.31597	.544	-66.1716	22.3938
			Ransom_Canyon	-3.22222	16.31597	.997	-47.5049	41.0605
		Mackenzie	Buddy Holly	104.34722*	16.81812	.000	58.7016	149.9928
			Dunbar	82.45833*	16.81812	.000	36.8127	128.1039
			Ransom_Canyon	101.12500*	16.81812	.000	55.4794	146.7706
		Dunbar	Buddy Holly	21.88889	16.31597	.544	-22.3938	66.1716
			Mackenzie	-82.45833*	16.81812	.000	-128.1039	-36.8127
			Ransom_Canyon	18.66667	16.31597	.666	-25.6161	62.9494
		Ransom_Canyon	Buddy Holly	3.22222	16.31597	.997	-41.0605	47.5049
			Mackenzie	-101.12500*	16.81812	.000	-146.7706	-55.4794
			Dunbar	-18.66667	16.31597	.666	-62.9494	25.6161

*. The mean difference is significant at the 0.05 level.

Table 3.8: One-way ANOVA for percent differences between total *Aeromonas* and ampicillin resistant *Aeromonas* in each canyon lake (Square-root transformed) for both samples, sample 1 and sample 2, and both samples combined using IBM SPSS 24. Shows values between lake groups, within lake groups, and total values for the sum of squares, df, mean square, F value, and Signifigance.

		Sum of Squares	df	Mean Square	F	Sig.
Percent Differences between Total *Aeromonas* and Ampicillin Resistant *Aeromonas* (Sqrt Transformed)- Sample 1	Between Groups	57.706	3	19.235	2.359	.091
	Within Groups	252.759	31	8.154		
	Total	310.465	34			
Percent Differences between Total *Aeromonas* and Ampicillin Resistant *Aeromonas* (Sqrt Transformed)- Sample 2	Between Groups	47.526	3	15.842	2.502	.078
	Within Groups	196.302	31	6.332		
	Total	243.828	34			
Percent Differences between Total *Aeromonas* and Ampicillin Resistant *Aeromonas* (Sqrt Transformed)- Samples Combined	Between Groups	36.271	3	12.090	1.512	.219
	Within Groups	527.646	66	7.995		
	Total	563.918	69			

CHAPTER IV

DISCUSSION

Although the overall trend between the Canyon Lakes displays an overall positive correlation in prevalence of antibiotic resistance among canyon lakes and a decrease in percent difference upon moving down the lake system, this cannot be confirmed statistically. While the correlation for the overall averages was strong, because the R value was less than that of the Critical value, the data could have been due to chance and the R value does not reach the threshhold of statistical significance (Hunt, 1986). Therefore, we cannot accept the proposed hypothesis 1 and instead must accept the null hypothesis.

One thing that was frequently observed in this study was the high degree of variation in both total *Aeromonas* counts and percent difference with ampicillin-resistant *Aeromonas.* This phenomenon is not unknown and has been observed frequently over the decades (Van Donsel, 1967, Warren, 2004). As can be seen in Table 3.3, some of the lakes such as Mackenzie, Dunbar, and Maxey have huge standard deviations for each, some almost as large as the mean itself. Also shown in Figure 3.10 where the percent differences are separated for each sample run, we see surprisingly almost opposite results. Correlation values of the best-fit line for each week's results were poor for both, which is an opposing trend to what we see in the overall averages. With both rounds of samples being taken 2 weeks apart, it raises the question if other factors may have caused this high degree of variation in both total cells and rate of antibiotic resistance. This is

especially true for the Mackenzie and Dunbar Canyon Lakes where the most variation is seen in the way of a large drop in overall *Aeromonas* going from Mackenzie to Dunbar along with a large drop in percent difference for combined samples. This high degree of variation is consistent with previous studies and can be due to several phenomena including various pH, temperature, hydrological retention time, landscape, and dissolved organic carbon (Lindstrom et al., 2005, Sommaruga, 2009, Yannarell, 2005, Yannarell, 2004). The variance in these results could be due to these reasons as well as the observation of large amounts of rain between the two weeks that samples were taken, which could skew certain factors such as hydrological retention time. More studies would need to be conducted at each of these lakes to learn more about any differences they might possess between each other. Because of this high degree of variation for each lake and possible differences in characteristics, more samples at different time points would need to be made in order to better understand what is going on as well as to have more power in the ANOVA by increasing the degrees of freedom (Hoaglin, 1976). Having samples at multiple points of the lakes with varying conditions could also assist us in better understanding the true nature for each lake.

Although this study was unable to show a significant trend of antibiotic resistance among the Canyon Lakes, the possibility is still present due to the fact that an overall negative correlation was observed with a high R value. Further studies can determine if there really is no relationship between rates of antibiotic resistance among the Canyon Lakes, or if there really is one. These studies might include using more samples and runs, as stated earlier due to our high degree of variance, as well as collecting from more of the

Canyon Lakes, which include Llano Estacado Lake, Comancheria Lake, and Buffalo Springs Lake. This would allow us to have a larger sample size and increase the overall precision of the data. Another option would also be metagenomic analysis by 16S rRNA gene identification (Muyzer, 1993) to more accurately identify *Aeromonas.* While in this study much caution and consistency went into identifying *Aeromonas* colonies on the agar plates, there is still the possibility of some *Aeromonas* colonies being missed or other non-aeromonads being counted. Genetic analysis would allow for even higher accuracy in the number of *Aeromonas* counted.

While no *Aeromonas* grew on any of the AIM+CIP plates, some other bacterial colonies were seen growing and were recorded. The highest numbers were found in Maxey Park Playa Lake at an average value of 1.8 colony-forming units (CFU)/mL. The reason for this could be due to the location of this lake and interaction with its immediate surroundings, which include a playground, baseball fields, and a hospital. Observations made when taking samples included seeing many people in the vicinity engaging in a variety of activities such as fishing, playing, sports, etc. However, comparison with Higginbotham Park Playa Lake, which was also observed to have a large amount of recreational activity, puts this into question. Focus then turns to Covenant Children's Hospital north of Maxey Park Playa Lake. Because the rate of antibiotic resistance is shown to be much higher in clinical isolates rather than environmental ones (Aravena-Roman et al., 2012), it might be possible that more clinical isolates are being passed into the nearby lake and transferring antibiotic-resistance genes to the environmental flora. A possible analysis in comparing genetic similarities between antibiotic-resistance genes in

clinical isolates from the hospital to those found in Maxey Park Playa Lake could help determine if this might be the case. Dunbar Historical Lake also showed a fairly high number of ciprofloxacin-resistant bacteria compared to the other lakes at 1.2 CFU/mL. While this lake is also seen to have high amounts of recreational activity, especially fishing, the reason behind this is unknown.

Literature Cited

Alonso, A., Sanchez, P., & Martinez, J. L. (2002). Environmental Selection of Antibiotic Resistance Genes. *Environmental Microbiology*, 3(1), 1-9.

Angulo, F. J., Baker, N. L., Olsen, S. J., Anderson, A., & Barrett, T. J. (2004). Antimicrobial Use in Agriculture: Controlling the Transfer of Antimicrobial Resistance to Humans. *Seminars in Pediatric Infectious Diseases*, 15(2), 78-85.

Aravena-Roman, M., Inglis, T. J. J., Henderson, B., Riley, T. V., & Chang, B. J. (2012). Antimicrobial Susceptibilities of *Aeromonas* Strains Isolated from Clinical and Environmental Sources to 26 Antimicrobial Agents. *Antimicrobial Agents and Chemotherapy*, 56(2), 1110-1112.

Ash, R. J., Mauck, B., & Morgan, M. (2002). Antibiotic Resistance of Gram-Negative Bacteria in Rivers, United States. *Emerging Infectious Diseases*, 8(7), 713-716.

Barel-Cohen, K., Shore, L. S., Shemesh, M., Wenzel, A., Mueller, J., & Kronfeld-Schor, N. (2006). Monitoring of Natural and Synthetic hormones in a polluted river. *Journal of Environmental Management*, 78(1), 16-23.

Baquero, F., Martinez, J. L., & Canton, R. (2008). Antibiotics and Antibiotic Resistance in Water Environments. *Current Opinion in Biotechnology*, 19(3), 260-265.

Berger, S. (2014). GIDEON Guide to Antimicrobial Agents (*2014 Edition*). GIDEON Informatics, INC, 2014.

Carmeli, Y. (2008). Strategies for Managing todays infections. *Clinical Microbiology and Infection*, 14(3), 22-32.

Chopera, A. K., Xu, X., Ribardo, D., Gonzalez, M., Kuhl, K., Peterson, J. W., & Houston, C. W. (2000). The Cytotoxic Enterotoxin of *Aeromonas hydrophila* Induces Proinflammatory Cytokines Production and Activates Arachidonic Acid Metabolism in Macrophages. *Infection and Immunity*, 68(5), 2808-2818.

Clardy J., Fischbach M. A., & Walsh, C. T. (2006). New Antibiotics from Bacterial Natural Products. *Nature Biotechnology*, 24, 1541-1550.

Colwell, R. R., MacDonell, M. R., & De Ley, J. (1986). Proposal to Recognize the Family Aeromonadaceae fam. nov. *International Journal of Systematic Bacteriology*, 36, 473-477.

Fleming, A. (1929). On the Antibacterial Action of Cultures of a *Penicillium*, with Special Reference to their Use in the Isolation of *B. influenzae*. *The British Journal of Experimental Pathology*, 10(3), 226-236.

Goni-Urriza, M., Capdepuy, M., Arpin C., Raymond, N., Caumette, P., & Quentin, C. (2000). Impact of an Urban Effluent on Antibiotic Resistance of Riverine *Enterobacteriaceae* and *Aeromonas spp. Applied and Environmental Microbiology*, 66(1), 125-132.

Gracey, M., Burke, V., & Robinson, J. (1982). Aeromonas-Associated Gastroenteritis. *The Lancet*, 320(8311), 1304-1306.

Gustavson, T. C. (1994). Development of Playa Basins, Southern High Plains Texas and New Mexico, p. 5-14. *In* L. V. Urban and A. W. Wyatt (ed.), Proceedings of the Playa Basin Symposium. Water Resources Center, Texas Tech University, Lubbock.

Hall, D. L. (1997). Species Diversity of Aquatic Macroinvertibrates in Playa Lakes: Island Biogeographic and Landscape Influences. Ph.D dissertation. Texas Tech University, Lubbock.

Hazen, T. C., Fliermans, C. B., Hirsch, R. P., & Esch, G. W. (1978). Prevalence and Distribution of *Aeromonas hydrophila* in the United States. *Applied and Environmental Microbiology,* 36(5), 731-738.

Hoaglin, D. C., & Welsch, R. E. (1976). The Hat Matrix in Regression and ANOVA. The *American Statician,* 31(1), 17-22.

Huddleston, J. R. (2003). Antibiotic and Metal-Resistant *Aeromonas* Isolated from Environmental Sources. Texas Tech University, Lubbock, Texas.

Huddleston, J. R., Zak, J. C., & Jeter, R. M. (2006). Antimicrobial Susceptibilities of *Aeromonas spp.* Isolated from Environmental Sources. *Applied and Environmental Microbiology,* 72(11), 7036-7042.

Huddleston, J. R., Zak, J. C., & Jeter, R. M. (2007). Sampling Bias Created by Ampicillin in Isolation Media for *Aeromonas. Canadian Journal of Microbiology,* 53(1), 39-44.

Huddleston, J. R., Brokaw, J. M., Zak, J. C., & Jeter, R. M. (2013). Natural Transformation as a Mechanism of Horizontal Gene Transfer among *Aeromonas* Species. *Systematic and Applied Microbiology* 36(4), 224-234.

Hunt, R. J. (1986). Percent Agreement, Pearson's Correlation, and Kappa as Measurers of Interexaminer Reliability. *Journal of Dental Research*, 65(2), 128-130.

Huys, G. (2014). The Family *Aeromonadaceae*. In: Rosenberg E., DeLong E.F., Lory S., Stackebrandt E., Thompson F. (eds.) The Prokaryotes. Springer, Berlin, Heidelberg

Janda, J. M., & Abbott S. L. (1996). Human Pathogens, p. 151-173. *In* B. Austin. M. Altwegg, P. J. Gosling, and S. Joseph (eds.), The Genus *Aeromonas*. John Wiley & Sons, Chichester, England.

Janda, J. M., & Abbott, S. L. (2010). The Genus *Aeromonas*: Taxonomy, Pathogenicity, and Infection. *Clinical Microbiology Reviews,* 23(1), 35-73.

Kirov, S. M. (1993). The Public Health Significance of *Aeromonas spp.* in Foods. *International Journal of Food Microbiology,* 20(4), 179-198.

Lederberg, J., Shope, R. E., & Oaks, S. C. (1992). Emerging Infections: Microbial Threats to Health in the United States. National Academy Press, Washington, D.C.

Lindstrom, E. S., Kamst-Van Agterveld, M. P., & Zwart, G. Distribution of Typical Freshwater Bacterial Groups is Associated with pH, Temperature, and Lake Water Retention Time. *Applied and Environmental Microbiology,* 71(12), 8201-8206.

Martinez, J. L. (2008). Antibiotics and Antibiotic Resistance Genes in Natural Environments. *Science* 321(5887), 365-367.

Muyer, G., de Waal, E. C., & Uitterlinder, A. G. (1993). Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rDNA. *Applied and Environmental Microbiology,* 59(3), 695-700.

Neu, H. C., (1992). The Crisis in Antibiotic Resistance. *Science.* 257(5073), 1064-1073.

Parker, J. L., & Shaw, J. G. (2011). *Aeromonas spp.* Clinical Microbiology and Disease. *Journal of Infection,* 62(2), 109-118.

Riesenfeld, C. S., Goodman, R. M., & Handelsman, J. (2004). Uncultured Soil Bacteria are a Reservoir of New Antibiotic Resistance Genes. *Environmental Microbiology,* 6(9), 981-989.

Smith, E. D., Sweazy, R. M., Whetstone, G. A., and Ramsey, R. H. (1979). A Study of the Reuse of Reused Water. *Groundwater,* 17, 366-374.

Smolinski, M. S., Hamburg, M. A., & Lederberg, J. (2003). Editors, Committee on Emerging Microbial Threats to Health in the 21st Century. Microbial Threats to Health: Emergence, Detection, and Response. Washington, D.C.: *The National Academies Press.*

Sommaruga, R., & Casamayo, E. Q. (2009). Bacterial 'Cosmopolitanism' and Importance of Local Environmental Factors for Community Composition in Remote High-altitude Lakes. *Freshwater Biology,* 55(5), 994- 1005.

Spellber, B., Guidos, R., Gilbert, D., Bradley, J., Boucher, H. W., Scheld, W. M., Bartlett, J. G., & Edwards, J. Jr. (2008). The Epidemic of Antibiotic-Resistance Infections: A Call to Action for the Medical Community from the Infectious Diseases Society of America. *Clinical Infectious Diseases,* 46(2), 155-164.

Spellberg, B., Powers, J. H., Brass, E. P., Miller, L. G., and Edwards, & J. E. Jr. (2004). Trends in Antimicrobial Drug Development: Implications for the Future. *Clinical Infectious Diseases,* 38(9), 1279-1286.

Stafford, T. (1981). Alluvial Geology and Archaeological Potential of the Texas Southern High Plains. *American Antiquity,* 46(3), 548-565.

Tenover, F. C. (2006). Mechanisms of Antimicrobial Resistance in Bacteria. *American Journal of Infection Control,* 34(5), S3-10.

Trust, T. J., & Chipman, D. C. (1979). Clinical Involvement of *Aeromonas hydrophila*. *Canadian Medical Association Journal,* 120(8), 942-946.

Van Donsel D. J., Geldreich E. E., & Clarke, N. A. (1967). Seasonal Variations in Survival of Indicator Bacterial in Soil and their Contribution to Storm-water Pollution. *Applied and Enivronmental Microbiology,* 15(6), 1362-1370.

VanLandeghem, M. M., Meyer, M. D., Cox, S. B., Sharma, B., & Patino, R. (2012). Spatial and Temporal Patterns of Surface Water Quality and Ichthyotoxicity in Urban River Basins in Texas. *Water Research,* 46(20), 6638-6651.

Warren, W. J., Jeter, R. M., Kimbrough, R. C., & Zak, J. C. (2004). Population Patterns and Antimicrobial Resistance of *Aeromonas* in Urban Playa Lakes. *Canadian Journal of Microbiology,* 50(6), 397-404.

Witte, W. (1998). Medical Consequences of Antibiotic Use in Agriculture. *Science,* 279(5353), 996-997.

Woodford N., & Ellington, M. J. (2006). The Emergence of Antibiotic Resistance by Mutation. *Clinical Microbiology and Infection,* 13(1), 5-18.

Yannarell, A. C., & Triplett E. W. (2005). Geographic and Environmental Sources of Variation in Lake Bacterial Community Composition. *Applied and Environmental Microbiology,* 71(1), 227-239.

Yannarell, A. C., & Triplett E. W. (2004). Composition of Bacterioplankton Communities: Investigations Using Multiple Spatial Scales. *Applied and Environmental Microbiology,* 70(1), 214-223.

Yu, G. B., Zhang, Y. L., Lau, Y. L., Yao, F., Vilches, S., Merino, S., Tomas, J. M., Howard, S. P., & Leung, K. Y. (2005). Identification and Characterization of Putative Virulence Genes and Gene Clusters in *Aeromonas hydophila* PPD134/91. *Applied and Environmental Microbiolgy,* 71(8), 4469-4477.

Zhang, X. X., Zhang, T., & Fang, H. H. P. (2009). Antibiotic Resistance Genes in Water Environments. *Applied Microbiolgy and Biotechnology,* 82(3), 397-414.