12-2013

The Incidence of Antibiotic Resistance in Mesophilic Aeromonas Isolated from the Buffalo River and from a Non-Urban Site Upstream

Amy L. Chapman
Buffalo State College, achapman13579@yahoo.com

Advisor

Gary W. Pettibone, Ph.D., Professor of Biology

First Reader

Douglas P. Easton, Ph.D., Professor of Biology

Second Reader

I. Martha Skerrett, Ph.D., Associate Professor of Biology

Department Chair

Gregory J. Wadsworth, Ph.D., Chair and Associate Professor of Biology

ABSTRACT OF THESIS

The Incidence of Antibiotic Resistance in Mesophilic *Aeromonas* Isolated from the Buffalo River and from a Non-Urban Site Upstream

Antibiotics are commonly used in agriculture and industry and their discharge is commonly seen in rivers, like the Buffalo River. This antibiotic discharge may cause a selective environment which favors the growth of antibiotic resistant *Aeromonas*. To study the effect of urban pollution on the antibiotic resistance in *Aeromonas,* 229 *Aeromonas* isolates were collected from fish tissues as well as sediment and water samples collected from the Buffalo River and a non-urban site (Cazenovia Creek). Seven different *Aeromonas* taxa were identified using biochemical tests. There were 124 (54%) isolates that were classified as atypical, which was the most commonly seen taxon. *Aeromonas veronii* biovar sobria was the most common species identified (63 isolates, 28%). All *Aeromonas* isolates were tested for their resistance to six different antibiotics (cephalothin, cefoxitin, ceftriaxone, nalidixic acid, piperacillin, and tetracycline). A total of 104 of 105 (> 99%) antibiotic-resistant isolates were resistant to cephalothin. All of the tested *Aeromonas* isolates had a cephalothin MIC greater than 32µg/ml. The cephalothin resistant isolates from the non-urban site all had an MIC greater than 256µg/ml. This study can be used to guide future studies in antibiotic resistance from the Buffalo River watershed.

State University of New York
College at Buffalo
Department of Biology

The Incidence of Antibiotic Resistance in Mesophilic *Aeromonas* Isolated from the Buffalo River and from a Non-Urban Site Upstream

A Thesis in
Biology

By

Amy Chapman

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Arts

December 2013

Approved by:

Gary Pettibone, Ph.D.
Professor in Biology
Chairperson of the Committee/Thesis Adviser

Gregory J. Wadsworth, Ph.D.
Chair and Associate Professor of Biology

Kevin J. Railey, Ph.D.
Associate Provost and Dean of the Graduate School

Table of Contents

List of Tables

List of Figures

1.0 Introduction

Aeromonas is a genus of gram negative facultative anaerobic bacilli (Warren *et al*., 2004) in the family Aeromonadaceae (Huddleston *et al*., 2006) which is found within the γ-3 subgroup of the class *Proteobacteria* (Abbott *et al*., 2003). Initially *Aeromonas* species were recognized as causing disease in poikilotherm animals. However, they also cause disease in immuno-compromised humans by means of a variety of exotoxins (Abbott *et al*., 2003; Bin Kingombe *et al*., 2010; Pablos *et al*., 2009). *Aeromonas* species are characterized as oxidase-positive (Chacón *et al*., 2002), capable of metabolizing glucose by fermentative pathways (Evangelista-Barreto *et al*., 2010), and motile by means of polar flagella (Yáñez *et al*., 2003).

1.10 Taxonomy

Physicians and microbiologists alike have difficulties determining phylogenetic relationships among *Aeromonas* species (Janda and Abbott, 1998). The taxonomy of *Aeromonas* over the past ten years has changed dramatically which has led to complications identifying species using biochemical methods alone (Chacón et al., 2002; Janda and Abbott, 1998). The genus *Aeromonas* was once proposed to accommodate rod-shaped bacteria possessing properties of enteric bacteria but motile by polar flagella (Popoff and Véron, 1976). In the 1970s the genus *Aeromonas* was considered to be a member of the family Vibrionaceae (Popoff and Véron, 1976) along with *Vibrio, Photobacterium,* and *Plesiomonas* (Abbott *et al*., 2003) until molecular techniques in the

mid-1970s indicated that these three genera are not closely related (Janda and Abbott, 1998; Janda and Abbott, 2010).

After the 1970s, *Aeromonas* species were divided into two groups: the mesophilic group with optimal growth between 35 to 37°C (Aberoum and Jooyandeh, 2010; Janda and Abbott, 2010) and the psychrophilic group with optimal growth between 22 to 28°C (Janda and Abbott, 1998). However, the accuracy of identification through chemotaxonomic methods alone was unreliable. A more comprehensive form of identification was required to characterize these species (Martino *et al*., 2011).

DNA hybridization studies revealed that within recognized mesophilic species, there are multiple hybridization groups, (Janda and Abbott, 2010) which are known as clusters or DNA groups (Abbott *et al*., 1992). Until these hybridization studies, the genus *Aeromonas* was considered to contain three motile species: *A. hydrophila*, *A. caviae*, and *A. sobria* (Borrell *et al*., 1997). Extensive DNA-DNA hybridization studies resulted in 11 additional defined species (Borrell *et al*., 1997). Early hybridization studies were expanded with 16S rRNA gene sequence analyses (Chacón et al., 2002; Martino *et al*., 2011; Nash *et al*., 2006) which could be conveniently stored in databases (Auch *et al*., 2010). As a result of 16S rRNA sequencing, the number of defined *Aeromonas* species increased from 11 to 14 with *Aeromonas veronii* containing two distinct biotypes, *A. veronii* biovar veronii and *A. veronii* biovar sobria (Janda and Abbott, 1998; Janda and Abbott, 2010). This 16S rRNA gene sequencing resulted in characteristic 16S rRNA sequences which could be used to identify known *Aeromonas* species (Borrell *et al*., 1997).

1.20 Disease

Several species of *Aeromonas* are known to cause disease in a variety of organisms. Motile, mesophilic species, especially *A. hydrophila*, have been linked to major fish die-offs around the globe resulting in economic losses (Janda and Abbott, 2010). *Aeromonas hydrophila* is a freshwater pathogen which causes disease, such as septicemia, in fish, frogs, and several other animals (Vivas *et al*., 2000; Vilches *et al*., 2009). *Aeromonas*-mediated fish die-offs included over 25,000 common carp in the St. Lawrence River in 2001 and 820 tons of goldfish in Indonesia in 2002, which resulted in a $37.5 million loss (Janda and Abbott, 2010).

Aeromonas veronii biovar sobria also is known to cause disease in fish. Epizotic ulcerative syndrome (EUS) is a fish disease that results in severe dermal ulcers on the body and dorsal regions of the fish. This disease has caused a substantial economic loss to fish farmers and the fisheries sector (Rahman *et al*., 2002). Rahman *et al*. (2002) found that all 14 *Aeromonas* isolates they obtained from EUS lesions in fish were identified through biochemical testing as *A. veronii* biovar sobria. In addition, these EUS isolates were unique in their ability to agglutinate fish erythrocytes. Huang *et al*. (2010) identified *A. veronii* as a new fish pathogen affecting channel catfish (*Ictalurus lunetaus*) in China. By using 16s rRNA sequencing, they identified *A. veronii* from 20 diseased fish from a commercial aquaculture farm in Sichuan province.

Motile *Aeromonas* species, such as *A. hydrophila*, *A. veronii* biovar sobria, and *A. caviae* can be pathogenic for humans as well (Graf, 1999). Diseases such as pneumonia

(Aberoum and Jooyandeh, 2010), diarrhea (Graf, 1999; Alavandi and Ananthan, 2009; Vilches *et al*., 2009), and septicemia (Graf, 1999; Evangelista-Barreto *et al*., 2010) that resulted from wound infections are linked to these species (Janda and Abbott, 1998; Pablos *et al*., 2009; Leblanc *et al*., 1981; Vilches *et al*., 2009). Many wounds infected by *Aeromonas* species are from exposure to contaminated water. These wound infections can progress quickly and become fatal if they become systemic (Huddleston *et al*., 2006).

1.30 The Environment

Aeromonas species have been isolated from a wide variety of sources (Janda and Abbott, 2010). The wide distribution of aeromonads in different aquatic ecosystems demonstrates their ability to inhabit environments of different trophic levels (Huddleston *et al*., 2006). The abundance of specific species of *Aeromonas* may be related to the trophic status of water which may make them useful indicators of water quality (Pianetti *et al*., 2005; Gugliandolo *et al*., 2009).

Aeromonas species may participate in biofilm formation which enables them to exist in a number of nutrient-deprived environments such as water distribution systems (Pianetti *et al*., 2005). Non-point sources of contamination, such as urban run-off, can be a major challenge for future research because the assessment of this contamination needs to include both the source of the run-off and the prevalence of the bacteria (Stewart *et al*., 2008).

1.31A Aquatic Ecosystems

Much research has been done on the presence of *Aeromonas* in aquatic environments due to their negative effect on fish and the resulting economy (Khajanchi *et al*., 2010). The input of large concentrations of nutrients, pollutants, and urban wastewater discharges can alter the quality of lakes, streams, rivers and coastal marine ecosystems (Gugliandolo *et al*., 2009). *Aeromonas hydrophila*, *A. caviae*, and *A. veronii* bv. sobria are the predominate mesophilic species that are isolated from fish and water samples (Janda and Abbott, 2010). Although *A. caviae* is predominately found in sewage and waters with high fecal pollution (Pianetti *et al*., 2005), it also can be found in less polluted waters in equal number to *A. hydrophila* (Adulhamd, 2009; Pianetti *et al*., 2005).

Mesophilic *Aeromonas* species have been isolated from lakes (Janda and Abbott, 2010), rivers (Huddleston *et al*., 2006), sewage (Maalej *et al*., 2003; Monfort and Baleux, 1990), surface, ground (Borchardt *et al*., 2003), drinking (Huddleston *et al*., 2006; Pablos *et al*., 2009; Ghenghesh *et al*., 2008), chlorinated (Borchardt *et al*., 2003), and polluted waters (Huddleston *et al*., 2006). In nutrient-rich waters, *Aeromonas* can grow to large numbers, peaking in the warmer temperatures of the summer months in both effluent ponds and freshwater lakes (Monfort and Baleux, 1990; Borchardt *et al*., 2003). *Aeromonas* species have the ability to tolerate antibiotic and metal-polluted environments (Huddleston *et al*., 2006). In addition, *Aeromonas* can be found in drinking water distribution systems even after chlorine treatment (Pablos *et al*., 2009).

Sisti *et al*. (1998) sought to obtain information on the chlorine resistance of motile *Aeromonas* species in drinking water. To study this, water samples that were

contaminated with bacteria were taken from a water distribution system and then exposed to various levels of chlorine for different times and at different temperatures. They discovered that the effect of chlorine was influenced by water temperature. The survival curve of *A. hydrophila* showed that free chlorine reduced the number of bacteria by one or more logs. However at 20°C, *A. hydrophila* displayed up to one log greater resistance to the oxidizing effects of the chlorine. The authors suggested that at 20°C the free chlorine combined with compounds that reduced microbicidal activity. This was supported in that other factors that could influence chlorine concentrations (pH, autochthonous microflora, and the presence of *Aeromonas* cells) were similar in chlorinated drinking water at both temperatures (Sisti *et al*., 1998).

Despite the many drinking water treatment strategies, which include rapid/slow sand filtration, hyperchlorination/direct filtration, and the use of granular activated carbon (Sen and Rodgers, 2004), many aeromonads are able to survive. The survival rate of *Aeromonas* species in these environments appears to be dependent on their ability to grow in biofilms (Sen and Rodgers, 2004; Borchardt *et al*., 2003). *Aeromonas* survival in the environment is enhanced by their ability to grow in pH ranges of 5.5 to 9.0, temperature ranges of 22-35°C, the presence of sunlight (UV irradiation), and in suspended solids (Martone-Rocha *et al*., 2010).

1.31B Non-Polluted vs. Polluted Waters

Aeromonas species are present in both non-polluted and polluted waters (Janda and Abbott, 2010). In many developing countries, health protection measures for irrigation of crops, such as the removal of pathogenic bacteria in stabilization ponds, may

be subpar leading to pathogenic bacteria consumption. Infection with antibiotic-resistant bacteria has lead to a number of deaths in these countries (Hassani *et al*., 1992).

Maalej *et al.* (2003) collected samples from a sewage treatment system to determine the prevalence of *Aeromonas* species in the treated effluent of a sewage treatment plant which the authors suggested could cause problems for public health. Water samples were collected from a stabilization pond which had an average depth of 3.2 meters and from seawater far from the wastewater outfall over the course of one year. Various environmental parameters including water temperature were recorded at the time of sampling. *Aeromonas* densities fluctuated over three logs during the study but the motile *Aeromonas* colony forming units (CFU) averaged 7.045 logs CFU 100 ml^{-1} in the treated effluent but only averaged 1.47 logs CFU 100 ml^{-1} in the marine water.

In December (cold weather period) the *Aeromonas* cell numbers decreased rapidly to undetectable levels in the marine water. However, the treated effluent had an increase of *Aeromonas* cell number. In the warm weather months (May to October), *Aeromonas* cell numbers increased in the marine water with the highest density recorded in the late summer/early autumn when the temperatures were around 22 to 23°C but cell density decreased in the treated effluent (Maalej *et al*., 2003). This shows that the presence of *Aeromonas* in treated effluent may not be temperature dependent as is in marine waters and that water treatment may be unable to remove all *Aeromonas* species from polluted waters.

However, motile *Aeromonas* species, such as *A. hydrophila*, *A. caviae*, and *A. sobria*, are seen in both polluted and unpolluted water often times related to water temperature. Monfort and Baleux (1990) determined the presence of these species in a

wastewater treatment plant in Mèze, France by taking water samples at five stations within sewage treatment ponds. This facility was studied because the city of Mèze is open to the Mediterranean Sea and can give information about the prevalence of these species in an urban wastewater treatment system from the inflow site to outflow site. There were a total of 247 *Aeromonas* isolates that were identified from the inflow and outflow of the sewage treatment pond. During the summer and fall, *A. caviae* was the most dominate species at the inflow site (51.5% in July and 42.8% in October). *Aeromonas sobria* was the second most common species at the inflow site (18.2% in July and 28.6% in October) and the most common species at the outflow site (85.2% in July and 96% in October). In the winter months, *A. caviae* was the most dominant at both the inflow (65.7%) and outflow (52.6%) sites. *Aeromonas hydrophila* was rarely seen at the inflow site (0 to 15%) or the outflow site (4 to 15%) (Monfort and Baleux, 1990). They concluded that the prevalence of *A. hydrophila*, *A. sobria*, and *A. caviae* may be dependent on environmental conditions such as water temperature and the concentration of pollutants in the aquatic environment.

In many countries, including the United States, wastewater is reused for recreational and economic purposes. Efficient removal of pathogens from wastewater is important especially since polluted water can serve as a reservoir for *Aeromonas* species. The presence of potential pathogens, including *Aeromonas*, in municipal wastewater may pose a health risk in countries that commonly reuse wastewater, such as the United States. To study the dynamics of *Aeromonas* species in wastewater, Martone-Rocha *et al*. (2010) determined the efficiency of microbiological removal in a wastewater treatment plant. They used biochemical methods to identify *Aeromonas* species from a

sanitary sewage stabilization pond treatment system in the City of Lins, Brazil. A total of 13 species were identified out of 203 isolates. The most prevalent mesophilic *Aeromonas* species found at the inflow site was *A. caviae* (53 isolates, 33.13%) followed by *A. allosaccharophila* (28 isolates, 17.5%). At the outflow site, *A. caviae* was again the most prevalent mesophilic species (12 isolates, 25%) identified followed by *A. media* (6 isolates, 12.5%). Overall, the most common species identified in all the samples was *A. caviae* (71 isolates, 25.09%) followed by *A. allosaccharophila* (69 isolates, 24.38%). They also used the most probable number (MPN) technique to calculate the number of *Aeromonas* in the pond influent, effluent, and the facultative pond (stabilization pond) outflow sites. They found that approximately 72% of the samples collected from the anaerobic pond inflow contained *Aeromonas* with MPN counts from 0 to $3.3x\ 10^9$ 100 ml^{-1}. Similarly, 55% of the samples from the pond effluent contained *Aeromonas* with MPN counts ranging from 0 to $1.1x10^9$ 100 ml^{-1}. Forty-eight percent of the samples from the facultative pond outflow contained *Aeromonas* and yielded MPN counts of 0 to $9.0x10^5$ 100 ml^{-1}. Therefore, although the number of *Aeromonas* decreased through the wastewater treatment process, total elimination did not occur. Also, this implies that *A. caviae* is commonly found in wastewater at both the inflow and outflow sites. Wastewater treatment is not always effective in removing *Aeromonas* from polluted water and this could be problematic for public health (Martone-Rocha *et al*., 2010).

Figueira *et al*. (2011) also studied *Aeromonas* species in a number of aquatic environments within an urban water cycle. Water samples were collected from a drinking water plant, a wastewater treatment plant (raw surface water, ground water, water treated by sand filtration and ozonation, and water treated with chlorine), and tap

water. A total of 121 *Aeromonas* isolates were collected and 11 species of the genus *Aeromonas* were identified using 16S rRNA gene sequence analysis. Ground water samples revealed an aeromonad count of 10^1 CFU ml^{-1} and drinking water samples were approximately 10^1 to 10^4 CFU ml^{-1}. *Aeromonas* isolates were found to be at a concentration of 10^4 to 10^6 CFU ml^{-1} in raw wastewater and treated wastewater water samples. They determined the most prevalent *Aeromonas* species in raw surface water was *A. veronii* (49.0%) followed by *A. media* (19.6%). *Aeromonas media* and *A. punctata* (*A. caviae*) were the most prevalent species found in the raw wastewater (both at 36.4%) and the treated wastewater (36.8% and 31.6%). Even after disinfection by ozonation, as part of wastewater treatment, several species of *Aeromonas* still were present (*A. aquariorum*-6.9%, *A. hydrophila* subsp. hydrophila-58.6%, *A. jandaei*-10.3%, *A. veronii*-24.1%) in the treated wastewater (Figueira *et al*., 2011).

The presence of motile *Aeromonas* species in treated tap water may indicate a potential source of infection for individuals. Kivanc *et al*. (2011) studied the presence of *Aeromonas* in an urban water treatment plant. They collected water samples from the Porsuk River, public drinking water, and tap water in the City of Eskisehir, Turkey. A total of 60 strains of *Aeromonas* were isolated from the Porsuk River. Identified species included *A. hydrophila*, *A. caviae*, *A. salmonicida*, and *A. media* by use of biochemical testing. They found that *Aeromonas* species occurred most frequently (82.53% of all isolates) in dry seasons from June to October in the Porsuk River. The tap and drinking water samples showed no *Aeromonas* species (Kivanc *et al*., 2011). It was shown that *Aeromonas* species in river water samples are commonly found in the warmer seasons of the summer and that water treatment helped to eliminate the presence of *Aeromonas*.

Aeromonas species were isolated from the Buffalo River to study the effect of urban pollution on the presence of *Aeromonas*. Pettibone (1998) sampled four sites within the upper Buffalo River watershed and one site in the Buffalo River. In addition, physical parameters of the water and the levels of *Aeromonas* were tested. The summer temperature averaged 17.9°C and the winter temperature averaged 2.6°C at all the sample sites of this study. The upper river sites had a wide range of total dissolved solids (0.4 to 453 mg l^{-1}) as well the downstream site (13.4 to 659 mg l^{-1}). The yearly mean values of *Aeromonas* at the five sample sites were one to two logs higher than the fecal coliform and fecal streptococci. Downstream sites that were located in more urbanized areas experienced higher levels of *Aeromonas* (120 to 140 ml^{-1}) than those in less populated areas (50-85 ml^{-1}) (Pettibone, 1998). Therefore, the presence of *Aeromonas* in the Buffalo River watershed might correlate to environmental factors such as temperature, total dissolved solids, and urban pollution.

1.40 Antibiotic Resistance

The overuse of antibiotics in medicine and agriculture is creating selective conditions for bacterial resistance (Warren *et al*., 2004). The indiscriminate use of antibiotics in medical, veterinary, and agricultural industries results in the discharge of antibiotics into the environment (Kümmerer, 2003; Goñi-Urriza *et al*., 2000a; Goñi-Urriza *et al*., 2000b). Antibiotic pollution is commonly seen in river waters because they receive sewage and urban effluents (Goñi-Urriza *et al*., 2000a; Goñi-Urriza *et al*., 2000b). Antibiotic pollution may contribute to the spread of bacterial antibiotic resistance (Goñi-

Urriza *et al*., 2000b). This may contribute to the antibiotic resistance in *Aeromonas* seen in river water.

According to Goñi-Urriza *et al*. (2000a), urban effluent resulted in an increase of the rates of antibiotic resistance of *Aeromonas* in the Arga River in Spain. They collected water samples at 16 sites near the wastewater discharge of the city of Pamplona. They isolated 118 strains of *Aeromonas* and 75% (88 isolates) of them showed antibiotic resistance. The isolates collected downstream of the wastewater discharge showed 50% more antibiotic resistant *Aeromonas* species than from upstream isolates. Nalidixic acid resistance (85 isolates, 72%) was most frequently found in *Aeromonas* species followed by tetracycline (25 isolates, 21%) and co-trimoxazole (17 isolates, 14%). Resistance to other antimicrobial agents, such as chloramphenicol, fosfomycin, beta-lactams, and aminoglycosides, were seen in fewer than 5% (6) of isolates (Goñi-Urriza *et al*., 2000a).

Antibiotic resistance in *Aeromonas* is also seen in wastewater. Hassani *et al*. (1992) identified 264 isolates of *Aeromonas* from wastewater collected from Marrakech, Morocco and tested their resistance to seven antibiotics. Three different species of *Aeromonas* were identified (163 *A. caviae*, 24 *A. hydrophila*, 54 *A. sobria)* as well as 23 atypical isolates that could not be identified to species level. None of the *Aeromonas* isolates showed resistance to polymyxin B and only *A. caviae* showed resistance to trimethroprim-sulfamethoxazole and nalidixic acid. However, all of the isolates were resistant to amoxicillin and 193 (73%) of the *Aeromonas* isolates were resistant to cephalothin. All of the *A. hydrophila* isolates showed multiple antibiotic resistance while 156 (96%) of *A. caviae* isolates were determined to be resistant to multiple antibiotics.

Multiple antibiotic resistances were not seen in the *A. sobria* and the atypical isolates (Hassani *et al*., 1992).

Antibiotic resistant *Aeromonas* is also commonly seen in river water that receives urban effluent. To further study this, Evangelista-Barreto *et al*. (2010) collected water samples from the River Cocó, Ceara, Brazil, which commonly received urban effluent, and found that 77% of the water samples contained *Aeromonas* species (*A. caviae*, *A. veronii* bv. sobria, *A. veronii* bv. veronii, *A. trota*, *A. media*, *A. sobria*, and *A. hydrophila*) and 60% of these were resistant to eight antibiotics. Almost all *A. caviae* strains that were tested were resistant to tetracycline, nitrofurantoin, cephalothin, ciprofloxacin, ceftriaxon, chloramphenicol, and nalidixic acid. This was followed by *A. veronii* bv. sobria which was resistant to the same antibiotics as *A. caviae* as well as to sulfamethoxazole-trimethoprim (Evangelista-Barreto *et al*., 2010).

Antibiotic uses in agriculture practices such as livestock production and fish farms can have a direct impact on the aquatic environment. Gordon *et al*. (2007) studied the effect of antibacterial treatments administered by feed in fish farms on *Aeromonas* resistance. Sediment samples were taken in a coastal river in a region of France known for its livestock and freshwater fish farming. *Aeromonas* isolates from this region were tested for their resistance to florfenicol, oxolinic acid, and oxytetracycline, which are antibiotics commonly used in these industries. The occurrence of florfenicol resistance was very low in isolates collected during both the fall and spring sample times (0.3%). Oxolinic acid resistance in the fall and spring occurred in about 0 to 34% of the isolates. However resistance to oxytetracycline showed the most variation. In fall there were between 0 to 22% of isolates resistant but in the spring resistance ranged to 99.8%

resistant. Upstream from these farms the *Aeromonas* isolates were not resistant to any of these antibiotics (Gordon *et al*., 2007). This demonstrated that antibiotics that are commonly used in aquaculture may increase the number of antibiotic resistant *Aeromonas* in that area.

Antibiotic resistance also is commonly seen in treated effluent that is reintroduced into the environment. Al-Bahry *et al.* (2009) studied the antibiotic resistance of 336 isolates collected from tertiary treated sewage effluent at a sewage treatment plant, used for irrigation. It was determined that *Aeromonas* was the second most common genus identified (55 isolates, 16%) with 100% resistant to ampicillin followed by streptomycin at 76.4% (42 isolates). However, no isolates were resistant to amikacin, cephotaxin, cloramphenicol, gentamycin, tetracycline, or trimethoprim (Al-Bahry *et al*., 2009).

Antibiotic and heavy metal resistant *Aeromonas* can also be seen in urban effluent. Matyar *et al*. (2010) studied the susceptibility patterns to 15 different antibiotics and six heavy metals in *Aeromonas* and *Pseudomonas* species isolated from Iskenderun Bay, Turkey. This area was of particular interest because it received domestic and hospital wastes as well as industrial wastes. A total of 198 isolates were tested, 60 isolates were identified as two different *Aeromonas* species and 138 isolates were identified as six different *Pseudomonas* species. Fifty-seven (95%) *Aeromonas* isolates were identified as *A. hydrophila* and three (5%) were *A. caviae*. About 67% (40 of 60) of all the *Aeromonas* isolates showed resistance to cefazolin and trimethoprim-sulphamethoxazole. Only about 14% (8 of 60) of isolates showed resistance toward gentamicin, chloramphenicol, and nalidixic acid and only about 3% (2 of 60) were resistant to amikacin (Matyar *et al*., 2010).

Environmental contamination with antibiotics and other pollutants may play a role for the spread of antibiotic resistant genes among *Aeromonas*. Subinhibitory concentrations of antibiotics may select for the genetic transfer of resistant genes (Kümmerer, 2003). In natural environments, unrelated bacteria may be able to pass resistance plasmids to each other (Huddleston *et al*., 2006). This is congruent with the fact that antibiotic and metal-resistant bacteria have been isolated in environments that have never been exposed to such pollutants (Huddleston et al., 2006). Antimicrobial agent resistance can be passed to bacteria in waters containing human and animal wastewater discharges (Goñi-Urriza *et al*., 2000a) as in the Buffalo River.

Sometimes resistance genes are inherited vertically by descendant cells within a single bacterial population via cellular division. Horizontal spread occurs between different bacterial populations through mobile genetic elements (plasmids, transposons, and integrons) (Warren *et al*., 2004). Plasmids containing multiple antimicrobial resistance determinants could be transferred between bacterial pathogens of fish, humans, and other animals which suggest the spread of mobile genetic elements between fish and human pathogens (Jacobs and Chenia, 2007). This is also the case in some instances of quinolone resistance (Ruiz, 2003). Resistance plasmids or R-plasmids can be used to monitor the incidence of antibiotic resistance and study the conjugative spread of resistance genes (Hedges *et al*., 1985; Schmidt *et al*., 2001).

Twenty-one *Aeromonas hydrophila* isolates from freshwater fish ulcers were tested for antibiotic resistance and their plasmids were characterized by Son *et al*. (1997). These *A. hydrophila* isolates were determined to be resistant to streptomycin (12 isolates, 57%), erythromycin (9 isolates, 43%), and tetracycline (10 isolate, 48%). About 33% (7)

of these resistant isolates contained antibiotic resistance (R) plasmids which ranged from 3 to 63.4 kb in size. One of the strains, *A. hydrophila* AH11, was shown to have an R plasmid that was transferred to a recipient *E. coli* K12 by a single step conjugation. This was demonstrated by electrophoresis where the plasmid bands were the same (6.2 and 63.4 kb) for both the donor and transconjugants. Conjugation studies were done to study the potential transfer of a resistance plasmid. These were performed *in vitro* with *A. hydrophila* isolates and nalixidic-acid resistant *E. coli* K12 and demonstrated that conjugal transfer of nalidixic acid occurred at a rate of $4.3x10^{-3}$ transconjugants per donor cells (Son *et al*., 1997).

Integrons are commonly studied in relation to gene transfer due to their association with mobile genetic elements and multi-resistance phenotypes (Moura *et al*., 2012). Integrons consist of integrase which catalyzes the incorporation or excision of gene cassettes by site-specific recombination. Moura *et al*. (2012) evaluated 697 isolates of Enterobacteriaceae and *Aeromonas* that were isolated from urban waste waters. They discovered that integrase-I1 (*intI*) positive isolates ranged from 4.6% (6 of 131) in treated effluents to 7.4% (7 of 95) in influents. Genes that encoded *intI*2 were present in only about 0.14% (1 of 697) of all isolates and were only seen in aeration tanks and no *intI*3 genes were detected. About 80% (21 of 26) of these *intI* positive isolates were *Aeromonas* species (*A. media, A. caviae, A. allosaccharophila, A. salmonicida,* and *A. veronii*). Their study determined that most integrases (*intI and intII*) in *Aeromonas* and Enterobacteriaceae were located on the chromosome, however about 33% were carried on plasmids. All of the 19 different *Aeromonas* strains identified had integrases located on the chromosome, although 21% (4 of 19) of strains (*A. media*-two strains, *A. caviae*,

A. allosaccharophila) also had integrases located on the plasmid. Antibiotic resistance of *intI*-postitive Enterobacteriaceae and *Aeromonas* revealed 84.2% (16 of 19) of multiresistant strains belonging to *Aeromonas* and 15.8% (3 of 19) belonging to Enterobacteriaceae. *Aeromonas* species were all resistant to nalidixic acid followed by cephalothin and ampicillin (90%) (Moura *et al*., 2012).

Resistance to some antibiotics, such as quinolones, can be chromosomally encoded (Ruiz, 2003). Figueira *et al*. (2011) studied quinolone resistance in *Aeromonas* isolated from wastewater plants. They studied mutations in chromosomal genes *gyrA* and *parC*, which can cause quinolone resistance. They found that 45 of 47 (96%) *Aeromonas* isolates had a *gyrA* mutation which resulted in nalidixic acid resistance and 15 (32%) isolates had a mutation in *parC* (Figueira *et al*., 2011).

Due to the high use of these broad spectrum antibiotics, there has been a rapid development of bacterial antibiotic resistance (Ruiz, 2003). Two types of chromosomally encoded mechanisms of resistance have been established: alterations in the targets of quinolones and decreased antibiotic concentrations inside the bacteria due to membrane impermeability and/or an expression of an efflux pump system (Ruiz, 2003). Since the target of quinolones is the inhibition of DNA gyrase, a type II topoisomerase, and topoisomerase IV, a mutation in these genes could lead to resistance (Ruiz, 2003).

1.50 Statement of Problem

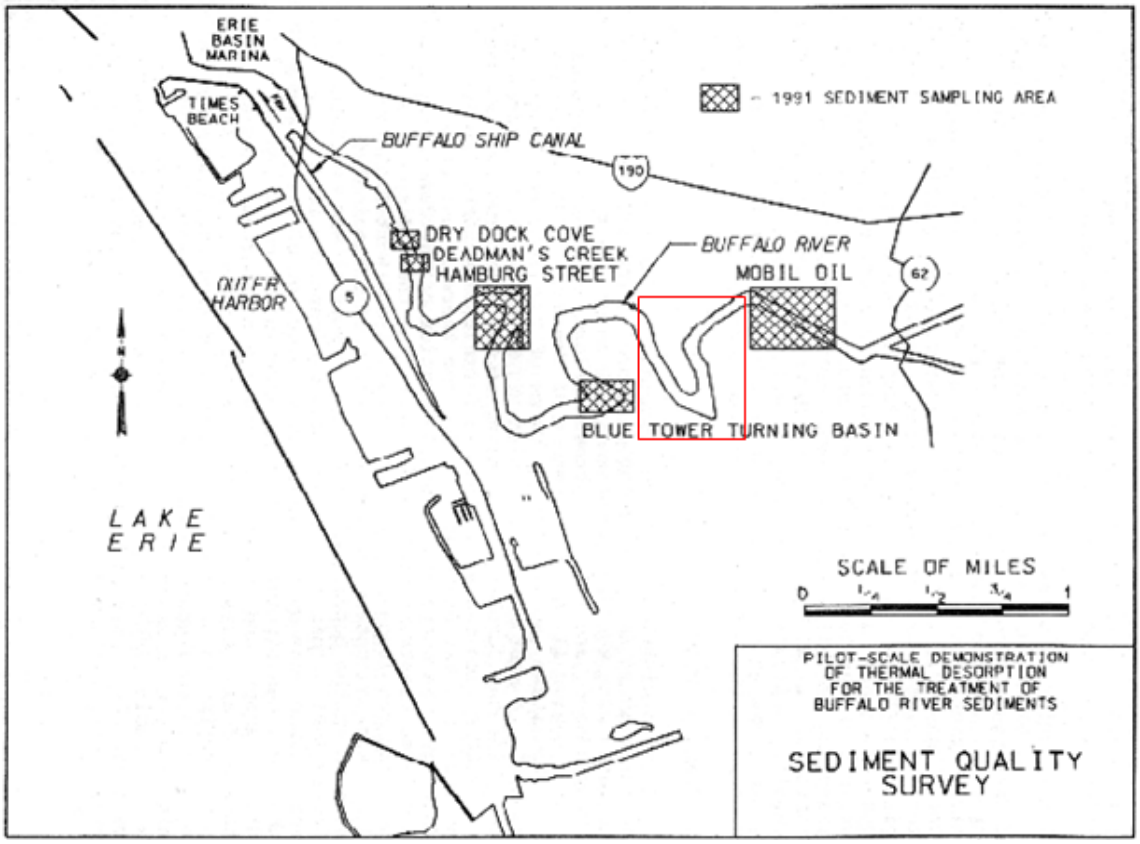
In 1972 the Clean Water Act was passed to regulate pollution discharges into waters of the United States and to regulate quality standards for surface waters. Under

this act, the Environmental Protection Agency has set wastewater standards for industry and set water quality standards for contaminants in surface waters (US Environmental Protection Agency, 2011). Bodies of water, like the Great Lakes, which provide recreation, a food source, and even employment, are considered ecosystems essential for our future well-being. They are impacted through pollution inputs, including externally introduced microbial contaminants (Stewart et al., 2008).

The Buffalo River is part of the Great Lakes Area of Concern (AOC). The Buffalo River watershed flows from the east and discharges into Lake Erie close to the Niagara River. The AOC "impact area" is 10 km long and is located between the mouth of the Buffalo River to the farthest point upstream at which the backwater condition exists during Lake Erie's highest monthly average lake level. There are three major streams located in the watershed that create the AOC source area: Cayuga Creek, Buffalo Creek, and Cazenovia Creek. Land use in these areas consists of residential communities, farmland, and commercial enterprises. The total drainage area for the Buffalo River watershed is about 708 square kilometers (440 square miles) (US Environmental Protection Agency, 2011).

The AOC impact area is characterized historically by heavy industrial development, especially between the Blue Tower Turning Basin and Mobil Oil, which is shown in Figure 1. The Buffalo River *Aeromonas* isolates used in this study were collected from this region of the river. In the 1981 Buffalo New York Area Sediment Survey (Rockwell 1984), the EPA found a number of organic substances in a concentration of 5 ppm or greater in grab sediment samples collected from the area.

Figure 1-Pollution sites within the Buffalo River. The isolates from the Buffalo River used in this study were collected in the boxed region between the Blue Tower Turning Basin and Mobil Oil (Rockwell 1984).


ERIE BASIN MARINA
TIMES BEACH
BUFFALO SHIP CANAL
1991 SEDIMENT SAMPLING AREA
190
DRY DOCK COVE
DEADMAN'S CREEK
HAMBURG STREET
BUFFALO RIVER
MOBIL OIL
62
OUTER HARBOR
5
BLUE TOWER TURNING BASIN
LAKE ERIE
SCALE OF MILES
0
1
PILOT-SCALE DEMONSTRATION OF THERMAL DESORPTION FOR THE TREATMENT OF BUFFALO RIVER SEDIMENTS
SEDIMENT QUALITY SURVEY

This region has been shown to have a high concentration of polycyclic aromatic hydrocarbons, shown in Table 1. There was also a high concentration of metals in the grab samples including chromium (36 mg/kg), copper (51 mg/kg), lead (90 mg/kg), zinc (210 mg/kg), and magnesium (500 mg/kg) (Rockwell, 1984).

Rising levels of pollution in the Buffalo River due to sewer overflow events and run-off may be causing an increase in some bacterial populations. Pettibone (1998) conducted a study on the Buffalo River demonstrating a link between storm events and levels of *Aeromonas* species. Between storm events, the number of *Aeromonas* upstream of the Buffalo River was between 230 bacteria/mL^{-1} and 20 bacteria/mL^{-1} in the Buffalo River. However, during storm events, the number of *Aeromonas* the upstream increased to between 420 bacteria/mL^{-1} and 760 bacteria/mL^{-1} in the Buffalo River (Pettibone, 1998). This decreased water quality may lead to loss of fish and wildlife habitat, degradation of fish wildlife populations, restrictions on fish and wildlife consumption, restrictions on drinking water consumption, and even added costs to agriculture and industry (Manninen, 2003).

Table 1-Concentration of polycyclic aromatic hydrocarbons found in the Buffalo River sediment in the study area (Rockwell 1984).

PAH Concentrations ($\mu g\ g^{-1}$) for Buffalo River Sediment Grab Samples

PAH	Blue Tower	Mobil Oil
Anthracene	0.13	0.14
Benzo(a)anthracene	0.45	0.46
Benzo(a)pyrene	0.86	0.74
Benzo(b)fluoranthene	1.49	1.21
Chrysene	0.63	0.52
Fluoranthene	1.21	1.05
Phenanthrene	0.59	0.60
Pyrene	0.76	0.83

The purpose of this study was to determine the incidence of antibiotic resistance in *Aeromonas* species isolated from urban and non-urban sites in the Buffalo River watershed. A number of studies have linked the increase in urban pollution seen in rivers to the rise of antibiotic-resistant *Aeromonas* (Hassani *et al*., 1992; Evangelista-Barreto *et al*., 2010; Al-Bahry *et al*., 2009; and Matyar *et al*., 2010). There has been little research done on antibiotic resistance in *Aeromonas* isolates from the Buffalo River watershed. *Aeromonas* isolates were collected from sediment, water, and fish in the Buffalo River watershed to study the prevalence of antibiotic resistant isolates in a region shown by Rockwell (1984) to contain urban pollution. These isolates were compared to sediment and water samples from a non-urban site (Cazenovia Creek), which was considered a control region, to determine the effect of urban pollution on the resistance of *Aeromonas* isolates. This study was done to determine if there is a link between the prevalence of urban pollution in the Buffalo River and the resulting antibiotic resistance in mesophilic *Aeromonas* species.

2.0 Materials and Methods

2.10 Sample Sites

A total of 229 *Aeromonas* isolates were previously collected from the Buffalo River watershed. These isolates were cryogenically stored at -70°C in tryptic soy broth and 20% glycerol on glass beads and were used as test organisms in this study. Ten of these isolates were taken from fish tissues (intestines (3 isolates), skin (2 isolates), kidney

(2 isolates), and liver (3 isolates)), 84 were from water samples from the Buffalo River, and 98 were from Buffalo River sediment samples, which is summarized in Figure 2. These *Aeromonas* isolates were collected from a polluted area of the Buffalo River, which is shown in Figure 3 as square.

Thirty-seven *Aeromonas* isolates also were collected from a non-urban site in the Western Branch of Cazenovia Creek indicated as a circle in Figure 3 which cuts through bedrock composed of Silurian and Devonian dolostones, limestones, and shales. Further downstream, Cazenovia Creek crosses glacial lake beach deposits and lacustrine silty clay soils (Wills and Irvine, 1996). Seventeen *Aeromonas* isolates were collected from the sediment and 20 were collected from water samples. This area is of importance because of its agricultural land usage (Inamdar, 2004) and was used as a non-urban control site.

The northernmost tributary in the Buffalo River watershed, Cayuga Creek, flows first through farmland and then through several residential communities. Buffalo Creek begins in Wyoming County and runs adjacent to the farmland. Cazenovia Creek is divided between two branches: the East Branch which begins in Sardina and the West Branch which begins in Concord. *Aeromonas* isolates that were used in this study were collected from Cazenovia Creek which has agricultural and wooded land adjacent to both branches. The agricultural land usage is shown in Figure 4. The *Aeromonas* isolates were collected from the area of the Cazenovia Creek in which 18-28% of the land is used for agriculture (Inamdar, 2004). Figure 5 shows the location of farms with farm edges within 1000 feet of a waterway in the Buffalo River watershed.

Figure 2- A flow chart representing the number of *Aeromonas* tested in the Buffalo River, fish, and non-urban site. The antibiotic resistance in each of these areas is shown as well as the number of multi-resistant isolates.

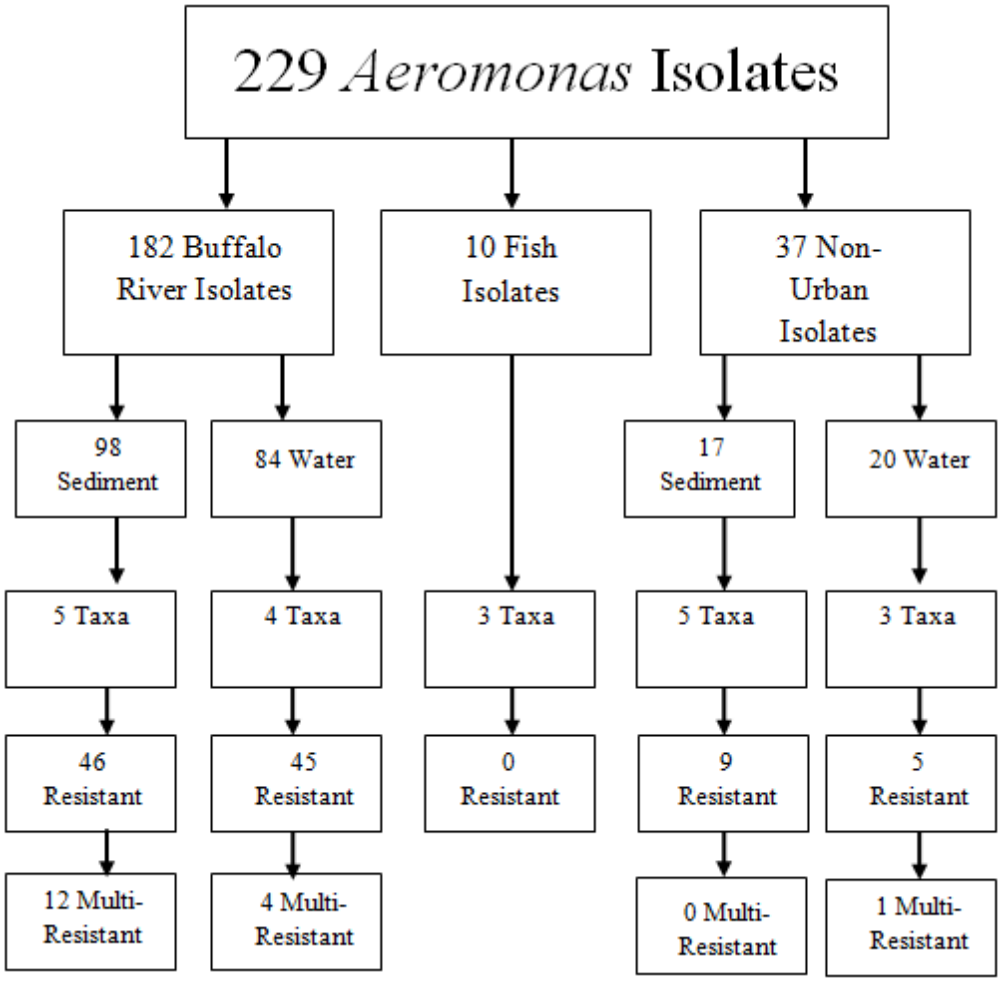

229 Aeromonas Isolates
182 Buffalo River Isolates
10 Fish Isolates
37 Non-Urban Isolates
98 Sediment
84 Water
17 Sediment
20 Water
5 Taxa
4 Taxa
3 Taxa
5 Taxa
3 Taxa
46 Resistant
45 Resistant
0 Resistant
9 Resistant
5 Resistant
12 Multi-Resistant
4 Multi-Resistant
0 Multi-Resistant
1 Multi-Resistant

Figure 3-The Buffalo River watershed showing the non-urban sampling site (circle) and the area in the Buffalo River (square) where water, sediment, and fish samples were collected.

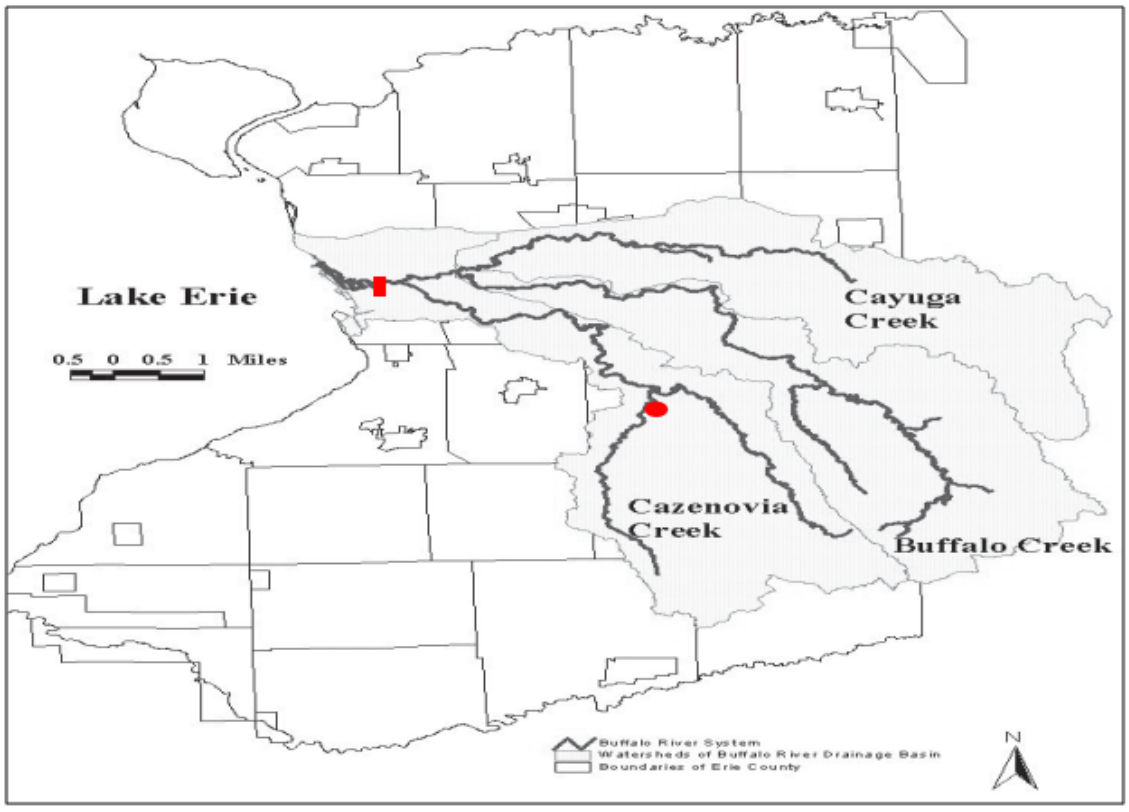

Lake Erie
0.5 0 0.5 1 Miles
Cayuga Creek
Cazenovia Creek
Buffalo Creek
Buffalo River System
Watersheds of Buffalo River Drainage Basin
Boundaries of Erie County
N

Figure 4-The distribution of agricultural land in individual sub-basins in the Buffalo watershed. The area shown with a red circle is the sample area for the non-urban site. A: The Cazenovia Creek sample area. B: The percentage of agricultural land use in the Cazenovia Creek sample area (Inamdar, 2004).

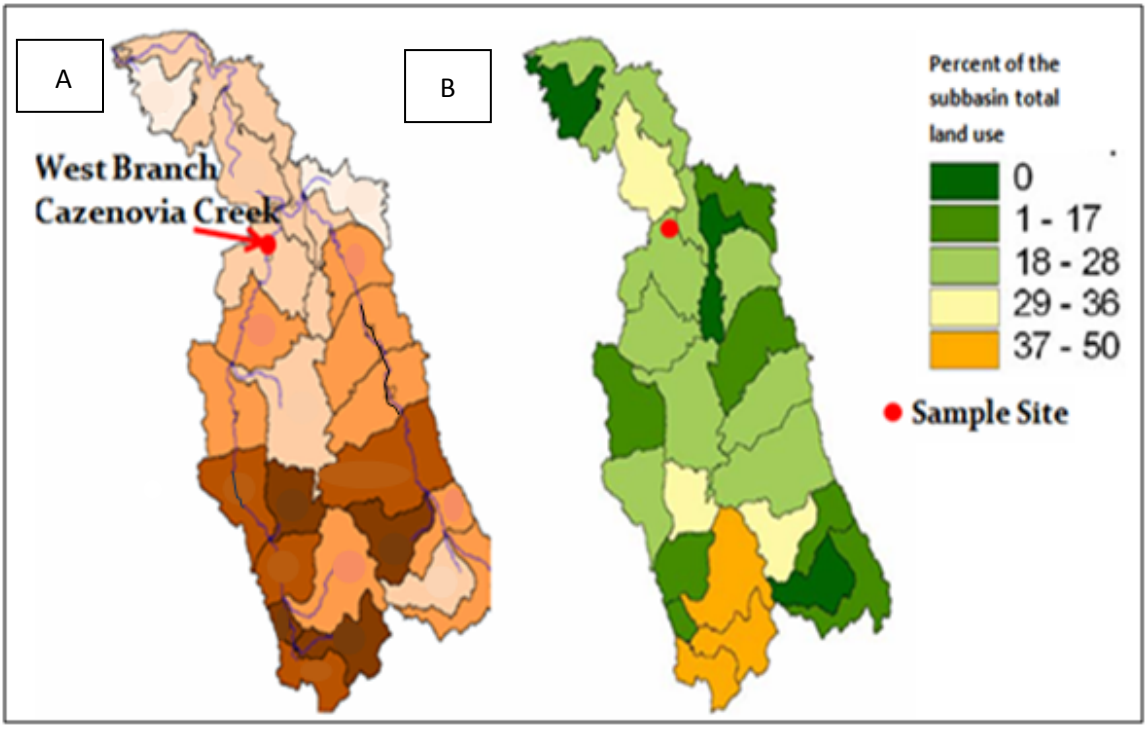
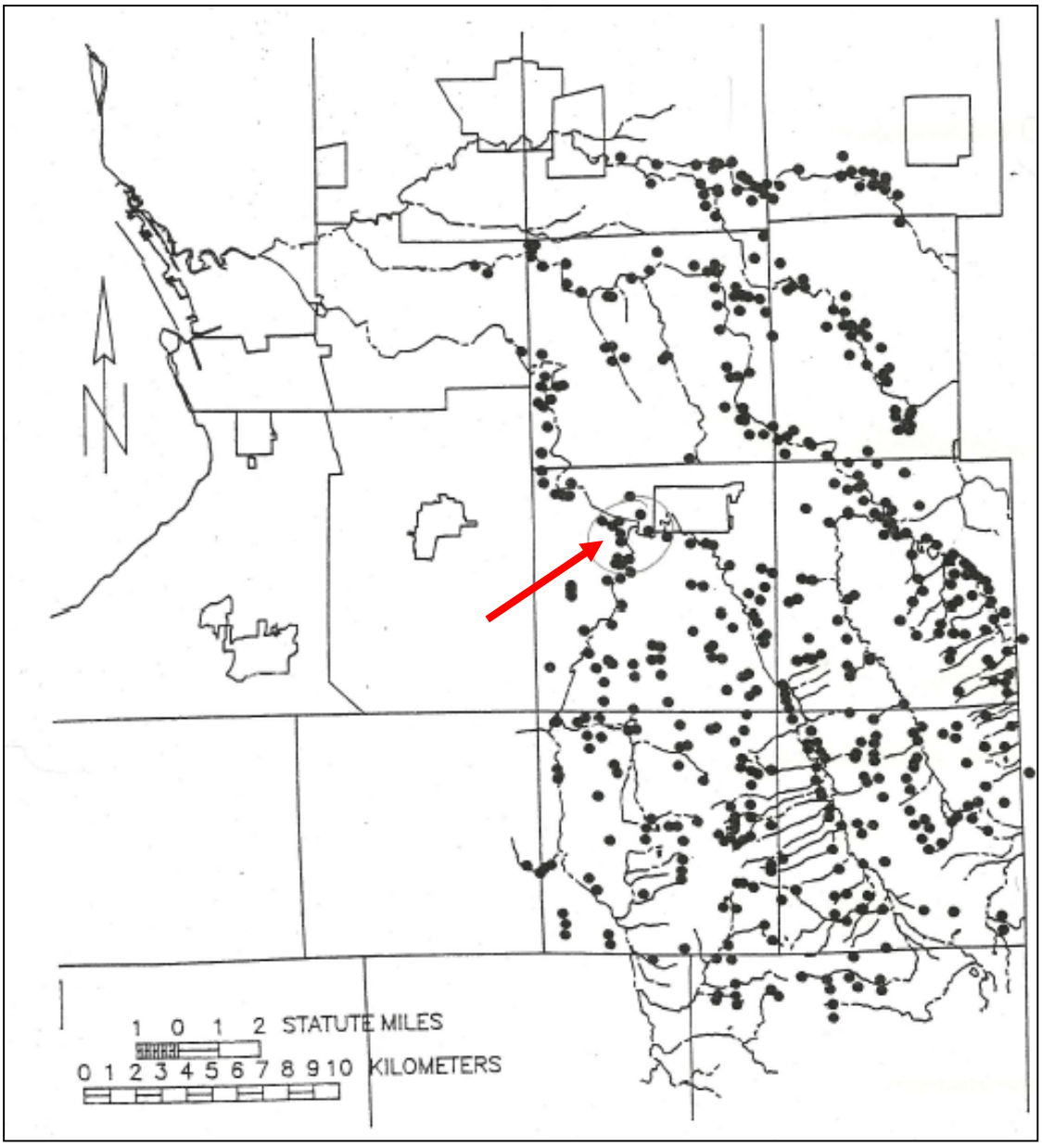

A
B
West Branch
Cazenovia Creek
Percent of the
subbasin total
land use
0
1 - 17
18 - 28
29 - 36
37 - 50
Sample Site

Figure 5- The location of farms with farm edges within 1000 feet of a waterway in the Buffalo River watershed. The arrow indicates the site where *Aeromonas* isolates were collected from both the sediment and water of the non-urban site of Cazenovia Creek (Irvine and Pettibone, 1996).


N
1 0 1 2 STATUTE MILES
0 1 2 3 4 5 6 7 8 9 10 KILOMETERS

2.20 Biochemical Testing

The *Aeromonas* isolates were removed from -70°C storage and were resuscitated by placing a glass bead coated with an *Aeromonas* isolate into one milliliter of nutrient broth containing ampicillin (10 µg/ml). After 24 hours of incubation at 25°C, the liquid cultures were streaked for isolation on nutrient agar plates containing ampicillin (10 µg/ml). After 24 hours of incubation at 35°C, a single, well isolated colony was selected and streaked onto nutrient agar slants containing ampicillin (10 µg/ml) which served as the working culture for biochemical testing.

Identification of *Aeromonas* isolates was determined based on the results of a number of biochemical tests. Biochemical tests included the production of acid and gas from the fermentation of glucose, sucrose, and mannitol as well as the organism's ability to decarboxylate lysine, ornithine, and arginine. Isolates also were tested for their ability to hydrolyze esculin, grow in 6% NaCl, and metabolize glucose via the Butanediol Pathway (Voges-Proskauer test). *Aeromonas hydrophila* ATCC7019, *Aeromonas caviae* ATCC15468, *Serratia marcescens* (laboratory strain), and *Proteus vulgaris* (laboratory strain) were used as controls in detecting esculin hydrolysis and lysine, arginine, and ornithine decarboxylation. The *Aeromonas* isolates were assigned to species by comparing test results with biochemical profiles for known *Aeromonas* species. After species identification, the isolates were cryopreserved on glass beads at -70°C in tryptic soy broth containing 20% glycerol until needed for antibiotic testing.

2.30 Kirby Bauer Disk Diffusion

Antibiotic susceptibility initially was assessed in *Aeromonas* isolates using the Kirby-Bauer disk diffusion method according to published ASM protocols (Wheat, 2001). Briefly, *Aeromonas* isolates were inoculated into 10 milliliters of tryptic soy broth and grown overnight at 35°C in a shaking incubator (Aros 160) at 150 rpm. After 12 to 16 hours of incubation, these cultures were inoculated into five milliliters of tryptic soy broth and incubated at 35°C for four to six hours in the shaking incubator (Aros 160) at 150 rpm to achieve log phase of growth. After incubation, these cultures were diluted, drop-wise using a sterile Pasteur pipette, into five milliliters of sterile 0.85% saline to achieve an optical density, as determined by visual comparison, corresponding to a 0.5 McFarland Standard. The 0.5 McFarland standard was made by adding 0.05 milliliters of 1% anhydrous barium chloride (BCl_2) and 9.95 milliliters of 1% sulfuric acid (H_2SO_4).

Within 15 minutes of preparation, a sterile cotton swab was used to evenly swab the organism three times onto the surface of a Mueller-Hinton plate, rotating the plate 60° after each swab application. A self-tamping six disc dispenser (BD Sensi-Disk Dispenser) was used to position the antibiotic disks (BD Sensi-Disk) onto the plate surface**.** The test antibiotics were cefoxotin (30µg), cepholothin (30µg), ceftriaxone (30µg), piperacillin (100µg), tetracycline (30µg), and nalidixic acid (30µg)**.** Plates were incubated at 35°C for 16 to 20 hours after which the diameter of the zone of growth inhibition was measured to the nearest millimeter using a digital caliper. Antibiotic-sensitive cultures of *Escherichia coli* ATCC25922, *Aeromonas hydrophila* ATCC7019,

and *Aeromonas sobria* ATCC9412 were included in each analysis as controls. Zone diameters were interpreted using published standards set by the National Committee for Clinical Laboratory Standards (Cavalieri *et al*., 2005).

2.40 Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of cephalothin-resistant *Aeromonas* isolates was assessed using the agar dilution method. A total of 0.2702 grams cephalothin sodium salt (Sigma-Aldrich) was added to 10 milliliters of sterile deionized and distilled water to make a cephalothin stock solution of 256,000µg/ml. Mueller Hinton agar plates were made to contain seven doubling dilutions of cephalothin (256µg/ml, 128µg/ml, 64µg/ml, 32µg/ml, 16µg/ml, 8µg/ml, and 4µg/ml). To make the first dilution (256µg/ml), one milliliter of the cephalothin stock (256,000µg/ml) was added to 99 milliliters of sterile deionized and distilled water. One milliliter of this dilution was added to 99 milliliters of Mueller Hinton agar and poured into plates. Subsequent doubling dilutions were made by adding five milliliters of the previous dilution into five milliliters of sterile deoinized and distilled water. One milliliter of each new dilution was added to 99 milliliters of Mueller Hinton agar and poured into plates.

Aeromonas isolates were inoculated into 10 milliliters of tryptic soy broth and grown overnight at 35°C in a shaking incubator (Aros 160) at 150 rpm. After 12 to16 hours of incubation, these cultures were inoculated into five milliliters of tryptic soy broth and incubated at 35°C for four to six hours in the shaking incubator (Aros 160) at

150 rpm to achieve log phase of growth. After incubation, these cultures were diluted, drop-wise using a sterile Pasteur pipette, into five milliliters of sterile 0.85% saline to achieve an optical density, as determined by visual comparison, corresponding to a 0.5 McFarland Standard.

A small volume, about 300 microliters, of each diluted culture was placed into a well in a 96-well plate using a sterile Pasteur pipette. An eight channel pipette inoculator (Eppendorf) was used to transfer one microliter of each culture onto previously made Mueller Hinton plates containing the cephalothin dilutions. Two Mueller Hinton plates containing no cephalothin were used as controls; one was inoculated in the beginning of the analysis and the other at the end, to ensure the cultures were transferred evenly to each plate. The plates were allowed to set for 10 minutes to ensure absorption of the cultures into the agar, after which they were inverted and incubated at 35°C for 16 to 20 hours. After incubation, the plates were assessed for the growth of each culture. The concentration of antibiotic in the first plate on which growth of an organism was not present was considered its minimum inhibitory concentration. *Aeromonas hydrophila* ATCC7019, *Aeromonas sobria* ATCC9412, *Escherichia coli* ATCC25922, and *Staphylococcus aureus* ATCC29213 were run with each analysis as control organisms.

2.50 Statistical Analysis

Statistical analysis was done on the data obtained from the Kirby Bauer disk diffusion test using GraphPad (www.graphpad.com). The two-tailed P values were

calculated using Fisher's exact test from 2x2 contingency tables comparing the number of cephalothin-resistant atypical *Aeromonas* taxa to the cephalothin-resistant identified taxa in the Buffalo River sediment (Appendix 1), Buffalo River water (Appendix 2), non-urban site sediment (Appendix 3), and the non-urban site water (Appendix 4). Analysis was also done to determine the significance of the number of cephalothin-resistant isolates in the sediment and water of the Buffalo River (Appendix 5) and of the non-urban site (Appendix 6). The number of cephalothin-resistant *Aeromonas* isolated from the Buffalo River was compared to the non-urban site using Fisher's exact test (Appendix 7).

3.0 Results

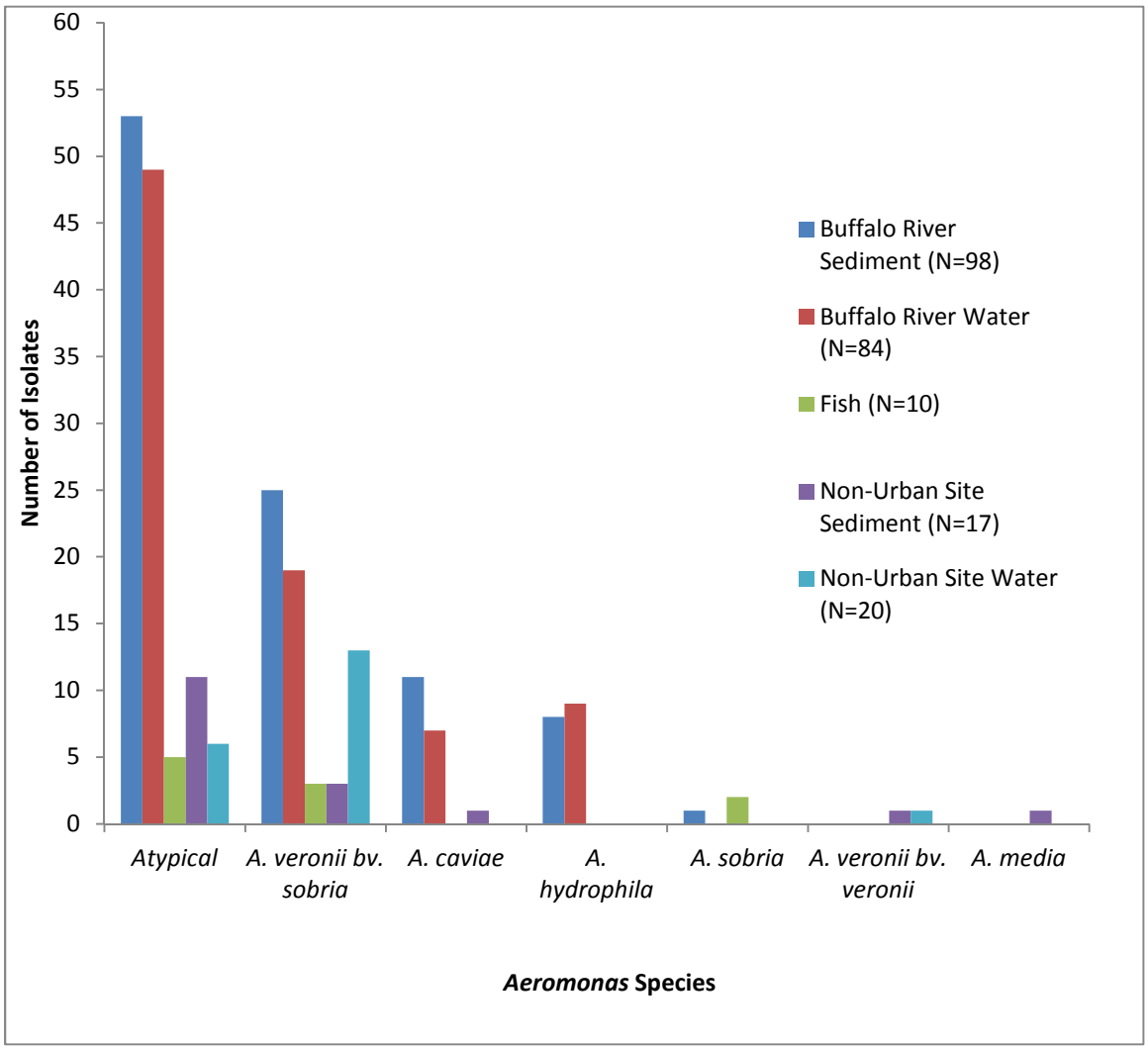
3. 10 Species Identification

Two hundred twenty-nine *Aeromonas* isolates were identified to the species level using 11 biochemical tests. All of the isolates were oxidase positive, produced acid from glucose metabolism, and were unable to grow in 6% NaCl. Most of the isolates (216 isolates, 94%) also produced acid from sucrose and mannitol metabolism. These characteristics were used to confirm that the isolate tested was *Aeromonas*. A total of 182 isolates were collected from the Buffalo River (98 sediment and 84 water isolates), ten *Aeromonas* isolates were collected from fish tissues in the Buffalo River watershed, and 37 isolates were collected from the non-urban site in Cazenovia Creek (17 sediment and 20 water isolates) (Figure 2).

Seven different *Aeromonas* taxa were identified among the 229 *Aeromonas* isolates. Unidentified *Aeromonas*, referred to as an "atypical isolate," was the most common taxon (124 isolates, 54%) (Figure 6). These atypical isolates were considered unidentifiable because they did not exactly match the published biochemical results of known *Aeromonas* species. Despite that, some atypical isolates shared common biochemical characteristics which will be explained in more detail. *Aeromonas veronii* biovar sobria was the most common species identified biochemically (63 isolates, 28%). *A. sobria* was found mainly in fish isolates (three isolates) but one was isolated from the Buffalo River sediment. *Aeromonas veronii* biovar veronii (two isolates) and *A. media* (one isolate) were found only in the non-urban site (Figure 6).

The biochemical characteristics for the Buffalo River *Aeromonas* isolates are summarized in Tables 2 and 3. There were 98 *Aeromonas* isolates collected from the sediment (Table 2) that were identified as one of five different *Aeromonas* taxa (atypical, *A. veronii* biovar sobria, *A. caviae*, *A. hydrophila*, and *A. sobria*). However, only four taxa were found among the 84 water *Aeromonas* isolates (atypical, *A. veronii* biovar sobria, *A. caviae*, and *A. hydrophila*) (Table 3). Atypical, or unidentified, isolates were found more commonly (102 of 182 isolates, 56%) in the Buffalo River than other taxon. Although many of these isolates differed from each other biochemically, a common biochemical pattern (arginine and ornithine decarboxylase, Voges-Praskeur, and glucose gas positive and negative for esculin hydrolysis) was noted in 16 (30%) of the atypical sediment isolates and 14 (29%) of the atypical water isolates.

Figure 6- The number of isolates in each *Aeromonas* taxa that were identified from the 229 *Aeromonas* isolates.


60
55
50
45
40
35
30
25
20
15
10
5
0
Number of Isolates
Buffalo River Sediment (N=98)
Buffalo River Water (N=84)
Fish (N=10)
Non-Urban Site Sediment (N=17)
Non-Urban Site Water (N=20)
Atypical
A. veronii bv. sobria
A. caviae
A. hydrophila
A. sobria
A. veronii bv. veronii
A. media
Aeromonas Species

There were three different *Aeromonas* taxa found in the 10 fish isolates: atypical, *A. veronii* biovar sobria, and *A. sobria* and their biochemical characteristics are shown in Table 4. Similar to the Buffalo River isolates, three of the atypical isolates (60%) had a similar biochemical pattern (arginine and ornithine decarboxylase, Voges-Praskeur, and glucose gas positive and negative for esculin hydrolysis). The biochemical characteristics of *A. veronii* biovar sobria and *A. sobria* were the same as those found in the Buffalo River isolates (Table 4).

The *Aeromonas* isolates collected from the non-urban site included two different taxa (*A. media* and *A. veronii* biovar veronii) than those found in the Buffalo River and fish isolates. There were five different *Aeromonas* taxa identified in the 17 sediment (atypical, *A. veronii* biovar. sobria, *A. caviae*, *A. veronii* biovar. veronii, and *A. media*) (Table 5). The only *A. media* isolate identified was a non-urban sediment isolate. There were only three *Aeromonas* species identified in the 20 non-urban water isolates (atypical, *A veronii* biovar sobria, and *A. veronii* biovar veronii) (Table 6). The atypical isolates from the sediment did not have a predominant biochemical pattern. However, there were two distinct biochemical patterns observed in the water isolates. Three non-urban water isolates (50%) were arginine and ornithine decarboxylase, Voges-Praskeur, and glucose gas positive, and negative for esculin hydrolysis. The other three water isolates (50%) were found to be arginine decarboxylase and VP positive only. The *A. media* isolate had the same biochemical characteristics as *A. veronii* biovar sobria but it

produced a brown pigment on the nutrient agar and ampicillin (10µg/ml) media (Tables 5 and 6).

3.20 Antibiotic Resistance

All 229 *Aeromonas* isolates were tested for antibiotic resistance to a first, second, and third generation cephalosporin (cephalothin, cefoxitin, and ceftriaxone, respectively), nalidixic acid, tetracycline, and piperacillin using the Kirby Bauer disk diffusion test. It is important to note that five *Aeromonas* isolates (three Buffalo River sediment isolates, one non-urban sediment isolate, and one non-urban water isolate) were not tested for antibiotic resistance because they did not survive resuscitation from storage at -70°C. All of the *Aeromonas* isolates tested were found to be susceptible to ceftriaxone and nalidixic acid. Similarly, all of the fish isolates were susceptible to all six of the test antibiotics (Figure 6).

A total of 105 of 229 (46%) *Aeromonas* isolates showed resistance to at least one of the test antibiotics. Resistance to cephalothin, which is a first generation cephalosporin, was the most common and was seen in 104 of the 105 (99%) resistant isolates. Resistance to any antibiotic was seen in 91 of 179 (51%) of the Buffalo River isolates (46 sediment and 45 water isolates) and in 14 of 35 (40%) of the non-urban isolates (nine sediment and five water isolates) (see Figures 6 and 7).

Table 2- Biochemical profiles for the *Aeromonas* isolated from Buffalo River sediment (n=98).

Species	Number of Isolates	Biochemical Test[a]				
		Esculin Hydrolysis	**Arginine Decarboxylase**	**Ornithine Decarboxylase**	**Voges Proskeur**	**Glucose Gas**
Atypical	53	+ (19%)	+ (83%)	+(55%)	+(79%)	+(57%)
A. veronii bv. sobria	25	-	+	-	+	+
A. caviae	11	+	+(64%)	-	-	-
A. hydrophila	8	+	+	-	+	+
A. sobria	1	-	-	-	-	+

a= 100% organisms showing trait is shown as a +
()=% of isolates demonstrating the trait.

Table 3- Biochemical profiles for the *Aeromonas* isolated from Buffalo River water (n=84).

Species	Number of Isolates	Biochemical Test[a]				
		Esculin Hydrolysis	**Arginine Decarboxylase**	**Ornithine Decarboxylase**	**Voges Proskeur**	**Glucose Gas**
Atypical	49	+(39%)	+(82%)	+(43%)	+(59%)	+(69%)
A. veronii bv. sobria	19	-	+	-	+	+
A. hydrophila	9	+	+	-	+	+
A. caviae	7	+	+(43%)	-	-	-

a= 100% organisms showing trait is shown as a +
()=% of isolates demonstrating the trait.

Table 4- Biochemical profiles for the *Aeromonas* isolated from fish (n=10).

Species	Number of Isolates	Biochemical Test[a]				
		Esculin Hydrolysis	**Arginine Decarboxylase**	**Ornithine Decarboxylase**	**Voges Proskeur**	**Glucose Gas**
Atypical	5	+(20%)	+(60%)	+(80%)	+(80%)	+
A. veronii bv. sobria	3	-	+	-	+	+
A. sobria	2	-	-	-	-	+

a= 100% organisms showing trait is shown as a +
()=% of isolates demonstrating the trait.

Table 5- Biochemical profiles of the *Aeromonas* isolated from non-urban sediment (n=17).

Species	Number of Isolates	Biochemical Test[a]				
		Esculin Hydrolysis	**Arginine Decarboxylase**	**Ornithine Decarboxylase**	**Voges Proskeur**	**Glucose Gas**
Atypical	11	+(18%)	+(55%)	+(36%)	+(64%)	+(45%)
A. veronii bv. sobria	3	-	+	-	+	+
A. caviae	1	+	+	-	-	-
A. veronii bv. veronii	1	-	-	+	+	+
A. media	1	-	+	-	+	+

a= 100% organisms showing trait is shown as a +
()=% of isolates demonstrating the trait.

Atypical *Aeromonas* isolates commonly showed antibiotic resistance (Figure 7). There were 54 of 102 (53%) atypical isolates from the Buffalo River (21 sediment and 31 water) that showed resistance to at least one antibiotic. Ten of 17 (59%) atypical isolates from the non-urban site (eight sediment and two water) showed resistance to at least one antibiotic. *Aeromonas hydrophila* was only found in the Buffalo River and 11 of 17 (65%) were found to be resistant to at least one antibiotic. Most of the *A. caviae* isolates from the Buffalo River (15 of 16 isolates, 94%) and the *A. caviae* from the non-urban site showed resistance to at least one test antibiotic. Eleven of 44 (25%) *A. veronii* bv. sobria isolates from the Buffalo River and three of 16 (19%) non-urban *A. veronii* bv. sobria isolates showed resistance to at least one antibiotic (Figure 7).

There were 17 *Aeromonas* isolates that showed resistance to more than one antibiotic. Sixteen of 17 (94%) of the multiple resistant isolates were collected from the Buffalo River (12 sediment and four water isolates) (Figure 6) and 15 (94%) of these were resistant to both cephalothin and cefoxitin. All the tested *Aeromonas* isolates in this study that showed cefoxitin resistance also showed cephalothin resistance. There was only one sediment isolate from the Buffalo River, identified as *A. hydrophila,* that was resistant to cephalothin and tetracycline. One water *A. veronii* biovar sobria isolate from the non-urban site showed resistance to three antibiotics: piperacillin, cefoxitin, and cephalothin.

Using the data obtained from the Kirby Bauer disk diffusion test, antibiotic profiles were made for each of the *Aeromonas* isolates for the Buffalo River (Table 7) and for the non-urban site (Table 8). Susceptibility to the test antibiotics was commonly

Table 6- Biochemical profiles for the *Aeromonas* isolated from non-urban water (n=20).

Species	Number of Isolates	Biochemical Test[a]				
		Esculin Hydrolysis	**Arginine Decarboxylase**	**Ornithine Decarboxylase**	**Voges Proskeur**	**Glucose Gas**
A. veronii bv. sobria	13	-	+	-	+	+
Atypical	6	-	+	+(50%)	+	+(50%)
A. veronii bv. veronii	1	-	-	+	+	+

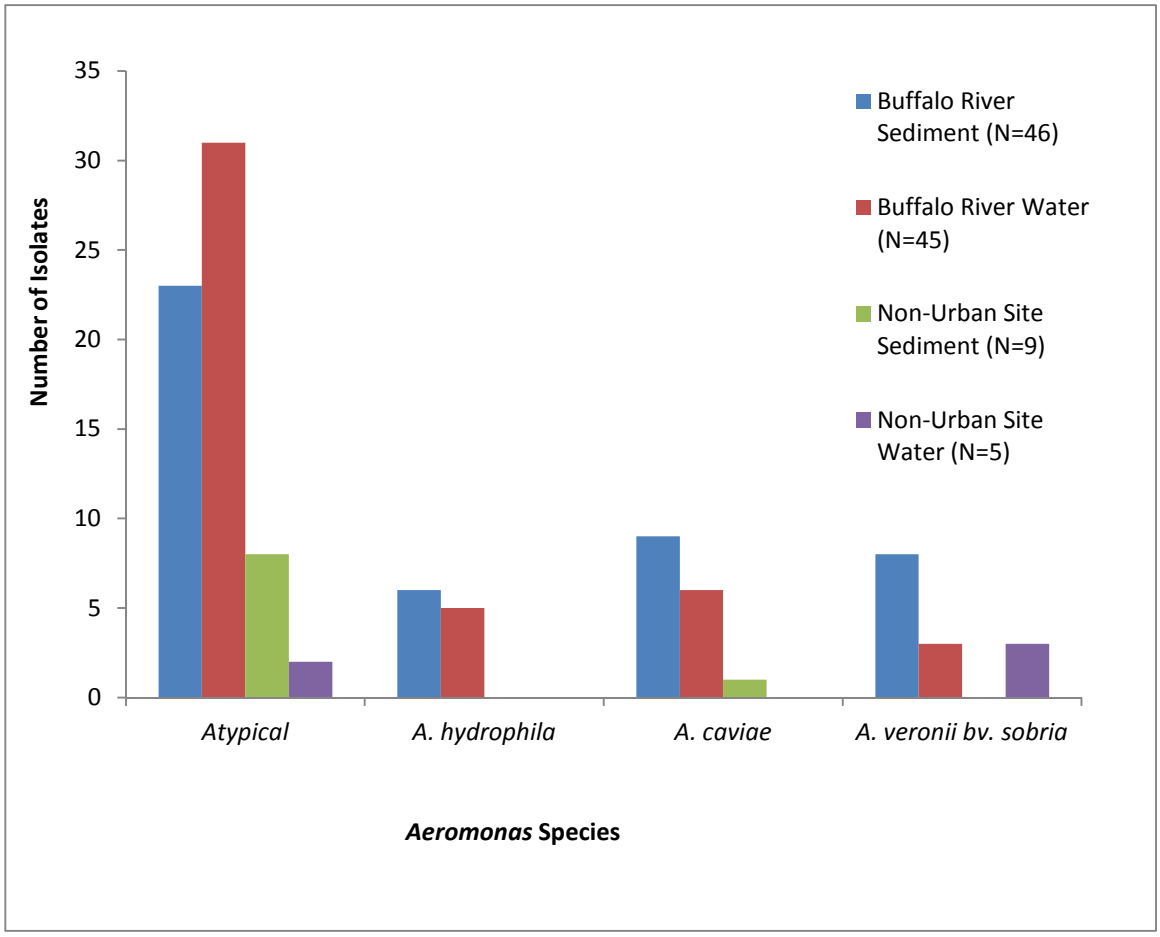
a= 100% organisms showing trait is shown as a +
()=% of isolates demonstrating the trait.

seen in both the Buffalo River (46 sediment isolates, 48% and 39 water isolates, 46%) and the non-urban site (seven sediment isolates, 44% and 14 water isolates, 70%). There were 90 isolates in the Buffalo River that were resistant to cephalothin. Seventy-four of these isolates (33 sediment and 41 water) showed only cephalothin resistance. Tetracycline resistance was seen only in two Buffalo River sediment isolates (Table 7). All 14 resistant isolates from the non-urban site were cephalothin resistant. One of these isolates also was resistant to piperacillin and cefoxitin (Table 8).

The degree of cephalothin resistance was determined for *Aeromonas* isolates that showed resistance to that antibiotic by the Kirby Bauer disk diffusion test. This was done by determining the minimum inhibitory concentration (MIC) of cephalothin, which is the lowest concentration of cephalothin that is needed to inhibit the growth of the organism. The MIC of cephalothin was tested because 104 of 105 (99%) of resistant isolates were cephalothin resistant. Only 76 of 90 cephalothin resistant Buffalo River isolates were tested because 14 of them did not survive long-term storage at 4°C. Likewise, only 13 of 15 cephalothin resistant non-urban isolates were tested because two isolates did not survive long term storage at 4°C.

Table 9 shows the cephalothin MIC results for the Buffalo River isolates. All of the tested isolates had an MIC greater than 32µg/ml. Only one isolate, identified as an atypical sediment isolate from the Buffalo River, had an MIC of 64µg/ml. Sixty-three of 76 (83%) of the Buffalo River isolates had an MIC greater than 256µg/ml, which was the maximum antibiotic concentration tested. All cephalothin-resistant isolates from the non-urban site (13 isolates) had an MIC greater than 256µg/ml (Table 10).

Figure 7- The total number of each *Aeromonas* taxa exhibiting resistance to at least one test antibiotic (n=105 isolates).


35
30
25
20
15
10
5
0
Number of Isolates
Buffalo River Sediment (N=46)
Buffalo River Water (N=45)
Non-Urban Site Sediment (N=9)
Non-Urban Site Water (N=5)
Atypical
A. hydrophila
A. caviae
A. veronii bv. sobria
Aeromonas Species

There was no significant difference between the number of cephalothin-resistant atypical and identified *Aeromonas* taxa in the Buffalo River sediment (p=0.4136) and in the non-urban water *Aeromonas* isolates (p=0.5696). However, in the Buffalo River water isolates, there was a statistical difference between the number of cephalothin-resistant atypical isolates and identified *Aeromonas* isolates (p=0.0465). In these water isolates, the number of cephalothin-resistant atypical *Aeromonas* (31 of 49) was higher than in the identified taxa (14 of 35). There was also a statistical difference between the number of cephalothin-resistant atypical *Aeromonas* (8 of 10) and identified *Aeromonas* (1 of 6) in the non-urban sediment (p=0.0350).

Analysis also was done on the number of cephalothin-resistant *Aeromonas* isolated from the sediment and water of both the Buffalo River and the non-urban site. It was determined that the number of cephalothin-resistant isolates collected from the sediment and water in the Buffalo River were not significantly different (p=0.4574). This was also true in the non-urban site (p=0.0937). When the number of cephalothin-resistant *Aeromonas* isolates found in all the Buffalo River isolates were compared to the non-urban *Aeromonas* isolates, it was determined that the difference was not statistically significant (p=0.2726).

Table 7- Antibiotic-resistance profiles for *Aeromonas* isolates from the Buffalo River as determined by the Kirby Bauer disk diffusion test (S: susceptible, R: resistant).

Buffalo River *Aeromonas* Isolates (n=179)

	Antibiotic						Number of Isolates	
	NA	**CT**	**PIP**	**TE**	**FOX**	**CF**	**Sediment (N=95)**	**Water (N=84)**
	S	S	S	S	S	S	49 (52%)	39 (46%)
	S	S	S	S	S	R	33 (35%)	41 (49%)
	S	S	S	S	R	R	11 (12%)	4 (5%)
	S	S	S	R	S	R	1 (1%)	0 (0%)
	S	S	S	R	S	S	1 (1%)	0 (0%)
Total	0	0	0	2	15	90	95 (100%)	84 (100%)

NA=Nalidixic Acid; CT=Ceftriaxone; PIP=Piperacillin; TE=Tetracycline; FOX=Cefoxitin; CF=Cephalothin

Table 8- Antibiotic-resistance profiles for *Aeromonas* isolates from the non-urban site as determined by the Kirby Bauer disk diffusion test (S: susceptible, R: resistant).

Non-Urban Site *Aeromonas* Isolates (n=35)

	Antibiotic						Number of Isolates	
	NA	**CT**	**TE**	**PIP**	**FOX**	**CF**	**Sediment (N=16)**	**Water (N=19)**
	S	S	S	S	S	S	7 (44%)	14 (70%)
	S	S	S	S	S	R	9 (56%)	4 (25%)
	S	S	S	R	R	R	0 (0%)	1 (5%)
Total	0	0	0	1	1	16	16 (100%)	19 (100%)

NA=Nalidixic Acid; CT=Ceftriaxone; PIP=Piperacillin; TE=Tetracycline; FOX=Cefoxitin; CF=Cephalothin

Table 9- Minimum inhibitory concentration of cephalothin among the Buffalo River isolates.

Buffalo River *Aeromonas* Cephalothin MIC (n=76)				
	Antibiotic Concentration			
Source	**64 µg/ml**	**128 µg/ml**	**256 µg/ml**	**>256 µg/ml**
Sediment (N=35)	1 (3%)	2 (6%)	3 (9%)	29 (83%)
Water (N=41)	0 (0%)	3 (7%)	4 (10%)	34 (83%)
Total	1 (1%)	5 (7%)	7 (9%)	63 (83%)

Table 10- Minimum inhibitory concentration of cephalothin among the non-urban site isolates.

Non-Urban Site *Aeromonas* Cephalothin MIC (n=13)				
	Antibiotic Concentration			
Source	**64 µg/ml**	**128 µg/ml**	**256 µg/ml**	**>256 µg/ml**
Sediment (N=8)	0 (0%)	0 (0%)	0 (0%)	8 (100%)
Water (N=5)	0 (0%)	0 (0%)	0 (0%)	5 (100%)
Total	0 (0%)	0 (0%)	0 (0%)	13 (100%)

4.0 Discussion

Aeromonas is commonly studied in aquatic environments, such as the Buffalo River watershed, because the prevalence of certain species can be linked to the trophic status of the water (Gugliandolo *et al*., 2009). The species found among the *Aeromonas* isolates were identified to determine if there was a correlation between the *Aeromonas* species and antibiotic resistance. Previous studies have found that *A. hydrophila* and *A. caviae* isolated from aquatic environments that receive urban pollution have a high incidence of antibiotic resistance (Hassani *et al.,* 1992 and Evangelista-Barreto *et al*., 2010).

In the present study, *A. vernoii* biovar sobria, *A. caviae*, and *A. hydrophila* were commonly isolated from both the Buffalo River and the non-urban sample site in Cazenovia Creek. These species typically are the most prevalent *Aeromonas* species identified from aquatic environments (Janda and Abbott, 2010). In particular, *A. veronii* biovar sobria was the most common species found in the Buffalo River watershed at 63 isolates (28%) which is agreement with other studies (Figueira *et al*., 2011 and Evangelista-Barreto *et al*., 2010).

Figueira *et al*. (2011) collected water samples from a wastewater treatment plant and identified 11 *Aeromonas* species using 16S rRNA gene sequence analysis. They found that the most prevalent species identified in raw surface water was *A. veronii* (25 of 51 isolates, 49%). They also found that *A. veronii* was the most common taxon found

among isolates collected from the wastewater treatment plant (33 of 121 isolates, 27%). Evangelista-Barreto *et al*. (2010) identified *A. caviae*, *A. veronii* biovar sobria, *A. veronii* biovar veronii, and *A. hydrophila* in 77% of water samples from River Cocó, Brazil. They also identified *A. trota*, *A. media*, and *A. sobria* in these water samples.

Most (124 of 229 isolates, 54%) of the *Aeromonas* isolates in the present study were not able to be identified to the species level with the biochemical tests used. The inability to accurately identify the *Aeromonas* species in this study made it difficult to determine the relationship, if any, between the identified *Aeromonas* species and the area that the isolate was collected from (Buffalo River and Cazenovia Creek). Although it is common to have some *Aeromonas* isolates that are unidentifiable with biochemical tests, the high number of atypical isolates in this study has not been seen in previously published data. Perhaps a greater number of atypical isolates could have been identified if different biochemical tests were used or genetic analysis was done.

Antibiotic-resistant bacteria are commonly seen in aquatic environments, like the Buffalo River, that receive urban effluent. Rivers are susceptible to the increase of antibiotic resistant bacteria because they receive water from wastewater treatment plants, industry, and farms, which often carry antibiotic discharge. Factors such as low-cost pharmaceuticals, the use of broad spectrum antibiotics, and inadequate waste-management of pharmaceuticals can be responsible for the pollution of rivers which may contribute to the selection of antibiotic-resistant bacteria (Lupo *et al*., 2012). The results of this study suggested that there may be a selective force in both the Buffalo River and Cazenovia Creek which favored the growth and antibiotic-resistant *Aeromonas*. This is

because approximately half (91 of 179 isolates, 51%) of the Buffalo River and about half (16 of 35 isolates, 46%) of the Cazenovia Creek *Aeromonas* isolates showed antibiotic resistance.

Urban populations strongly influence the water quality of aquatic environments, especially rivers because they often receive urban effluent. Aquatic bacteria, such as *Aeromonas*, can be useful indicators of the concentration of these pollutants in the aquatic environment. The higher the concentration of antibiotic pollution in an aquatic environment, the greater the selective pressure is favoring the growth of antibiotic resistant *Aeromonas* (Gugliandolo *et al*., 2009).

The antibiotics that were chosen for this study were four different β-lactams (piperacillin, and a first, second, and third generation of cephalosporin), tetracycline, and nalidixic acid, all of which are antibiotics commonly used in healthcare and agriculture. Three different generations of cephalosporins were used to study the emergence of antibiotic resistance in *Aeromonas*. Antibiotic resistance was seen in 105 of 229 *Aeromonas* isolates (46%). In particular, 104 of 105 (99%) antibiotic resistant *Aeromonas* isolates showed resistance to cephalothin indicating that resistance to first generation cephalosporins was common in the *Aeromonas* isolates. Cephalosporins, a type of β-lactam antibiotic, are commonly grouped together based on their antimicrobial properties and when they were developed (Pacifici, 2011). First generation cephalosporins, such as cephalothin, were the first group in this class to be introduced into healthcare. Since these antibiotics have been used for a longer period than second and third generation cephalosporins, resistance to first generation cephalosporins would

be expected to be more commonly seen in *Aeromonas,* which was a trend observed in this study.

Cephalothin resistance in *Aeromonas* has been shown in previous studies (Matyar *et al*., 2010; Hassani *et al*., 1992; and Evangelista-Barreto *et al.,* 2010). Matyar *et al*. (2010) found a high percentage (43 to 67%) of *Aeromonas* isolates collected from Iskenderun Bay, Turkey were resistant to four generations of cephalosporins. In particular, *Aeromonas* species showed a higher resistance to cefazolin, a first generation cephalosporin (59 to 86%) than later generation cephalosporins (12.3 to 71.4%). Hassani *et al.* (1992) found that 193 of 264 *Aeromonas* isolates (73%) collected from wastewater were resistant to cephalothin. They also found that all of the *A. hydrophila* isolates and 156 of 163 (96%) *A. caviae* isolates had multiple antibiotic resistances. In the present study, a high percentage of *A. hydrophila* and *A. caviae* showed resistance to cephalothin (11 of 17 *A. hydrophila* isolates, 65% and 15 of 16 *A. caviae* isolates, 94%). Evangelista-Barreto *et al*. (2010) found that almost all *A. caviae* strains collected from river water in Brazil were resistant to cephalothin as well as ciprofloxacin, ceftriaxon (third generation cephalosporin), chloramphenicol, and nalidixic acid. *Aeromonas veronii* biovar sobria also was resistant to these antibiotics as well as to sulfamethoxazole-trimethoprim, which was not seen in this study.

In this study, only two *Aeromonas* isolates showed tetracycline resistance and no isolates were found to be resistant to nalidixic acid. Previous studies (Huddleston *et al*., 2006 and Al-Bahry *et al*., 2009) also have shown that tetracycline and nalidixic acid resistance in *Aeromonas* species is rare. Huddleston *et al.* (2006) collected *Aeromonas*

isolates from urban and rural playa lakes in Lubbock, Texas and from several rivers in West Texas and New Mexico. They found that there were no *Aeromonas* isolates that were resistant to tetracycline and nalidixic acid. Al-Bahry *et al.* (2009) also found no tetracycline resistant *Aeromonas* species in water samples collected from tertiary treated sewage effluent collected from the Sultan Qaboos University sewage treatment plant. This system was studied because it received antimicrobial discharge from an on-site hospital and several laboratories.

Due to the low toxicity of β-lactams and their broad spectrum of action, cephalosporins are the most commonly prescribed antibiotic drug class (Lupo *et al*., 2012 and Dancer, 2001). Gram negative bacteria, including *Aeromonas*, may develop β-lactam resistance by the production of β-lactamases, especially by the expression of *ampC* genes located on the chromosome (Lupo *et al.,* 2012). Antibiotic resistance is seen in bacteria as part of natural selection which allows them to survive in different environments. Bacteria, like *Aeromonas*, are able to adapt to changes in the environment, such as an increase in antibiotic concentration, which often results in the development of mutations allowing them to survive in unfavorable conditions. Also, bacteria are able to transfer resistant genes to one another via vertical and horizontal transfer which aids in their ability to adapt to their environment (Kümmerer, 2009; Gugliandolo *et al*., 2009). This genetic transfer can be mediated by plasmids, bacteriophages, transposons, genomic islands, and transformations (Lupo *et al*., 2012). Son *et al*. (1997) found that the conjugal transfer of the nalidixic acid resistance gene occurred at a rate of $4.3x10^{-3}$ transconjugates per donor cells in *A. hydrophila* isolates collected from fish tissues. Future research on

the *Aeromonas* isolates used in this study should include identifying the cephalothin-resistance gene as well as its ability to transfer the resistance gene among *Aeromonas* and other bacteria in the environment.

An organism, such as *Aeromonas*, that is resistant to a cephalosporin may also demonstrate a reduced susceptibility to other antibiotics (Dancer, 2001). This may help to explain why some isolates that showed cephalothin resistance were also resistant to cefoxitin. Antibiotic resistance in *Aeromonas* to older antibiotics, such as first generation cephalosporins, is more common than resistance to newer antibiotics, such as second, third, and fourth generation cephalosporins. This is because the longer the antibiotic is used a greater concentration of it could be seen in urban effluent. This can lead to a greater number of *Aeromonas* that developed resistance towards the antibiotic and spread this resistance gene to other bacteria. Matyar *et al*. (2010) identified resistance in *Aeromonas* isolates to first generation cephalosporins was higher (59 to 86%) than in later generations of cephalosporins (14 to 54%). This was also the case in the present study in which 104 of 105 (99%) antibiotic resistant *Aeromonas* showed resistance to cephalothin (first generation cephalosporin). Only 16 of 105 (15%) isolates showed resistance to cefoxitin (second generation cephalosporin) and no isolate showed resistance to ceftriaxone (third generation cephalosporin). It is also important to note that all of the *Aeromonas* isolates that showed cefoxitin resistance were also resistant to cephalothin. Perhaps the cefoxitin and cephalothin-resistance genes are both located on the same plasmid or integron, which could be studied further.

Although antibiotic resistance in bacteria occurs naturally in the environment, factors like pollution, which is commonly seen in aquatic environments such as the Buffalo River, could speed its evolution (Lupo *et al.*, 2012). More research has to be done to understand the emergence of antibiotic resistance in *Aeromonas* species in the Buffalo River. It was hypothesized that there would be a greater number of cephalothin-resistant *Aeromonas* isolates from the Buffalo River than from the non-urban site. However, only an 11 percent difference between the number of antibiotic resistant *Aeromonas* isolates in the Buffalo River (91 of 179 isolates, 51%) and in the non-urban site (14 of 35 isolates, 40%) (p=0.2726).

In this study, more antibiotic-resistant isolates were found in the non-urban site than was originally hypothesized. Perhaps the reason for this is agricultural discharge in Cazenovia Creek. About 18-28% of the land in the sample area of Cazenovia Creek is used for agriculture and antibiotics are commonly used in farming and cattle rearing. Although there was no data present at the time of this study on the antibiotic usage in this area, the high number of antibiotic-resistant *Aeromonas* isolates found in this area may suggest that there is a heavier antibiotic load that is being discharged into the creek than was originally thought. Since the Buffalo River watershed also flows through residential communities, the improper disposal of personal antibiotics also may be causing a greater concentration of antibiotic discharge into the water.

Goñi-Urriza *et al.* (2000a) found that there was an increase in the number of antibiotic-resistant *Aeromonas* in urban effluents, unlike the present study. They found that *Aeromonas* isolates collected downstream from the wastewater discharge of

Pamplona, Spain showed 50% more antibiotic resistant *Aeromonas* species than those collected upstream. Although it is unknown why antibiotic resistance was not more commonly seen in the Buffalo River *Aeromonas* isolates, one can speculate that there may be a higher concentration of antibiotics in the agricultural discharge from Cazenovia Creek than originally thought. This would cause an increase in the number of antibiotic resistant *Aeromonas* isolates from the non-urban site.

Often times antimicrobial treatments used in fish farming and agriculture can have an impact on the incidence of antibiotic resistant *Aeromonas*. Gordon *et al.* (2007) studied the antibiotic resistance in *Aeromonas* isolates collected from sediment samples upstream and downstream of fish farms. They found that oxolinic acid resistance, a common antibiotic used in these farms, occurred in up to 34% of the isolates downstream of the farms and in none of the isolates collected upstream of the fish farms. This suggested that antibiotics used in agriculture can persist in the aquatic environment and select for antibiotic-resistant *Aeromonas*. This could explain the number of antibiotic-resistant *Aeromonas* isolated from the non-urban site in this study. Perhaps antibiotics used in these farms persist in the Buffalo River watershed and the increase in their concentration is responsible for the number of antibiotic-resistant *Aeromonas*. The most common antibiotics used in plant agriculture are streptomycin, oxytetracycline, gentamicin, and oxilinic acid. In the United States, about 40% of the antibiotics used are in animal husbandry (McManus *et al*., 2002).

This study analyzed previously collected *Aeromonas* isolates from the Buffalo River watershed for antibiotic resistance. The non-urban *Aeromonas* isolates were

collected from an area, shown in Figure 4b, in which 18 to 28% of the land was used for agriculture (Inamdar, 2004). Cazenovia Creek passes through land that is also used for residential and commercial activities in several small communities along the creek and industrial activities further downstream towards the city of Buffalo (Wills and Irvine, 1996). The upper branches of Cazenovia Creek (East and West Branches) have a steeper stream gradient (0.31 to 2.9%) than the lower creek and an average slope gradient of 9 to 12% (Wills and Irvine, 1996 and Inamdar, 2004).

Inamdar (2004) suggested that subbasins that have a greater percentage of agricultural land use and steeper slope gradients also produce the most sediment concentrations. It was determined that Cazenovia Creek was the largest contributor of discharge to the Buffalo River watershed (38%) and the second largest runoff contributor (34%) in the watershed (Inamdar, 2004). Wills and Irvine (1996) determined that the water quality on the East Branch of Cazenovia Creek significantly decreased immediately downstream of the village of East Aurora, which was downstream of the sample site in the present study (Figure 2a). They suggested that the local urban runoff and inputs from the sewage treatment plant may have a negative effect on the water quality (Wills and Irvine, 1996). This run-off may include antibiotic discharge and could create a selective environment downstream of the sample site favoring the growth of antibiotic resistant *Aeromonas*.

It was hypothesized that the *Aeromonas* isolates collected from Cazenovia Creek would show less antibiotic resistance than the Buffalo River isolates because the site is upstream of the urban discharge, thus the concentration of antibiotic pollution in

Cazenovia Creek would be less than the Buffalo River. However, there is no information, at the time of this study, about antibiotic usage in the farms located near the sample site in Cazenovia Creek. If more information were gathered about these farms and their antibiotic usage, the large number of antibiotic resistant isolates in this area might be explained.

Sediment isolates were hypothesized to contain more antibiotic resistant *Aeromonas* isolates. This is because most antibiotics are hydrophobic molecules which would settle on the sediment layer causing an increase the concentration of antibiotic pollution. This increase in antibiotic concentration would select for antibiotic resistant *Aeromonas*. The concentration of antibiotics in solid surfaces such as sediments, sewage sludge, and soil is typically higher if the active compounds are persistent and are able to accumulate. Thus, past usage of antibiotics could be indicated by the increase of antibiotic resistance among bacteria found in the sediments, especially in areas of fish farms (Kümmerer, 2009).

Antibiotic discharge seen in the Buffalo River should, in theory, cause a higher concentration of antibiotic pollution in the sediment layer. This should select for antibiotic-resistant *Aeromonas*, thus causing the number of these isolates to be higher than in the Buffalo River water isolates and the non-urban site isolates. However, when the number of cephalothin-resistant water and sediment isolates from the Buffalo River were compared, it was found that there was no significant difference between them ($p=0.4574$). This suggests that there may not be a selective force favoring cephalothin resistance in the sediment isolates as was originally hypothesized. In other words,

cephalothin resistance occurs at the same rate in both the sediment and water isolates in the Buffalo River. This was also the case in the non-urban site. There was no significant difference between the number of cephalothin-resistant sediment and water isolates from the non-urban site (p=0.0937).

The *Aeromonas* taxa (atypical or identified species) had no effect on the number of cephalothin-resistant isolates in the Buffalo River sediment (p=0.4136) and the non-urban site water (p=0.5696). However this was not the case in the Buffalo River water and non-urban site sediment. There were more atypical isolates (31 of 49 isolates) in the Buffalo River water that showed cephalothin resistance than identified isolates (14 of 35 isolates, p=0.0465). This was also seen in the non-urban sediment where eight of 10 atypical isolates showed cephalothin resistance as opposed to only one of six identified isolates (p=0.0350). This suggests that in the Buffalo River water and the non-urban site sediment, there may be a selective force favoring cephalothin resistance in the atypical isolates as opposed to the identified isolates. However to prove this hypothesis, further research has to be done on a larger sample of isolates from these two areas.

More research needs to be done on the concentration of antibiotics in the Buffalo River and their effect on the incidence of antibiotic-resistance *Aeromonas*. If there is a greater concentration of an antibiotic in the environment, the greater the selective pressure would be which favors the growth of *Aeromonas* that are resistant to that antibiotic. This might include studies on mobile genetic element transfer among *Aeromonas* species in the Buffalo River watershed, which might suggest that antibiotic resistance genes are passed from resistant *Aeromonas* to susceptible *Aeromonas* isolates.

Antibiotic resistance is a growing problem among *Aeromonas* species due to the overuse of antibiotics. This study serves as a model for studying the incidence of antibiotic resistance in a river that receives urban effluent. These results can be used to guide future studies on the incidence of antibiotic resistance in the Buffalo River. These studies can include determining the presence and concentration of antibiotic pollution in the Buffalo River watershed. This information could be used to study the direct effects of antibiotic pollution on the incidence of antibiotic-resistant *Aeromonas* isolates. Research also should be done on the ability of these resistance genes, in particular cephalothin-resistant genes, to transfer to non-resistant *Aeromonas* isolates and other clinically important bacteria. The information gathered from these experiments can be used to understand the problem of urban pollution in the Great Lakes Area of Concern (AOC). By studying the effects of antibiotic pollution on the incidence of antibiotic-resistant *Aeromonas*, more strict water quality guidelines can be set.

Resistance to a first generation cephalosporin is commonly seen in this study probably because this generation has been used for a longer period of time than later generations. An extension of this study would be to study the evolution of resistance to later generations of cephalosporin over time. Perhaps *Aeromonas* isolates would show resistance toward second and third generation cephalosporins over the course of several years after the concentration of these antibiotics have had the chance to accumulate in the aquatic environment. This information could be used to study the ability of *Aeromonas* to evolve with the environment. The incidence of antibiotic resistance in *Aeromonas* suggests that antibiotic pollution which decreases water quality can lead to a rise in

resistant *Aeromonas* which could have serious consequences in both healthcare and the economy.

5.0 Literature Cited

Abbott, S. L., Cheug. W. K. W., & Janda, J. M. (2003). The Genus *Aeromonas*: Biochemical Characteristics, Atypical Reactions, and Phenotypic Identification Schemes. *Journal of Clinical Microbiology*, 41, 2348–2357.

Abbott, S. L., Cheung, W. K. W., Kroske-Bystrom, S., Malekzadeh, T., & Janda, J. M. (1992). Identification of *Aeromonas* Strains to the Genospecies Level in the Clinical Laboratory. *Journal of Clinical Microbiology*, 30, 1262-1266.

Aberoum, A. & Jooyandeh, H. (2010). A Review on Occurrence and Characterization of the *Aeromonas* Species from Marine Fishes. *World Journal of Fish and Marine Sciences*, 2, 519-523.

Adulhamd, Ashraf T. (2009). Characterization of *Aeromonas hydrophila* Isolated from Aquatic Environments Using Phenotypic and Genotyping Methods. *Research Journal of Agriculture and Biological Sciences*, 5, 923-931.

Alavandi, S.V. & Ananthan, S. (2009). Biochemical Characteristics, Serogroups, and Virulence Factors of *Aeromonas* Species Isolated From Cases of Diarrhea and Domestic Water Samples in Chennai. *Indian Journal of Medical Microbiology*, 21, 233-238.

Al-Bahry, S. N., Mahmoud, I. Y., Al-Khaifi, A., Elshafie, A. E., Al-Harthy, A. (2009). Viability of Multiple Antibiotic Resistant Bacteria in Distribution Lines of Treated Sewage Effluent Used for Irrigation. *Water Science and Technology*, 60, 2939-2948.

Auch, A. F., von Jan, M., Klenk, H. P., Göker, M. (2010). Digital DNA-DNA Hybridization for Microbial Species Delineation by Means of Genome-to-Genome Sequence Comparison. *Standards in Genomic Sciences*, 2**,** 117-134.

Bin Kingombe, C. I., D'Aoust, J. Y., Huys, G., Hofmann, L., Rao, M., & Kwan, J. (2010). Multiplex PCR Method for Detection of Three *Aeromonas* Enterotoxin Genes. *Applied and Environmental Microbiology*, 76, 425–433.

Borchardt, Mark A., Stemper, Mary E., & Standridge, Jon H. (2003). *Aeromonas* Isolates from Human Diarrheic Stool and Groundwater Compared by Pulsed-Field Gel Electrophoresis. *Emerging Infectious Diseases*, 9, 224-228.

Borrell, N, Actinas, S. G., Figueras, M. J., & Martínez-Murcia, A. J. (1997). Identification of *Aeromonas* Clinical Isolates by Restriction Fragment Length Polymorphism of PCR-Amplified 16S rRNA Genes. *Journal of Clinical Microbiology*, 35, 1671-1674.

Cavalieri, S. J., Rankin, I. D., Harbeck, R. J., Sautter, R. L., McCarter, Y. S., Sharp, S. E., Ortez, J. H., and Spiegel, C. A. *Manual of Antimicrobial Susceptibility Testing.* Library of Congress Cataloging-in-Publication Data, (2005) ISBN: 1-55581-349-6.

Chacón, M. R., Castro-Escarpulli, G., Soler, L., Guarro, J., & Figueras, M. J. (2002). A DNA Probe Specific for *Aeromonas* Colonies. *Diagnostic Microbiology and Infectious Disease*, 44, 221-225.

Dancer, S. J. (2001). The problem with cephalosporins. *Journal of Antimicrobial Chemotherapy*, 48, 463-478.

Evangelista-Barreto, N. S., Teles de Carvalho, F. C., dos Fernandes Vieira, R. H. S., dos Reis, C. M. F., Macrae, A., & dos Prazeres Rodrigues, D. (2010). Characterization of *Aeromonas* Species Isolated from an Estuarine Environment. *Brazilian Journal of Microbiology*, 41, 452-460.

Faller, A. & Schleifer, K. H. (1981). Modified Oxidase and Benzidine Tests for Separation of Staphylococci from Micrococci. *Journal of Clinical Microbiology*, 13, 1031.

Figueira, V., Vaz-Moreira, I., Silva, M., & Manaia, C. M. (2011). Diversity and Antibiotic Resistance of *Aeromonas* Species in Drinking and Waste Water Treatment Plants. *Water Research*, 45, 5599 -5611.

Ghenghesh, Khalifa Sifaw, Ahmed, Salwa F., El-Khalek, & Rania Abdel. (2008). *Aeromonas*-Associated Infections in Developing Countries. *Journal of Infections Developing Countries*, 2, 81-98.

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

Goñi-Urriza, M., Pineau, L., Capdepuy, M., Roques, C., Caumette, P., & Quentin, C. (2000b). Antimicrobial Resistance of Mesophilic *Aeromonas* Species Isolated from Two European Rivers. *Journal of Antimicrobial Chemotherapy*, 46, 297-301.

Gordon, L., Giraud, E., Ganière, J. P., Armand, F., Bouju-Albert, A., de la Cotte, N., Mangion, C., Le Bris, H. (2007). Antimicrobial Resistance Survey in a River Receiving Effluents from Freshwater Fish Farms. *Journal of Applied Microbiology*, 102, 1167–1176.

Graf, J. (1999). Symbiosis of *Aeromonas veronii* Biovar sobria and *Hirudo medicinalis*, the Medicinal Leech: a Novel Model for Digestive Tract Associations. *Infection and Immunity*, 67, 1-7.

GraphPad. Retrieved from www.graphpad.com (2013, July 17).

Gugliandolo, C., Lentini, V., Fera, M. T., La Camera, E., & Maugeri, T. L. (2009). Water Quality and Ecological Status of the Alcantara River Estuary (Italy). *New Microbilogica*, 32, 77-87.

Hassani, L., Imziln, B., Boussaid, A., & Gauthier, M. J. (1992). Seasonal Incidence of and Antibiotic Resistance among *Aeromonas* Species Isolated from Domestic Wastewater Before and After Treatment in Stabilization Ponds. *Microbiology Ecology*, 23, 227-237.

Hedges, R. W., Smith, P., & Brazil, G. (1985). Resistance Plasmids of Aeromonads. *Journal of General Microbiology*, 131, 2091-2095.

Huang, X., Wang, K., Zong-Jun, D., Geng, Y., Deng, Y. (2010). Identification, Isolation and *In Vitro* Antimicrobial Susceptibility Testing of *Aeromonas veronii* Associated with an Acute Death of Channel Catfish *(Ictalurus lunetas*) in China. *African Journal of Biotechnology*, 9, 2161-2164.

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

Inamdar, Shreeram. (2004). Sediment Modeling for the Buffalo River Watershed. Great Lakes Center, Buffalo State College, 1-59.

Irvine, K. N. and Pettibone, G. W. (1996). Planning Level Evaluation of Densities and Sources of Indicator Bacteria in a Mixed Land Use Watershed. Environmental Toxicology, 17, 1-12.

Jacobs, L., & Chenia, H. Y. (2007). Characterization of Integrons and Tetracycline Resistance Determinants in *Aeromonas* Species Isolated from South African Aquaculture Systems. *International Journal of Food Microbiology*, 114, 295–306.

Janda, J. M., & Abbott, S. L. (1998). Evolving Concepts Regarding the Genus *Aeromonas*: An Expanding Panorama of Species, Disease Presentations, and Unanswered Questions. *Clinical Infectious Diseases*, 27, 332–44.

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

Khajanchi, B. K., Fadl, A. A., Borchardt, M. A., Berg, R. L., Horneman, A. J., Stemper, M. E., Joseph, S. W., Moyer, N. P., Sha, J., & Chopra, A. K. (2010). Distribution of Virulence Factors and Molecular Fingerprinting of *Aeromonas* Species Isolates from Water and Clinical Samples: Suggestive Evidence of Water-to-Human Transmission. *Applied and Environmental Microbiology*, 76, 2313–2325.

Kivanc, Merih, Yilmaz, Meral, & Demir, Filiz. (2011). The Occurrence of *Aeromonas* in Drinking Water, Tap Water and the Porsuk River. *Brazilian Journal of Microbiology*, 42, 126-131.

Kümmerer, K. (2003). Significance of Antibiotics in the Environment. *Journal of Antimicrobial Chemotherapy*, 52, 5-7.

Kümmerer, K. (2009). Antibiotics in the Aquatic Environment- A Review- Part II. *Chemosphere*, 75, 435-441.

Leblanc, D., Mittal, K. R., Oliver, G., & Lallier, R. (1981). Serogrouping of Motile *Aeromonas* Species Isolated from Healthy and Moribund Fish. *Applied and Environmental Microbiology*, 42, 56-60.

Lupo, A., Coyne, S., and Berendonk, T. U. (2012). Origin and Evolution of Antibiotic Resistance: The Common Mechanisms or Emergence and Spread in Water Bodies. *Frontiers in Microbiology*, 3, 1-13.

Maalej, S., Mahjoubi, A., Elazri, C., & Dukan, S. (2003). Simultaneous Effects of Environmental Factors on Motile *Aeromonas* Dynamics in an Urban Effluent and in the Natural Seawater. Water Research, 37, 2865-2874.

Manninen, Christine. (2003). "Beneficial Uses in the Great Lakes Area of Concern." Great Lakes Information Network. Retrieved from http://www.great-lakes.net/envt/pollution/benefuse.html.

Martino, M. E., Fasolato, L., Montemurro, F., Rosteghin, M, Manfrin, A., Patarnello, T., Novelli, E., & Cardazzo, B. (2011). Determination of Microbial Diversity of *Aeromonas* Strains on the Basis of Multilocus Sequence Typing, Phenotype, and Presence of Putative Virulence Genes. *Applied and Environmental Microbiology*, 77, 4986–5000.

Martone-Rocha, S., Piveli, R. P., Matté, G. R., Doria, M. C., Dropa, M., Morita, M., Peternella, F. A., & Matté, M. H. (2010). Dynamics of *Aeromonas* Species Isolated from Wastewater Treatment System. *Journal of Water and Health*, 8, 703-711.

Matyar, F., Akkan, T., U□ak, Y., Eraslan, B. (2010). *Aeromonas* and *Pseudomonas*: Antibiotic and Heavy Metal Resistance Species from Iskenderun Bay, Turkey (Northeast Mediterranean Sea). *Environmental Monitoring and Assessment*, 167, 309–320.

McManus, P. S., Stockwell, V. O., Sundin, G. W., and Jones, A. L. (2002). Antibiotic Use in Plant Agriculture. *Phytopathology*, 40, 443-465.

Monfort, P. and Baleux, B. (1990). Dynamics of *Aeromonas hydrophila*, *Aeromonas sobria*, and *Aeromonas caviae* in a Sewage Treatment Pond. *Applied and Environmental Microbiology*, 56, 1999-2006.

Moura, A., Pereira, C., Henriques, I., & Correia, A. (2012). Novel gene Cassettes and Integrons in Antibiotic-Resistant Bacteria Isolated from Urban Wastewaters. *Research in Microbiology*, 163, 92-100.

Nash, J. HE, Findlay, W. A., Luebbert, C. C., Mykytczuk, O. L., Foote, S. J. Taboada, E. N., Carrillo, C. D., Boyd, J. M., Colquhoun, D. J., Reith, M. E., & Brown, L. L. (2006). Comparative Genomics Profiling of Clinical Isolates of *Aeromonas salmonicida* using DNA Microarrays. *BMC Genomics*, 7.

Pablos M., Rodríguez-Calleja, J. M., Santos, J. A., Otero, A., & García-López, M. L. (2009). Occurrence of Motile *Aeromonas* in Municipal Drinking Water and Distribution of Genes Encoding Virulence Factors. *International Journal of Food Microbiology*, 135, 158–164.

Pacifici, G. M. (2011). Pharmacokinetics of Cephalosporins in the Neonate: A Review. Clinics, 66, 1267-1274. Doi: 10.1590/S1807-59322011000700024

Pettibone, G. W. (1998). Population Dynamics of *Aeromonas* species in an Urban River Watershed. *Journal of Applied Microbiology*, 85, 723-730.

Pianetti, A., Falcioni, T., Bruscolini, F., Sabatini, L., Sisti, E., & Papa, S. (2005). Determination of the Viability of *Aeromonas hydrophila* in Different Types of Water by Flow Cytometry, and Comparison with Classical Methods. *Applied and Environmental Microbiology*, 71, 7948-7954.

Popoff, M. & Véron, M. (1976). A Taxonomic Study of the *Aeromonas hydrophila-Aeromonas punctata* Group. *Journal of General Microbiology*, 94, 11-22.

Rahman, M., Colque-Navarro, P., Kühn, I., Huys, G., Swings, J., & Möllby, R. (2002). Identification and Characterization of Pathogenic *Aeromonas veronii* Biovar sobria Associated with Epizootic Ulcerative Syndrome in Fish in Bangladesh. *Applied and Environmental Microbiology*, 68, 650-655.

Rockwell, David. C., Claff, R. E., and Kuehl, D. W. (1984). 1981 Buffalo, New York, Area Sediment Survey (BASS). Environmental Protection Agency, Chicago, IL. Great Lakes National Program Office. EPA 905/3-84-001.

Ruiz, J. (2003). Mechanisms of Resistance to Quinolones: Target Alterations, Decreased Accumulation and DNA Gyrase Protection. *Journal of Antimicrobial Chemotherapy, 51,* 1109–1117.

Schmidt, A. S., Bruun, M. S., Dalsgaard, I., & Larsen, J. L. (2001). Incidence, Distribution, and Spread of Tetracycline Resistance Determinants and Integron Associated Antibiotic Resistance Genes among Motile Aeromonads from a Fish Farming Environment. *Applied and Environmental Microbiology*, 67, 5675–5682.

Sen, K., & Rodgers, M. (2004). Distribution of Six Virulence Factors in *Aeromonas* Species Isolated from US Drinking Water Utilities: a PCR Identification. *Journal of Applied Microbiology*, 97, 1077–1086.

Sisti, M., Albano, A., Brandi, G. (1998). Bactericidal Effect of Chlorine on Motile *Aeromonas* Species in Drinking Water Supplies and Influence of Temperature on Disinfection Efficacy. *Letters in Applied Microbiology*, 26, 347-351.

Son, R., Rusul, G., Sahilah, A. M., Zainuri, A., Raha, A. R., Salmah, I. (1997). Antibiotic Resistance and Plasmid Profiling of *Aeromonas hydrophila* Isolates from Cultured Fish, Telapia (*Telapia mossambica*). *Letters in Applied Microbiology*, 24, 479-482.

Stewart, J. R., Gast, R. J., Fujioka, R. S., Solo-Gabriele, H. M., Meschke, J. S., Amaral-Zettler, L. A., del Castillo, E., Polz, M. F., Collier, T. K., Strom, M. S., Sinigalliano, C. D., Moeller, P. DR., & Holland, A. F. (2008). Proceedings: The Coastal Environment and Human Health: Microbial Indicators, Pathogens, Sentinels and Reservoirs. *Environmental Health*, 7 (Suppl. 2), S3.

U.S. Environmental Protection Agency. (2011, December 15). "Great Lakes". Retrieved from http://www.epa.gov/greatlakes/index.html.

Vivas, J., Saa, A. I., Tinajas, A., Barbeyto, L., & Rodriguez, L. A. (2000). Identification of Motile *Aeromonas* Strains with the MicroScan WalkAway System in Conjunction with the Combo Negative Type 1S Panels. *Applied and Environmental Microbiology*, 66, 1764-1766.

Vilches, S., Jimenez, N., Tomás, J. M., Merino, S. (2009). *Aeromonas hydrophila* AH-3 Type III Secretion System Expression and Regulatory Network. *Applied and Environmental Microbiology*, 75, 6382–6392.

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

Wheat, P. F. (2001). History and Development of Antimicrobial Susceptibility Testing Methodology. *Journal of Antimicrobial Chemotherapy*, 48, 1-4.

Wills, M. and Irvine, K. N. (1996). Application of the National Sanitation Foundation Water Quality Index in the Cazenovia Creek, NY, Pilot Watershed Management Project. *Middle States Geographer*, 1996, 95-104.

Yáñez, M. A., Catalán, V., Apráiz, D., Figueras, M. J., and Martínez-Murcia, A. J. (2003). Phylogenetic Analysis of Members of the Genus *Aeromonas* Based on gyrB Gene Sequences. *International Journal of Systematic and Evolutionary Microbiology*, 53, 875-883.

6.0 Appendix 1: Buffalo River Sediment Analysis

	Cephalothin Resistant	Not Cephalothin Resistant
Atypical *Aeromonas*	23	29
Identified *Aeromonas*	23	20
p=0.4136		

Appendix 2: Buffalo River Water Analysis

	Cephalothin Resistant	Not Cephalothin Resistant
Atypical *Aeromonas*	31	18
Identified *Aeromonas*	14	21
p=0.0465		

Appendix 3: Non-Urban Sediment Analysis

	Cephalothin Resistant	Not Cephalothin Resistant
Atypical *Aeromonas*	8	2
Identified *Aeromonas*	1	5
p=0.0350		

Appendix 4: Non-Urban Water Analysis

	Cephalothin Resistant	Not Cephalothin Resistant
Atypical *Aeromonas*	2	3
Identified *Aeromonas*	3	11
p=0.5696		

Appendix 5: Buffalo River Analysis

	Cephalothin Resistant	Not Cephalothin Resistant
Sediment	45	49
Water	45	39
p=0.4574		

Appendix 6: Non-Urban Analysis

	Cephalothin Resistant	Not Cephalothin Resistant
Sediment	9	7
Water	5	14
p=0.0937		

Appendix 7: Total *Aeromonas* Analysis

	Cephalothin Resistant	Not Cephalothin Resistant
Buffalo River	90	88
Non-Urban	14	21
p=0.2726		