Abstract

Impact of Wastewater Effluent on Antibiotic Resistance in Sediment-Associated Aeromonas

Because certain antibiotics can be excreted largely as the parent compound in human waste, questions have been raised concerning the possibilities of antibiotic resistance generation in microbes within and as a consequence of discharges by wastewater treatment plants (WWTPs). The release of low doses of antibiotics into receiving surface waters by WWTPs has therefore been identified as a possible contributor to the problem of resistance. The overarching concern surrounding the constant release of these pharmaceuticals from WWTPs is that this may give rise to the creation of a background level of resistance within environmental bacterial populations, which in turn may be transferred to more virulent human pathogens. The goal of this investigation therefore is to contribute to the body of knowledge surrounding the development of drug resistant bacteria as a consequence of the pervasive subtherapeutic concentrations of antibiotics in the environment. More specifically, the question examined in this study was whether wastewater effluent being discharged into environmental waters contributed to an increase in the numbers of resistant bacteria found downstream.

The ubiquitous environmental genus *Aeromonas* was employed as a marker of the impacts of residual antibiotics on riparian bacterial populations. Opportunistic pathogens themselves, *Aeromonas* spp. have been shown to be able to transfer resistance to other pathogens such as *Vibrio cholera*, and they are included on the U.S. Environmental Protection Agency's Unregulated Contaminant Monitoring Rule List 2. Since both antibiotics and bacteria migrate between aqueous and sediment phases, sediment grab

samples were taken around the point of effluent discharge from a local WWTP which uses activated sludge treatment and chlorination/dechlorination. Three sample collection points were located approximately 600 meters upstream of the effluent outfall, close to the point of effluent discharge, and 965 meters downstream of the outfall. Water column samples taken contemporaneously with sediment at the up- and downstream sampling points revealed antibiotic residuals in the latter but not the former site. Sediment samples were processed for bacterial isolation. An algorithm of several standard biochemical tests was employed to identify 50 *Aeromonas* isolates from each sampling site; these isolates were subjected to resistance testing to four commonly used antibiotics detected in the WWTP effluent, namely ciprofloxacin, tetracycline, trimethoprim, and sulfamethoxazole. Changes in the susceptibility to the synergistic combination trimethoprim/sulfamethoxazole were also assessed.

The results of antibiotic resistance tests indicate that the *Aeromonas* bacteria found at points close to the effluent and downstream of the outfall exhibit an increase in both absolute and intermediate resistance relative to the same genus found upstream at the unimpacted sampling point. Statistical analyses revealed these differences to be significant, and since no other identifiable environmental impact exists at this site, the wastewater treatment plant effluent was identified as the source of this resistance increase. This finding highlights the need for both the implementation of conservative antibiotic prescription practices and disposal that reduce the levels of antibiotics reaching WWTPs and the consideration of alternate treatment processes that remove the last vestiges of these compounds before the effluent enters receiving streams.

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1 Introduction *

It is widely accepted by medical and public health professionals that the rise of drug resistant bacteria is attributable to the misuse and overprescription of antibiotics. Widespread and repeated exposure to these compounds coupled with improper dosing practices serve to select for and inure pathogens to subsequent drug therapies. Not only are pathogens developing defenses against commonplace antibiotics such as tetracycline, but many of the most potent last-line antibiotics such as ciprofloxacin are failing in the face of emerging resistant infections. Multiple drug resistance patterns are also becoming more and more prevalent (CDC 2004). Consequently, concerns about the increase of antibiotic resistance have given rise to a number of studies investigating possible sources and contributors to this phenomenon (Guardabassi, Petersen et al. 1998; Goni-Urriza, Capdepuy et al. 2000; Aarestrup, Seyfarth et al. 2001; Anderson, Nelson et al. 2003; Boxall, Kolpin et al. 2003; Kummerer and Henninger 2003). Hospitals certainly figure prominently as propagators of antimicrobial resistance, as pathogens and drugs interact in these institutions under optimal conditions for drug resistance acquisition and transfer. Confined animal feeding operations, or CAFOs, have also received much attention as they are known for their constant low-dose feeding of antibiotics to cattle, swine, and poultry in an attempt to promote growth and stave off infections within the herd or flock. These continuous, therapeutic and sub-therapeutic exposures have led to much research into CAFOs and the agricultural use of antibiotics as a potential source for drug resistant *Salmonella*, *E. coli*, *Campylobacter*, and other such food-borne bacterial pathogens

* Please note: throughout the text, "mcg" and "µg" are used interchangeably to represent "micrograms".

(Khachatourians 1998; Mathew, Saxton et al. 1999; Chee-Sanford, Aminov et al. 2001; Hayes, English et al. 2004).

Because the minimum necessary conditions under which resistance may be acquired are not fully understood, the study of resistance has extended beyond the scope of the aforementioned scenarios to encompass sources that are less obvious and poorly characterized. Hospitals and CAFOs represent settings of both high concentrations of antibiotics and dense bacterial populations, creating ideal conditions for resistance acquisition. However, because certain antibiotics can be excreted largely as the parent compound in human waste (Kummerer 2003), questions have also been raised concerning the possibilities of resistance generation within wastewater treatment plants (WWTPs) themselves. The release of low doses of antibiotics into the receiving surface waters by WWTPs, hospitals, and pharmaceutical industries has, therefore, been identified as an additional possible contributor to the overall problem of antimicrobial resistance among both pathogenic and non-pathogenic bacteria (Szewzyk and Feuerpfeil, 2000; Halling-Sorensen, Nielsen et al. 1998; Kummerer and Henninger 2003). The overarching concern surrounding the constant release of pharmaceuticals from WWTPs is that this may give rise to the creation of a background level of antimicrobial resistance within environmental bacterial populations, which in turn may be transferred to human pathogens (Goni-Urriza, Pineau et al. 2000; Ash, Mauck et al. 2002; Biyela, Lin et al. 2004).

The aforementioned exploration of the sources of antibiotics in the environment, the potential for development of resistance in environmental bacterial populations, various facets of the public health consequences of antibiotic resistance, the concerns

surrounding environmental resistance, and the nature of *Aeromonas*, culminated in this study. The goal of this investigation is to contribute to the body of knowledge surrounding the development of drug resistant bacteria as a consequence of the pervasive presence of subtherapeutic concentrations of antibiotics in the environment. Human antimicrobial usage and the potential impacts of drug residuals resulting from current levels of community use are the primary focus of this undertaking. Members of the ubiquitous environmental genus *Aeromonas* were employed as markers for changes in antibiotic resistance profiles of bacteria found in riparian sediments upstream, downstream, and at the point of a municipal WWTP effluent discharge. The hypothesis behind this approach was that subtherapeutic concentrations of antibiotics in the wastewater effluent will result in a notable increase of antimicrobial resistance in the bacteria downstream of the effluent outfall. It is possible that the increase could arise from the presence of antimicrobially resistant bacteria in the effluent, from indigenous bacteria in the receiving water acquiring antimicrobial resistance because of the presence of antimicrobials in the wastewater effluent, or both.

2 Literature Review

2.1 Environmental Studies of Impacts on Resistance

This study's conception, design and implementation were predicated on a review of previous studies regarding the prevalence of antibiotic resistance in the environment and contributing factors to this phenomenon. Environmental antibiotic occurrence studies and the impacts on bacterial resistance trends can be divided roughly into two categories. The first concerns the influence that agricultural use of antibiotics has had on pathogens that are part of the natural enteric flora of animals, such as *E. coli*, *Salmonella*

and *Campylobacter*; the second area of research is concerned with primarily human antibiotic use which includes both clinical and outpatient settings, pharmaceutical production and waste generated therefrom, and fate and transfer of antibiotics in WWTPs. While this present study addresses the latter area, a brief discussion of the agricultural milieu is warranted.

While no precise statistics are kept, it is estimated that over half of the antibiotics produced in the United States are appropriated by the agricultural industry and are employed both for therapeutic treatment of acute infections and subtherapeutic use as growth promoters (Lipsitch, Singer et al. 2002). The latter form of dosing, which relies on a constant low-level exposure of the animals to the drugs, gave rise to concerns about the possibility of resistant bacteria being generated within the animals themselves. In addition, these bacteria are excreted in substantial quantities by the animals, primarily in their fecal wastes. These wastes are held in treatment and storage lagoons, and their contents may seep into surrounding soil accidentally or may purposely be land-applied as fertilizer and soil amendment. In both cases the potential for intrusion into groundwater by both unmetabolized antibiotics and resistant bacteria is high, and several studies have demonstrated that this phenomena is widespread in areas of high agricultural activity. Tetracycline resistance and contributions made thereto by lagoons was addressed (Chee-Sanford, Aminov et al. 2001) in a study examining the prevalence of tetracycline resistance genes in swine farm lagoons and underlying groundwater. Not only were all tetracycline resistance conferring genes detected in lagoon water, but it was shown that lagoon seepage was contributing to the mobilization of tetracycline resistance through indigenous soil bacteria and into groundwater supplies. In another study, bacteria

isolated from soil samples taken at farms where cattle were treated subtherapeutically were compared to bacteria isolated from soils around dairy farms, where regulations for antibiotic use are stricter. Tylosin resistance in the former farms was shown to be statistically significantly higher, leading to the authors' conclusion that subtherapeutic antibiotic dosing leads to higher rates of resistance (Onan and LaPara 2003). Finally, a more recent study evaluated resistance of enterococci from 75 animal (cattle, swine, and poultry) farms, most of which employed antibiotics subtherapeutically. While there was indeed some resistance among enterococci isolates from farms that did not employ antibiotics, it was shown that the relative rates of resistance were significantly higher on those farms with drug dosing practices (Hershberger, Oprea et al. 2005).

The agricultural studies cited above are but a tiny fraction of the wealth of studies conducted with respect to antibiotic use in farming and its environmental impacts. Most agricultural studies seem to correlate well the rise of resistant bacteria with heavy antibiotic usage. However, analogous correlations to WWTP effluent and the environmental impacts of human antibiotic usage are less well documented and more difficult to establish. Lagoons, which serve to concentrate waste and in turn the antibiotics contained within, have been relied upon as the source of much of the data concerning the amount of antibiotics detected in agricultural practices. Consequently, reported pharmaceutical concentrations contributed by agriculture are much higher than those being detected in urban WWTP effluents and are also much closer to concentrations that are perceived to exert selective pressure on bacteria to become resistant. There are also the difficulties associated with distinguishing between resistant bacteria excreted by the animals and bacteria that have acquired resistance in the lagoons

or other waste matrices as a result of conjugative transfer instigated by the antibiotics present. Furthermore, disinfection practices at WWTPs tend to alleviate the problems associated with discriminating between older bacteria present in the wastewater discharge and newer resistant bacteria created in the receiving water. In contrast, most animal manures are not disinfected, leading to the continued environmental presence of high concentrations of enteric and other bacteria, some antimicrobial resistant, fecally shed by the animals.

In 2002 the U.S.Geological Survey (USGS), which had been examining the concentrations of antibiotics in agricultural waste lagoons, undertook to quantify the amounts of various pharmaceuticals being discharged by WWTPs (Kolpin, Furlong, et al. 2002). Until this time, most reconnaissance work of this type had been conducted abroad, and there had been several studies, primarily in Europe, investigating the prevalence of these pharmaceutical micropollutants in the environment (Halling-Sorensen, Lutzhoft et al. 2000; Jorgensen and Halling-Sorensen 2000; Stuer-Lauridsen, Birkved et al. 2000; Kummerer 2002). Surface water samples from 139 water bodies in 30 states were analyzed by the USGS for pharmaceutical residues among other organic contaminants, and antibiotics were frequently found in concentrations of ecological importance. Of the antibiotics relevant to this study, sulfamethoxazole was detected at maximum concentrations of approximately 0.5 µg/L and trimethoprim at a minimum of 0.2 µg/L. (Kolpin, Furlong et al. 2002). Yang and Carlson (2003) sought to parallel the USGS study on a condensed scale by evaluating point and non-point discharges and their contributions made to concentrations of tetracyclines and sulfonamides in a stretch of impacted river. The results of their study showed a dramatic variance in the distribution

of tetracyclines, ranging from no incidence upstream at the control point, to maximum concentrations of 0.3 μg/L for diverse members of the tetracycline class downstream of urban effluent and agricultural lots. Sulfonamides were also detected but only downstream of the wastewater discharge and at half the tetracycline concentration, approximately 0.16 μg/L.

Some of the cited occurrence studies broached the subject of antibiotic resistance as a possible consequence of antibiotic-laden discharges (wastewater treatment plants that service hospitals and pharmaceutical manufacturers were cited as particularly likely candidates for resistant bacteria generation and propagation), but most of the studies did not directly examine the resistance patterns of bacteria subjected to these discharges. Analysis to examine more closely the relationship between these anthropogenic drug sources and resistance in the environment has been less commonly reported. A study conducted in a stretch of river heavily impacted by WWTP effluent showed increased resistance in *E. coli* isolates downstream of WWTP outfalls (Iwane, Urase et al. 2001); however, the study sought to attribute resistance not to antibiotic concentrations in the effluent but rather resistant *E. coli* being discharged into the receiving waters. This conclusion was based on microbial analysis and resistance testing of samples taken throughout the wastewater treatment plants, including effluent. However, there was no concurrent chemical analysis of the effluent to verify the presence of antibiotics. In addition, the bacteria examined were isolated from water column samples, and there was no attempt to assess the impacts on indigenous bacterial populations. Another study sought to correlate the resistance patterns of enteric bacteria found in industrially impacted surface waters with clinical isolates from diarrheal patients. The conclusions

pointed to both anthropogenic influences on resistance patterns and direct public health consequences, as there was a strong correlation between clinical and environmental isolates (Lin, Biyela et al. 2004).

Similar studies have included ubiquitous environmental organisms as indicators of increasing background levels of resistance. Goni-Urriza, Capdepuy et al. in 2000 sampled waters at a control site upstream of a sewage outfall and several points downstream; samples were microbially analyzed for both *Aeromonas* and *Enterobacteraciae* spp. Resistance to a battery of antibiotics was tested, and marked decreases in susceptibility to antibiotics were detected for both genera. In addition, conjugation experiments were conducted for a select group of resistant bacteria and transfer of resistance characteristics was demonstrated even interspecially. Again, however, there was no analysis of either effluent or the surface water samples for antibiotics.

Aeromonas spp. were specifically targeted again as environmental indicators in a survey of the prevalence of antibiotic resistance in the environment. Fresh water samples of rivers in France and Spain were analyzed for resistant *Aeromonas,* and surprising levels of resistance to quinolones were detected. Susceptibility to fluoroquinolones was still evident although the minimum inhibitory concentrations (MICs) did increase. However, the study did not link the presence of antimicrobial resistant bacteria to any sort of anthropogenic influences, i.e. samples were not specifically taken with respect to wastewater discharges (Goni-Urriza, Pineau et al. 2000). Another study employing ubiquitous environmental bacteria as indicators found antimicrobial resistance increased as a consequence of hospital and pharmaceutical waste discharge into sewers. Samples

collected from points in the sewers up- and downstream of the discharges were analyzed for resistance patterns of *Acinetobacter* spp. Surprisingly, hospital discharge seemed to result in very transient increases in oxytetracycline resistance, while pharmaceutical effluent resulted in multiple-antibiotic resistance increases that persisted further downstream. Both residual antibiotic concentrations and potential release of resistant bacteria with the pharmaceutical waste were suggested as causes for this increase (Guardabassi, Petersen et al. 1998). The putative antibiotic concentrations were not measured as part of the study, however.

The cited studies are primarily concerned with aqueous bacterial concentrations, and many of them rely on fecal coliforms as markers of the effluent impacts. However, the use of fecal coliforms as indicators of the influence of anthropogenic activities on changes in background environmental resistance is complicated and source identification is difficult. In addition, because of considerations that will be discussed in this chapter, Section 2.2, sediments may provide a better type of environmental sample for the examination of the effects of wastewater discharges on the selection and occurrence of antimicrobial resistant bacteria. Studies examining the prevalence of resistance in sediment dwelling indigenous bacteria as a consequence of human impacts are relatively rare (Andersen and Sandaa 1994; Sandaa and Enger 1994; Chelossi, Vezzulli et al. 2003) and usually examine marine as opposed to freshwater sediments. Therefore, this current study seeks to fill a gap in the aforementioned work by combining the detection of ubiquitous environmental indicator organisms in fresh water sediments with impacts from municipal wastewater effluents. In addition, contemporaneous water column samples

were taken and analyzed for antibiotic concentrations in order to provide some correlative basis for explaining the potential bacterial resistance levels.

2.2 Research Components

2.2.1 Aeromonas spp.

The *Aeromonas* genus is comprised of Gram negative, oxidase positive rods found commonly in many environmental waters, sediments, and soils. Several factors contributed to the decision to investigate *Aeromonas* species in this study. Chiefly, it was thought that the use of typical enteric bacteria of humans and animals such as *E. coli* and *Enterococci* as indicators of environmental antibiotic resistance was too likely to result in unsuccessful efforts to reach bacterial source tracking conclusions. The background to this decision is explained in further detail below, as this premise is particularly important to the study.

It is already difficult to distinguish with great reliability the differences in antimicrobial resistance patterns between fecal coliforms of human and animal origins. The average rate of correct classification of these bacteria can be as low as 72% when comparisons are made between environmental isolates of human and nonhuman sources (Simpson, Santo Domingo et al. 2002). Using antibiotic resistance profiles of fecal organisms is a recent area of research designed to better address this microbial source-tracking question, based on the assumption that wild-type strains of bacteria will demonstrate greater susceptibility to a broader range of antibiotics than bacteria of enteric origin within humans and animals. However, vast reference databases ("libraries") containing resistance profiles of many thousands of isolates from known environmental fecal contamination sources are required for comparison, and correlation between

environmental isolates and these databases remains low (Wiggins, Cash et al. 2003). In addition, these databases must vary among geographical areas because of faunal variations and consequent changes in fecal contamination sources (Simpson, Santo Domingo et al. 2002). These factors can have significant consequences when attempting to make the distinction between fecal coliforms that have acquired resistance in the environment, where it was induced by antibiotic concentrations introduced through WWTP discharges, or conversely, drug resistance acquired in the intestinal tracts of humans. Therefore, because acquisition of antibiotic resistance by enteric bacteria in the intestinal tract is highly probable and could result in mistaken attribution of origination in the environment, *Aeromonas hydrophila* and *Aeromonas caviae* were chosen as markers instead. These bacteria are less likely to have come from human reservoirs, and they occur naturally in the aquatic environment. This will not entirely eliminate the possibility of attributing enterically acquired resistance to environmental impacts, but will substantially reduce the probability of this error. It should be mentioned that while the study sought to initially identify and use two species of *Aeromonas*, this proved to be too difficult because of the continuing discoveries made about *Aeromonas* taxonomy and the inconsistency with which biochemical tests identify *Aeromonas* species (Abbott, Cheung, et al. 2003). See Chapter 3 Section 3.5 for more detail on the speciation approach.

Another reason for the use of *Aeromonas* spp. was their proven environmental proliferation. Because these bacteria are ubiquitous in nature, their detection and isolation at great frequency and in high numbers from environmental matrices are highly likely. In addition, because of their ubiquity, *Aeromonas* spp. could serve as a substantial contributor to the potential reservoir of resistance that in turn makes it possible to pass

resistance genes to more and varying kinds of bacteria. Previous studies have already demonstrated the ability for *Aeromonas* to conjugatively transfer plasmids to other Gram negative bacteria such as *E. coli* and *V. cholera* (Kruse, Soerum et al. 1995; Goni-Urriza, Capdepuy et al. 2000).

Not only might aeromonads transmit antimicrobial resistance to other pathogens, these bacteria are also emerging as a human pathogen of some consequence. Several *Aeromonas* species, including those investigated in this study, have been implicated in such diverse clinical presentations as gastroenteritis, wound infections, septicemia, and, rarely, more severe illnesses such as meningitis and necrotizing fascitis (Minnaganti, Patel et al. 2000). Both as pathogens and as environmental bacteria, *Aeromonas* species still show a relatively broad susceptibility to many antibiotics; fluoroquinolones and cephalosporins are still very active against this genus and tetracyclines are still capable of inactivating *Aeromonas* (Goni-Urriza, Pineau et al. 2000). Penicillins are however of little use, and most *Aeromonas* species are inherently resistant to some aminopenicillins such as ampicillin (Vila, Marco et al. 2002). *Aeromonas* species have been isolated from post-chlorination drinking water distribution systems, both in the water phase and as a genus that frequently colonizes pipe biofilms (Kuhn, Allestam et al. 1997; Bomo, Storey et al. 2004). Finally, because of the frequency with which *Aeromonas* spp. are found in drinking water sources and distribution systems, the U.S. Environmental Protection Agency (U.S.EPA) placed them on the Contaminant Candidate List (CCL), conducted occurrence monitoring for these organisms in 2003, and added them to the U.S. EPA's Unregulated Contaminant Monitoring Rule Monitoring List (U.S.EPA 2004). Recently, the U.S. EPA has proposed to exclude them from the monitoring requirements of

Unregulated Contaminant Monitoring Rule Monitoring List due to difficulties in meeting the sample holding time limits for microbial analysis and the challenges of *Aeromonas* speciation (U.S.EPA, 2005). Nevertheless, these bacteria are considered important and in need of further study in drinking water and its sources.

2.2.2 Sediments

The focus in this study on sediments rather than water is predicated on a number of assumptions. Perhaps most importantly is the evidence that identifies sediments as areas of accumulation for the antibiotics in question. Several studies have shown that tetracycline in particular has a propensity for solid-phase partitioning, particularly in highly organic matrices such as silty sediments where cation exchange figures prominently as a mechanism for this phenomena (Figueroa, Leonard et al. 2004). Ciprofloxacin has also been shown to partition to sediments and, given its synthetic origins, is less likely to be biodegraded (Nowara, Burhenne et al. 1997; Jarnheimer, Ottoson et al. 2004).

Another important feature of sediments is that bacteria also tend to accumulate in sediments and that bacterial activity is concentrated there as opposed to the water column (White 1995). This increases both the likelihood of isolation and detection of the desired organism and the possibility for antimicrobial resistance acquisition. This latter hypothesis is based on a number of observations. First, the high bacterial density in sediments increases the likelihood of conjugative transfer because large bacterial communities are in such close proximity. Another proposed method of resistance acquisition, both in clinical and environmental settings, occurs through transformation; fragments of DNA, in this case, genes that confer antibiotic resistance, are released as a

result of cell death and are taken up by other cells (Davison 1999). While DNA is unstable in the environment and subject to enzymatic breakdown, as with many organic and inorganic compounds adsorption to particles such as sediment prolongs the survival time of DNA, thereby enhancing the possibility of sediment-associated bacteria encountering and absorbing resistance genes (Davison 1999). Consequently, sediments in which both antibiotic and bacterial concentrations are high can provide a rich milieu for environmental resistance acquisition by a number of available mechanisms.

2.2.3 Antibiotics Employed in This Study

The antibiotics employed in this study were chosen based on a number of factors. A brief examination of each drug and its relevant characteristics is warranted.

2.2.3.1 Tetracycline Hydrochloride

The tetracycline class of antibiotics comprises a set of broad-spectrum pharmaceuticals that have long been used therapeutically in humans against a number of infections. They are also frequently employed by agriculture and aquaculture as growth promoters and for acute treatment of disease. Because of their heavy use in all of these arenas, tetracyclines are often included in the suite of pharmaceutical compounds targeted for environmental detection (Lindsey, Meyer et al. 2001; Jacobsen, Halling-Sorensen et al. 2004). In addition, the repeated finding in several extra-clinical settings of drugs from this antibiotic class has led to its common inclusion in studies striving to ascertain the possible impacts of subtherapeutic antibiotic use on resistance profiles of environmental bacteria (Andersen and Sandaa 1994; McKeon, Calabrese et al. 1995; Guardabassi, Petersen et al. 1998). Therefore, both the high level of therapeutic use and prior inclusion

in a number of similar studies make tetracycline a relevant antimicrobial compound for this particular study.

Several studies have also indicated the propensity for tetracyclines to partition to marine sediments, dependent on the sediments' physical properties such as organic content. This particular partitioning characteristic has been shown to relate to a significant influence on the adsorption coefficient for tetracyclines (Pouliquen and Le Bris 1996). Examinations of this sediment association tendency have been particularly concerned with aquaculture, as tetracyclines are very commonly employed in this industry (Bjorklund, Bondestam et al. 1990; Bjorklund, Rabergh et al. 1991; Coyne, Hiney et al. 1994; Petersen, Andersen et al. 2002; Hatha, Vivekanandhan et al. 2005). Tetracyclines, particularly oxytetracycline, in concentrations as high as 4000 µg/kg have been detected in sediments below fish farms (Thiele-Bruhn 2003), and oxytetracycline residues have been found to persist as intact, active antibiotics for up to 10 months (Capone, Weston et al. 1996). In addition, the concentrations of drugs detected under fish farms have been correlated to higher numbers of resistant bacteria residing in the sediments (Samuelsen, Torsvik et al. 1992; Schmidt, Bruun et al. 2001; Chelossi, Vezzulli et al. 2003). Fewer studies are found for riverine, freshwater sediments, but a recent examination of Chesapeake Bay detected oxytetracycline concentrations up to 3.3 µg/g in sediments impacted by a municipal WWTP (Simon 2005), comparable to those being detected in fish farm environs. These concentrations may contribute to resistance, but because aquaculture practices usually more closely parallel CAFO impacts when compared to WWTP discharges in terms of both antibiotic and animal concentration, there are important differences that do not permit direct comparison when examining

environmental impacts of fish farms and WWTPs. Finally, the National Committee for Clinical Laboratory Standards (NCCLS) has approved tetracycline hydrochloride as representative of the entire class of tetracyclines, meaning that resistance to tetracycline is indicative of resistance to oxytetracycline, doxycycline, and all other antibiotics in this group.

2.2.3.2 Ciprofloxacin

Ciprofloxacin, a member of the fluoroquinolone antibiotic class, is another broad-spectrum drug capable of treating a number of infections. Unlike tetracycline, however, ciprofloxacin is a much newer, synthetic compound and consequently, resistance to this pharmaceutical is less common. Until recently, because of the prevalence of resistance to penicillins, tetracyclines, and other front line antibiotics, ciprofloxacin was one of the last line defenses against many bacterial diseases. An increased use consistent with a greater reliance on ciprofloxacin and other fluoroquinolones has contributed to a more frequent detection of bacteria resistant to these compounds. A national surveillance on the resistance trends in the opportunistic pathogen, *P. aeruginosa* demonstrated a marked decrease in ciprofloxacin susceptibility, from an 85% susceptibility rate in 1993 to one of only 68% in 2002 (Obritsch, Fish et al. 2004). This trend is evident not only in opportunistic organisms, but has also been seen in such pathogenic organisms as *S. aureus* (Cuevas, Cercenado et al. 2004). Therefore, an examination of environmental bacteria that may have reduced susceptibility to this powerful and strategically important drug is crucial.

Fluoroquinolones are also being detected in surface waters (Golet, Alder et al. 2002; Kolpin, Furlong et al. 2002). They are considered to be among the more stable

environmental compounds because of their resistance to both biodegradation and hydrolysis, thereby permitting a longer exposure of bacteria to the drug (Thiele-Bruhn 2003). Studies conducted in Switzerland on surface waters considerably impacted by urban effluents detected wastewater concentrations of ciprofloxacin as high as 553 ng/L, which were attenuated in the receiving water column to around 15 ng/L, presumably by phototransformation and/or adsorption to particles (Golet, Alder et al. 2002). Similar studies in the United States examining less impacted surface waters also detected a number of fluoroquinolones, but at much lower concentrations (Kolpin, Furlong et al. 2002). Relevant to this current study is the assertion by the latter study that the low concentrations in water column samples were probably attributable to the tendency for these compounds to partition to sediments. Removal rates of fluoroquinolones in WWTPs have been shown to be as high as 80% because of their tendency to adsorb to sludge and biosolids (Xia, Bhandari et al. 2005). Nevertheless, the wastewater effluent can contain concentrations of these compounds that, after accumulation in downstream sediments, may reach levels capable of exerting selective pressure on bacteria.

2.2.3.3 Trimethoprim/Sulfamethoxazole

Trimethoprim and sulfamethoxazole are synthetic antibiotics that inhibit folic acid synthesis and have synergistic properties when coupled together, enhancing each other's antimicrobial capacity. They are therefore commonly administered together in one dose that is typically lower than the dose of either one alone. While resistance to sulfa drugs is fairly widespread, trimethoprim/sulfamethoxazole still may be relied upon for broad spectrum use, but resistance to even this therapy is on the rise (Masters, O'Bryan et al. 2003). Because *Aeromonas* spp. still show susceptibility to the combination antibiotic, a

survey of this particular genus with respect to its potentially increasing resistance is appropriate.

Neither trimethoprim nor sulfamethoxazole occurs naturally and so their presence is indicative of anthropogenic or agricultural impacts. Sulfonamides are employed heavily in agriculture as well as clinical practice and are frequently detected in surveys attempting to quantify antibiotic concentrations in animal waste lagoons and sewage as well as fresh waters (Campagnolo, Johnson et al. 2002; Kolpin, Furlong et al. 2002). Although susceptible to photodegradation, sulfa drugs are shielded from this effect when introduced into waters with high dissolved organic content. Important for this study is the tendency for sulfamethoxazole in particular to persist for longer in the environment relative to other members of the sulfa class (Boreen, Arnold et al. 2004). In addition, studies have shown that sulfonamides resist biodegradation (Al-Ahmad, Daschner et al. 1999; Kummerer, Al-Ahmad et al. 2000). These compounds are not as readily adsorbed by soils and sediments as the tetracyclines or fluoroquinolones (Thiele-Bruhn, Seibicke et al. 2004), but their high usage rates coupled with stability in the environment contribute to a fairly consistent background level of these drugs in receiving waters. This in turn may lead to accumulation in sediments to concentrations that would exert selective pressure on bacteria.

Trimethoprim is also detected in environmental matrices and at various points in sewage treatment plants (Kolpin, Furlong et al. 2002; Lindberg, Wennberg et al. 2005). The inefficacy of treatment plants to remove trimethoprim from influent was well illustrated by Lindberg, Wennberg et al., who reported a median removal rate from raw sewage to final effluent of only 3%. Trimethoprim has not been as extensively examined

with respect to its environmental longevity, but a controlled study included trimethoprim in a suite of antibiotics examined for persistence in marine sediments. While tetracyclines predictably outlived trimethoprim, the half-life of the latter antibiotic was determined to be approximately 75 days in the topmost centimeter of sediment (Hektoen, Berge et al. 1995).

3 Materials and Methods

3.1 Preliminary Site Background and Site Description

Prior to the acquisition of Morgan Creek data on which this study is based, another site was investigated similarly and will be referred to as the "preliminary" site to distinguish it from the "primary" site in the following text. It should be noted that the majority of the data collected at the preliminary site comprised only *Aeromonas* identification and speciation. After a small subset of the isolates was subjected to antibiotic resistance testing, it was determined that the project would benefit from relocation to what was to become the primary site. Consequently, the only data presented from the preliminary site is a comparison of bacterial concentrations in the water and sediment. A brief description of the preliminary site and its environs follows:

- Effluent samples of the wastewater treatment plant discharging to this stream had been analyzed by another laboratory for pharmaceutically active compounds (PhACs), and antibiotics were among the trace pollutants found.
- Minimal agricultural impacts to the receiving waters enabled the study to focus more narrowly on urban effluent effects.
- There were minimal upstream point and non-point pollution sources that would impact the antimicrobial compound quality of the upstream control samples.

- The wastewater treatment plant services a 238-bed hospital and an 81-bed nursing home, plus a number of textile industries.
- Treatment at the plant begins with screening, flow equalization, and primary sedimentation. This is followed by an activated sludge process coupled with a biological nutrient removal (BNR) system. Secondary clarifiers remove and recycle most of the suspended solids, and tertiary treatment consists of final gravity sand filtration prior to disinfection with chlorine.

3.1.1 Sediment

3.1.1.1 Sampling

Four points were chosen around this site for sampling purposes. What was considered to be the unimpacted control station was located approximately 1600 meters upstream of the wastewater plant outfall. Points at the effluent discharge, 300 meters and 800 meters downstream of the effluent discharge were also sampled.

Duplicate samples at this site were initially taken with a mesh scoop grab sampler; the mesh containing the sample was inverted over an opened sterile 125 mL Nalgene high-density polyethylene (HDPE) wide-mouthed bottle which was then placed immediately in a cooler for transport back to the laboratory. As the sampler was permitted intrusion of water into the collected sediments, an examination of the relative concentrations of bacteria in both sediments and water was conducted and is explained in this chapter, Section 2. An Eckmann dredge was employed in subsequent sampling excursions, and the sample collection procedure was refined into that which will be described in this chapter, Section 3.1.1. The samples collected at this site were not processed immediately but placed in a 4°C walk-in cooler overnight.

3.1.1.2 *Preparation and Procedure for Spread Plating at Preliminary Site*

The first few sampling excursions at this site collected sediment that was centrifuged at 3000 rpm for 5 minutes at a temperature of approximately 15°C. This step was ultimately removed from the protocol. Diluent in these studies was not phosphate buffered water but autoclaved environmental water taken at the same time as the sediment samples; a grab sample of this water was taken and stored with the sediment samples in a one-liter HDPE wide-mouth bottle. To this diluent water, the surfactant Tween 80 (Fisher Scientific, Fair Lawn, NJ) was added in a ratio of 0.05 mL Tween 80 to 500 mL environmental water before autoclaving. Aside from these alterations, the sediment processing followed that described in this chapter, Section 3.1.2. A water content analysis was performed as per this chapter, Section 3.3.

3.1.2 *Biochemical Test Preparation and Performance on Bacterial Isolates from the Preliminary Site*

The biochemical methods of identification employed at the preliminary site were almost identical to those at the primary site; more detailed methods may be found in the section on primary site sample processing, including specific biochemical tests. After the isolates were subjected to a battery of biochemical tests, species confirmation of isolates at the preliminary site was conducted through the use of API 20E strips (Marcy l'Etoile, France) as per the manufacturer's instructions. Briefly, presumptive isolates were incubated overnight on tryptic soy agar (TSA) plates, after which individual colonies were chosen and vortexed in 5 mL of sterile physiological saline. The bacterial suspension was adjusted to a 0.5 McFarland turbidity standard, and aliquots were pipetted into the wells of the API 20E strip. Mineral oil was added to the appropriate wells in order to create anaerobic conditions, and the strips were incubated overnight at 35°C.

Reagents of the API 20E system were added as per manufacturer's instructions after incubation. The strips were then left to incubate overnight on the bench at ambient conditions and reexamined (Koksal, 2005). This was in an effort to allow completion of the carbohydrate fermentations, which often required the full 48-hr incubation period (24 hours at 35°C plus 24 hours at room temperature). Aside from the API test, the only alteration made in the biochemical analysis was the inclusion of an O/129 Vibriostatic disk in the primary study. Isolates were archived as described in this chapter, Section 3.2.

3.2 Sediment vs. Water Bacterial Concentration

3.2.1 Sampling

The mesh grab-sampling device employed by this study was subject to minor water column intrusion. Consequently, the relative bacterial concentrations in sediment versus water were examined so as to dispel doubts concerning the possibility of mistaking sedimentary bacteria for those suspended in and originating from the water column. One liter water samples were taken during one of the sediment sampling excursions at the primary site from three points in the water column: at the surface, at mid-depth, and at the bottom (sediment-water interface). The last two samples were obtained with a Van Doren sampler, while a grab sample sufficed for the surface water. The Van Doren was suspended from a rope on which meter lengths had been marked; therefore, the middle of the water column was measured as approximately half the depth that was indicated by the rope when the Van Doren sampler was allowed to rest on the riparian bottom. The sediment-water interface sample was taken slightly above the depth at which the sampler was felt to touch the bottom sediment. The siphon on the Van

Doren was used to dispense the collected sample into autoclaved 1-L HDPE wide mouth bottles. All sample bottles were placed immediately in a cooler for transport back to the laboratory, where they were stored overnight in a 4°C walk-in refrigerator.

3.2.2 Sample Preparation for Bacterial Primary Isolation and Enumeration

Water samples from the preliminary site were homogenized by wrist shaking for five seconds before ten-fold serial dilutions of the water samples were conducted in pre-prepared phosphate buffered solution. 100 µL volumes of the undiluted, 10^{-1} and 10^{-2} dilutions were spread-plated in triplicate onto ADA-V agar (see this chapter, Section 3.1.2 for description of the agar). Sediment samples were processed as described in this chapter, Section 3.1.2. The plates were incubated overnight at 35°C. The results of these analyses are presented in Chapter 4, Section 1 of this report.

3.3 Primary Site Description

The wastewater treatment plant around which samples were taken is described as follows:

- Minimal agricultural impacts to the receiving waters enabled the study to focus more narrowly on urban wastewater effluent effects.
- There were minimal upstream point and non-point pollution sources that would impact the quality of the upstream control samples.
- Treatment at the wastewater plant includes screening, primary and secondary sedimentation, activated sludge, biological nutrient removal, and disinfection with chlorine; the treatment plant has a 12 million gallon per day (MGD) capability and treats on average 8 MGD.

- The effluent of a 688-bed university hospital and a large research university is serviced by the WWTP.
- The upstream sampling site (US) was located approximately 600 meters before the effluent discharge. Samples were also taken at points 33 and 965 meters downstream, referred to hereafter as effluent (E) and downstream (DS) respectively. These points were chosen for their accessibility, but the fluvial morphology of the sampling location also figured prominently in the selection of the sites. Areas of lower flow possibly resulting in longer residence times for water and sediment accumulation were selected to allow for the greatest possible antibiotic partitioning to sediments and highest antibiotic exposure time for bacteria.

3.3.1 Sediment

3.3.1.1 Sampling

The US sampling site was on the upstream side of a bridge where flow in the stream was slightly impeded. At E, a large log that had fallen across the stream created a downstream pool into which effluent intruded at a much slower flow rate. Finally, a concrete ford dammed the stream slightly at DS, and samples were taken a few feet upstream of the ford.

Duplicate grab samples from US, E and DS were taken with the aid of an Eckmann dredge. Although the water column never reached depths greater than 1 meter, the dredge maintained, to a large extent, the integrity of the sediment strata. The top centimeter of sediment retrieved by the dredge was removed with the aid of a spatula, transferred to 125 mL autoclaved Nalgene HDPE bottles, and placed immediately in a cooler for transport back to the laboratory; the spatula was rinsed thoroughly with

deionized water (Dracor, North Carolina) between samples. The amount of sediment sample varied, but approximated 30-50 grams, which was enough to both employ in serial dilutions for bacteriological analysis and for volatile solids (VS) analysis. Samples were processed immediately upon return to the laboratory.

3.3.1.2 Preparation and Procedure for Spread Plating at Primary Site

The Eckmann dredge employed in the sediment sampling can lend itself to inadvertent water collection. In the event that the sediment was overlaid with water after transport back to the lab, the supernatant was pipetted out of the Nalgene bottle before sediment homogenization. After the water was removed, the bottle was recapped and the sediment samples were wrist-shaken for one minute. One gram of the homogenized sediment was added to a 15 mL plastic conical tube containing 9 mL of phosphate buffered water with magnesium and vortexed for ten seconds; this was considered to be a 10^{-1} dilution. The phosphate buffered water had final phosphate and magnesium ion concentrations of approximately 59 and 97 mg/L, respectively (see Appendix A for more detailed calculations). Briefly, stock solutions of potassium dihydrogen phosphate (34 g/L) and magnesium chloride (81.1 g/L) were prepared and stored in 125 mL glass bottles at 4°C until use. When needed, 1.25 mL of phosphate stock and 5 mL of magnesium chloride stock were added to a 1-L glass bottle containing 500 mL of deionized water and autoclaved. (Standard Methods, 20th ed., 1998). The 10^{-1} sediment dilutions were prepared first so that by the time further dilution commenced, the 10^{-1} dilution tubes had been sitting out on the bench for at least 20 minutes. The tube containing sediment was vortexed again for ten seconds to ensure homogenization of the dilution before 1 mL of the supernatant was removed and added to the next tube of 9 mL

phosphate buffer; ten-fold serial dilutions were prepared until the 10^{-4} dilution was reached. The 10^{-2} through 10^{-4} dilutions were vortexed for five seconds before removing 1 mL for subsequent dilutions, again to ensure homogenization of the solution. Volumes of 100 µL of the 10^{-2}, 10^{-3}, and 10^{-4} dilutions were spread-plated in duplicate onto ampicillin-dextrin agar (m-Aeromonas Isolation Agar, Biolife, Milan, Italy) supplemented with vancomycin (ADA-V). Briefly, the 100 µL of dilution was evenly distributed across the surface of the agar using a flame sterilized glass hockey stick and a small horizontally mounted wheel. The agar plate was placed on the wheel and spun while the glass hockey stick was used to smear the sample across the agar surface. ADA-V plates with the spread-plated sample were incubated for 24+/- 2 hours at 35°C.

The ADA-V agar was employed and prepared as recommended by EPA Method 1605 for the isolation of Aeromonads from drinking water (U.S.EPA, 2001). Briefly, the agar was prepared according to manufacturer's agar-to-deionized water ratios, sterilized by autoclaving, and allowed to cool in a 50° water bath. Ten milligrams of ampicillin sodium salt and 2 mg of vancomycin hydrochloride (Sigma Aldrich, St. Louis, MO) were dissolved into two separate 10 mL volumes of deionized water, and each 10 mL volume was filter-sterilized through a 0.22 µm-pore-sized filter into one liter of cooled agar. The agar was wrist-shaken gently to uniformly distribute the antibiotics before dispensing 10-ml volumes into individual sterile 100 mm Petri dishes.

3.3.2 Biochemical Test Preparation and Performance on Bacterial Isolates from the Primary Site

All agars were manufactured by Becton Dickinson (Sparks MD) unless otherwise noted. Dispensing of liquid agars and aliquotting of broths was conducted in a horizontal laminar flow, clean bench hood; 10 mL of agar were added to each plate unless otherwise

noted. All incubations took place at 35° +/- 2° C. See Appendix B for the composition and agar-to-water ratios for the dehydrated media.

After the incubation of the ADA-V plates, presumptive colonies were triple-streaked onto tryptic soy agar (TSA) plates to which a 150 µg disk of O/129 Vibriostatic Agent (Remel, Lenexa, KS) was added in the densest part of the initial streak. The disk was added with tweezers, which were flame-sterilized between each isolate. The TSA and all following agars were prepared by employing manufacturer-recommended ratios of dehydrated agar to deionized water. The appropriate amount of agar plus water was wrist shaken until ingredients were dispersed uniformly in a 1-L glass bottle and then autoclaved immediately thereafter. However, the agars were neither heated with agitation nor boiled for one minute. These latter steps were not thought necessary because the agars would dissolve into solution during the autoclaving process. After autoclaving, agars in capped bottles were gently inverted to ensure homogenization of ingredients.

The inoculated TSA plates were incubated overnight. If the isolates proved resistant to the vibriostatic agent, they were archived at –80°C in a freezing solution comprised of tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD) and glycerol (Fischer Scientific, Fair Lawn, NJ) to a final concentration of 25%. One hundred and fifty mL of TSB was prepared according to manufacturer's instructions to which was added 50 mL of glycerol. This solution was sterilized by autoclaving and, after cooling, 1 mL aliquots were added to sterile 1.5-ml capacity microfuge (Eppendorf) tubes. Using a sterilized wooden applicator, one isolated colony from each TSA plate was added to one microfuge tube and vortexed in order to homogenize the bacterial suspension. Sets of

the archived isolates were then removed as needed from the -80° freezer, and a sterile pre-packaged 1 mL glass pipette was used to remove a small portion of the frozen bacterial suspension which was dotted onto a TSA plate. Sterile wooden applicators were then used to triple-streak the inoculum for bacterial colony isolation. These TSA plates were incubated overnight, and isolated colonies from these plates were subjected to a number of subsequent biochemical tests. An individual colony was picked up with a sterile wooden applicator and both aerobically and anaerobically inoculated on Kliger iron agar (KIA) slants by fishtail streaking on the slant and stabbing into the butt of the agar slant. Slants were prepared by adding 5 mL of agar to sterile glass 16x100mm tubes and allowing them to dry in an inclined position. Towards the end of the study, problems with the KIA were encountered, and a change to triple sugar iron agar (TSI) was implemented. Individual isolates were also streaked onto plates of bile esculin (BE) agar (Oxoid, Lenexa, KS), and subjected to spot indole and spot oxidase tests; the reagents for the last two tests were purchased from Remel, (Lenexa KS) and Becton-Dickinson, (Sparks, MD), respectively. Spot indole and oxidase tests were conducted according to the following procedure: a 45 mm media pad (Millipore, Benton MA) was saturated with the reagent and a small bit of colony material was smeared with a sterile wooden applicator onto the reagent –soaked portion of the pad. Colonies were also streaked onto MacConkey agar plates and inoculated into sterile glass tubes containing 5 mL of purple broth base (PBB) (BBL, New Jersey) containing a concentration of 1% L-arabinose. For this test, a stock solution of 10% L-arabinose was prepared by adding 10 grams of L-arabinose to a final volume of 100 mL deionized water and dissolved into solution with the aid of a magnetic stir bar and stir plate. Volumes appropriate to achieve a final

concentration of 1% L-arabinose in the broth base were filter sterilized through a 0.22 µm-pore-sized filter into a sterile graduated cylinder, to which was added sterile PBB to a final desired volume. For example, 25 mL of 10% L-arabinose would be added to 225 mL of PBB for a final concentration of 2.5 grams of L-arabinose in a total volume of 250 mL. Tubes were vortexed gently in order to evenly distribute the bacteria. Finally, colonies were introduced with sterile wooden applicators into sterile glass tubes containing 1 mL of Methyl-red Voges-Proskauer (MRVP) broth (BBL, New Jersey). All tests requiring the use of glass tubes were capped with sterile loose fitting plastic caps in order to allow for aerobic conditions within the tube. Bile esculin plates and L-arabinose tubes were incubated at 35 °C for 7 days, and VP tubes were allowed to incubate at 35 °C for 2 days before reagents were added. After incubation, 6 drops each of 6% α-naphthol and 40% potassium hydroxide were added to the tubes containing VP broth and allowed to react for 10-15 minutes; the addition of reagents was conducted in a fume hood. Initially, these reagents were obtained from BioMerieux (Marcy l'Etoile, France); later they were prepared independently in the laboratory and used in control studies before being employed in the discriminatory analysis. For the VP tests, a 40 % potassium hydroxide (KOH) solution was prepared by adding 40 grams of KOH pellets (EM Scientific, Cherry Hill, NJ) to a small amount of deionized water and stirred into solution. The dissolved KOH was then transferred to a 100 mL volumetric flask to which was added enough deionized water to bring the final volume to 100 mL. This reagent was stored in 20 mL plastic screw-top vials at 4°C until use. A 6% α-naphthol solution was prepared by dissolving 6 grams of α-naphthol (Sigma-Aldrich, St. Louis, MO) in a small amount of denatured HPLC grade ethanol (Sigma-Aldrich, St. Louis MO) and

transferring this solution to a 100 mL volumetric flask. Ethanol was then added to a final volume of 100 mL. After aliquotting the α-naphthol reagent into 40 mL screw-top amber vials, the vials were sealed additionally with Teflon tape to prevent volatilization and stored at 4°C until use. See Table 1 for a summary of test descriptions and positive results for each test.

Table 1: Biochemical Tests and Positive Results

Reagent/Agar	*Test*	*Positive Results*
ADA	Dextrin fermentation	Orange/yellow colonies
150 mcg O129	Motility	No inhibited growth near disk
MacConkey	Lactose fermentation	Growth of pink colonies
TSA	Growth	Isolated colonies
Spot Oxidase	Presence of oxidase enzymes	Deep blue color upon exposure to reagent
Spot Indole	Tryptophan oxidation	Deep blue color upon exposure to reagent
Bile Esculin	Esculin hydrolysis	Black-brown colonies, darkening of agar
Voges-Proskauer	Acetoin production from glucose fermentation	Pink line at interface of reagent and broth
L-Arabinose	Arabinose fermentation	Broth color change from red to yellow
KIA/TSI	Glucose fermentation with gas production	Alkaline/acid, gas production

A random sample of 15 isolates was subjected to a Gram stain to confirm their physiology as Gram-negative rods. Isolates were streaked on TSA and incubated overnight before staining. Reagents were supplied by Biochemical Sciences, Swedesboro, NJ, and stains were conducted according to standard procedures (MacFaddin, 2000).

3.3.3 Water and Volatile Solids (VS) Content Analysis

After 1 g from each sediment sample had been removed for the serial dilutions and bacterial analysis described above, the remaining sediment was again wrist shaken in the sample container for a minute and then added to an aluminum weigh boat. The wet weight was recorded (A) before the weigh boat was dried in an 110°C oven overnight. Weigh boats were cooled on the laboratory bench until the dry weight was recorded (B), and water content (WC) analysis was calculated according to the following:

$$WC = \frac{A-B}{A} \; X\ 100$$

The dried sediment was transferred to a ceramic crucible, which was weighed (C). The crucible was then placed in a 550°C muffle furnace for 4 hours and weighed again (D), after being allowed to cool on the laboratory bench top. Volatile solids (VS) content was calculated according to the following:

$$VS = \frac{C-D}{C} \; X\ 100$$

A summary of the water and VS content for the sediment samples can be found in Appendix C.

3.3.4 Antibiotic Partitioning to Sediments

Table 2 summarizes sorption capacity as a function of percent organic carbon of the sediments for antibiotics representative of those investigated in this current study. It should be noted that the data in Table 2 are part of a broader publication (Thiele-Bruhn

2003) and that some of the compounds described in this publication were either omitted or have only partial information provided.

Table 2: Relevant Properties of Antibiotics with Respect to Sediment Partitioning

Antibiotic	*Soil Description*	*%Organic Carbon*	*Kd (L/kg)*	*Koc (L/kg)*
Oxytetracycline	Sand	1.5	417	27790
Sulfamethazine	Sand	0.9	1.2	174
Ciprofloxacin	Loamy Sand	0.7	427	61000

Because of the nature of the sediment in this study (refer to Appendix C), soils mentioned in the cited study with low % organic carbon content were chosen as analogous reference points from which to predict a concentration of antibiotic adsorbed to sediment.

Important to the discussion are also the concentrations of antibiotics that were found in the water column downstream of the wastewater treatment plant (see this chapter, Section 3.7 for a description of water column sampling, and see Appendix D for the sample analysis procedure). The concentrations of antibiotics used in this study found in the downstream water samples can be found in Appendix E. However, because the sampling excursions spanned only two months, seasonal variability was not considered, and the concentrations were averaged over all samples to give a representative value of each antibiotic for the purposes of this analysis; this number is reported in the last column of the table found in Appendix E. Appropriate unit conversions were made from ng/L to mg/L and the antibiotic concentration in solution was multiplied by the soil-water partitioning coefficient (K_d) value reported in Table 2 to yield a predicted concentration in the sediments in mg/kg. Because the units at this stage in the calculations (mg antibiotic/kg sediment) did not reflect those that might be

compared to MIC concentrations (µg antibiotic/mL solution), the density of the sediment was used to calculate a concentration in mg/cm^3; a conversion of 1 cm^3 to 1 mL was used to approximate a final concentration of drug adsorbed to the sediments in units that were analogous to those of MIC values. The density of the sediment was obtained through separate analyses conducted by colleagues and reported to the author.

3.3.5 Bacterial Isolation and Confirmation Background

The following biochemical tests were chosen because of their recognized capability as identification tests for *Aeromonas* spp. Previously published biochemical algorithms for identification of these bacteria employ these tests in their analysis and have shown them to be the tests of choice when isolating for aeromonads. Figure 1 below is a flowchart describing Aerokey II, an *Aeromonas* biochemical speciation algorithm as developed by Carnahan, Behram et al. (1991). In spite of continuing discoveries in *Aeromonas* taxonomy this scheme is still widely accepted when attempting to identify the members of this bacterial family.

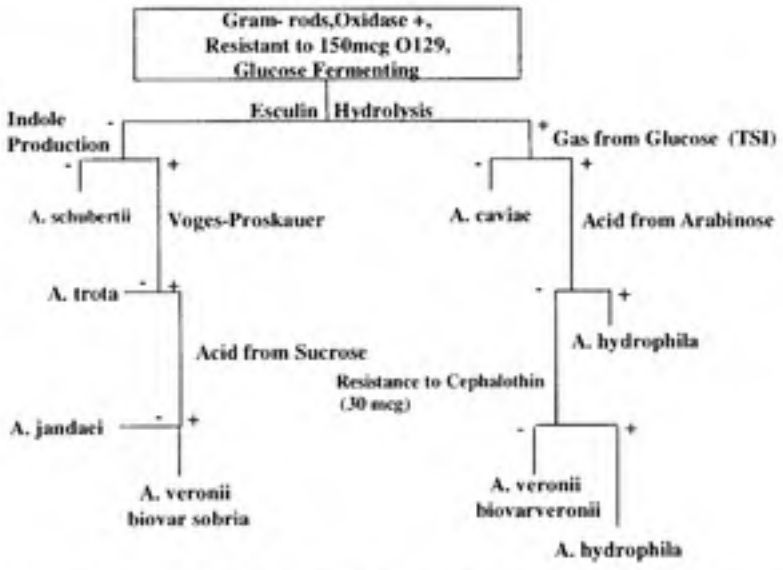


Figure 1: Aerokey II Biochemical Testing Algorithm for Aeromonas spp.

Gram stains were initially not included at the beginning of this study, as it was presumed that the application of all other biochemical tests being conducted would, in fact, prove the presence of *Aeromonas* spp. A random sample of isolates was, however, Gram stained in order to validate this assumption as being true, and the methods for this test are described in this chapter, Section 3.2. Cephalothin resistance was also not measured, as it was low enough in the flowchart sequence that this study's isolate would have been already identified as an *A. caviae* or *A. hydrophila*. The same rationale was applied to the test for acid from sucrose. Although the Aerokey II scheme aids substantially in distinguishing possible *Aeromonas* species, biochemical results are inherently subjective, and aeromonads present a considerable challenge when attempts are made to identify them with these methods. Studies that examine the reactions of known *Aeromonas* strains in biochemical tests consistently report variable results. It was, therefore, thought that the addition of molecular methods to identify the genes of *Aeromonas* species would eliminate this ambiguity. However, as will be discussed in Chapter 4, Section 3, the polymerase chain reaction (PCR) techniques employed by this study were not as reliable as hoped in identifying isolates to the species level. Consequently, in addition to guidance provided by the Aerokey II method, biochemical results of this study's environmental isolates were compared to those obtained by Abbot, Cheung et al. (2003) who sought to complex in species sub-groups rather than individually speciate isolates. The primary table consulted from that study is reproduced below.

Table 3: Reproduction of Biochemical Identification of Aeromonas to Complex Level

Biochemical identification of *Aeromonas* to complex level

Test	Number of strains identified as belonging to[a]:		
	A. hydrophila complex (*A. hydrophila, A. bestiarum, A. salmonicida*)	*A. caviae* complex (*A. caviae, A. media, A. eucrenophila*)	*A. sobria* complex (*A. veronii* HG8, *A. jandaei, A. schubertii, A. trota*)
Esculin	87 (92, 81, 85)	71 (76, 55, 78)	0
Voges-Proskauer	74 (88, 63, 62)	0	54 (88, 87, 17, 0)
Glucose (gas)	81 (92, 69, 77)	16 (0, 0, 78)	87 (92, 100, 0, 69)
L-Arabinose	93 (84, 100, 100)	96 (100, 100, 78)	4 (12, 0, 0, 0)

[a] The first number is the overall percent positive for each complex for a given trait; the numbers in parentheses are percent positive for each species listed within that complex.

In this current study, the results of the biochemical tests mentioned in Table 3 for *Aeromonas* complexation were heavily relied upon in the event that results from the other biochemical tests and PCR analyses were inconclusive. In these cases, speciation was omitted in favor of complexation only. This is explained in more detail in Chapter 4, Section 3.

3.3.6 PCR Confirmation

Isolates whose biochemical test results were indicative of *Aeromonas* spp. were subjected to PCR analysis for the confirmation of species. The detection of a specific lipase gene has been employed by others as a marker for *A. hydrophila* (Cascon, Anguita et al. 1996), and these methods were employed by this study in an attempt to speciate the environmental isolates. Briefly, presumptive aeromonads were streaked onto a TSA plate and incubated overnight. A 12.5 µL volume of HotStart Taq Mastermix (Qiagen, Valencia CA), 0.4µL of the forward and reverse primers and RNAse free water were mixed together and aliquotted into PCR reaction tubes. The primer set employed by this study reflected that of the Cascon study, which based their selection on the nucleotide sequence of the lipase gene; primer 1 and primer 2 were, respectively, 5'-

AACCTGGTTCCGCTCAAGCCGTTG-3' AND 5'-TTGCTCGCCTCGGCCCAGCAGCT-3'. An inoculating loopful of bacteria was added to 100 µL water and DNA was heat released at 99° C for 10 minutes. A 2 µL volume of the heat-released bacteria DNA was added to Eppendorf amplification reaction tubes containing the primer mixture. The tubes were then transferred to a pre-heated PTC-thermocycler and a PCR program was run for the detection of the *A. hydrophila* lipase gene. The PCR products were visualized on an agarose gel stained with ethidium bromide. Nayduch, Honko et al. (2001) identified *Aeromonas caviae* not by its lipase genes but rather by direct sequencing of the 16S ribosomal RNA gene and comparison to GenBank sequences. The procedure for *A. caviae* PCR detection followed the same protocol; the primer set used was as follows: (forward) 5'-TAGCTTGCTACTTTTGCCGG-3', (reverse) 5'-CACAGCCAGCAGRTATTAGCYACT –3'. Amplification conditions for these genes and more detailed methods can be found in Appendix F.

3.3.7 Water Column Antibiotic Residual Analysis

In addition to microbial analysis, water samples were taken for the detection and quantification of antibiotic residuals in the stream. One liter grab samples in duplicate were collected in amber glass bottles by submerging the bottle in the stream; the bottles were capped tightly with no headspace and stored on ice in a cooler until transport to the laboratory. The first sampling run included samples from the US and DS sampling sites, in addition to effluent samples collected at the discharge of the wastewater treatment plant. Effluent was collected with a grab sampler at the plant before discharge into the stream, and the sample was transferred to amber glass bottles filled to zero headspace.

Subsequent samples were collected only at US and DS points. Sample bottles were placed in a refrigerator upon return to the laboratory, where they were held for no longer than 4 hours before extraction. Analytical procedures are in Appendix D.

3.3.8 Antibiotic Resistance Testing

3.3.8.1 Microdilution Protocol: Plate Preparation and Layout

The microdilution method was employed as per NCCLS standards as the primary method of determining antibiotic resistance patterns (NCCLS, 2003a). Plates for antibiotic resistance testing were made to the project's specifications with the assistance of Betty Crews and Dr. Christopher Woods of the Durham Veteran's Administration Medical Center, Durham, North Carolina.

3.3.8.2 Antibiotic Stock Preparation for Microdilution

Ciprofloxacin (CIP) and trimethoprim (TMP) were purchased from (Fluka/Sigma Aldrich, St. Louis MO) with reported purities of 98% and 98.5%, respectively. Sulfamethoxazole (SMX) and tetracycline hydrochloride (TET) were purchased from Sigma Aldrich (St. Louis, MO) with purities of 95% and 98%, respectively. Antibiotic standards were stored at –20° C until use. Stock solutions of ciprofloxacin, tetracycline, trimethoprim, and sulfamethoxazole at ten times the highest desired concentration were prepared immediately prior to use as follows. The amount of antibiotic powder to be weighed was calculated according to NCCLS criteria, the equation for which follows:

$$Weight\ (mg) = \frac{Volume\ (mL) \times Desired\ Concentration\ (mcg/mL)}{Potency} \times 10$$

where volume is constant at 100 mL, the desired concentration is relative to the antibiotic in question (ciprofloxacin=16, sulfamethoxazole=1024, tetracycline=64, trimethoprim=32 μg/mL) and potency is determined to be the percent purity of the antibiotic as reported by the manufacturer (NCCLS, 2003a). This approach was adjusted for tetracycline hydrochloride as the provided reagent was in a salt form. The percent by weight of tetracycline was determined by dividing the molecular weight of tetracycline HCl by the molecular weight of tetracycline, and the result (92.4%) was used as potency instead of the reported 95%. The weight was multiplied by 10 as indicated because the stock solutions were to be ten times the highest desired concentration. Actual weights of antibiotics employed in the stock solutions are given in Appendix G.

Three of the antibiotics required dissolution in an acid or base before subsequent dilution with water. Appropriate quantities of antibiotic powders were added to sterile sample cups provided by the Duke VA hospital, and 1 mL of pre-prepared 2.5 M sodium hydroxide (NaOH) was added to the sulfamethoxazole powder; 10 mL of pre-made 0.05 M hydrochloric acid was added to the trimethoprim. It should be noted that while the NCCLS protocols did not require a solvent for ciprofloxacin (NCCLS, 2002), it was found to be necessary. Because protocol indicated that other antibiotics in the fluoroquinolone class required small amounts of NaOH as a solvent, this was assumed to be acceptable for ciprofloxacin. A 1.5 mL volume of pre-prepared 2.5 M NaOH was added to the ciprofloxacin powder and found to be sufficient for dissolution. After the powders were dissolved, the volume was brought up to 100 mL as indicated on the sample cup with sterile water, also provided by the VA hospital. Stocks were then diluted with cation-adjusted Mueller-Hinton broth; the broth had been prepared at the VA

hospital early in the day so as to allow for thorough cooling. Ten-fold dilutions of the initial stock were prepared for the highest desired concentration, (row A, columns 1-4 in Figure 2), and these concentrations were sequentially diluted two-fold to obtain all subsequent concentrations. Because only four antibiotics were employed, triplicate volumes of each antibiotic were prepared for each sterile, lidded 96-well plate (Fisher Scientific, Hampton NH), resulting in the plate layout shown in Figure 2.

	1	2	3	4	5	6	7	8	9	10	11	12
*	CIP	SMX	TET	TMP	CIP	SMX	TET	TMP	CIP	SMX	TET	TMP
A	16	1024	64	32	16	1024	64	32	16	1024	64	GC
B	8	512	32	16	8	512	32	16	8	512	32	32
C	4	256	16	8	4	256	16	8	4	256	16	16
D	2	128	8	4	2	128	8	4	2	128	8	8
E	1	64	4	2	1	64	4	2	1	64	4	4
F	0.5	32	2	1	0.5	32	2	1	0.5	32	2	2
G	0.25	16	1	0.5	0.25	16	1	0.5	0.25	16	1	1
H	0.125	8	0.5	0.25	0.125	8	0.5	0.25	0.125	8	0.5	SC

Figure 2: Antibiotic Resistance Testing Plate Layout Showing Antibiotics (Columns) and Concentrations in µg/mL (Rows)

- *Ciprofloxacin (CIP), Sulfamethoxazole (SMX), Tetracycline hydrochloride (TET), Trimethoprim (TMP)*
- *GC – growth controls; SC – sterility controls*

The numbers in each block correspond to the antibiotic concentration in µg/mL. Although the NCCLS has not established MICs for *Aeromonas* spp., the concentrations for the 96-well plate were chosen based on an examination of inhibitory concentrations established for *V. cholera*, *P. aeruginosa* and *Enterobacter*, the MICs of which may be found in Appendix H; the 96-well plates were designed to include maximum and

minimum ranges of antibiotic concentrations for these bacteria. Top right and bottom right wells were left as growth controls (GC) and sterility controls (SC), respectively. Both of these types of wells were filled with pure culture broth only; growth controls were inoculated with a control strain of *E. coli* ATCC 25922 while the sterility control was left uninoculated in order to monitor for unexpected growth due to possible bacterial contamination. Plates were wrapped in plastic bags, taped, and stored at –20°C until use.

3.3.8.3 *Microdilution Protocol: Isolate Inoculation and Incubation*

Confirmed *Aeromonas* isolates were streaked on TSA plates and incubated overnight at 35°C. Colonies from these plates were then added to a thin-walled glass tube containing 4 mL of sterile physiological saline (4.25g NaCl/500 mL deionized water) and vortexed to achieve a 0.5 McFarland turbidity standard (Remel, Lenexa KS). This corresponds to a concentration of 10^8 colony forming units (CFU)/mL. The turbidity – adjusted bacterial suspension was diluted 1:100 in sterile saline to achieve a 10^6 CFU/mL solution, and 10 µL aliquots of this dilution were used as the inoculum for each well of the 96-well plates, resulting in a final concentration of 10^4 CFU/well (NCCLS 2003a). After inoculation, the plates were incubated overnight at 35°C and examined for growth as indicated by any increase in turbidity of the well as measured by the naked eye. The MICs were reported as the highest concentration of antibiotic which did not show any bacterial growth. For example, a reported tetracycline MIC of 16 µg/mL represents a bacterial isolate that will grow in concentrations of tetracycline as high as 8 µg/mL as per the microdilution plate employed by this study, but not at the next highest concentration of 16 µg/mL. In the event that the bacterial isolate was capable of growing in the most concentrated antibiotic well, the MIC was defined as the concentration that would be the

next in the series, which is double the final growth well concentration. This results in some bacteria with MICs that are not representative of antibiotic concentrations in the 96-well plate.

3.3.8.4 *Disk Diffusion Protocol: Media Preparation and Isolate Inoculation*

The disk diffusion method was employed for a 25 µg combination trimethoprim-sulfamethoxazole disk (Becton-Dickinson, New Jersey). Mueller-Hinton agar (Becton-Dickinson, New Jersey) was prepared according to the manufacturer's dehydrated medium-to-water-ratio. The pH of the agar was tested by allowing a small amount to solidify before submerging a pH meter probe into the aliquot. Volumes of 25 mL of agar were dispensed per plate and plates were stored at 4°C for no more than three days before use.

Testing was conducted as per NCCLS protocol (NCCLS, 2003b). Bacterial isolates were incubated on TSA for 18-24 hours before being tested. Colonies from these plates were added to a thin-walled glass tube containing 4 mL of sterile physiological saline and vortexed to achieve a 0.5 McFarland turbidity standard. One hundred µL aliquots of the bacterial solution were then spread-plated on the Mueller-Hinton surface in order to create a uniform lawn of growth. Trimethoprim-sulfamethoxazole disks were placed on the agar plate using flame-sterilized tweezers, and plates were incubated at 35°C for 16-18 hours. The zone diameter around the disks was then measured with a ruler and recorded. Because the disk creates a zone of antibiotic around itself, the more resistant the bacterium is to the antibiotic, the closer it can grow to the disk.

3.4 Data Collection, Management, and Analysis

3.4.1 Biochemical Test Results

The isolates were assigned a label that documented from which sampling station they originated (US, E, DS), the date of the sampling excursion, and a number based on how many isolates were obtained from that station. Worksheets were generated in Excel with rows assigned for the isolate label and a column for each biochemical test. Hard copies were printed and used in the laboratory, where the results of the biochemical tests for each isolate were recorded in the appropriate column. This data was then re-entered electronically into the spreadsheet. Isolates whose biochemical results conformed to the aforementioned classification schema (Aerokey II, Abbott's complexation) were considered to be *Aeromonas* spp.

3.4.2 Antibiotic Resistance Testing Results

Excel was again employed to create representations of the 96-well plate complete with antibiotic abbreviation and concentration (see Figure 2). Hard copies of these figures were used in the lab to score the actual inoculated plates after incubation, and these hard copies were stored in a data collection folder. The zone diameter around each antibiotic disk was recorded in a laboratory notebook along with the corresponding isolate label.

3.4.3 Analysis of Antibiotic Resistance Data Based on a Variation of Discriminant Analysis

After the isolate was satisfactorily determined to be an *Aeromonas* and was tested for resistance, an electronic spreadsheet was again organized with the isolate label, the recorded MIC from the 96-well plate for each isolate, and whether the isolate was susceptible, intermediate or resistant to the trimethoprim/sulfamethoxazole disk. In order

to better approach possible associations between higher MICs/resistance levels and sampling site vis a vis the effluent discharge, three categories of *Aeromonas* resistance were created based on the antibiotic resistance patterns seen in the 96-well plate and on the Mueller-Hinton disk diffusion plates. If the isolate was clearly susceptible to the antibiotic, i.e. no growth in any well or the presence of distinct zones around the antibiotic disk, a score of "0" was assigned for this particular antibiotic. When there was any growth in the antibiotic well, or the zone diameter fell short of complete susceptibility, a score of "1" was assigned, regardless of whether growth was in a concentration still within the range of susceptibility. An intermediate score such as this was decided upon as a category of resistance/susceptibility primarily to be able to include ciprofloxacin in this analysis, as almost every ciprofloxacin MIC was still well within ranges of antibiotic susceptibility relative to the level of clinically relevant resistance. Finally, resistance to the antibiotic, as indicated by growth close to the antibiotic disk or in a well with high antibiotic concentration, was given a score of "2". This was done across each antibiotic for every isolate subjected to resistance testing, and a total antibiotic resistance score (ARS) comprising the sum of all individual antibiotic scores was assigned to the isolate. Another column of these scores was included in the spreadsheet of resistance results; this data is found in Appendix I. Scores were then averaged within the sampling sites and compared. This type of examination, that of classification and comparison of resistance patterns, is a modification and simplification by Dr. Mark Sobsey (University of North Carolina, Environmental Sciences and Engineering Department, personal communication, 2005) based on the much more data-rich and sophisticated discriminant analysis described by Wiggins (1996). The results are

usually employed in an attempt to differentiate sources of fecal pollution by comparing wild type, human, and agricultural isolates and their resistance patterns. This study, however, only employs this technique to formulate a structure for comparison of results from each sampling site.

3.5 Statistical Analyses

All statistical analysis at the primary site was conducted through the use of an online statistical program that can be found at the following: http://www.physics.csbsju.edu/cgi-bin/stats (in order to access this program, one must first open http://www.physics.csbsju.edu/, then follow the link to statistics, after which one may choose from a list of statistical tests to perform).

Unpaired t-tests between the results obtained from three sample pairings were conducted on the ARSs referred to in Section 4.3 of this chapter in order to establish the significance of the difference between the means. A one-way analysis of variance (ANOVA) was also conducted on the ARSs for the isolates at each of the sampling stations (US, E and DS) in order to assess the statistical difference among the frequency of antibiotic resistance in the three groups. The null hypothesis (H_o) in both the t-tests and ANOVA was allowed to represent the possibility that there no real effect, i.e., that the difference in resistance patterns is solely due to chance; the factor under study is presumed to be the effect of the wastewater treatment plant outfall, or rather, how the ARSs vary in relation to the location of isolate collection around the point of effluent discharge. The benefit of using the ANOVA in conjunction with the t-tests is primarily the ability to collapse the data into one overall comparison. By conducting multiple t-tests on the same data set, in order to maintain the significance of the entire examination,

the p, or probability that the observed difference is due to random chance, for each test should equal 0.05/n where n is the number of tests conducted (Dr. Mike Symons, University of North Carolina Biostatistics Department, personal communication).

4 Results

4.1 Sediment vs. Water Column Bacteria Concentrations

The sediments were found to have a substantially greater number of bacteria than the water column. Figure 3 displays the $\log_{10}$ concentrations of bacteria in CFU/mL; these units were chosen because the supernatants of the resuspended and diluted sediment sample were spread plated as opposed to the actual sediment, which would have reported units of CFU/gram sediment. The sampling station identifications are different from those labels assigned to the primary site as this facet of the research was conducted at the initial (preliminary) rather than the primary site.

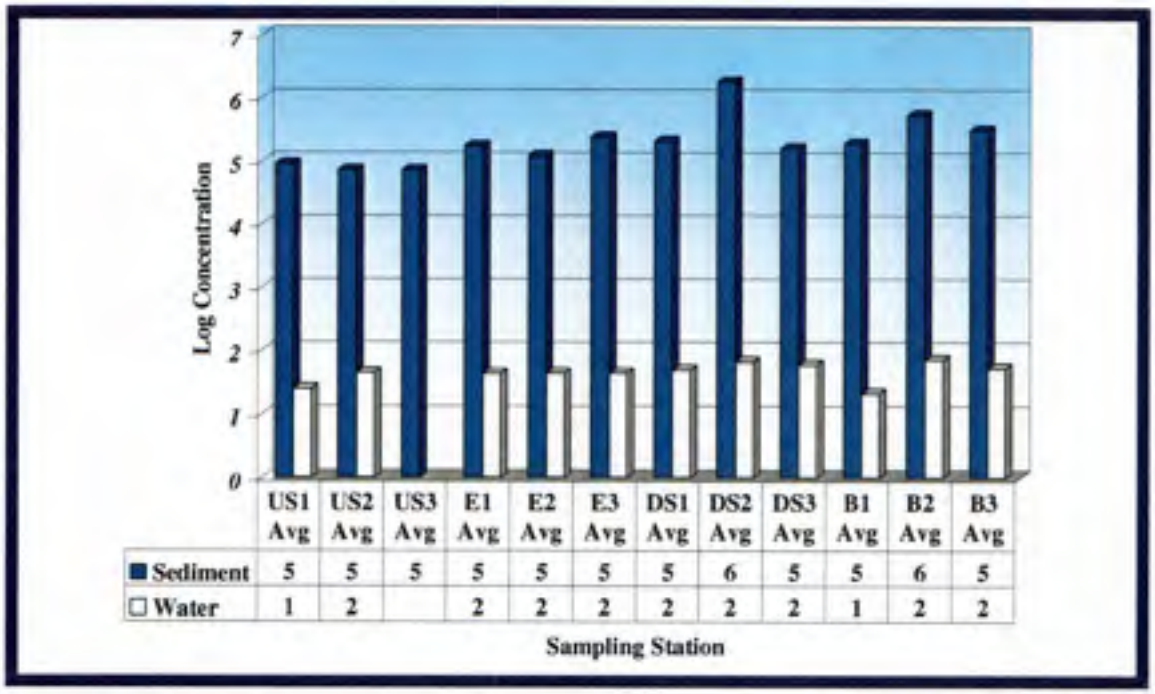


Figure 3: Bacterial Concentrations in Sediment and Various Points in the Water Column at the Preliminary Site

The concentrations in the sediment supernatant are much higher than those in even the undiluted water; on average the difference between the two is approximately 3 $\log_{10}$. A t-test conducted between the averages of the $\log_{10}$ concentrations in sediment and water shows the difference to be statistically significant, with a probability of the difference being attributable to pure chance equal to less than 0.0001 (more detailed results can be found in Appendix J). Therefore, even if minor water column intrusion into the sediment did occur, its contribution to the whole of the sediment bacterial population can be considered negligible. Although sediment processing methods testing was not conducted as part of this work, the results of the sediment/water concentration analysis could be employed as a surrogate. Since concentrations of bacteria found in the sediment supernatant are consistently much higher than those found in the surface water, it is evident that the methods used to isolate bacteria from sediment were efficient.

4.2 Prediction Estimates of Antibiotic Concentrations in the Sediments Collected from the Primary Site

Table 4 displays the results of the calculations made to predict the concentrations of antibiotics that might be found in the sediments collected at the primary site.

Table 4: Predicted Concentrations of Antibiotics in the Sediments Sampled

Antibiotic	*Avg. Concentration (mg/L)*	*Sorbed (mg/kg)*	*Sorbed (mcg/mL) at Sediment Density 2.6 g/cm3*
Ciprofloxacin	0.02	6.4	16.7
Sulfamethoxazole	0.63	0.8	2.0
Tetracycline	0.02	6.9	18.0

Refer to Chapter 3, Section 3.4 for details on how the predicted concentrations were calculated. Recall that rather than report the concentration in mg of antibiotic/kg sediment, appropriate conversions were made using the sediment density and approximation of 1 cm^3/mL to result in the µg/mL units reported in the last column of Table 4. This was in order to better compare, through consistent units, the predicted concentration in the sediment to the antibiotic concentrations that were used in resistance testing. Chapter 5, Section 3.1 discusses the ramifications of this examination in more detail.

4.3 Aeromonas Species Distribution

A total of 465 isolates was obtained from the primary site and biochemically tested according to the previously described methods. A total of 103 isolates was identified through PCR testing as *A. caviae* or *A. hydrophila*. Although it was frequently the case that the biochemical results and the PCR results did not correspond, it was thought initially that this was due in large part to the limitations and inaccuracies of

biochemical testing schemes particularly when applied to this genus. However, it was later discovered that in spite of literature to the contrary (Cascon, Anguita et al. 1996), the use of 16S ribosomal RNA to correctly identify *Aeromonas* to the species level is considered highly suspect by some investigators (Morandi, Zhaxybayava et al., 2005), and perhaps only the genus of this particular organism may be confirmed using this technique (Dr. Amy Carnahan, University of Maryland, personal communication). Therefore, those isolates that were thought to have been genetically identified to the species level were considered only to be *Aeromonas* genus, and the biochemical results of those isolates were consulted again in order to speciate the organisms. The results of the biochemical tests for all isolates can be found in Appendix K.

When the aggregate results of biochemical tests were capable of identifying the species, this information was assigned to the isolate, otherwise, the isolate was classified by complex according to Abbot, Cheung et al., 2003. *A. caviae* and *A. hydrophila* were the only definite species assigned. After PCR identities were considered unreliable based on recent publications, the acquisition of new PCR data was terminated because of the possible unreliability of the employed genetic confirmation method coupled with budgetary limitations. Thereon, the remaining bacteria that were isolated for this study were identified only by the biochemical tests outlined in Section 3.2 of Chapter 3. Fifty isolates from each site, upstream (US), at point of effluent discharge (E), and downstream (DS), were identified as aeromonads. This sample size was calculated on the basis of an assumed difference in proportions of resistance within the bacterial populations of 25%, an α value of 0.01 and power of 0.9. The alpha parameter describes the probability that a statistically significant difference is found between the groups tested when there is in fact

no difference. The power represents the probability that an extant difference is detected. Therefore, these constraints define the sample size required to detect with a probability of 0.9 a 25% difference in antibiotic resistance prevalence, with a 0.01 chance of error. There was no attempt in this study to quantitatively enumerate the *Aeromonas* populations at each sampling station. Table 5 shows the number of isolates identified to the complex level by site, the number of isolates assigned a definite species, and the percent of the isolates whose genus was PCR confirmed.

Table 5: Distribution of Aeromonas by Sampling Site

Station ID	% Identified to Species (Biochemical)	% Identified as Aeromonas (PCR)	Number of Isolates per Complex at Each Sampling Point*		
			A. hydrophila (*A. hydrophila*, *A. bestiarum*, *A. salmonicida*)	***A. caviae*** (*A. caviae*, *A. media*, *A. eucrenophila*)	***A. sobria*** (*A. veronii* HG8, *A. jandaei*, *A. shubertii*, *A. trota*)
US	62	78	18	25	7
E	62	72	4	34	12
DS	66	74	11	32	2

*Complexation taken from Abbot, Cheung et al., 2003; Upstream (US), Effluent (E) Downstream (DS)

In the case of the DS site, four of the isolates were confirmed by PCR to the genus only and were not complexed. There was, however, a total of 50 isolates from the DS station that were tested for increased MICs.

The distribution of complexes is consistent with prior observations of the incidence of *Aeromonas* species in the environment. While the percentage of isolates between *A. hydrophila* and *A. caviae* at the US site is similar, note the increase at E in the *A. caviae* proportion. This is in agreement with past studies that have drawn correlations between high organic loading and an increase in environmental populations of *A. caviae* (Araujo, Arribas et al. 1991). However, it is not necessarily the case that the bacteria are

being released into the stream with the effluent. Instead, it is possible that *A. caviae* have an intrinsic advantage in proliferating or surviving in eutrophied environments (Warren, Jeter et al. 2004).

4.4 Antimicrobial Analysis of Isolates Based on Minimum Inhibitory Concentrations

Those isolates whose genus was confirmed as *Aeromonas* by PCR and those remaining isolates that were biochemically speciated without the PCR confirmation were tested for their antimicrobial resistance properties as measured by MICs. Again, it must be reiterated that the NCCLS has not identified MICs for *Aeromonas* spp. However, in order to establish some form of classification for the levels of resistance displayed by the environmental isolates, for the purposes of this project the NCCLS MIC guidelines for the aforementioned bacteria (Chapter 3, Section 3.8.2) are used to define "susceptibility" and "resistance" in the following results. The profiles were also compared to a control *A. hydrophila* ATCC 7966 strain, which showed susceptibility to ciprofloxacin, tetracycline and the trimethoprim/sulfamethoxazole combination disk. In the subsequent plots, lighter-colored columns relate to lower MICs (Note that darker columns represent the proportion of isolates with increasing resistance; legends are concentrations of antibiotic in µg/mL). So as not to confuse general expressions describing relative positions of up- and downstream, the designations of US, E and DS will be employed to refer to characteristics of bacteria isolated from the specific sampling points. A distinction is also made between isolates with merely higher MICs, even if increased MICs may still fall within ranges of antibiotic susceptibility, and those that may be deemed resistant according to NCCLS standards previously mentioned. It is important in this study to identify any increase in MICs, as this may be indicative of the possibility for the

subsequent creation of clinically resistant bacteria. This study will primarily rely on statistical analyses between the differences in antibiotic resistance profiles of bacteria isolated up and downstream of the wastewater effluent discharge. Appendix L records the MIC for all isolates.

4.5 Incidence of Resistance or Increased MIC

The following describes only raw proportional increases in MICs calculated as the percent of isolates displaying any increase in MICs relative to the total number of isolates tested from each sampling site.

4.5.1 Discrepancies in MIC Profiles of Bacteria Relative to Tetracycline

Approximately a third of both the effluent and downstream isolates tested for tetracycline resistance showed increased minimum inhibitory concentrations; resistance accounted for 24% and 20% of the total E and DS isolates respectively. Figure 4 illustrates the dramatic difference between the susceptibility of upstream isolates and increase in inhibitory concentrations at sample points downstream of effluent discharge.

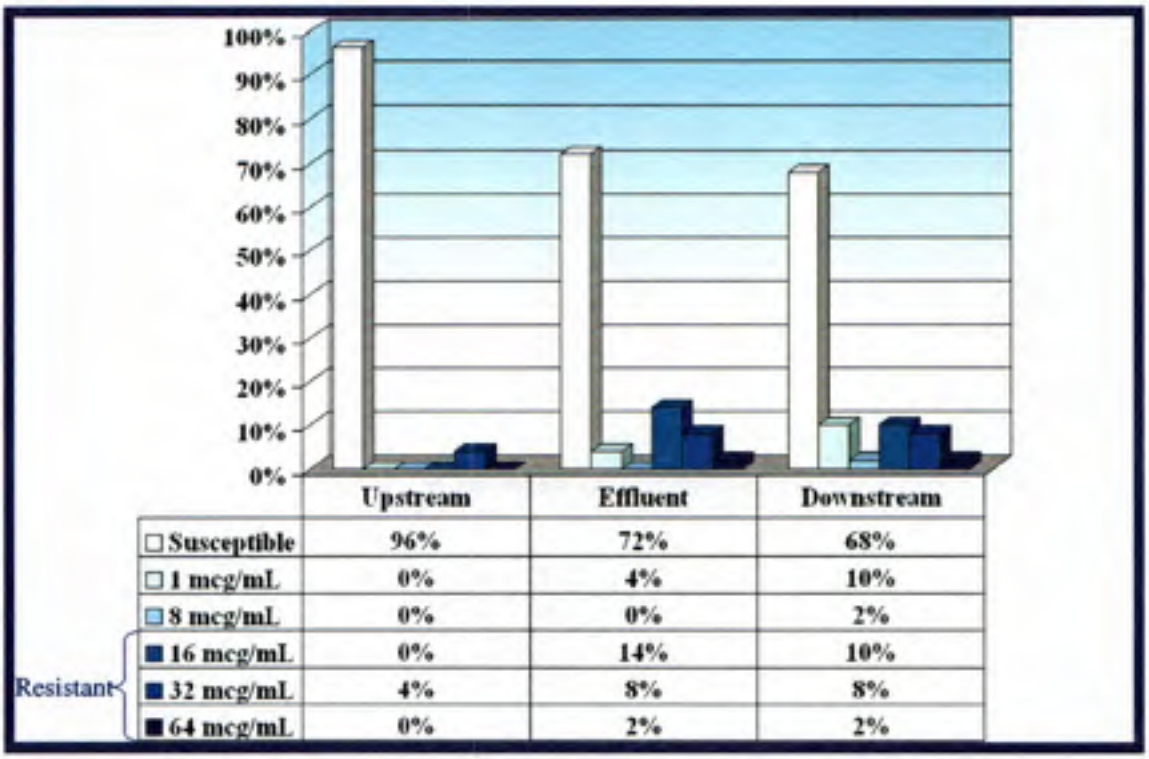


Figure 4: Percent Incidence of Tetracycline Resistance MICs (μg/mL) in Aeromonas Isolates from Upstream, Effluent and Downstream Sampling Stations

There appears to be some resistance to tetracycline (4% of isolates) among the upstream isolates; however, the greater percentage of isolates with increased tetracycline MICs downstream as shown in Figure 4 is significant. These results implicate the wastewater effluent discharge as having a considerable impact on tetracycline resistance profiles of *Aeromonas* bacteria in stream sediments. This pattern is demonstrated not only with tetracycline but also with other the antibiotics examined by this study. These findings are consistent with impacts from the effluent discharge that influence the antimicrobial resistance of bacteria in downstream waters.

4.5.2 MIC Profiles of Bacteria Relative to Trimethoprim and Sulfamethoxazole Independently and in Combination

Trimethoprim (TMP) and sulfamethoxazole (SMX) showed some very minor differences in levels of resistance found in *Aeromonas* species when examined individually. For the two highest concentrations of TMP and SMX tested, the percent of isolates resistant at stations E and US combined averaged 63% and 85%, respectively. In comparison, *Aeromonas* resistance to the two highest concentrations of TMP and SMP at station US was somewhat lower at 58 and 78% respectively. It is important to note that NCCLS criteria for resistance testing of TMP and SMX using the broth microdilution method do not allow for a classification of intermediate resistance; disk diffusion methods for the combination antibiotic are, however, categorized according to all three classifications: susceptible, intermediate and resistant.

Both changes in MICs and changes in percent increased resistance were examined for these drugs. The MICs of SMX and TMP were recorded for each isolate tested and tallied, thereby creating bins of concentrations and the number of isolates for which this concentration was determined to be the MIC. For each sampling site, the percent of isolates at a given MIC was plotted. Tables 6 and 7 show the incidence of trimethoprim and sulfamethoxazole MICs respectively at each sampling point.

Table 6: Number of Isolates with a Given Trimethoprim MIC at Each Sampling Station

	Trimethoprim MIC						
	64	***32***	***16***	***8***	***4***	***2***	***1***
	Number of Isolates at Each Sampling Station						
US	21	8	9	8	2	2	0
	42%	16%	18%	16%	4%	4%	0%
E	29	4	11	5	1	0	0
	58%	8%	22%	10%	2%	0%	0%
DS	27	4	9	5	2	3	0
	54%	8%	18%	10%	4%	6%	0%

Table 7: Number of Isolates with a Given Sulfamethoxazole MIC at Each Sampling Station

	Sulfamethoxazole MIC				
	2048	***1024***	***512***	***256***	***128***
	Number of Isolates at Each Sampling Station				
US	22	17	8	2	1
	44%	34%	16%	4%	2%
E	24	17	5	2	2
	48%	34%	10%	4%	4%
DS	19	25	6	0	0
	38%	50%	12%	0%	0%

Figure 5 displays the plot of MIC incidence at each sampling site for trimethoprim.

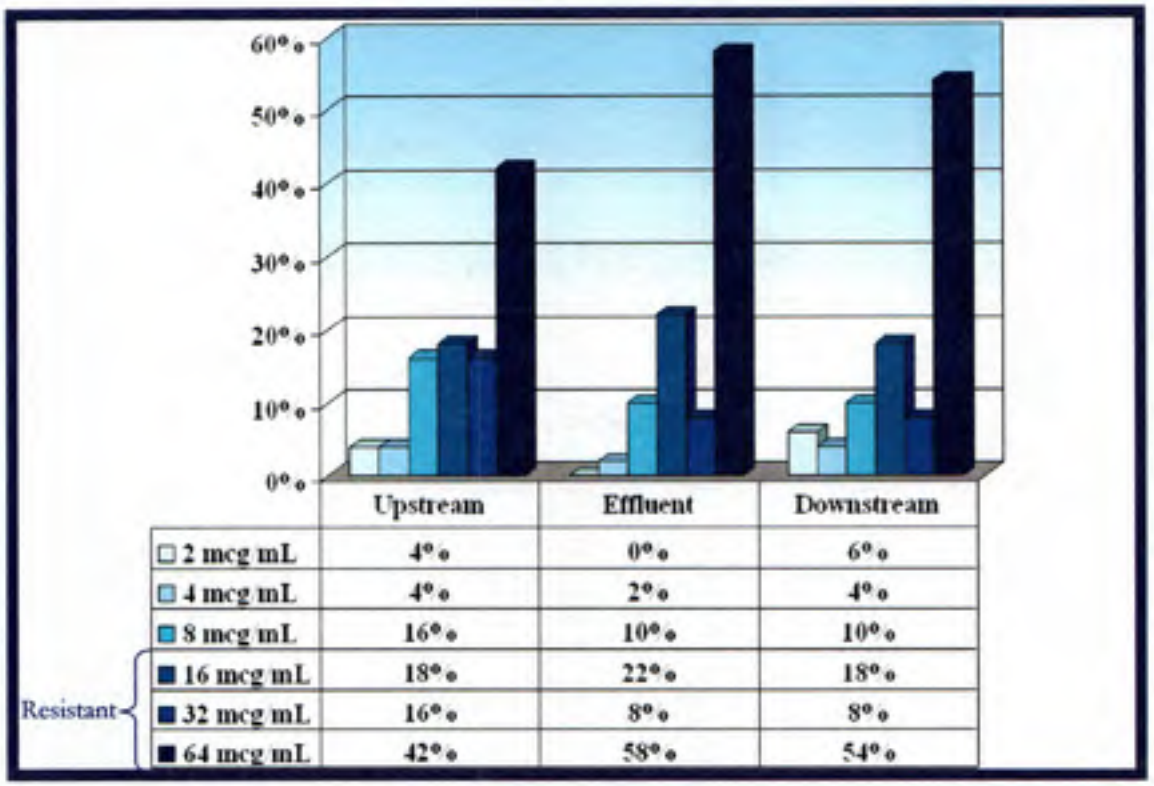


Figure 5: MICs of Isolates for Trimethoprim in Upstream, Effluent, and Downstream Sampling Stations

By examination of Table 6 and Figure 5, there is little in the way of a discernable trend of trimethoprim MICs by sample site. However, it is interesting to note the increase in the

occurrence at station E as compared to station US of the highest MIC of 64 µg/mL, an occurrence that decreases slightly at station DS. When these MIC classifications are collapsed into broader groups of resistant vs. susceptible isolates, this increase in 64 µg/mL frequency is reflected in a commensurate increase in those isolates deemed resistant. Of the US isolates, 76% are resistant, and this increases to 88% resistant at sampling point E. Differences in MICs by sample station are less notable for sulfamethoxazole.

Figure 6 shows the MICs of isolates relative to sampling station.

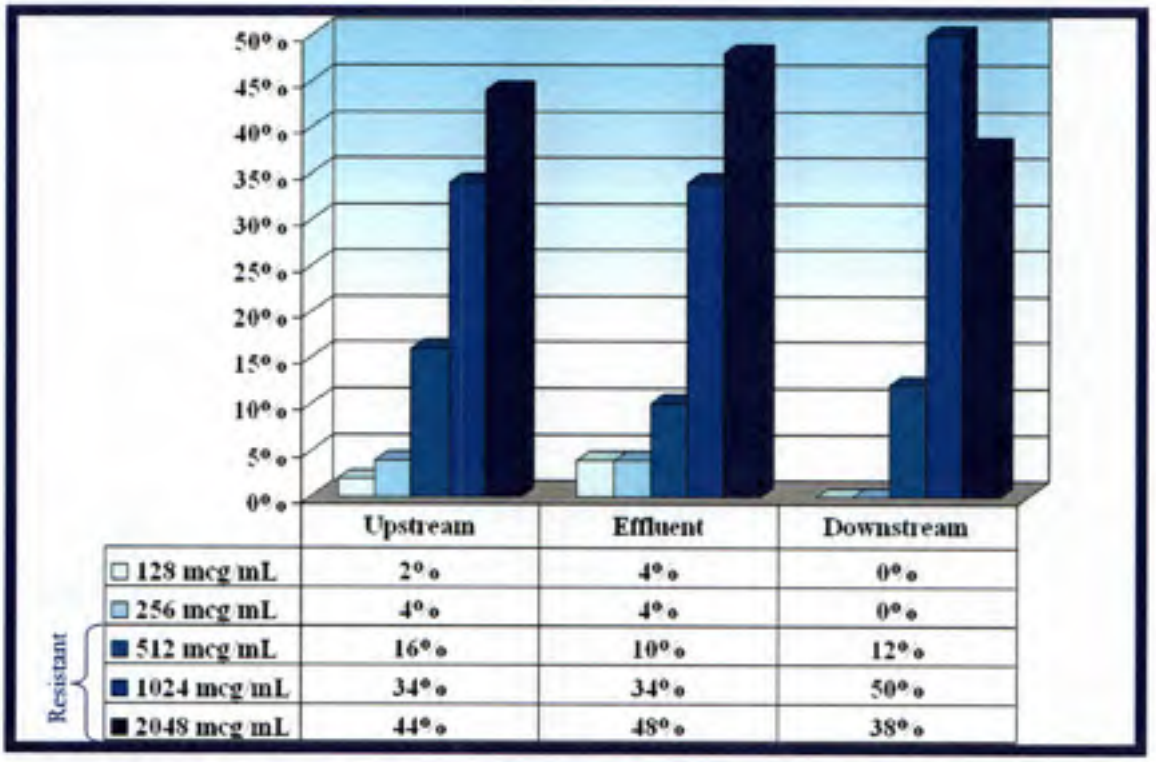
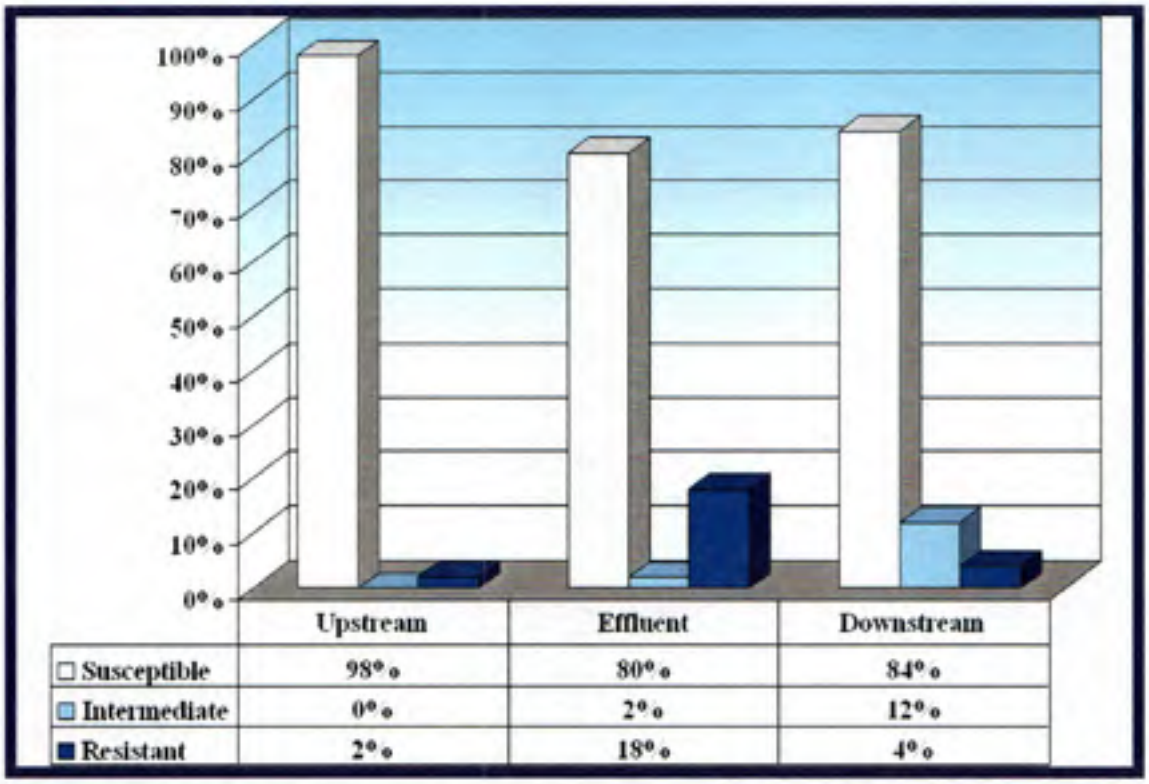


Figure 6: MICs of Isolates for Sulfamethoxazole in Upstream, Effluent and Downstream Sampling Stations

The proportion of isolates capable of being classified as resistant was similar over all sampling points, although there are marginally higher percentages of isolates resistant to

the two highest concentrations at stations E (82%) and DS (88%) compared to station US (78%).

Resistance to the combination of trimethoprim/sulfamethoxazole was also examined. Zone diameters exceeding 16mm were considered susceptible; intermediate resistance was attributed to isolates whose zone diameters ranged between 11-15 mm. Resistance to the combination of drugs was recorded if the diameter around the disk was measured as less than 11 mm; these diameters were employed in gauging susceptibility as per NCCLS standards for *V. cholera*. Figure 7 shows the proportional incidence of trimethoprim/sulfamethoxazole resistance in relation to sampling station.



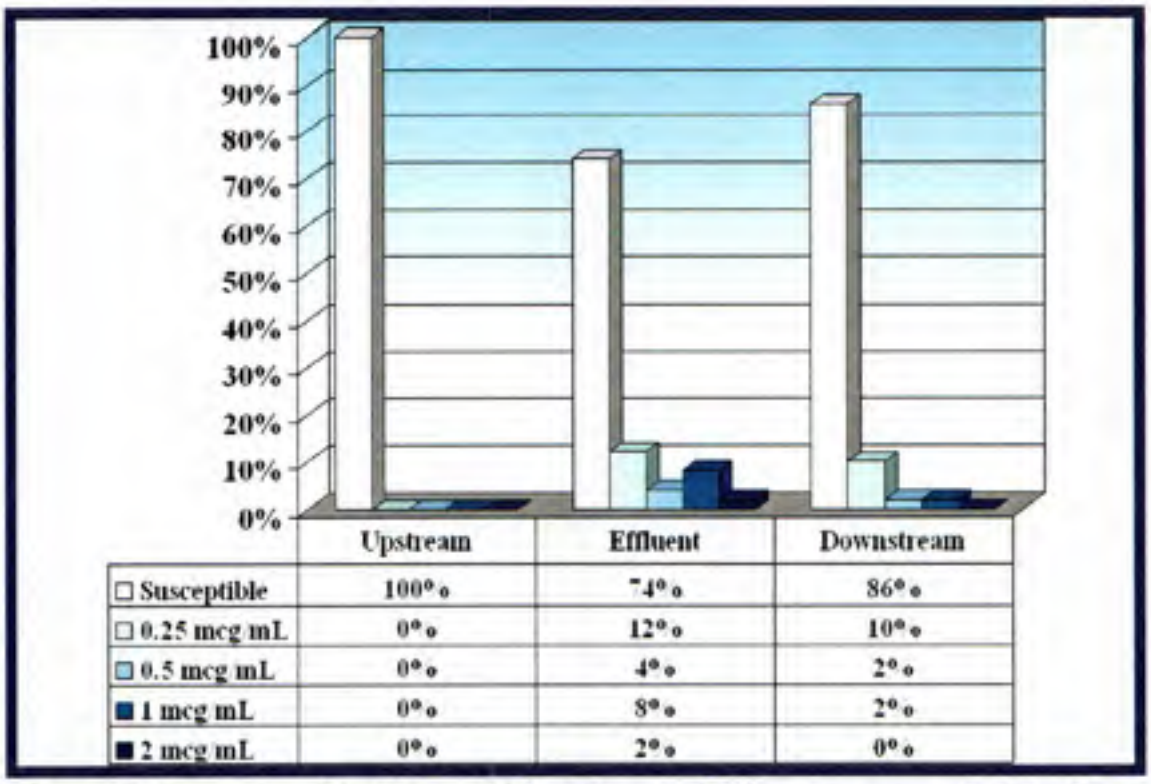
	Upstream	Effluent	Downstream
Susceptible	98%	80%	84%
Intermediate	0%	2%	12%
Resistant	2%	18%	4%

Figure 7: Susceptibility of Isolates to Trimethoprim/Sulfamethoxazole at Upstream, Effluent, and Downstream Sampling Stations

Resistance was shown to be most prevalent in isolates closest to the effluent discharge (at E), while intermediate resistance predominated further downstream (at DS). Again, the proportion of bacteria either resistant or showing decreased susceptibility to trimethoprim/sulfamethoxazole is notably higher in isolates found downstream of the wastewater effluent discharge compared to upstream. In addition, a pattern that was seen in tetracycline, that of intermediate resistance predominating at DS instead of absolute resistance predominating at E, can also be seen here.

4.5.3 MIC Profiles of Bacteria Relative to Ciprofloxacin

Very few isolates showed any increase in minimum inhibitory concentrations for ciprofloxacin. Of the few isolates that exhibited any increase in resistance, the vast majority grew in the most dilute concentration of antibiotic in the 96-well plate. However, this growth was usually heavy, and is worth noting since it alludes to the beginnings of increased resistance in environmental isolates. In addition, every isolate that exhibited even marginally decreased susceptibility was found either close to the effluent discharge or at the downstream point, as shown by Figure 8.



	Upstream	Effluent	Downstream
Susceptible	100%	74%	86%
0.25 mcg mL	0%	12%	10%
0.5 mcg mL	0%	4%	2%
1 mcg mL	0%	8%	2%
2 mcg mL	0%	2%	0%

Figure 8: Proportional Incidence of Ciprofloxacin MICs in Aeromonas Isolates from Upstream, Effluent, and Downstream Sampling Stations

No isolate exhibited absolute resistance to ciprofloxacin, however, growth in the presence of 2 µg/mL is defined as "intermediate" resistance by NCCLS. It can be seen from Figure 8 again that resistance is lowest in upstream isolates, that effluent has a greater proportion of isolates with higher MICs, and that incidence of resistance decreases somewhat at the downstream sampling site. The percentage of isolates displaying any ciprofloxacin MIC is very low. Yet because fluoroquinolones show excellent activity against *Aeromonas* infections (Vila, Marco, et al. 2002), any decrease in susceptibility is a concern. In addition, given that all upstream isolates show complete susceptibility to this antibiotic, the effluent discharge appears to be the point of origin for the rise in *Aeromonas* MICs in this short stream reach. While the aforementioned trend of lower MICs predominating at the downstream point is observed, the effect is not as dramatic as with trimethoprim/sulfamethoxazole and tetracycline.

5 Analysis and Discussion

The rise in antibiotic resistance has been at the forefront of public health concerns for years. While many advances have been made in identifying the sources and conditions that precipitate the acquisition of resistance by bacteria, the vast majority of prevailing conclusions in the literature stem from research conducted in clinical and agricultural settings. Much remains to be determined with respect to the impacts these pharmaceuticals have when introduced into the environment at much lower levels than found in hospitals and on cattle feeding lots. To this end, the following discussion seeks to examine the topic of environmental resistance acquisition in light of the new data collected and generated by this study for these potentially significant but as yet relatively unexplored sources and environmental conditions.

5.1 Antibiotic Resistance Scores Based on Discriminant Analysis

The averages of the antibiotic resistance scores (ARS) based on a variation of discriminant analysis are shown in Figure 9.

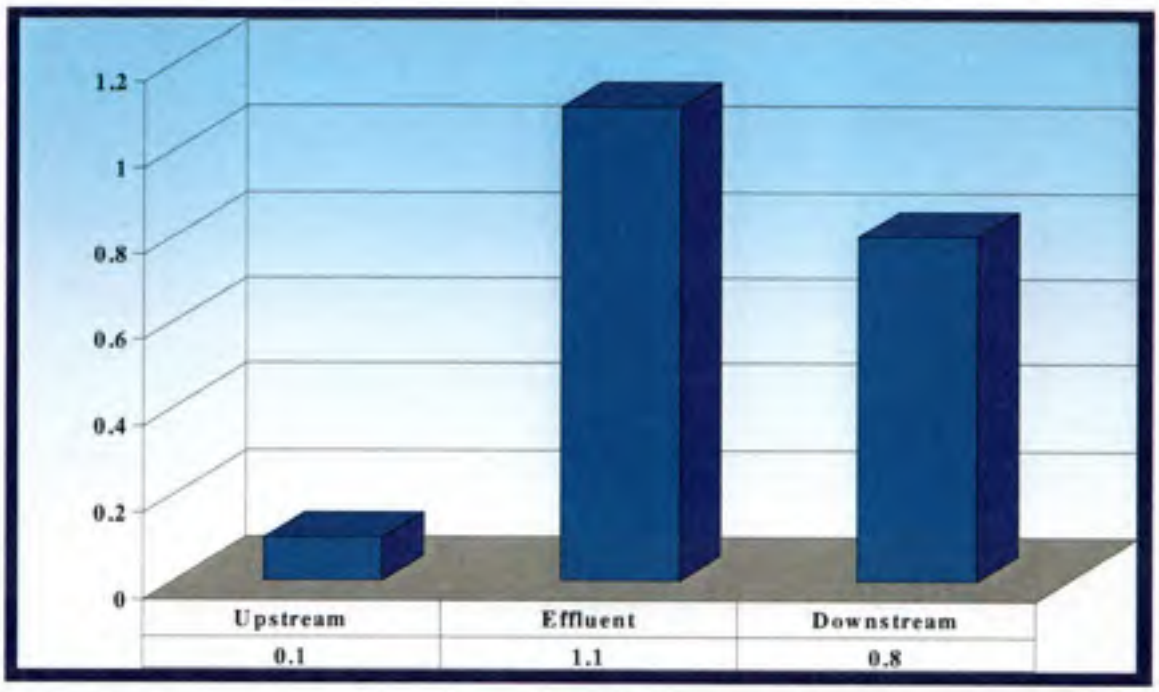


Figure 9: Categorical Analysis of Antibiotic Resistance Levels of Aeromonas Isolates at each Sampling Station

Only resistance to the combination of trimethoprim and sulfamethoxazole is used in computation since inherent resistance to the independent drugs only served to increase the averages without revealing much additional information. The resulting averages are much lower but still display a markedly increasing trend from upstream to downstream. Once again, *Aeromonas* isolates found close to the effluent discharge display the most resistance (score = 1.1) compared to those downstream (score = 0.8) and especially upstream (score = 0.1). Upstream *Aeromonas* spp. were relatively susceptible to all antibiotics employed in this study compared to those isolates further downstream and were influenced by the sewage effluent discharge. Because the sampling sites are spaced closely, the only plausible and clearly identifiable point or non-point impact as a possible source of this increase in resistance is again the wastewater treatment plant.

The averages of these scores are reproduced below in addition to their standard deviation and variance.

Table 8: Comparison of ARS Means, Standard Deviations and Variances for Each Sampling Site

	US		*E*		*DS*	
Mean	3.5	0.1	4.7	1.1	4.4	0.8
St. Dev.	1.2	0.6	2.3	1.8	1.6	1.2
Variance	1.6	0.4	5.1	3.4	2.6	1.5

*Upstream (US), Effluent (E), Downstream (DS)

The first column under each station heading refers to the scores calculated when trimethoprim and sulfamethoxazole are analyzed as independent drugs. Because of *Aeromonas*' natural decreased susceptibility to these antibiotics, the score is higher. For further analyses, only the combination version of these drugs was used. While there was a significant increase in the percent of isolates with higher MICs downstream of the effluent outfall, the majority of the isolates still displayed susceptibility and were assigned a score of 0, which may account for the high variability within each sample. Further analysis on both the scores assigned to the isolates and the variability in MICs alluded to in Chapter 4 follows. However it is apparent from the differences in average values of resistance for each station that the effluent outfall has some bearing on downstream ARS.

5.2 Statistical Analyses

5.2.1 Unpaired t-tests

Unpaired t-tests between the results obtained from three sample pairings were conducted on the total ARS, in order to establish the significance of the difference between the means. The results are summarized Table 9.

Table 9: P and t values For Unpaired t-tests Between Sampling Stations

	Compared Groups		
	US/E	US/DS	E/DS
t	-3.62	-3.41	1.09
P	0.0005	0.0009	0.28

**Tests between Upstream/Effluent (US/E), Upstream/Downstream (US/DS) and Effluent/Downstream (E/DS) isolates*

The t-test is employed in an attempt to measure the degree to which differences in group means may be attributable to random chance, or if in fact an environmental impact is exacting an effect on the groups. In this case, the impact under investigation was the presence of the wastewater effluent relative to the groups in question. If the null hypothesis (H_o) is constructed to define the difference between groups as due to chance, then the P value in Table 9 represents the possibility of the differences in groups occurring assuming H_o. Hence, the lower the value of P, the greater the likelihood that the change in means between the two groups is attributable to the impact of the wastewater discharge. Usually, the effect is considered "significant" at P values of <0.05. Consequently, from Table 9 one can infer that the difference between the upstream and both downstream points is most assuredly statistically significant, different, and not random. Because the sampling points are closely spaced and little else may be identified as an impact, the wastewater effluent is most likely the cause of the increase in MICs downstream. There does not, however, appear to be a statistically significant difference

in the downstream isolates from stations E and DS relative to each other's total ARS (P = 0.28).

5.2.2 ANOVA of Total Scores

A one-way analysis of variance was conducted on the isolates and their assigned scores for stations US, E, and DS in order to assess the statistical difference among the three groups. Again, the factor under study is presumed to be the effects of the wastewater treatment plant outfall, or rather, how the ARSs vary relative to their position vis a vis the effluent discharge. The results of an ANOVA are reported as a ratio of actual versus expected variation within the groups (F) and a P value that allows us to determine whether the ratio F is large enough to reject the null hypothesis. In this case, when the total scores from each station were compared, the returned F equaled 7.3 with a P value of 0.0009. This result implies that the actual variation of scores among stations far exceeds what would be expected and thus permits the rejection of the hypothesis that these groups are only differing by mere chance. Consequently, even with these basic statistical comparisons, an obvious statistically significant difference is observed in the levels of antimicrobial resistance in the bacterial populations tested with respect to their AR profiles and their station of origin. The detailed results of the aforementioned statistical analyses are in Appendix M.

5.3 Discussion

The described statistical analysis would indicate strongly that the wastewater effluent outfall in question is influencing the antibiotic resistance profiles of environmental *Aeromonas* bacteria. However, the question still remains as to the source of origin of such resistance. It is possible that this study isolated bacteria that had already

acquired their resistance from humans or from other sources in the sewage and were being discharged into the environment. An alternative explanation is that these antimicrobial resistant aeromonads were created or selected for in the stream water or sediment. The fundamental question of whether it is in fact the residual concentrations of antibiotics inducing this resistance in the sewage treatment plant or in the receiving water and its sediment is unaddressed by the statistical comparisons thus far presented. A definitive answer as to the origin of the antimicrobial resistant aeromonads would most likely require genetic analysis of isolates both in the receiving waters and the WWTP itself in an attempt to identify genetic identities or similarities, such as chromosomal or in plasmids etc. However, the data presented in this report and their analyses identify a correlation between actual antibiotic concentrations and antimicrobial resistance profiles of *Aeromonas* species, as further discussed in this chapter, Section 3.2.

5.3.1 Partitioning to Sediment

Earlier in this report the tendency for several of these antibiotics to adsorb to and concentrate in sediments was discussed briefly. The point was also made that this tendency was heavily a function of the organic content of the sediment. While this is obviously not the only property on which antibiotic adsorption depends, it figures prominently and was employed as a basis for the following comparisons.

Refer to Tables 2 and 4 for the soil/water partitioning coefficients and the predicted antibiotic concentrations in the sediments. The concentrations of ciprofloxacin and tetracycline are comparable to those against which the bacteria are tested, however, even with sulfamethoxazole's relatively high concentration in the liquid phase, the partitioning coefficient reduces this substantially in the sediments. These predictions

must be interpreted as being based on presumed virgin sediment; that is, there is no accounting for accumulation over time. Degradation coefficients for these antibiotics may be found for sewage sludge and other highly organic matrices, which do not suit these purposes. The persistence, though, of these compounds in marine sediments is documented, with oxytetracycline proving to be very stable, followed by quinolones, sulfadiazine and trimethoprim. Half lives of these compounds ranged from 151 days for oxytetracycline to 50 days for sulfadiazine (Hektoen, Berge et al. 1995) in the first centimeter of sediment. Therefore, one must consider the accumulation of the pharmaceuticals in addition to the partitioning. The wastewater treatment plant is a continuous source of these compounds, constantly exposing the bacteria, water and sediments to pharmaceuticals. Also, the sampling sites downstream of the wastewater outfall afforded some measure of protection from high flow rates, and the propensity for resuspension and movement of the sediments was somewhat reduced. Although the predictions are based on one parameter of many that govern the partitioning of antibiotics to sediment, given the characteristics of the sediment, antibiotics and sampling site, it is highly likely that the sediments in this study are indeed accumulating antibiotics to which the sediment-associated bacteria are being exposed. The sediments are also hosting and probably allowing proliferation of an increased number of bacteria relative to the water column. The higher levels of bacteria in water than in sediments was supported by evidence for this phenomenon based on determinations of bacterial concentrations in water and sediments as conducted in this study. High densities of culturable bacteria, coupled with the proposed concentrations of drugs create an environment in which the probability for resistance genes acquisition or selection is high. This does not entirely

answer the question posed previously about the origins of the resistant aeromonads, but it does offer some evidence that the environmental scenario lends itself to the increase of resistant *Aeromonas* bacteria.

5.3.2 Selective Pressure and Gene Transfer

An examination of another aspect of this phenomenon follows, but a brief introduction into the microbiological underpinnings is warranted. Antimicrobial resistance is a familiar topic in the medical and public health communities. It is identifiable and measurable, but the precise mechanisms by which bacteria defend themselves against drugs still elude thorough explanation. It is generally accepted, however, that whatever the defense, it exacts a physiological burden on the bacteria (Bjorkman and Andersson 2000). This is thought to be supported by studies that report the return to antibiotic susceptibility after the selective pressure of the drugs is removed. Presumably, bacteria will not expend the energy in maintaining resistance when the threat of antibiotic exposure is diminished. One of the most famous cases demonstrating this hypothesis is that of the marked reduction in resistance rates on European poultry and pig farms that correlated to the ban of growth promoting (referred to in Chapter 1 as subtherapeutic growth promoters) antibiotics from feed. The resistance of broiler and swine enteric bacteria to erythromycin, vancomycin and avilamycin, all antibiotics which had been previously employed in the Danish agricultural industry, was monitored before and after the 1995-1997 ban. Substantial increases over 5 years in susceptibility were documented (Aarestrup, Seyfarth et al. 2001). Another study examined *Enterococcus* spp. isolates from Swiss swine as soon as 5-6 months after a complete removal in that country of all antibiotic growth promoters. Even after this brief period, significant

decreases in resistance to erythromycin, spiramycin, and clindamycin were noted and perhaps more relevant to this study, to tetracycline as well. It should be noted that tetracycline resistance was strongly correlated to macrolide resistance, a class of antibiotics to which erythromycin belongs, and this increase in tetracycline susceptibility was implicated as a result of the removal of macrolides from the feed (Boerlin, Wissing et al. 2001). Cross-resistance of drugs is of serious concern, because the presence of one antibiotic may unpredictably induce resistance to an entirely different class of antibiotics. This topic however cannot be addressed meaningfully within the scope of this study.

The notion that the removal of the antibiotic will naturally affect the commensurate reduction in resistance has met with some criticism. One admonition reminds us that antibiotic resistance conference may not be the primary or only function of a gene and that the gene's expression can therefore have no relation to environmental drug concentrations. Second, there are indications that other drugs, such as antihypertensives, which were not designed specifically with microbial toxicity in mind, as well as other widely present chemicals, such as quaternary compounds and heavy metals, are in fact harmful to bacteria which might engender resistance development. Cross-resistance to these sorts of pharmaceuticals, anthropogenic chemicals, and antibiotics has been studied. Mechanisms of resistance can also be instigated by a number of environmental contaminants; a prime example is that of efflux pumps, which are designed by the bacteria to pump out all manner of unrelated compounds. The fact that the compound is an antibiotic may be a mere secondary consideration (Heinemann, Ankenbauer et al. 2000). This last point has been examined in some detail with respect to heavy metal resistance in bacteria and the frequency with which it is detected in

conjunction with various antibiotic resistance traits (Timoney, Port et al. 1978). Also, that the exchange of these genes will occur without the selective pressure of antibiotics is well-documented for a variety of laboratory and environmental situations (Sandaa and Enger 1994; Lorenz 1998; Paul 1999; Rhodes, Huys et al. 2000; Arana, Justo et al. 2001; L'Abee-Lund and Sorum 2001; Schmidt, Bruun et al. 2001). Finally, there is ample evidence that compensatory gene mutations occur with resistance acquisition, which results not in bacteria that revert to being susceptible, but rather bacteria which maintain the resistance without antibiotic selective pressure (Andersson and Levin 1999; Bjorkman 2000; Levin, Perrot et al. 2000; Summers 2002). Therefore, the precise identification of the source of antibiotic resistance acquisition and pinpointing the reasons for maintenance of those genes within the microbial community remains profoundly difficult to determine. It is impossible to attribute its origin or the reasons for its perpetuation to one factor. In spite of all of these unanswered questions, a hypothesis may be formulated with respect to this study in particular as to what accounted for the results presented here.

There is evidence that the presence of small concentrations of antibiotics promotes the transfer of resistance genes at higher frequencies. A study examining a number of simulated natural environments included conjugation experiments between *Aeromonas salmonicida* and *V. cholera* or *E. coli* with plasmids harboring multiple-antibiotic genes. Seawater with and without subtherapeutic concentrations of tetracycline (0.5-1.0 µg/mL) was used as the medium rather than standard culture media in order to mimic environmental conditions. Even in suboptimal conditions, i.e. conditions not rich in nutrients, the addition of tetracycline increased the transfer of antimicrobial resistance from *V. cholera* to the *Aeromonas* recipient, although this was

not the case with respect to the *E. coli/Aeromonas* pairing (Kruse and Sorum 1994). There are painfully few controlled laboratory studies that combine the probability of resistance gene transfer with environmental conditions, but the results shown here are promising from the perspective of providing a plausible explanation for this phenomenon. More studies in this vein are crucial.

Mentioned numerous times in Chapter 4 of this report is the observation that intermediate resistance seems to be skewed towards the DS isolates; there also seems to be a trend with respect to MICs and their decrease to intermediate values from DS to E. T-tests were conducted between the E and DS sampling station antimicrobial resistance scores for trimethoprim/sulfamethoxazole and ciprofloxacin. Tetracycline was analyzed in this way by using both the MIC values and the antimicrobial resistance score, but there was no significant difference between the two sampling stations. Both trimethoprim/sulfamethoxazole and ciprofloxacin however fall within the realm of interest with the difference between the ARS, yielding a P value=0.056 for the former and 0.058 for the latter. These values skirt the fringes of significance as defined previously, but this trend should not be ignored. The fact that the difference between the E and DS can be considered almost statistically significant could be indicative of the possibility that the less absolute resistance, or heightened intermediate resistance, noted downstream is a function of the lower concentrations of antibiotics in the water, and the commensurately lower residuals that could be expected in the sediments. The biological premise for this hypothesis follows.

In *in vitro* studies of antibiotic efficacy and resistance, bacteria are passaged from progressively lower to higher concentrations of antibiotics; this technique is employed to

both measure the susceptibility of bacteria to new drugs for pharmaceutical studies and to select for resistant bacteria for future study. Bacteria with more and more resistance to the antibiotic are gradually isolated from those with only intermediate resistance or slightly increased MICs. *In vitro* resistance development experiments that combine both the antibiotics and bacteria employed by this study are unknown to the author, but there are numerous examinations of this phenomena for other bacteria (Davies, Dewasse et al. 2000; Gilbert, Kohlhepp et al. 2001). It seems that these studies are more frequently performed with respect to more clinically relevant bacteria such as *Staphylococcus aureus*, which has drawn much attention lately for its increased resistance to last-line antibiotics (Cunha 2005). Most of the studies rely on 24-48 hour incubation periods for each passage, indicating that the bacteria resistant to the drug concentration being assessed only require this brief period of time for selection. Only one direct effluent sample was taken in this study, but the percent decrease in drug concentration from E to DS ranged from approximately 36% in sulfamethoxazole to 80% for ciprofloxacin. Tetracycline and trimethoprim were attenuated by 65% and 56% respectively. The decrease in drug concentration coupled with a seeming trend in decreasing resistance over distance may suggest that the bacteria with increased MICs at the DS point are being selected with respect to the amount of antibiotic in the environment; the higher the concentration of antibiotics in the water (for example, at the effluent discharge), the greater the resistance in the bacteria. Conversely, as the concentration diminishes further downstream, so too do the MICs of the organisms. This presents an analogous situation to the *in vitro* resistance development studies. Further fieldwork would require the investigation of more isolates at the E and DS sample points and perhaps the inclusion of

another downstream sampling site further away from the effluent outfall. More important is the analysis of the sediment for antibiotic concentrations, the methods for which are still being refined. The correlation between the drug levels in the sediment to resistance levels in the bacteria would add substantially to a more definitive determination of the causes and origins of *Aeromonas* resistance. In addition to field work, controlled laboratory studies that are able to track resistance acquisition rates relative to antibiotic residual concentration, gene transfer, and liquid-solid partitioning of the antibiotic and the bacteria would be the cornerstone for better understanding of this phenomenon and its public health consequences.

The hypotheses presented here require further investigation before the mechanisms of environmental antibiotic resistance acquisition and maintenance can be understood. What they both indicate though is that antimicrobial resistance in the environment is potentially minimally attributable to bacterial releases from the wastewater treatment plant. In addition, even if resistant fecal coliforms were in fact part of the effluent flora, it seems that resistance is more a function and a reaction on the part of the bacteria to the residual levels of drugs in the receiving waters, which influence perhaps both the rate of gene transfer and the development of bacterial drug resistance as a MIC. Consequently, environmental remediation of this phenomenon would require the removal of the drug residuals at the point of effluent discharge. Ozonation looks promising as a way of degrading these pharmaceuticals, but the doses required to remove the compounds below detection limits are substantial; 10 and15 mg/L were reported as requisite doses versus 1-5 mg/L usually required for disinfection (Ternes, Stuber et al. 2003).

5.3.3 Recommendations for Future Studies

The primary focus of future research should be the controlled examination of the minimum necessary conditions for the acquisition of resistance by bacteria. A laboratory controlled microcosm would have to include, at a minimum, the following:

- Environmental waters, filter sterilized to remove any indigenous bacterial populations, and characterized for nutrient concentration
- Characterized sediments with known K_d values for the chosen antibiotics
- Refined sediment extraction methods for quantifying precisely the quantities of antibiotics partitioned to the sediments
- Spikes of known, susceptible bacterial populations
- Spikes of antibiotics at known subtherapeutic concentrations

The microcosm would be sampled over specific periods of time in an attempt to discern seasonal variability of the resistance profiles of the bacteria, and the concentrations of spiked antibiotics would gradually decrease over the course of the study in an attempt to determine the minimum concentration at which the resistance profiles begin to change. This could be done for multiple combinations of waters, sediments and antibiotics. Gene transfer experiments should also be conducted under these conditions. Being able to explain the biological processes that occur in a more ecological setting would serve to better tailor a treatment at the WWTP that could ameliorate the problem of resistance as a function of antibiotic discharge.

6 Conclusions

Although considerable advances have been made in explaining the origins of antibiotic resistance and factors that contribute to its perpetuation, there remains much to be studied and learned. Physiological traits and genetic properties of bacteria, and the effect of environmental influences to which the organisms are subjected, still present questions with respect to the acquisition of resistance. Wastewater treatment plants and their ecological influence figure prominently as a potential source of this public health concern. One thing is clear, however, and that is the contribution that over-prescription and misuse of antibiotics have made in the propagation and perhaps inception of this massive public health problem. Conservative prescription practices, drug cycling, and the removal of subtherapeutic antibiotics from commercial cattle and poultry farm feeds would be an excellent start to diminishing the levels of antibiotic resistance both in the clinical and environmental settings.

Sewage effluent-impacted sediment, which is known to concentrate both bacteria and certain antibiotics, was examined as a possible arena for the acquisition by bacteria of increased resistance to antimicrobial pharmaceuticals. Environmental bacteria, *Aeromonas* spp., were isolated from these sediments, characterized for resistance to 4 antimicrobials and these data were used to measure the differences in up- and downstream drug susceptibility. This study concluded that the impacts of a wastewater treatment plant discharge contribute markedly to the antimicrobial resistance of *Aeromonas* spp. in the environment. This was shown by an approximately 30% increase in the number of downstream isolates demonstrating either absolute or intermediate resistance to a number of antibiotics. Unfortunately, it was not possible to definitively

pinpoint the precise reason for this increase in *Aeromonas* resistance. It is highly likely and most plausible that the wastewater effluent facilitates this phenomenon.

Further examination should include fieldwork in the form of collecting additional isolates at points further downstream. More importantly, however, is the development of controlled laboratory studies that model the environmental conditions in the receiving waters.

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Appendices

Appendix A: Calculations of Phosphate and Magnesium Ion Concentrations in Sediment Diluent

Molecular Formulas and Weights in Grams of Potassium Dihydrogen Phosphate and Magnesium Chloride

Formula	KH_2PO_4	$MgCl_2$-$6H_2O$
Weight	136.07	202.4

Element	***Molecular Weights***
Potassium (K)	39.1
Phosphorous (P)	30.97
Oxygen (O)	16
Magnesium (Mg)	24.3
Chlorine (Cl)	35.05
Hydrogen (H)	1

Ratios of Ions to the Respective Compounds

PO_4/KH_2PO_4	$Mg/MgCl_2$-$6H_2O$
0.70	0.12

Phosphate Stock Solution

KH_2PO_4 (g/L)	34
PO_4 (g/L)	23.73

Magnesium Stock Solution

$MgCl_2$-$6H_2O$ (g/L)	81.1
Mg (g/L)	9.74

Phosphate in 1.25 mL Stock Solution (g)	0.03
Magnesium in 5 mL Stock Solution (g)	0.05
Final Phosphate Concentration in 500 mL DI g/L	0.06
Final Magnesium Concentration in 500 mL DI g/L	0.10

Final Phosphate Concentration in 500 mL DI mg/L	59.33
Final Magnesium Concentration in 500 mL DI mg/L	97.37

Appendix A: Calculations of Phosphate and Magnesium Ion Concentrations in Sediment Diluent

Molecular Formulas and Weights in Grams of Potassium Dihydrogen Phosphate and Magnesium Chloride

Formula	KH_2PO_4	$MgCl_2$-$6H_2O$
Weight	=B7+(2*B12)+B8+(4*B9)	=B10+(2*B11)+(12*B12)+(6*B9)

Element	***Molecular Weights***
Potassium (K)	39.1
Phosphorous (P)	30.97
Oxygen (O)	16
Magnesium (Mg)	24.3
Chlorine (Cl)	35.05
Hydrogen (H)	1

Ratios of Ions to the Respective Compounds

PO_4/KH_2PO_4	$Mg/MgCl_2$-$6H_2O$
=(B8+(4*B9))/B4	=B10/C4

Phosphate Stock Solution

KH_2PO_4 (g/L)	34
PO_4 (g/L)	=34*A16

Magnesium Stock Solution

$MgCl_2$-$6H_2O$ (g/L)	81.1
Mg (g/L)	=81.1*B16

Phosphate in 1.25 mL Stock Solution (g)	=B20*1.25/1000
Magnesium in 5 mL Stock Solution (g)	=B24*5/1000
Final Phosphate Concentration in 500 mL DI g/L	=C26/0.5
Final Magnesium Concentration in 500 mL DI g/L	=C27/0.5

Final Phosphate Concentration in 500 mL DI mg/L	=C29*1000
Final Magnesium Concentration in 500 mL DI mg/L	=C30*1000

Tryptic Soy Agar*

Approximate Formula Per Liter

Pancreatic Digest of Casein	15.0 g
Enzymatic Digest of Soybean Meal	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g

Suspend 40 g of the powder in 1 L of purified water.

MacConkey Agar without Crystal Violet*

Approximate Formula Per Liter

Pancreatic Digest of Casein	10.0 g
Peptic Digest of Animal Tissue	10.0 g
Lactose	10.0 g
Bile Salts	5.0 g
Sodium Chloride	5.0 g
Agar	12.0 g
Neutral Red	0.05 g

Suspend 52 g of the powder in 1 L of purified water.

Mueller Hinton II Agar*

Approximate Formula Per Liter

Beef Extract	2.0 g
Acid Hydrolysate of Casein	17.5 g
Starch	1.5 g
Agar	17.0 g

Suspend 38 g of the powder in 1 L of purified water.

Triple Sugar Iron Agar*

Approximate Formula Per Liter

Pancreatic Digest of Casein	10.0 g
Peptic Digest of Animal Tissue	10.0 g
Dextrose	1.0 g
Lactose	10.0 g
Sucrose	10.0 g
Ferrous Ammonium Sulfate	0.2 g
Sodium Chloride	5.0 g
Sodium Thiosulfate	0.2 g
Agar	13.0 g
Phenol Red	25.0 mg

Suspend 59.4 g of the powder in 1 L of purified water.

Kligler Iron Agar*

Approximate Formula Per Liter

Pancreatic Digest of Casein	10.0 g
Peptic Digest of Animal Tissue	10.0 g
Lactose	10.0 g
Dextrose	1.0 g
Sodium Chloride	5.0 g
Ferric Ammonium Citrate	0.5 g
Sodium Thiosulfate	0.5 g
Agar	15.0 g
Phenol Red	25.0 mg

Suspend 52 g of the powder in 1 L of purified water.

Methyl Red-Voges Proskauer Broth*

Approximate Formula Per Liter

Pancreatic Digest of Casein	3.5 g
Peptic Digest of Animal Tissue	3.5 g
Potassium Phosphate	5.0 g
Dextrose	5.0 g

Dissolve 17 g of the powder in 1 L of purified water.

Purple Broth Base*

Approximate Formula Per Liter

Pancreatic Digest of Gelatin	10.0 g
Sodium Chloride	5.0 g
Bromcresol Purple	0.02 g

Suspend 15 g of the powder in 1 L of purified water.

****http://www.bd.com/ds/productCenter/DehydratedCultureMediaAndIngredients.asp***

Bile Aesculin Agar**

Approximate Formula Per Liter

Peptone	8.0 g
Bile salts	20.0 g
Ferric citrate	0.5 g
Aesculin	1.0 g
Agar	15.0 g

Suspend 44.5g in 1 L of distilled water.

*****http://www.oxoid.com/uk/blue/index.asp***

mAeromonas Agar***
Approximate Formula Per Liter

Tryptose	5.0 g
Dextrin	11.4 g
Yeast Extract	2.0 g
Sodium Chloride	3.0 g
Potassium Chloride	2.0 g
Magnesium Sulfate	0.1 g
Iron Chloride	0.06 g
Bromothymolblue	0.08 g
Sodium Desoxycholate	0.1 g
Agar	13.0 g

Suspend 18.4 g in 500ml of purified water.

*******http://www.emdchemicals.com/analytics/Micro_Manual/TEDISdata/prods/1_07621_0500.html#composition***

Appendix C: Water and Volatile Solids Content for Sediments at the Primary Site

All weights in grams

Samples Taken May 17, 2005

	Pan+Sample			Crucible+Sample		
	Pan	Wet	Dry	Water	Dry	Burned	VS
US1	0.95	46.39	36.04	22.3%	51.34	50.96	0.74%
US2	0.96	38.87	31.09	20.0%	47.62	47.35	0.57%
E1	0.96	48.41	38.46	20.6%	55.31	54.91	0.72%
E2	0.96	37.00	29.60	20.0%	47.07	46.73	0.72%
DS1	0.97	43.52	36.17	16.9%	53.32	52.86	0.86%
DS2	0.96	42.86	34.95	18.5%	52.38	51.95	0.82%

Samples Taken May 26, 2005

	Pan+Sample			Crucible+Sample		
	Wet	Dry	Water	Dry	Burned	VS
US1	58.37	37.15	36.4%	52.64	50.75	3.59%
US2	52.66	43.44	17.5%	59.64	59.18	0.77%
E1	64.2	51.21	20.2%	58.21	57.81	0.69%
E2	72.51	56.56	22.0%	61.52	60.9	1.01%
DS1	67.88	52.34	22.9%	55.33	50.53	8.68%
DS2	70.26	55.55	20.9%	55.03	54.43	1.09%

Samples Taken June 8, 2005

	Pan+Sample			Crucible+Sample		
	Wet	Dry	Water	Dry	Burned	VS
US1	58.07	36.42	37.3%	52.93	51.36	2.97%
US2	65.05	55.27	15.0%	65.4	64.96	0.67%
E1	53.46	41.49	22.4%	55.99	55.45	0.96%
E2	52.82	34.84	34.0%	49.91	48.63	2.56%
DS1	63.77	50.76	20.4%	61.8	60.69	1.80%
DS2	58.51	45.65	22.0%	57.56	56.93	1.09%

Samples Taken June 22, 2005

	Pan + Sample			Crucible+Sample		
	Wet	Dry	Water	Dry	Burned	VS
US1	40.43	25.7	37.9%	43.03	41.98	2.44%
US2	65.77	55.93	16.2%	64.37	63.74	0.98%
E1	53.67	42.16	22.8%	51.62	51.11	0.99%
E2	55.49	44.82	20.6%	60.22	59.65	0.95%
DS1	62.34	52.74	16.7%	62.22	61.63	0.95%
DS2	62.69	52.16	18.0%	63.84	62.81	1.61%

Samples Taken July 6, 2005

	Pan+Sample			Crucible+Sample		
	Wet	Dry	Water	Dry	Burned	VS
US1	86.35	67.57	21.7%	61.31	60.74	0.93%
US2	54.82	45.35	17.3%	58.14	57.5	1.10%
E1	70.43	53.48	24.1%	65.74	64.76	1.49%
E2	44.96	36.10	19.7%	52	51.45	1.06%
DS1	42.76	33.69	21.2%	49.58	49.03	1.11%
DS2	58.23	38.35	34.1%	51.86	50.41	2.80%

Appendix D: Materials and Methodology for Antibiotic Analysis of Water Column Samples Taken at Primary Site

Materials. Ciprofloxacin was purchased from ICN Biochemicals (Irvine, CA), enrofloxacin from Fluka (Buchs, Switzerland) and simatone from Accustandards (New Haven, CT). All other standards were obtained from Sigma (St. Louis, MO). Disodium ethylenediamine tetraacetic acid dehydrate (Na_2EDTA) was obtained from Aldrich (Milwawkee, WI). Formic acid, HPLC grade water, acetonitrile, and methanol were purchased from EM Science (Gibbstown, NJ). Laboratory grade water (LGW) was prepared in a water purification system from Pure Water Solutions (Hillsborough, NC) which pre-filters fines (1 µm), removes chlorine, reduces total organic carbon to less than 0.2 ppm with an activated carbon resin, and removes ions to 18 Mohm with mixed bed ion-exchange resins.

Solid Phase Extraction (SPE). Before extraction, the water sample was filtered through 0.7 µm glass fiber filters (Ahlstrom, Windsor Locks, CT) and then 0.45 µm nylon filters to remove non-dissolved particles. The sample was then extracted using a 200 mg hydrophilic-lipophilic balance (HLB) resin, a copolymer of [poly(divinylbenzene)-*co*-*N*-vinylpyrrolidone)] (Waters Inc., Milford, MA), contained in a 6 mL cartridge which was preconditioned with 6 mL methanol, 3 mL acidified methanol (0.1% formic acid in HPLC grade methanol, v/v), and 2 × 6 mL LGW. To each 250 mL sample, 1 mL of a 2.5 g/L Na_2EDTA solution was added, the solution pH adjusted to 6 with 1% sulfuric acid, and the solution then extracted through the HLB cartridge at a flow rate of ~ 5 mL/min using a 12-position vacuum manifold (Fisher Scientific, Pittsburg, PA). After extraction, the cartridge was rinsed with 2 × 6 mL LGW and vacuum dried for about 5 minutes. The retained analytes were subsequently eluted with 4 × 2 mL acidified methanol into a glass test tube. The eluent from this solid phase extraction was reduced to ~ 100 µL under a gentle stream of nitrogen at ~ 45°C and the extract then reconstituted to ~ 250 µL in a solvent mixture of LGW/methanol (9:1) to which 10 µL of a 1.25 mg/L simatone solution in methanol was added as the internal standard (I.S.). The extract was then filtered through a 4 mm i.d. 0.45 µm pore size cellulose acetate syringe filter (National Scientific, Duluth, CA), transferred to an amber autosampler vial, and stored at -15°C until LC-MS/MS analysis which was carried out within 3 days of extraction.

Liquid Chromatography Tandem Mass Spectrometry. The LC-MS system consists of a ProStar 210 solvent delivery module equipped with a ProStar 430 autosampler and a Varian 1200L triple stage quadrupole mass spectrometry with a dual off-axis electrospray ionization (ESI) interface (Varian Inc., Walnut Creek, CA). The 12 antibiotics were separated in a single chromatographic run on a Pursuit C-18 guard (3 cm × 2 mm, 3 µm) and analytical (15 cm × 2 mm, 3 µm) column (Varian Inc., Walnut Creek, CA) using the gradient method shown in Table D1. Mobile phase A was 0.1% formic acid (v/v) in water and phase B was 100% acetonitrile and the total flow rate was 0.2 mL/min. The injection volume was 20 µL.

All analytes were ionized in positive ion mode. The nebulizer needle voltage and shield voltage were set at 5000 V and 600 V, respectively. The optimal ion-transfer voltage was 60 V. Nitrogen was used as drying gas at a flow rate of 4 L/min at 300°C and was alsoused as the nebulizer gas at a flow rate of 1 L/min. The ESI chamber temperature

was 50°C. The analytes were detected in MS/MS using the method in Table D2 at a collision gas (argon) pressure of 2.6 mTorr.

Detection, Quantitation, and Quality Control. Detection was based on the identification of the most sensitive product ion with a signal-to-noise ratio higher than 3 and comparison of the retention time of the unknown to that of the standard compound. The method of standard addition was used for calibration in which the sample was divided into several equal portions with two of them not spiked and the others spiked with analytes for two concentrations. Calibration curves were constructed by plotting the ratio of the analyte quantifying ion response to the response of ion for the I.S. against the spiked concentrations. The concentration of the analyte in each non-spiked sample was obtained by extrapolation of the calibration curve.

Table D1. Gradient Method for LC Separation of Antibiotics

Time (min)	A (%)	B (%)
0	85	15
18	0	50
21	0	100
23	0	100
25	85	15
38	85	15

Table D2. MS/MS method

Compound	Precursor Ion $[M+H]^+$	Product Ions (Optimal Collision Voltage, eV)	
Tetracycline	445.0	410.0 (18);	427.0 (12)
Chlortetracycline	479.0	444.0 (19);	154.0(24)
Doxycycline	445.0	428.0 (17);	154.0 (24)
Norfloxacin	320.0	276.0 (17);	233.0 (23)
Ciprofloxacin	332.0	288.0 (17);	245.0 (23)
Oxolinic acid	262.0	244.0 (15);	216.0 (27)
Flumequine	262.0	244.0 (15);	202.0 (30)
Trimethoprim	291.0	230.0 (23);	123.0 (21)
Sulfamethoxazole	254.0	156.0 (14);	108.0 (20)
Erythromycin-H_2O	716.4	158.0 (22);	558.0 (16)
Simatone (IS)	198	128 (18)	

Appendix E: Concentrations of Antibiotics Pertinent to This Study Found in the Effluent and Downstream Sampling Station at the Primary Site

Antibiotic	*Concentration (ng/L) in Effluent**	*Date of Downstream Sample (DS)** and Concentration (ng/L)*				
		10-May	*31-May*	*8-Jun*	*22-Jun*	*Average*
Ciprofloxacin	140	26	10	14	10	15
Tetracycline	13	5	0	8	38	17
Trimethoprim	309	136	147	48	117	112
Sulfamethoxazole	1514	976	432	315	817	635

*Effluent samples taken May. 10 2005; all samples taken in 2005.

**DS corresponds to the sampling station DS referred to in text.

Template Preparation

Colonies were grown overnight on non-selective TSA plates. One loopful of bacteria was added to separate 100µL portions of RNAse-free water (Sigma, St. Louis, MO) in PCR reaction tubes. The samples were added using a sterile stick and the suspension is mixed by stirring. Bacterial DNA was released by heating the tubes for 10 min. at 99°C. After centrifuging the tubes at 13,000 x g for 30 seconds, the supernatant was either used directly in the PCR or stored at -20°C.

Identification of *Aeromonas* isolates by PCR

DNA of all *Aeromonas* isolates was tested by PCR amplification of a region of the lip gene for *A. hydrophila* (Cascon et al., 1996) and of a region of the 16S rRNA gene for *A. caviae* (Nayduch et al. 2001). For each bacterial sample, a mixture of 12.5 µL of the HotStar Taq Mastermix from the Qiagen Kit (Valencia, CA), 20 pmol of forward and reverse primer(s) (see Table F-1), and enough RNAse free water to reach a final volume of 25.0 µL was prepared. A large mixture of both the Mastermix and primers was individually prepared, dispensed in 50 µL aliquots into 0.6 mL thin PCR reaction tubes (Fisher Scientific, Pittsburgh, PA) and frozen at -20°C for future use. When needed, the Mastermix and primers were thawed slowly on ice and mixed by flicking the containers. Primers and water are mixed into the Mastermix by pipeting up and down. Pipet tips are changed after each use.

Two µL of the bacterial template DNA were added to each PCR reaction tube containing the PCR Mastermix and primers. The solution was mixed by pipeting up and down. The PCR was carried out in a PTC-200 thermal cycler (MJ Research, Watertown, MA) and consisted of activation of the Taq polymerase at 95°C for 15 min., followed by 40 cycles of 94°C for 30 sec., 50°C for 30 sec., 72°C for 30 sec., and a 7 min. final extension at 72°C for the amplification of a 760 bp fragment of the lip gene of *A. hydrophila* and 40 cycles of 94°C for 1 min., 60°C for 1 min., 72°C for 1 min., and a 10 min. final extension at 72°C for the amplification of a 450 bp product of the 16S gene of *A. caviae*.

The PCR products (5µL) were electrophoresed on a 2% agarsose gel which was prepared as follows: 100 mL of Tris-Acetate-EDTA (TAE) buffer solution (Sigma Aldrich, St. Louis, MO) was added to a 250 mL glass Erlenmeyer flask with 2 g of agarose gel (Invitrogen, Carlsbad, CA) and the solution was swirled to mix. The flask was covered and the solution heated in a microwave (Goldstar) for 1 minute. The flask was removed and the solution swirled, then heated again for another minute in order for the media to dissolve into solution. The mixture was cooled slightly and 4 µL of ethidium bromide (10 mg/ml, Promega, Madison, WI) was added. The solution was poured into the electrophoresis gel chamber and combs were placed in solution to form wells. After the solution cooled for 40 minutes, the combs were removed and the gel covered with single strength TAE buffer.

Using a 20 µL unfiltered pipet, 1 µL of 6X loading dye was mixed in a well plate with 5 µL of PCR product from each sample. The loading dye was prepared in the laboratory, and consists of 30% glycerol (Fisher Scientific, Pittsburgh, PA) and 0.25% bromophenol blue (Sigma-Aldrich, St. Louis, MO) in DI water; this preparation was autoclaved to ensure sterilization. Each sample was then added to separate wells in the gel, being careful not to contaminate between separate lanes. A molecular weight DNA

standard (100 bp marker) (Promega, Madison, WI) was run on each gel to mark the size of the products. The chamber was covered and the electrodes connected to the power supply. The samples ran on the gel for approximately 45 minutes at 120 volts after which the gel was viewed under a 300nm UV light to visualize the PCR products. *A. hydrophila* ATCC 7966 and *A. caviae* ATCC 7966 were used for each PCR experiment as positive controls, and a water blank was included as a negative control. Samples that displayed a visible band of the appropriate size (760 bp for the lip PCR and 450 bp for the 16S PCR) were considered *Aeromonas* positive.

DNA sequencing and sequence analysis

PCR products (both strands) were sequenced using the PCR primers after purification with a QIAquick PCR purification kit (Qiagen). DNA sequencing was performed at the Lineberger Sequence facility (UNC campus, Chapel Hill, NC) using the BigDye Terminator Cycle Sequencing Ready reaction Kit (PE Applied Biosystems). Multiple alignments of nucleotides and deduced amino acid sequences will be generated using Clustal W and compared to reference strains obtained from GenBank.

Table F-1 Oligonucleotide Primers Used in This Study

Primer ID	DNA sequence (5'-3')	Target Gene	Positions
Lip1	AACCTGGTTCCGCTCAAGCCGTTG	lipase *A. hydrophila*	442- 467
Lip2	TTGCTCGCCTCGGCCCAGCAGCT	lipase *A. hydrophila*	1181-1205
16S-fw	TAGCTTGCTACTTTTGCCGG	16S *A. hydrophila*	75- 94
16S-rv	CACAGCCAGCAGRTATTAGCYACT	16S *A. hydrophila*	412- 435

Appendix G: Weights of Antibiotics Used in Stock Solutions for Microdilution Plates

Antibiotic	Required Weight (mg)	Actual Weight (mg)
Ciprofloxacin	16.3	16.8
Sulfamethoxazole	1045	1044.5
Tetracycline	69.3	69.6
Trimethoprim	32.5	32.5

Appendix H: Minimum Inhibitory Concentrations of Antibiotics Pertinent to this Study for Control Bacteria

Enterobacteriaceae/E.coli*

	Susceptible	Intermediate	Resistant
	Antibiotic Concentration (mcg/mL)		
Tetracycline	<=4	8	>=16
Ciprofloxacin	<=1	2	>=4
Trimethoprim/Sulfamethoxazole	<=2/38	-	>=4/76
Trimethoprim	<=8	-	>=16
Sulfamethoxazole	<=256	-	>=512

P. aeruginosa/V. cholera*

	Susceptible	Intermediate	Resistant
	Antibiotic Concentration (mcg/mL)		
Tetracycline	<=4	8	>=16
Ciprofloxacin	<=1	2	>=4
Trimethoprim/Sulfamethoxazole	<=2/38	-	>=4/76
Sulfamethoxazole	<=256	-	>=512

V.cholera**

	Susceptible	Intermediate	Resistant
	Zone Diameter (mm)		
Trimethoprim/Sulfamethoxazole	>=16	11-15	<=10

*National Committee for Clinical Laboratory Standards, "Performance Standards for Antimicrobial Susceptibility Testing"; 12th Informational Supplement, M100-S12, Vol. 22 No. 1, Wayne, Pa: National Committee for Clinical Laboratory Standards; 2002

**National Committee for Clinical Laboratory Standards, "Performance Standards for Antimicrobial Disk Susceptibility Tests". 8th ed. Approved Standard M2-A8, Vol. 23 No. 1, Wayne, Pa: National Committee for Clinical Laboratory Standards; 2003

Appendix I: Antibiotic Resistance Scores for Primary Site Isolates

Legend for Appendix I	
Isolate Label	
MC#	Morgan Creek, #th sampling excursion
US	Upstream sediment sample
E	Sediment sample taken close to effluent discharge
DS	Downstream sediment sample
Numbers following the sample station labels refer to the replicate sample number (1 of 2, 2 of 2), and the number assigned to the isolate	
Corresponding Dates of Sample Excursions	
MC2	April 12, 2005
MC3	May 17, 2005
MC4	May 26, 2005
MC5	June 8, 2005
MC6	June 22, 2005
MC7	July 6, 2005
Antibiotic Label	
CIP	Ciprofloxacin
SUL	Sulfamethoxazole
TET	Tetracycline
TRI	Trimethoprim
TMP/SMX	Trimethoprim/Sulfamethoxazole
Score Results	
0	Susceptible
1	Intermediate
2	Resistant
Mean	Mean
StDev	Standard Deviation
Var	Variance
Total	Sum of all scores
Total, no sul, no tri	Sum of scores using TMP/SMX combo only, not as independent drugs.

Appendix I: Antibiotic Resistance Scores for Primary Site Isolates

Isolate	Antibiotic						Total no res, tri
	CIP	SUL	TET	TRI	TMP SMX	Total	
MC3 US1 2	0	2	0	0	0	2	0
MC3 US1 3	0	2	0	2	0	4	0
MC3 US1 6	0	2	0	2	0	4	0
MC3 US1 9	0	2	0	2	0	4	0
MC3 US1 10	0	2	0	2	0	4	0
MC3 US1 11	0	2	0	2	0	4	0
MC3 US1 12	0	2	0	2	0	4	0
MC3 US1 16	0	2	0	0	0	2	0
MC3 US2 1	0	2	0	2	0	4	0
MC3 US2 2	0	2	0	2	0	4	0
MC3 US2 3	0	0	0	2	0	2	0
MC3 US2 5	0	2	0	2	0	4	0
MC3 US2 10	0	2	0	2	0	4	0
MC3 US2 12	0	2	0	2	0	4	0
MC3 US2 13	0	2	0	2	0	4	0
MC3 US2 14	0	2	0	2	0	4	0
MC3 US2 15	0	2	0	2	0	4	0
MC3 US2 17	0	2	0	2	0	4	0
MC3 US2 20	0	2	0	2	0	4	0
MC3 US2 22	0	2	0	2	0	4	0
MC3 US2 23	0	0	0	2	0	2	0
MC3 US2 24	0	2	0	2	0	4	0
MC4 US1 1	0	2	0	0	0	2	0
MC4 US1 3	0	2	0	0	0	2	0
MC4 US1 4	0	2	0	2	0	4	0
MC4 US1 5	0	2	0	2	0	4	0
MC4 US1 6	0	2	0	2	0	4	0
MC4 US1 7	0	2	0	2	0	4	0
MC4 US1 10	0	2	0	2	0	4	0
MC4 US1 11	0	2	0	2	0	4	0
MC4 US1 12	0	2	0	2	0	4	0
MC4 US1 13	0	2	2	2	2	8	4
MC4 US1 14	0	2	0	2	0	4	0
MC4 US1 16	0	2	0	0	0	2	0
MC4 US2 2	0	2	0	2	0	4	0
MC4 US2 5	0	2	0	2	0	4	0
MC4 US2 6	0	2	0	0	0	2	0
MC4 US2 10	0	2	0	2	0	4	0
MC4 US2 12	0	2	0	0	0	2	0
MC4 US2 13	0	2	0	0	0	2	0
MC4 US2 16	0	2	2	2	0	6	2
MC5 US1 1	0	2	0	2	0	4	0
MC5 US1 3	0	2	0	0	0	2	0
MC5 US1 6	0	2	0	2	0	4	0
MC5 US1 8	0	2	0	2	0	4	0
MC5 US1 13	0	2	0	0	0	2	0
MC5 US1 15	0	2	0	2	0	4	0
MC5 US1 18	0	0	0	0	0	0	0
MC5 US2 5	0	2	0	0	0	2	0
MC5 DS2 12	0	2	0	2	0	4	0
					Mean	0.1	
					StDev	0.6	
					Var	0.393	

Isolate	Antibiotic						Total no res, tri
	CIP	SUL	TET	TRI	TMP SMX	Total	
MC3 E1 1	0	2	0	2	0	4	0
MC3 E1 2	0	2	0	2	0	4	0
MC3 E1 4	0	2	0	2	0	4	0
MC3 E2 1	0	2	0	0	0	2	0
MC3 E2 2	0	2	0	0	0	2	0
MC3 E2 3	0	2	0	2	0	4	0
MC3 E2 4	0	2	0	2	0	4	0
MC3 E2 5	0	2	0	2	0	4	0
MC3 E2 6	1	2	1	2	2	8	4
MC3 E2 7	1	2	2	2	2	9	5
MC3 E2 8	0	2	0	2	0	4	0
MC3 E2 9	0	2	0	0	0	2	0
MC3 E2 10	0	2	1	2	0	5	1
MC3 E2 14	0	2	0	2	0	4	0
MC3 E2 15	0	2	0	0	0	2	0
MC3 E2 16	1	2	1	2	2	8	4
MC4 E1 1	1	2	0	2	0	5	1
MC4 E1 2	0	2	0	2	1	5	1
MC4 E1 3	0	2	0	2	0	4	0
MC4 E1 4	1	2	1	2	0	6	2
MC4 E1 7	1	2	1	2	0	6	2
MC4 E1 10	0	0	0	2	0	2	0
MC4 E1 14	0	2	0	2	0	4	0
MC4 E1 16	0	2	0	2	0	4	0
MC4 E2 1	1	2	2	2	2	9	5
MC4 E2 2	1	2	1	2	2	8	4
MC4 E2 3	0	2	0	2	0	4	0
MC4 E2 5	0	2	1	2	1	6	2
MC4 E2 7	0	2	0	2	0	4	0
MC4 E2 8	1	2	1	2	2	8	4
MC4 E2 9	0	2	0	2	0	4	0
MC4 E2 13	0	0	0	0	0	0	0
MC4 E2 14	0	2	0	2	1	5	1
MC4 E2 15	0	2	0	2	0	4	0
MC4 E2 16	0	2	0	0	0	2	0
MC5 E1 2	0	2	0	2	0	4	0
MC5 E1 4	0	2	0	2	0	4	0
MC5 E1 12	1	2	1	2	2	9	5
MC5 E1 15	0	2	0	2	0	4	0
MC5 E1 17	1	2	2	2	2	9	5
MC5 E1 19	0	2	0	2	0	4	0
MC5 E2 1	1	2	2	2	2	9	5
MC5 E2 2	0	2	0	2	0	4	0
MC5 E2 4	0	2	0	2	0	4	0
MC5 E2 5	0	0	0	2	0	2	0
MC5 E2 6	1	2	2	2	2	9	5
MC5 E2 7	0	2	0	2	0	4	0
MC5 E2 9	0	0	0	2	0	2	0
MC5 E2 10	0	2	0	2	0	4	0
MC5 E2 13	0	2	0	2	0	4	0
					Mean	1.1	
					StDev	1.8	
					Var	3.414	

Isolate	Antibiotic						Total no res, tri
	CIP	SUL	TET	TRI	TMP SMX	Total	
MC3 DS1 1	1	2	1	2	0	6	2
MC3 DS1 2	0	2	0	2	0	4	0
MC3 DS1 4	0	2	0	0	0	2	0
MC3 DS1 8	0	2	0	2	0	4	0
MC3 DS1 9	0	2	0	2	0	4	0
MC3 DS1 10	0	2	0	0	0	2	0
MC3 DS1 11	0	2	0	0	0	2	0
MC3 DS1 12	0	2	0	2	0	4	0
MC3 DS1 13	0	2	0	0	0	2	0
MC3 DS1 14	0	2	1	0	1	4	2
MC3 DS1 16	0	2	2	2	0	6	2
MC3 DS2 1	0	2	0	2	0	4	0
MC3 DS2 2	0	2	0	2	0	4	0
MC3 DS2 3	0	2	0	2	0	4	0
MC3 DS2 5	0	2	1	2	0	5	1
MC3 DS2 7	0	2	0	2	0	4	0
MC3 DS2 8	0	2	0	2	0	4	0
MC3 DS2 9	0	2	0	2	0	4	0
MC3 DS2 10	1	2	1	2	0	6	2
MC3 DS2 13	0	2	0	0	0	2	0
MC3 DS2 16	0	2	1	2	0	5	1
MC4 DS1 2	0	2	0	2	0	4	0
MC4 DS1 4	0	2	0	0	0	2	0
MC4 DS1 6	0	2	0	2	0	4	0
MC4 DS1 12	0	2	0	2	0	4	0
MC4 DS1 16	1	2	0	2	1	6	2
MC4 DS2 1	0	2	0	2	0	4	0
MC4 DS2 4	0	2	1	2	0	5	1
MC4 DS2 6	1	2	2	2	2	9	5
MC4 DS2 9	0	2	2	2	1	7	3
MC5 DS1 4	0	2	0	0	0	2	0
MC5 DS1 5	0	2	2	2	1	7	3
MC5 DS1 7	0	2	0	2	0	4	0
MC5 DS1 8	0	2	0	2	0	4	0
MC5 DS1 11	0	2	2	2	0	6	2
MC5 DS1 14	0	2	0	2	0	4	0
MC5 DS1 17	0	2	0	2	0	4	0
MC5 DS2 1	1	2	1	2	0	6	2
MC5 DS2 2	0	2	0	2	1	5	1
MC5 DS2 3	0	2	0	0	0	2	0
MC5 DS2 5	0	2	0	2	0	4	0
MC5 DS2 6	0	2	0	2	0	4	0
MC5 DS2 8	0	2	2	2	2	8	4
MC5 DS2 10	1	2	1	2	0	6	2
MC5 DS2 11	0	2	0	2	0	4	0
MC5 DS2 12	0	2	0	0	0	2	0
MC5 DS2 13	0	2	1	2	1	6	2
MC5 DS2 16	1	2	1	2	0	6	2
MC5 DS2 17	0	2	0	2	0	4	0
MC7 DS1 2	0	2	0	2	0	4	0
						Mean	0.8
						StDev	1.7
						Var	1.5

Appendix J: t-Test Comparison of Bacterial Concentrations in the Water Column and Sediments of the Preliminary Site

Student's *t*-Test: Results

Comparison of the Averages of the Log Bacterial Concentrations in Water and Sediment

The results of an unpaired t-test performed at 13:11 on 11-OCT-2005
t= 21.0
sdev= 0.389
degrees of freedom = 22 The probability of this result, assuming the null hypothesis, is less than .0001

Group A: Number of items= 12 **(Sediment Concentrations)**
5.00 5.00 5.00 5.00 5.00 5.00 5.00 5.00 5.00 5.00 6.00 6.00
Mean = 5.17
95% confidence interval for Mean: 4.934 thru 5.400
Standard Deviation = 0.389
Hi = 6.00 Low = 5.00
Median = 5.00
Average Absolute Deviation from Median = 0.167

Group B: Number of items= 12 **(Water Concentrations)**
1.00 1.00 2.00 2.00 2.00 2.00 2.00 2.00 2.00 2.00 2.00 2.00
Mean = 1.83
95% confidence interval for Mean: 1.600 thru 2.066
Standard Deviation = 0.389
Hi = 2.00 Low = 1.00
Median = 2.00
Average Absolute Deviation from Median = 0.167

Legend for Appendix K	
Isolate Label	
MC#	Morgan Creek, #th sampling excursion
US	Upstream sediment sample
E	Sediment sample taken close to effluent discharge
DS	Downstream sediment sample
Numbers following the sample station labels refer to the replicate sample number (1 of 2, 2 of 2), and the number assigned to the isolate	
Corresponding Dates of Sample Excursions	
MC2	April 12, 2005
MC3	May 17, 2005
MC4	May 26, 2005
MC5	June 8, 2005
MC6	June 22, 2005
MC7	July 6, 2005
Biochemical Test Label	
Indole	Spot indole test
Oxidase	Spot oxidase test
MAC	MacConkey agar
KIA/TSI	Kliger Iron Agar/Triple Sugar Iron Agar
VP	Voges-Proskauer
Larab	L-arabinose fermentation
BE	Bile Esculin
Complex	Grouping according to Abbott, et al. 2003
Legend for KIA/TSI Test Results	
+	For "KIA": red slant, yellow butt, gas production
~	For "KIA": red slant, yellow butt
-	For "KIA": yellow slant, yellow butt, gas production
y/y	For "KIA": yellow slant, yellow butt
a/a	For "TSI": yellow slant, yellow butt
a/a g	For "TSI": yellow slant, yellow butt, gas production
red tip g (ah)	For "TSI": slant tip red, gas production, (identical to *A. hydrophila* control)
(ah)	For "TSI": Identical to *A. hydrophila* control
	No data for these biochemical results but PCR confirmed to the genus level

Appendix K: Biochemical Test Results for Primary Site Isolates

Isolate	*Indole*	*Oxidase*	*MAC*	*KIA*	*VP*	*Larab*	*BE*	*Complex*
MC3 US1 2	+	+	-	+	+	+	+	A. hydrophila
MC3 US1 3	+	+	-	+	+	+	+	A. hydrophila
MC3 US1 6	+	+	-	-	-	+	+	A. caviae
MC3 US1 9	+	+	-	-	-	+	+	A. caviae
MC3 US1 10	+	+	-	-	-	+	+	A. caviae
MC3 US1 11	+	+	-	-	-	+	+	A. caviae
MC3 US1 12	+	+	-	-	-	+	+	A. caviae
MC3 US1 16	+	+	-	+	+	+	+	A. hydrophila
MC3 US2 1	+	+	-	-	-	+	+	A. caviae
MC3 US2 2	+	+	-	-	-	+	+	A. caviae
MC3 US2 3	+	+	-	-	-	+	+	A. caviae
MC3 US2 5	+	+	-	-	-	+	+	A. caviae
MC3 US2 10	+	+	-	-	-	+	+	A. caviae
MC3 US2 12	-	+	-	+	+	+	+	A. hydrophila
MC3 US2 13	+	+	-	-	-	+	+	A. caviae
MC3 US2 14	+	+	-	-	-	+	+	A. caviae
MC3 US2 15	+	+	-	-	-	+	+	A. caviae
MC3 US2 17	+	+	-	-	+	-	-	A. sobria
MC3 US2 20	+	+	-	-	+	-	-	A. sobria
MC3 US2 22	+	+	-	-	+	-	-	A. sobria
MC3 US2 23	+	+	-	-	-	+	+	A. caviae
MC3 US2 24	+	+	-	-	+	-	-	A. sobria
MC4 US1 1	+	+	-	-	+	+	-	A. hydrophila
MC4 US1 3	+	+	-	+	-	+	+	A. hydrophila
MC4 US1 4	+	+	-	-	+	+	+	A. hydrophila
MC4 US1 5	+	+	-	-	+	+	+	A. hydrophila
MC4 US1 6	+	+	-	-	+	+	+	A. hydrophila
MC4 US1 7	+	+	-	+	+	-	-	A. hydrophila
MC4 US1 10	+	+	-	-	-	+	+	A. caviae
MC4 US1 11	+	+	-	-	-	+	+	A. caviae
MC4 US1 12	+	+	-	-	+	+	+	A. hydrophila
MC4 US1 13	+	+	-	-	-	+	+	A. caviae
MC4 US1 14	-	+	-	-	-	+	+	A. caviae
MC4 US1 16	+	+	-	+	+	+	+	A. hydrophila
MC4 US2 2	+	+	-	-	+	-	-	A. sobria
MC4 US2 5	+	+	-	-	-	+	+	A. caviae
MC4 US2 6	+	+	-	-	-	+	+	A. caviae
MC4 US2 10	+	+	-	-	+	-	-	A. sobria
MC4 US2 12	+	+	-	-	-	+	+	A. caviae
MC4 US2 13	+	+	-	+	+	+	-	A. sobria
MC4 US2 16	+	+	-	-	-	+	+	A. caviae
MC5 US1 1	+	+	-	a/a	-	+	+	A. caviae
MC5 US1 3	+	+	-	a/a g	-	+	+	A. hydrophila
MC5 US1 6	+	+	-	a/a	-	+	+	A. caviae
MC5 US1 8	+	+	-	a/a	-	+	+	A. caviae
MC5 US1 13	+	+	-	red tip g (ah)	+	+	+	A. hydrophila
MC5 US1 15	+	+	-	red tip g (ah)	+	+	+	A. hydrophila
MC5 US1 18	+	+	-	red tip g (ah)	-	+	+	A. hydrophila
MC5 US2 5	+	+	-	a/a	+	+	+	A. hydrophila
MC5 US2 12	+	+	-	a/a g	-	+	+	A. hydrophila

Appendix K: Biochemical Test Results for Primary Site Isolates

Isolate	*Indole*	*Oxidase*	*MAC*	*KIA*	*VP*	*Larab*	*BE*	*Complex*
MC3 E1 1	+	+	-	+	-	-	-	A. sobria
MC3 E1 2	+	+	-	~	-	+	+	A. caviae
MC3 E1 4	+	+	-	-	-	+	-	A. caviae
MC3 E2 1	+	+	-	~	-	-	-	A. sobria
MC3 E2 2	+	+	-	~	+	-	-	A. sobria
MC3 E2 3	+	+	-	+	-	-	-	A. sobria
MC3 E2 4	+	+	-	y/y	-	+	+	A. caviae
MC3 E2 5	+	+	-	~	-	+	+	A. caviae
MC3 E2 6	+	+	-	-	-	-	-	A. sobria
MC3 E2 7	+	+	-	~	-	+	+	A. caviae
MC3 E2 8	+	+	-	+	-	-	+	A. sobria
MC3 E2 9	+	+	-	~	+	+	+	A. hydrophila
MC3 E2 10	+	+	-	-	-	-	-	A. sobria
MC3 E2 14	+	+	-	~	-	+	+	A. caviae
MC3 E2 15	+	+	-	~	-	-	-	A. sobria
MC3 E2 16	+	+	-	-	-	-	-	A. sobria
MC4 E1 1	+	+	-	-	-	+	+	A. caviae
MC4 E1 2	+	+	-	~	-	+	+	A. caviae
MC4 E1 3	+	+	-	-	-	+	+	A. caviae
MC4 E1 4	+	+	-	~	-	+	-	A. caviae
MC4 E1 7	+	+	-	y/y	-	+	+	A. caviae
MC4 E1 10	+	+	-	~	+	+	+	A. hydrophila
MC4 E1 14	+	+	-	y/y	-	+	+	A. caviae
MC4 E1 16	+	+	-	-	+	+	+	A. hydrophila
MC4 E2 1	+	+	-	~	-	+	+	A. caviae
MC4 E2 2	+	+	-	+	+	-	+	A. hydrophila
MC4 E2 3	+	+	-	~	-	+	+	A. caviae
MC4 E2 5	+	+	-	-	-	+	+	A. caviae
MC4 E2 7	+	+	-	~	-	+	+	A. caviae
MC4 E2 8	+	+	-	~	-	-	-	A. sobria
MC4 E2 9	+	+	-	+	+	-	-	A. sobria
MC4 E2 13	+	+	-	~	-	+	+	A. caviae
MC4 E2 14	+	+	-	~	-	+	+	A. caviae
MC4 E2 15	+	+	-	+	+	-	-	A. sobria
MC4 E2 16	+	+	-	~	-	+	+	A. caviae
MC5 E1 2	+	+	-	a/a	-	+	+	A. caviae
MC5 E1 4	+	+	-	a/a	-	+	+	A. caviae
MC5 E1 12	+	+	-	a/a	-	+	+	A. caviae
MC5 E1 15	+	+	-	a/a	-	+	+	A. caviae
MC5 E1 17	+	+	-	a/a	-	+	+	A. caviae
MC5 E1 19	+	+	-	a/a	-	+	+	A. caviae
MC5 E2 1	+	+	-	a/a	-	+	+	A. caviae
MC5 E2 2	+	+	-	a/a	-	+	+	A. caviae
MC5 E2 4	+	+	-	a/a	-	+	+	A. caviae
MC5 E2 5	+	+	-	a/a	-	+	+	A. caviae
MC5 E2 6	+	+	-	a/a	-	+	+	A. caviae
MC5 E2 7	+	+	-	a/a	-	+	+	A. caviae
MC5 E2 9	+	+	-	a/a	-	+	+	A. caviae
MC5 E2 10	+	+	-	a/a	-	+	+	A. caviae
MC5 E2 13	-	+	-	a/a	-	+	+	A. caviae

Appendix K: Biochemical Test Results for Primary Site Isolates

Isolate	*Indole*	*Oxidase*	*MAC*	*KIA*	*VP*	*Larab*	*BE*	*Complex*
MC3 DS1 1	+	+	-	-	-	+	+	A. caviae
MC3 DS1 2	+	+	-	-	-	+	+	A. caviae
MC3 DS1 4	+	+	-	+	-	-	+	A. sobria
MC3 DS1 8	+	+	-	-	-	+	+	A. caviae
MC3 DS1 9	+	+	-	-	-	+	+	A. caviae
MC3 DS1 10	+	+	-	+	+	+	+	A. hydrophila
MC3 DS1 11	+	+	-	+	-	+	-	A. hydrophila/A. caviae
MC3 DS1 12	+	+	-	-	-	+	+	A. caviae
MC3 DS1 13	+	+	-	+	+	+	+	A. hydrophila
MC3 DS1 14	+	+	-	+	-	+	+	A. hydrophila
MC3 DS1 16	+	+	-	-	-	+	+	A. caviae
MC3 DS2 1	+	+	-	-	-	+	+	A. caviae
MC3 DS2 2	+	+	-	-	-	+	+	A. caviae
MC3 DS2 3	+	+	-	-	-	+	+	A. caviae
MC3 DS2 5	+	+	-	+	-	+	+	A. hydrophila
MC3 DS2 7	+	+	-	-	-	+	+	A. caviae
MC3 DS2 8	+	+	-	-	-	+	+	A. caviae
MC3 DS2 9	+	+	-	-	-	+	+	A. caviae
MC3 DS2 10	+	+	-	-	-	+	+	A. caviae
MC3 DS2 13	+	+	-	-	-	+	+	A. caviae
MC3 DS2 16	+	+	-	-	-	+	+	A. caviae
MC4 DS1 2	+	+	-	+	-	+	+	A. hydrophila
MC4 DS1 4	+	+	-	-	-	-	-	A. sobria
MC4 DS1 6	+	+	-	-	-	+	+	A. caviae
MC4 DS1 12	+	+	-	-	-	+	+	A. caviae
MC4 DS1 16	+	+	-	-	-	+	+	A. caviae
MC4 DS2 1	+	+	-			+	+	
MC4 DS2 4	-	+	-			+	+	
MC4 DS2 6	+	+	-			+	+	
MC4 DS2 9	+	+	-			+	+	
MC5 DS1 4	+	+	-	red tip g(ah)	+	+	+	A. hydrophila
MC5 DS1 5	+	+	-	a/a	-	+	+	A. caviae
MC5 DS1 7	+	+	-	a/a g	-	+	+	A. hydrophila
MC5 DS1 8	+	+	-	a/a	-	+	+	A. caviae
MC5 DS1 11	+	+	-	a/a	-	+	+	A. caviae
MC5 DS1 14	+	+	-	a/a g	-	+	+	A. hydrophila
MC5 DS1 17	+	+	-	a/a	-	+	+	A. caviae
MC5 DS2 1	+	+	-	a/a	-	+	-	A. caviae
MC5 DS2 2	+	+	-	a/a	-	+	+	A. caviae
MC5 DS2 3	+	+	-	(ah)	+	+	+	A. hydrophila
MC5 DS2 5	+	+	-	(ah)	-	+	+	A. hydrophila
MC5 DS2 6	+	+	-	a/a	-	+	+	A. caviae
MC5 DS2 8	+	+	-	a/a	-	+	+	A. caviae
MC5 DS2 10	+	+	-		-	+	-	A. caviae
MC5 DS2 11	-	+	-		-	+	+	A. caviae
MC5 DS2 12	+	+	-	a/a g	-	+	+	A. hydrophila
MC5 DS2 13	+	+	+	a/a	-	+	+	A. caviae
MC5 DS2 16	+	+	-		-	+	-	A. caviae
MC5 DS2 17	+	+	-	a/a	-	+	+	A. caviae
MC7 DS1 2	+	+	-	a/a	-	+	+	A. caviae

Appendix L: Minimum Inhibitory Concentrations for Primary Site Isolates

Legend for Appendix L	
Isolate Label	
MC#	Morgan Creek, #th sampling excursion
US	Upstream sediment sample
E	Sediment sample taken close to effluent discharge
DS	Downstream sediment sample
Numbers following the sample station labels refer to the replicate sample number (1 of 2, 2 of 2), and the number assigned to the isolate	
Corresponding Dates of Sample Excursions	
MC2	April 12, 2005
MC3	May 17, 2005
MC4	May 26, 2005
MC5	June 8, 2005
MC6	June 22, 2005
MC7	July 6, 2005
Antibiotic Label	
CIP	Ciprofloxacin
SUL	Sulfamethoxazole
TET	Tetracycline
TRI	Trimethoprim
TMP/SMX	Trimethoprim/Sulfamethoxazole
MICS and Results for TMP/SMX	
All MICS are antibiotic concentrations in mcg/mL	
S	Susceptible
I	Intermediate
R	Resistant

Appendix L: Minimum Inhibitory Concentrations for Primary Site Isolates

Isolate	CIP	SUL	TET	TRI	TMP SMX	Isolate	CIP	SUL	TET	TRI	TMP SMX	Isolate	CIP	SUL	TET	TRI	TMP SMX
MC3 US1 2	0	1024	0	8	S	MC3 E1 1	0	1024	0	32	S	MC3 DS1 1	0.5	1024	1	64	S
MC3 US1 3	0	1024	0	32	S	MC3 E1 2	0	1024	0	64	S	MC3 DS1 2	0	1024	0	64	S
MC3 US1 6	0	1024	0	64	S	MC3 E1 4	0	1024	0	16	S	MC3 DS1 4	0	1024	0	8	S
MC3 US1 9	0	1024	0	64	S	MC3 E2 1	0	512	0	8	S	MC3 DS1 8	0	2048	0	64	S
MC3 US1 10	0	2048	0	64	S	MC3 E2 2	0	512	0	4	S	MC3 DS1 9	0	2048	0	64	S
MC3 US1 11	0	2048	0	64	S	MC3 E2 3	0	1024	0	32	S	MC3 DS1 10	0	1024	0	2	S
MC3 US1 12	0	2048	0	64	S	MC3 E2 4	0	2048	0	64	S	MC3 DS1 11	0	1024	0	8	S
MC3 US1 16	0	1024	0	8	S	MC3 E2 5	0	1024	0	16	S	MC3 DS1 12	0	2048	0	64	S
MC3 US2 1	0	2048	0	64	S	MC3 E2 6	0.25	2048	16	64	R	MC3 DS1 13	0	1024	0	8	S
MC3 US2 2	0	1024	0	64	S	MC3 E2 7	0.5	2048	32	64	R	MC3 DS1 14	0	2048	16	2	I
MC3 US2 3	0	128	0	64	S	MC3 E2 8	0	512	0	16	S	MC3 DS1 16	0	2048	32	64	S
MC3 US2 5	0	1024	0	32	S	MC3 E2 9	0	1024	0	8	S	MC3 DS2 1	0	1024	0	16	S
MC3 US2 10	0	2048	0	64	S	MC3 E2 10	0	1024	1	16	S	MC3 DS2 2	0	2048	0	64	S
MC3 US2 12	0	2048	0	32	S	MC3 E2 14	0	2048	0	32	S	MC3 DS2 3	0	1024	0	64	S
MC3 US2 13	0	2048	0	64	S	MC3 E2 15	0	512	0	8	S	MC3 DS2 5	0	1024	1	16	S
MC3 US2 14	0	2048	0	64	S	MC3 E2 16	0.25	2048	16	64	R	MC3 DS2 7	0	2048	0	32	S
MC3 US2 15	0	2048	0	32	S	MC4 E1 1	0.5	1024	0	64	S	MC3 DS2 8	0	2048	0	64	S
MC3 US2 17	0	1024	0	64	S	MC4 E1 2	0	2048	0	64	S	MC3 DS2 9	0	2048	0	64	S
MC3 US2 20	0	1024	0	16	S	MC4 E1 3	0	2048	0	64	S	MC3 DS2 10	0.25	1024	1	32	S
MC3 US2 22	0	1024	0	32	S	MC4 E1 4	0.25	1024	16	16	S	MC3 DS2 13	0	512	0	4	S
MC3 US2 23	0	256	0	64	S	MC4 E1 7	0.25	1024	16	64	S	MC3 DS2 16	0	1024	1	64	S
MC3 US2 24	0	1024	0	32	S	MC4 E1 10	0	256	0	16	S	MC4 DS1 2	0	1024	0	16	S
MC4 US1 1	0	2048	0	4	S	MC4 E1 14	0	2048	0	64	S	MC4 DS1 4	0	1024	0	4	S
MC4 US1 3	0	2048	0	8	S	MC4 E1 16	0	512	0	16	S	MC4 DS1 6	0	1024	0	64	S
MC4 US1 4	0	2048	0	16	S	MC4 E2 1	1	2048	64	64	R	MC4 DS1 12	0	1024	0	64	S
MC4 US1 5	0	2048	0	16	S	MC4 E2 2	0.25	2048	16	64	R	MC4 DS1 16	0.25	2048	0	64	I
MC4 US1 6	0	2048	0	16	S	MC4 E2 3	0	2048	0	64	S	MC4 DS2 1	0	2048	0	64	S
MC4 US1 7	0	512	0	16	S	MC4 E2 5	0	2048	1	32	I	MC4 DS2 4	0	1024	1	64	S
MC4 US1 10	0	1024	0	64	S	MC4 E2 7	0	2048	0	64	S	MC4 DS2 6	1	2048	32	64	R
MC4 US1 11	0	2048	0	64	S	MC4 E2 8	0.25	2048	16	64	S	MC4 DS2 9	0	2048	32	32	I
MC4 US1 12	0	2048	0	32	S	MC4 E2 9	0	1024	0	16	S	MC5 DS1 4	0	1024	0	8	S
MC4 US1 13	0	2048	32	64	R	MC4 E2 13	0	256	0	8	S	MC5 DS1 5	0	1024	32	64	I
MC4 US1 14	0	2048	0	32	S	MC4 E2 14	0	2048	0	16	S	MC5 DS1 7	0	1024	0	16	S
MC4 US1 16	0	1024	0	8	S	MC4 E2 15	0	1024	0	16	S	MC5 DS1 8	0	1024	0	64	S
MC4 US2 2	0	1024	0	16	S	MC4 E2 16	0	1024	0	8	S	MC5 DS1 11	0	2048	16	64	S
MC4 US2 5	0	1024	0	64	S	MC5 E1 2	0	2048	0	64	S	MC5 DS1 14	0	512	0	16	S
MC4 US2 6	0	512	0	8	S	MC5 E1 4	0	2048	0	64	S	MC5 DS1 17	0	1024	0	32	S
MC4 US2 10	0	512	0	16	S	MC5 E1 13	2	2048	32	64	R	MC5 DS2 1	0.25	1024	16	16	S
MC4 US2 12	0	512	0	8	S	MC5 E1 15	0	2048	0	64	S	MC5 DS2 2	0	1024	0	64	I
MC4 US2 13	0	512	0	2	S	MC5 E1 17	1	2048	32	64	R	MC5 DS2 3	0	1024	0	2	S
MC4 US2 16	0	2048	32	64	S	MC5 E1 19	0	1024	0	64	S	MC5 DS2 5	0	2048	0	16	S
MC5 US1 1	0	2048	0	64	S	MC5 E2 1	1	2048	16	64	R	MC5 DS2 6	0	512	0	64	S
MC5 US1 3	0	512	0	8	S	MC5 E2 2	0	1024	0	64	S	MC5 DS2 8	0	2048	64	64	R
MC5 US1 6	0	2048	0	64	S	MC5 E2 4	0	2048	0	64	S	MC5 DS2 10	0.25	512	16	16	S
MC5 US1 8	0	2048	0	64	S	MC5 E2 5	0	128	0	64	S	MC5 DS2 11	0	2048	0	64	S
MC5 US1 13	0	512	0	4	S	MC5 E2 6	1	2048	32	64	R	MC5 DS2 12	0	512	0	8	S
MC5 US1 15	0	512	0	16	S	MC5 E2 7	0	1024	0	64	S	MC5 DS2 13	0	1024	8	64	I
MC5 US1 18	0	256	0	2	S	MC5 E2 9	0	128	0	64	S	MC5 DS2 16	0.25	512	16	16	S
MC5 US2 5	0	1024	0	8	S	MC5 E2 10	0	2048	0	64	S	MC5 DS2 17	0	2048	0	64	S
MC5 US2 12	0	1024	0	16	S	MC5 E2 13	0	1024	0	16	S	MC7 DS1 2	0	2048	0	64	S

Appendix M: Statistical Analysis Results of Antibiotic Resistance Score Comparisons as Reported by the Statistical Program

Student's **t**-Test: Results

Between Total Antibiotic Scores of Upstream and Effluent Isolates

The results of an unpaired t-test performed at 21:50 on 11-AUG-2005
t= -3.62
sdev= 1.38
degrees of freedom = 98 The probability of this result, assuming the null hypothesis, is 0.0005

Group A: Number of items= 50 **(Upstream Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 2.00 4.00
Mean = 0.120
95% confidence interval for Mean: -0.2672 thru 0.5072
Standard Deviation = 0.627
Hi = 4.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 0.120

Group B: Number of items= 50 **(Effluent Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 1.00 1.00 1.00 1.00 2.00 2.00
2.00 4.00 4.00 4.00 4.00 5.00 5.00 5.00 5.00 5.00 5.00
Mean = 1.12
95% confidence interval for Mean: 0.7328 thru 1.507
Standard Deviation = 1.85
Hi = 5.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 1.12

Appendix M: Statistical Analysis Results of Antibiotic Resistance Score Comparisons as Reported by the Statistical Program

Between Total Antibiotic Scores of Upstream and Downstream Isolates

The results of an unpaired t-test performed at 21:52 on 11-AUG-2005
t= -3.41
sdev= 0.968
degrees of freedom = 98 The probability of this result, assuming the null hypothesis, is 0.0009

Group A: Number of items= 50 **(Upstream Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 2.00 4.00
Mean = 0.120
95% confidence interval for Mean: -0.1517 thru 0.3917
Standard Deviation = 0.627
Hi = 4.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 0.120

Group B: Number of items= 50 **(Downstream Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 1.00 1.00 1.00 1.00 2.00 2.00 2.00 2.00
2.00 2.00 2.00 2.00 2.00 2.00 3.00 3.00 4.00 5.00
Mean = 0.780
95% confidence interval for Mean: 0.5083 thru 1.052
Standard Deviation = 1.22
Hi = 5.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 0.780

Appendix M: Statistical Analysis Results of Antibiotic Resistance Score Comparisons as Reported by the Statistical Program

Between Total Antibiotic Scores of Effluent and Downstream Isolates

The results of an unpaired t-test performed at 21:53 on 11-AUG-2005
t= 1.09
sdev= 1.56
degrees of freedom = 98 The probability of this result, assuming the null hypothesis, is 0.28

Group A: Number of items= 50 **(Effluent Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 1.00 1.00 1.00 1.00 2.00 2.00
2.00 4.00 4.00 4.00 5.00 5.00 5.00 5.00 5.00 5.00

Mean = 1.12
95% confidence interval for Mean: 0.6809 thru 1.559
Standard Deviation = 1.85
Hi = 5.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 1.12

Group B: Number of items= 50 **(Downstream Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 1.00 1.00 1.00 1.00 2.00 2.00 2.00 2.00
2.00 2.00 2.00 2.00 2.00 2.00 3.00 3.00 4.00 5.00
Mean = 0.780
95% confidence interval for Mean: 0.3409 thru 1.219
Standard Deviation = 1.22
Hi = 5.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 0.780

Between Antibiotic Scores of Effluent and Downstream Isolates Relative to Ciprofloxacin

The results of an unpaired t-test performed at 22:00 on 11-AUG-2005
t= 1.91
sdev= 0.300
degrees of freedom = 98 The probability of this result, assuming the null hypothesis, is 0.058

Group A: Number of items= 50 **(Effluent Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.250 0.250 0.250 0.250 0.250 0.250 0.500 0.500 1.00 1.00 1.00
1.00 2.00
Mean = 0.170
95% confidence interval for Mean: 8.5705E-02 thru 0.2543
Standard Deviation = 0.389
Hi = 2.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 0.170

Group B: Number of items= 50 **(Downstream Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.250 0.250 0.250 0.250 0.250 0.500 1.00
Mean = 5.500E-02
95% confidence interval for Mean: -2.9295E-02 thru 0.1393
Standard Deviation = 0.170
Hi = 1.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 5.500E-02

Appendix M: Statistical Analysis Results of Antibiotic Resistance Score Comparisons as Reported by the Statistical Program

Between Antibiotic Scores of Effluent and Downstream Isolates Relative to Trimethoprim/Sulfamethoxazole

The results of an unpaired t-test performed at 22:03 on 11-AUG-2005
t= 1.93
sdev= 0.673
degrees of freedom = 98 The probability of this result, assuming the null hypothesis, is 0.056

Group A: Number of items= 50 **(Effluent Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 1.00 1.00 1.00 2.00 2.00 2.00 2.00 2.00 2.00 2.00 2.00 2.00 2.00

Mean = 0.460
95% confidence interval for Mean: 0.2711 thru 0.6489
Standard Deviation = 0.813
Hi = 2.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 0.460

Group B: Number of items= 50 **(Downstream Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 1.00
1.00 1.00 1.00 1.00 1.00 2.00 2.00
Mean = 0.200
95% confidence interval for Mean: 1.1057E-02 thru 0.3889
Standard Deviation = 0.495
Hi = 2.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 0.200

ANOVA: Results

Between Total Antibiotic Scores From All Sampling Stations

The results of a ANOVA statistical test performed at 21:56 on 11-AUG-2005

Source of Variation	*Sum of Squares*	*d.f.*	*Mean Squares*	*F*
between	25.85	2	12.93	7.333
error	259.1	147	1.763	
total	285.0	149		

The probability of this result, assuming the null hypothesis, is 0.0009

Group A: Number of items= 50 **(Upstream Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
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0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 2.00 4.00
Mean = 0.120
95% confidence interval for Mean: -0.2511 thru 0.4911
Standard Deviation = 0.627
High = 4.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 0.120

Group B: Number of items= 50 **(Effluent Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 1.00 1.00 1.00 1.00 2.00 2.00
2.00 4.00 4.00 4.00 4.00 5.00 5.00 5.00 5.00 5.00 5.00
Mean = 1.12
95% confidence interval for Mean: 0.7489 thru 1.491
Standard Deviation = 1.85
High = 5.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 1.12

Group C: Number of items= 50 **(Downstream Antibiotic Scores)**
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00

0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00 0.000E+00
0.000E+00 0.000E+00 0.000E+00 0.000E+00 1.00 1.00 1.00 1.00 2.00 2.00 2.00 2.00
2.00 2.00 2.00 2.00 2.00 2.00 3.00 3.00 4.00 5.00
Mean = 0.780
95% confidence interval for Mean: 0.4089 thru 1.151
Standard Deviation = 1.22
High = 5.00 Low = 0.000E+00
Median = 0.000E+00
Average Absolute Deviation from Median = 0.780