ABSTRACT

A Comparison of Speciation Methods on Environmentally Isolated, Sediment Associated Aeromonas using Matrix Assisted Laser Desorption/Ionization Mass Spectrometry and Three Housekeeping genes

Background: The taxonomy of Aeromonas is complex and has confounded researchers for years. Over the past twenty years, technological advances have allowed researchers to attempt to clarify the taxonomy through genetic and proteomic techniques. Several of these advances include PCR amplification and sequencing of numerous housekeeping genes and small sub-unit ribosomal DNA (*16S* rDNA) in order to determine the small taxonomic differences between the species in the genus *Aeromonas*. Another method that has recently been employed in this endeavor is matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). Since it is a relatively new method used for aeromonads, no one to date has sought to compare the genetic and proteomic approaches to see which has more utility for the determination of species within the genus. **Methods:** 133 isolates were characterized using multiple methods that have been reported in literature as having the resolving power to determine species level data within the genus *Aeromonas*. These methods included biochemical phenotyping, PCR amplification of three genomic loci: *16S* rDNA (*16S*), *gyrB* (*gyrB*), and the σ^{70} factor of RNA polymerase (*rpoD*), and the characterization and fingerprinting of the 133 isolates using MALDI-MS. The sequence and protein fingerprint data generated using the aforementioned methods were used to construct phylogenetic trees to be used for the determination of species within the isolate set. The species calls were then used to construct a species profile of each respective sampling site. **Results:** For all PCR protocols tested, only the *16S* and *gyrB* were able to amplify product that was able to be sequenced in 73% and 80% of the isolates, respectively. From the sequence data following alignment, both the *16S* and *gyrB* loci had limited genetic variability (19% and 33%). While the limited genetic variability of the *16S* locus limited its resolution, the *gyrB* locus was able to

speciate 86% of the isolates tested. Additionally, MALDI-MS was able to produce much more valid results, characterizing all 133 isolates into nine species. **Conclusion:** *16S* and *gyrB* do provide a significant level of resolution to make species level determinations for this isolate set; more research needs to be done using other housekeeping genes sequences in order to have a PCR based method that is capable of delineating species within the genus, including further characterization of these isolates with *rpoD*; MALDI-MS provided robust and internally validated data that was able to determine nine species within the isolate set; the effluent outfall is addative to the overall species diversity downstream when compared to the upstream species profile; and finally, potential pathogenic species are present in the effluent outfall and downstream indicating the potential for regrowth in the environment of some strain of *Aeromonas*, and that further work needs to be done to characterize that virulence of these isolates.

ACKNOWLEDGEMENTS

Many aspects of this project were aided and supported by others. I owe many thanks to my committee, Drs. Louise Ball, Lola Stamm, Chip Simmons, and Mark Sobsey, for their continued support and facilitation of this project. I would like to extend my deepest thanks to the members of the Sobsey laboratory at UNC- Chapel Hill, especially Lisa Casanova, Mark Elliott, Dave Love, and Whitney Lyman who helped a relative newcomer to the world of microbiology get his feet wet and build a large repertoire of microbiological skills. I would also like to thank Doug Wait and Dorothy Thompson for their valuable insight and knowledge with this project and others. Additional thanks goes to Mike Folan, who was my partner in this project and was extraordinarily helpful in the past two months with the completion of my research. I would also like to thank Dr. Howard Weinberg for introducing me to this project, and for his support, guidance, and oversight. More thanks goes to Tom Randall from the Center for Bioinformatics at UNC – Chapel Hill, who introduced and guided me through the world of phylogenetics. Without his help and weekly meetings, I would still only have a handful of sequences. A huge thanks goes to Dr. Maura Donohue from the Environmental Protection Agency who graciously agreed to use her time and MALDI-MS platform to analyze my isolates. She also was a source of constant inspiration and guidance when I had questions about this infernal microbe. Additional thanks goes to Dr. Colin Stein, who helped to get my genetic analysis on track. I would finally like to thank my wife, Erin McClain, for her grammar skills and her willingness to feign interest about *Aeromonas* – I truly believe that I could not have completed this without her.

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LIST OF ABBREVIATIONS

ADA-V	ampilicllin-dextrin agar with vancomycin
BE	bile esculin
CCL	Candidate Contaminate List
CFU	colony forming unit
DS	downstream sampling site
E	effluent sampling site
EPA	Environmental Protection Agency
GAC	granular activated carbon
HG	hybridization group
KIA	Kliger iron agar
KOH	potassium hydroxide
MALDI-MS	matrix-assisted laser desorption/ionization-mass spectrometry
MGD	million gallons per day
MLST	multi-locus sequence typing
MRVP	Methyl red Voges-Proskauer
OWASA	Orange Water and Sewer Authority
PAUP*	Phylogenetic Analysis Using Parsimony
PBB	purple broth base
PCR	polymerase chain reaction
PI	phylogenetically important
SWDA	Safe Drinking Water Act of 1996
TSA	tryptic soy agar
TSB	tryptic soy broth
TSI	triple sugar iron
UMCR	Unregulated Contaminate Monitoring Rule
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
US	upstream sampling site
VBNC	viable but non-culturable state
WWTP	wastewater treatment plant

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1.0 Introduction

1.1 Background

Members of the genus *Aeromonas* are an economically, environmentally, and medically significant group of bacteria that have proven to be very perplexing to microbiologists and physicians alike because of the evolving nature of their taxonomy, the ever changing spectra of diseases that they cause, and their shifting phylogenetic relationships. First isolated over 100 years ago, the taxonomy of *Aeromonas* has undergone numerous changes, but not so much as in the past 20 years. These recent changes have been due to advances in the knowledge of the genus, an explosion in the discovery of numerous new species, taxa, and biogroups within the genus, and the utilization of new technologies for species identification.

In the 1970s, most aeromonads were grouped into two major, distinct groups: the mesophiles (35°C – 37°C) and the psychrophiles (22°C – 28°C). The mesophiles were responsible for a variety of human infections and were referred to as *A. hydrophila*. Conversely, the psychrophiles primarily caused infections in fish and were designated as *A. salmonicida*. The taxonomy of the latter has remained fairly stable, but the number of mesophilic species has expanded to 16 distinct DNA hybridization groups. Indeed, according to the last edition of *Bergey's Manual of Systematic Bacteriology* (Martin-Carnahan & Joseph, 2005), the genus comprises the following 18 validated species: *A. hydrophila, A. bestiarum, A. salmonicida, A. caviae, A. media, A. eucrenophila, A. sobria, A. veronii (biovars sobria and veronii), A. jandaei, A. schubertii, A. trota, A. allosaccharophila, A. encheleia, A. popoffii* and the two DNA homology groups, *A. sp.* HG11, *A. sp.* HG13 (formerly Enteric Group 501) which remain without a species name. Of these 18, only five species have known human clinical and public health significance and are considered pathogenic because they have been isolated in pure culture from clinical isolates. These five species are *A. hydrophila, A. caviae, A. veronii* bv

sobria (*A. sobria*) and bv *veronii, A. jandaei, and A. schubertii*, with the first three representing the majority of isolates causing disease (Janda and Abbott, 1998).

A. hydrophila, A. caviae, and A. sobria are considered cytopathogenic because of their ability to elaborate enterotoxins, cytotoxin, hemolysins/aerolysins, hemagglutinin, and invasive properties (Moyer, 1987; Morgan et al, 1985). More recently, research conducted by Albert, et al (2000), on clinical isolates from children with diarrhea has discovered three novel toxins that may act synergistically to produce severe diarrhea – a heat-labile cytotoxic enterotoxin (alt), a heat-stable cytotoxic enterotoxin (ast), and a cholera-like cytotoxic enterotoxin (act). Additionally, aeromonads have been implicated in a spectrum of gastrointestinal illnesses, wound infections, and ocular infections, though it remains unclear whether or not *Aeromonas* is the causative organism. A study conducted in Peru found that there was a high frequency of co-infection with other enteropathogenic microbes, including rotavirus, ETEC, *Campylobacter*, *Shigella*, and *Salmonella*, in hospitalized infants with diarrhea. These results suggest that aeromonads might be transient flora or opportunist and not primary pathogens (Pazzaglia et al, 1991). Moreover, a volunteer challenge study conducted by Morgan et al (1985) failed to induce diarrheal illness in 55 of 57 volunteers even at the highest challenge doses (10^{10}) with the five strains tested, and only certain strains were associated with colonization and shedding These results suggest that these aeromonads may lack more than one factor or critical pathway in pathogenesis.

The epidemiological data are also unclear as it relates to the pathogenicity of different aeromonads. Despite the association of virulence factors with certain aeromonads, there is evidence that strains isolated from clinical specimens generally belong in different groups than those strains isolated from environmental sources. Havelaar, et al (1992) demonstrated that there

was little similarity between strains from stools and strains isolated from drinking-waters – a purported exposure source of aeromonads causing gastroenteritis. Other studies have shown that *A. hydrophila* prevalence is related to hybridization groups, with HG1 associated with clinical specimens, while HG3 and HG2 being the predominant groups in water and other environmental samples (Kirov et al, 1994; Hänninen, 1994).

There still has been no confirmed, significant epidemiological evidence that a relationship exists between aeromonads presence in drinking water supplies and gastrointestinal illness from such water. Indeed, current evidence has shown that isolates from clinical specimens and isolates from drinking water supplies are not even in the same DNA homology groups. Furthermore, the levels of aeromonads found in finished drinking water in the distribution system are still relatively low, and volunteer studies have previously indicated that high doses ($>10^{10}$) may be needed to initiate disease in a healthy host. All of these observations indicate that aeromonads present in drinking water represent a relatively low risk to the health of human populations. However, the risk may be higher for certain at-risk groups, including the very young, the elderly, and the immunocompromised, though this has not been thoroughly studied. In light of this, and invoking the precautionary principle, it would be prudent to control excessive levels of aeromonads in drinking water supplies.

One way that this issue is being addressed in the governmental regulatory area is that *A. hydrophila* has been placed on the US Environmental Protection Agency (EPA) Candidate Contaminate List (CCL) under the Unregulated Contaminate Monitoring Rule (UMCR) 1 and 2 promulgated under the Safe Drinking Water Act (SDWA) of 1996. Under this rule, the EPA requires public water systems to conduct a screening survey of contaminants listed on the current CCL, and the agency compiles the findings to determine if any contaminants should be regulated.

In regard to the presence of *Aeromonas* in wastewater treatment processes, the risk is considered variable. With the recent push for more sustainable uses of water, more plants have been diverting some of their effluent to reclamation purposes. The EPA has stated that the level of disinfection should be commensurate with the effluent's intended use (i.e. lower levels of disinfection for irrigation of forage crops, and higher levels of disinfection for use in irrigation of crops and lands for which there would be unrestricted public access), and that regulation is to be done at the state level (EPA, 2004)

While these regulations seem to be sufficient to protect public health from aeromonads in water and wastewater, studies sited previously have found that aeromonads are consistently found in higher quantities than coliforms and have proven to be resistant to or able to overcome (perhaps by regrowth) multiple treatment processes. Given this information, the risk of waterborne exposure to aeromonads is considered significant. In effluent discharges from wastewater treatment plants, the risk is also likely to be minimal because a majority of aeromonads are likely eliminated by treatment, including disinfection, prior to discharge and are not contributing to an appreciable increase above the background, environmental levels of aeromonads in receiving waters.

The risk associated with incidental environmental contact with aeromonads may be the most significant potential source of human exposure. This is because it is difficult to control the levels of aeromonads in recreational waters or the public's use of these waters for primary contact recreation and other exposure routes. Again, like other potential exposures, it is difficult to quantify the risk from recreational water because there is a lack of sufficient epidemiological or dose-response data on *Aeromonas* infection or illness. Aeromonad concentrations in ambient waters and human infection as a consequence of ambient water exposures are not routinely

measured. To date, there has been no large outbreak of *Aeromonas* –associated gastroenteritis or wound infections The risks from occasional, sporadic occurrences of exposure and health effects are likely to be low, based on further details to be presented in subsequent sections of this report.

The lack of definitive data on human health risks and environmental exposure and documented evidence of possible health risks from such exposure has been considerably limited by the lack of robust methods to detect, quantify and characterize the different aeromonads present in water. Therefore, better methods to address these current limitations and deficiencies in characterizing exposures leading to possible health effects are still lacking and much needed. Furthermore, the need to identify and apply effective methods of detection, quantification and characterization (e.g., exposure assessment), is a critical early step in the US EPA CCL process.

1.2 Objectives

The goals of this study were to:

(1) develop a profile of waterborne, sediment-associated *Aeromonas* isolates within a previously obtained collection of isolates from an urban stream near a wastewater treatment plant discharge, and

(2) determine if the treated sewage effluent discharge contributes to the presence of these *Aeromonas* in stream sediments based on the *Aeromonas* at this site being inherently different in their species profile and genetic properties compared to *Aeromonas* isolates from upstream and downstream collection sites. Specific objectives to characterize these isolates include the following:

- PCR amplification and nucleotide sequencing of three genomic loci: *16S* rDNA (*16S*), gyrase B subunit (*gyrB*), and the σ^{70} factor of RNA polymerase (*rpoD*);

- characterization of ~ 133 isolates using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and the generation of a species profile from selected mass ion (m/z) signatures that are common biomarkers for *Aeromonas* species;
- generation of a phylogenetic tree for each isolate from the sequence and m/z data obtained from each of the loci and MALDI-MS, respectively;
- analysis and comparison of each tree to the others for the determination a species profile for each sampling station.

2.0 Literature Review

2.1 Occurrence of Aeromonads in Natural, Waste and Drinking Waters

In almost all aquatic environments throughout the world, mesophilic aeromonads may be found. They are often found in numbers as low as 10^{-2} CFU/ml in drinking water plants and distribution systems to as high as 10^{4} CFU/ml in rivers receiving sewage discharges (Holmes, et al., 1996). The occurrence of aeromonads and the environmental factors effecting their growth and persistence are of particular interest to environmental and drinking water microbiologists, as well as to public health officials and epidemiologists.

This concern about mesophilic aeromonads is especially true in the United States, where *A. hydrophila* has been placed on the candidate contaminate list by the EPA because they have been associated with gastroenteritis and wound infections acquired from contaminated water. The addition of *A. hydrophila* to the CCL has lead to an increased interest the public health significance of their presence in drinking waters and recreational waters where human contact is most likely.

The distribution of the mesophilic species in the aquatic environment may be related to the level of pollution in the water. Several researchers (Ajaujo, et al, 1991; Schubert, 1975; Stecchini and Domenis, 1994) reported that *A. caviae* predominates in sewage and waters with a high degree of fecal pollution. In less polluted waters, either fresh or marine, *A. caviae* and *A. hydrophila* were almost equally distributed. *A. sobria* can be found in unpolluted waters, brackish waters, and sewage effluents. Additionally, the numbers of mesophilic aeromonads have been reported to show seasonal variation, with population increases in the warmer months for both temperate freshwater lakes and chlorinated drinking water (Rippey and Cabelli, 1989; Burke et al, 1984).

The aquatic environments that are commonly investigated have been divided into four convenient types: Seawater, freshwater, sewage, and potable water. Within each of these categories there are a wide range of habitats and variations in physical and chemical conditions to which *Aeromonas* is adaptable and able to live. The following sections will cover these four environments in more detail.

2.1.1 Seawater

The majority of studies of *Aeromonas* in saline habitats have been undertaken as a result of them being obligate fish pathogens and as a potential pathogen for humans. Therefore, these studies tend to focus on commercial and sport fisheries and recreational waters in coastal and estuarine waters. Holmes et al (1996), estimated the typical numbers of aeromonads in seawater to be between 10^{-2} and 10^{2} CFU/ml, although some studies have shown an estimated prevalence of as high as 10^{1}-10^{10}/100ml in southern California coastal waters (Ardi, 2005). A prevalence study conducted by Hazen et al (1978), found that *Aeromonas hydrophila* was quite halotolerant, and that it demonstrated robust growth in a saline environment and was able to persist in a wide range of salinities, except the most extreme (>100‰), over a large range of pH (5.2-9.8), and a

large range of temperatures (4°C and 45°C). Hazen et al (1978), also found that that saline habitats had a much higher density, of *Aeromonas*, up to 9 x 10^5 CFU/100ml, than did freshwater environments, but with much larger variation in their numbers (Table 1). Studies undertaken around Japan demonstrated that the most abundant species was *A. caviae*, with *A. sobria* predominant in brackish waters (Nanako, et al, 1990). They also found that *Aeromonas* numbers correlated with those for fecal coliforms, but not with temperature. Other prevalence studies conducted in Italian seawaters demonstrated that the presence of aeromonads was associated with the presence of zooplankton, such as copepods, and that they were found in higher numbers in sediment, which harbors allocthonous microflora, making it possible to withstand environmental stresses and multiply (Dumontet, et al, 2000).

A phenomenon particularly associated with saline aquatic environments is the viable but non-culturable state (VBNC). Laboratory experiments have demonstrated a 1 log decrease in viable cell count after 2 days (Brandi et al, 1999) and have demonstrated that *Aeromonas* species, like other Gram negative bacteria, are capable of entering the VBNC state when stressed and elaborating new proteins to help to deal with the additional stress of a nutrient-poor saline environment (Maajel, et al, 2004).

2.1.2 Freshwater

As stated previously, the fresh water environment covers a wide range of habitats, including eutrophic rivers and lakes, oligotrophic groundwater, and alpine lakes. The prevalence of *Aeromonas* in rivers has been estimated to be between 1 and 10^2 CFU/ml in clean rivers to levels as high as 10^4 CFU/ml in rivers receiving sewage effluent (Holmes, Niccolis, and Sartory, 1996). Studies conducted on two major urban rivers (Pettibone, 1998; Seidler, et al, 1980) suggest that there is a high prevalence of aeromonads in these waters at levels between 3 and 4000 CFU/ml depending on the season, with higher numbers observed in the warmer months.

Additionally, it was observed that both of these rivers, the Buffalo and the Anacostia, have high levels of fecal pollution from multiple effluent discharges and other anthropogenic sources (Pettibone, 1998; Seidler, et al, 1980). Interestingly though, there was not a correlation between the levels of fecal indicator organisms and *Aeromonas*. In Pettibone's study (1998), it was observed that the geometric mean of *Aeromonas* counts was almost an order of magnitude higher than fecal coliform and streptococci. This observation could indicate that the levels of aeromonads in the river waters are not dependent on these point sources upstream, but could be linked to another parameter such as nutrient levels (Rippey and Cabelli, 1979). This observation also seems to be corroborated by the fact that following heavy rainfall events, when the organic content and total suspended solids increases, the levels of aeromonads that are detectable in the waters tends to increase (Pettibone, 1998; Hazen et al, 1978). Alternatively, the increase in detectable levels of aeromonads could be attributed to soil run-off depositing aeromonads into environmental waters. Brandi, et al (1996) observed that aeromonads could survive and maintain their virulence for up to 42 days in unsterilized soils, acting as a potential reservoir and non-point source of contamination of environmental waters.

According to Holmes, et al (1996), the estimated prevalence of *Aeromonas* is $1 - 10^2$ CFU/ml in pristine lakes and reservoirs. Hazen et al (1978) demonstrated in a prevalence survey in the US of a wide range of freshwater habitats that the mean concentration of *A. hydrophila* was 20 CFU/ml with a range of 0.1-205 CFU/ml in lentic habitats compared to a mean of 196 CFU/ml for lotic habitats (Table 1). He concluded from his study that there was a negative correlation between numbers of *A. hydrophila* and dissolved oxygen, while also demonstrating a positive correlation with numbers of fecal coliforms and heterotrophic plate count bacteria.

As with lotic habitats, lentic waters are also prone to being the receiver of non-point source pollution, such as run-off from soils, agricultural lands, pet and waterfowl feces, and other anthropogenic inputs. The higher the level of these inputs, the more prone the lake is to eutrophication. Rippey and Cabelli (1989) surveyed 68 lakes in the US, each with a different trophic status, and found that increasing levels of *Aeromonas* spp. positively correlated to increasing trophic state (oligotrophic to hypereutrophic) and were seasonally variable with the highest numbers occurring in the warmer months.

Unlike surface waters, groundwaters are normally poor in nutrients with mineral content determined mainly by leaching from the overlying rock and subsurface sediment. Yet, even in these nutrient poor environments *Aeromonas* can be found. While there is a lack of data from pristine boreholes, some studies have recovered *Aeromonas* from deep aerobic and anerobic groundwaters in low numbers with a maximum count of 35 CFU/100ml in the absence of coliforms (Havelaar, et al, 1990). *A. hydrophila* was predominant in these waters, although *A. caviae* made up 30-40% of the population. Similarly, Kuhn and colleagues (1997) found *Aeromonas* at levels <10 CFU/100ml groundwater and artificially recharged groundwater wells in Sweden. Havelaar and colleagues (1990) conducted another survey of 64 still mineral wells in Europe where they were unable to recover any aeromonads. Six of the wells surveyed were inoculated with a tap-water derived isolate of *A. hydrophila* that was pregrown under low nutrient conditions. The isolate only survived and multiplied when the autochthonous bacteria had been removed by pasteurization. They inferred from this data that most groundwaters do not have sufficient nutrients to support and sustain populations of *Aeromonas* at levels that can compete with the natural flora.

Table 1: Comparison of densities of A. hydrophila by water habitat (Hazen, et al, 1978)

Habitat	Density of *A. hydrophila* (CFU/ml)			Number of sites sampled
	Mean	Std. error	Range	
Freshwater				
Lotic	161	46	3600 – 0.4	96
Lentic	20	8	205 – 0.1	26
All	130	36	3600 – 0.1	122
Saltwater	746	688	9000 – 0.1	13
Total	189	73	9000 – 0.1	135

2.1.3 Wastewater and Sewage

Aeromonads are not generally considered to be normal flora of the human gastrointestinal tract, although estimates demonstrate that approximately 1% of healthy adults carry them (Geldrich, 1978). This number is likely variable by world region, diet, and sanitation. In comparison to marine and fresh waters, the highest levels of *Aeromonas* occur in and around wastewater treatment processes. As Table 2 illustrates, domestic sewage sludge has greater than 10^8CFU/ml, and wastewater has between 10^3 and 10^7CFU/ml. Poffe and Op de Beeck (1991) found that between 10^4 and 10^6CFU/ml of *A. hydrophila* were present in domestic wastewaters and, on average, 99.975% were removed by activated sludge and 98.25% by trickling filters. Furthermore, they found that 20.9% of initial *A. hydrophila* became part of the primary sludge, which contained up to 10^7 CFU/g dry sludge and, after 3 months, anaerobically fermented and partially dried sludge from trickling filters contained more than 10^6 CFU/g dry sludge (Poffe and Op de Beeck, 1991). Surface water receiving raw sewage contained several hundreds of *A. hydrophila*/ml, comparable with the numbers found in sewage effluent waters, while surface waters receiving no municipal wastewater contained only small and perhaps negligible numbers. Despite these figures, large numbers of aeromonads are discharged with wastewater into

receiving waters. In a study conducted by Maalej and co-workers (2003), they found *Aeromonas* CFU counts fluctuated between 1.48×10^5 and 2.2×10^8 CFU/100 ml in the effluent from an urban wastewater treatment plant.

Table 2: Typical Numbers of Aeromonas species in sewage and wastewater **(Holmes, Niccolis and Sartory, 1996)**

Environment	Typical Counts (CFU/ml)
Domestic Sewage Sludge	$>10^8$
Crude (Untreated) Sewage	10^5 - 10^8
Treated Sewage	10^3 – 10^5
Wastewater	10^2 – 10^7

2.1.4 Drinking Water and Distribution Systems

Aeromonas can occur in high numbers in some water sources, especially lowland lakes and rivers, from which they can enter the drinking water supply as a result of ineffective or incomplete treatment. Control of aeromonads and other bacteria is typically achieved with increased disinfection and, like other, similar bacteria, free floating aeromonads are susceptible to commonly used chlorine-based disinfectants. A study conducted on clinical and environmental strains of *Aeromonas* found that mesophilic aeromonads were generally more susceptible to chlorine and monochlorine than a control group of enterobacteria and pseudomonads (Knochel, 1991). This was corroborated by Sisti et al (1998) who tested the susceptibility of toxigenic aeromonads to chlorine in drinking water supplies. Through the course of their study, they tested the effectiveness several different residual chlorine concentrations at two temperature levels (5°C and 20°C) on *A. hydrophila.* Table 3 shows that free chlorine is much more effective at inactivation at the lower temperatures and with increasing concentrations.

Table 3: Times required for a 50% (T_{50}) and 95% (T_{95}) reduction in the number of viable A. hydrophila cells from the initial population (7.4 x 10^4 cells/ml) after exposure to increasing concentrations of free chlorine in drinking water at 5°C and 20°C (Sisti et al, 1998)

Chlorine (mg/L)	T_{50} 5°C	T_{50} 20°C	T_{95} 5°C	T_{95} 20°C
0.05	6 min	>90 min	68 min	>90 min
0.1	53 sec	>90 min	66 min	>90 min
0.2	23 sec	>90 min	64 min	>90 min
0.25	21 sec	>90 min	41 min	>90 min
0.3	14 sec	11.5 min	31 min	82 min
0.35	14 sec	30 sec	28 min	67 min
0.4	8 sec	20 sec	7 min	57 min
0.5	<5 sec	<5 sec	<5 sec	5 min
0.6	<5 sec	<5sec	<5 sec	3 min

Another study found that aeromonads are also susceptible to inactivation by chlorine dioxide at levels $\leq$ 0.2mg/L (Medema, et al, 1991). Other findings in this study concluded that in most drinking waters a chlorine dioxide dose of 0.2 mg/L is completely consumed within 10 minutes. Chlorine dioxide can be an effective agent for post-disinfection, provided the chlorine dioxide demand of the receiving water is low. Significant die-off of *Aeromonas* occurs only in the presence of a residual chlorine dioxide concentration. For effective protection, a chlorine dioxide residual should be maintained throughout the network. In most water types, this can only be established by addition of higher chlorine dioxide doses (0.5-1 mg/L) (Medema et al, 1991).

Once in the distribution system, aftergrowth is possible and contamination subsequent to treatment is possible, especially when associated with biofilms (Block, 1992). The levels of aeromonads found in the post-treatment waters and the distribution system range from 10^{-2}-10

CFU/ml and 10^{-2}-10^{3} CFU/ml, respectively (Holmes, Niccolls, and Sartory, 1996). These results indicate that aftergrowth is likely to occur in finished water in the distribution system, and that treatment may be ineffective in providing an adequate residual chlorine level that will eliminate aeromonads. In a study conducted in Belgium (Kersters, et al, 1995) that surveyed several drinking water plants, the average reduction of aeromonads following flocculation-sedimentation and chlorination was 99.7% and slow sand filtration reduced the numbers by 98.9%. Interestingly, they also observed an increase in the levels of aeromonads following filtration by granular activated carbon (GAC), and postulated that *Aeromonas* species might inhabit these filters and other filter media by forming or inhabiting biofilms (Kersters, et al, 1995). This phenomenon was also observed previously by Wilcox (1983), who observed that aeromonads, in addition to other bacteria, were present in higher numbers in the effluent than the influent waters treated with a GAC-filter.

Other studies have suggested that the formation of biofilms and the resistance to chlorine or failure to maintain a high residual chlorine level in the distribution system might also contribute to the aftergrowth of aeromonads. Free aeromonads are generally susceptible to typical chlorine-based disinfectants, however, when associated with biofilms, increased contact times and elevated residual levels of disinfectants at > 0.2mg/L are necessary to control aeromonads within a distribution system (Edge, et al, 1987). This was substantiated by Holmes and Niccolls (1995), who investigated biofilms from exhumed pipe lengths, found that 30% contained aeromonads with an average population of 118 CFU/g wet weights. Following disinfection with 1mg/L of chlorine, *Aeromonas* could still be isolated from 10% of pipe lengths with an average population of 51 CFU/g wet weights. This indicates that although free cells of *Aeromonas* are susceptible to disinfection, populations of aeromonads may survive high chorine

dosing when associated with biofilms. Havelaar and colleagues (1990) reported re-growth of aeromonads in 16 out of 20 Dutch distribution systems. The geometric mean counts varied between 1 and 440 CFU/100ml, with a maximum count of 3300 CFU/100ml obtained from a river water sourced supply. Growth occurred in the peripheral parts of the distribution system and was particularly associated with water derived from anaerobic groundwater containing methane. In a study conducted in a drinking water supply in northeast Scotland (Garivel, Landre, and Lamb, 1998), it was observed that the probability of detecting aeromonads increased with decreasing levels of chlorination in the distribution system, but, that even in areas of high residual chlorine (≥0.2mg/L), aeromonads were still detected, indicating a protective mechanism for *Aeromonas*. Garivel, Landre, and Lamb (1998) posed two possibilities for this occurrence: The first is that a biofilm is present within the distribution network, providing a protective matrix surrounding the aeromonads, thereby shielding them from oxidation by chlorine and providing nutrients that promote aftergrowth. The alternative explanation is that aeromonads are acquiring tolerance to chlorine through a physiological adaptation, either through exposure to sub-lethal doses or through cell-starvation.

Similar observations were made by van der Kooij and Hinjen (1998), who demonstrated that *A. hydrophila* could utilize a variety of organic compounds, including carbohydrates, amino acids, and long-chain fatty acids, at low concentrations (10μg/L). Mixtures of compounds at individual concentrations of 0.1-1 μg/L enhanced growth, demonstrating that aeromonads are capable of growth in the presence of the low concentrations of nutrients that would be available in biofilms and sediments within a distribution system (van der Kooij, Hinjen, 1998).

2.2 Rationale for Environmental Research on *Aeromonas* and Choice of Analytical Methods

2.2.1 *Aeromonas* Species

Aeromonads are ubiquitous, oxidase positive, facultatively anaerobic, glucose fermenting, Gram-negative bacilli that are autochthonous to aquatic environments. They have been isolated from brackish, fresh, estuarine, marine, and chlorinated and unchlorinated water supplies. They have also been isolated from diseased cold and warm blooded animals and from humans since the early 1950s (Carnahan, 2001). Additionally, aeromonads are able to tolerate a wide range of temperatures, salinities, and pH conditions. Traditionally, they have been separated in two distinct groups: Mesophilic, motile and psychophilic, non-motile aeromonads. Both are capable of causing disease in fish, although it is the motile, mesophilic aeromonads that have been implicated as agents of human disease.

Aeromonas have been selected for this study because of their ubiquity in the environment, their relatively easy detection and isolation, their inclusion on the EPA's CCL 1 and 2, and because some of them, notably *A. hydrophila*, are considered potential human pathogens. Additionally, they have been shown to be resistant to multiple antibiotics (Folan, 2007; Bronsted, 2005; Goni-Urriza, et al, 1999).

2.2.2 Recovery and Detection of *Aeromonas* in the Environment

The most often used procedure for the detection, isolation, and enumeration of *Aeromonas* from treated and environmental waters is membrane filtration and culture on ampicillin-dextrin agar with vancomycin (ADA-V) medium (EPA method 1605). There are also other similar media, such as m-*Aeromonas* agar, which also contain ampicillin (Rippey and Cabelli, 1979) and are considered equally selective and efficient at isolating most aeromonads

from environmental sources when used in combination with standard membrane filtration techniques. However, there are a few non-pathogenic species of aeromonads that are susceptible to ampicillin and will be selected against using these media. Spread and pour plate culture techniques and liquid enrichment culture methods can also be utilized for the recovery of *Aeromonas* from environmental sources (Moyer et al., 1992).

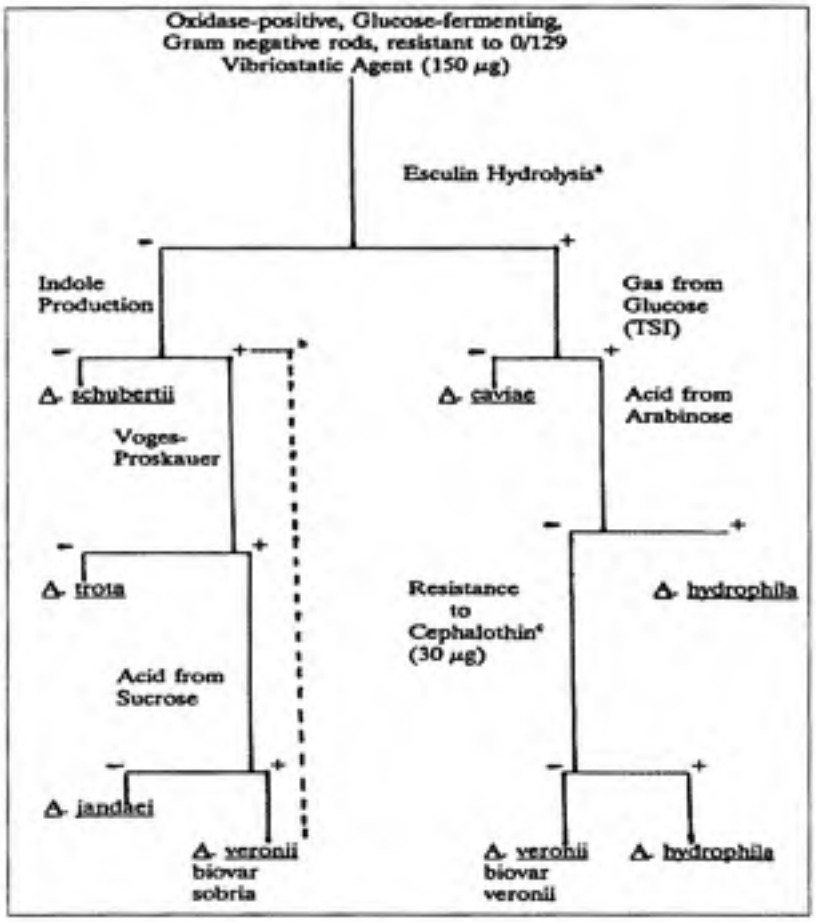
2.2.3 Speciation Methods for *Aeromonas*

The identification of *Aeromonas spp.* is crucial but has proven to be difficult. Over the past twenty years, the taxonomy of the family *Aeromonadaceae* has grown from four known species in 1984: *A. hydrophila* (HG1), *A. caviae* (HG4), *A. sobria* (HG7), and *A. salmonicida* (HG3) (Popoff, 1984), to seventeen species by 2004. The seventeen species are also referred to as hybridization groups (HG) based on DNA–DNA hybridization studies. The newer species include *A. bestiarum* (HG2), *A. media* (HG5), *A. eucrenophila* (HG6), *A. veronii bv sobria* (HG 8), *A. jandaei* (HG9), *A. veronii bv veronii* (HG10), *A. sp.*(HG11), *A. schubertii* (HG12), *Aeromonas sp.* (HG13), *A. trota* (HG14), *A. allosaccharophila* (HG15), *A. encheleia* (HG16), and *A. popoffii* (HG17) (Martin-Carnahan & Joseph, 2005). This evolving taxonomy has prevented the development of an unambiguous and rapid means to identify species of *Aeromonas* species.

Speciation of isolates can be done in several ways, including DNA-DNA hybridization, conventional biochemical analysis, multi-locus sequence typing (MLST) and matrix assisted laser desorption/ionization mass spectrometry. The first two are older, more conventional methods, with biochemical characterization/phenotyping being the most widely used in clinical and environmental laboratories because of their ease and practicality. Several biochemical schemes have been described, but the most useful and practical for the identification of the five pathogenic species is the Aerokey II (Carnahan, Berham and Joseph, 1991) (Figure 1). Other

biochemical methods have been described by Abbot and colleagues (2003). These schemes are a good deal more extensive and require numerous tests in order to elucidate more than complex level identification of aeromonads.

Figure 1: Aerokey II scheme for the phenotypic speciation of Aeromonas. a, agar formulation only; b, Aerokey II can be modified to end here with an identification of A. veronii bv. sobria; c, Bauer-Kirby disk diffusion method only (Carnahan, Berham and Joseph, 1991)



DNA-DNA hybridization studies on *Aeromonas* were first described by Popoff and co-workers (1981). In this study, three species were initially described: *A. hydrophila* (type strain = ATCC 7966), *A. caviae* (type strain = ATCC 15468), and *A. sobria* (type strain = CIP 7433). Within these groups, they found that each contained two to three DNA hybridization groups (HGs) that could not be separated from each other by phenotypic traits. *A. hydrophila*

phenospecies resided among three genospecies referred to as DNA HGs 1-3. DNA HGs 4-6 contained aeromonads that resembled the phenospecies *A. caviae,* and DNA HGs 7-8 resembled *A. sobria.* Not included in the initial description by Popoff was the non-motile psychrophile *A. salmonicida*, but it was included in the first edition of *Bergey's Manual of Systematic Bacteriology* in 1984 (Popoff, 1984). This list of genotypically distinct *Aeromonas* genospecies has since expanded to 17 HGs (Martin-Carnahan & Joseph, 2005).

MLST is relatively new way to characterize bacteria using molecular methods (Enright and Spratt, 1999). It augments current biochemical identification methods and is particularly useful for rare species of *Aeromonas* found in environmental waters. MLST utilizes a set of genes in order to differentiate species differences within a genus. The genes that have been used for the identification of aeromonads are *16S*, recA, DNA gyrases (gyrA and *gyrB*), chitinase and other housekeeping genes that are thought to be less conserved than *16S* and have more genetic diversity to help separate the species. For *Aeromonas*, a γ-proteobacterium, previous studies have documented chitinase genes as powerful loci for taxonomic identification of Proteobacteria (Cotrrell et al, 2000) and are included along with several other loci for the numerous enzymes that are elaborated by aeromonads (Pemberton et al, 1997).

Other studies have used the highly conserved *16S* ribosomal RNA gene sequences for phylogenetic analyses. As described by Woese (1987), because this region is highly conserved, it is reflective of bacterial phylogeny between different genera. Martinez-Murcia et al (1992; 1999; 2005), have done extensive work on the *16S* genes of aeromonads, plesiomonads and *E. coli*. They have determined sequences for most of the respective species of the genus *Aeromonas* and constructed phylogenetic trees based on the data. Most species exhibited high

levels of overall sequence similarity (98-100%) to each other, but dissimilarities from other genera in the gamma subclass of the proteobacteria.

Recent studies have reported success in differentiation of species of *Aeromonas* with the use of only three genetic loci:*16S*, *gyrB*, and *rpoD* (Soler, et al, 2004; Kupfer, et al, 2006). They found nucleotide sequences of *rpoD* and *gyrB* had similar nucleotide substitution rates (<2%) and a similar number of variable positions (34% for *rpoD* versus 32% for *gyrB*). Strain groupings by analysis of *rpoD*, *gyrB* and a combination of both genes were consistent with the taxonomic organization of all *Aeromonas* species described to date. They additionally observed that the simultaneous analysis of both molecular (evolutionary) clocks improved the reliability and the power to differentiate closely related taxa (Soler, et al, 2004; Kupfer, et al, 2006).

The use of mass spectrometry for microbial analysis is an attractive alternative to the more traditional phenotypic and molecular biological approaches to bacterial taxonomy. For over 60 years, analytical chemists have used a wide assortment of mass spectrometers and mass spectrometry-based approaches, including pyrolysis mass spectrometry, gas chromatography-mass spectrometry, fast-atom bombardment mass spectrometry, and electrospray- tandem mass spectrometry to characterize a number of microorganisms by their protein content or by their fatty acid composition (Anhalt and Fenselau, 1975; Wieten et al, 1981; Magee et al, 1993; Cain et al, 1994). The most recent instrument employed in this field of bacterial classification has been matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS). Due to the MALDI-MS "soft" ionization process and its ability to simultaneously detect a number of intact analytes (peptides, proteins and other biological molecules), this instrument is very attractive for the analysis of bacterial whole cells. In recent years, there have been a number of studies demonstrating the capability of MALDI-MS to differentiate between a wide assortment of

microbes based on the mass spectral output of the MALDI-MS. This technique generates a mass spectral fingerprint, or *m/z* signature (between *m/z* 2000 and 26,000), that can be used to identify the genus and species of a microorganism due to conservation of protein expression within a taxonomic group (Donohue, et al, 2005; 2007).

The taxonomy of *Aeromonas* is complex and has confounded researchers for years. Over the past twenty years, technological advances have allowed researchers to attempt to clarify the taxonomy through genetic and proteomic techniques. Several of these advances include PCR amplification and sequencing of numerous housekeeping genes and small sub-unit ribosomal DNA (*16S* rDNA) in order to determine the small taxonomic differences between the species in the genus *Aeromonas*. Another method that has recently been employed in this endeavor is matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). Since it is a relatively new method used for aeromonads, no one to date has sought to compare the genetic and proteomic approaches to see which has more utility for the determination of species within the genus. The following section will provide a detailed description of the methods used to meet the objectives mentioned in Section 1.

3.0 Methods

Site selection, sampling and biochemical testing of the bacterial isolates of this study were performed previously by Ms. Embrey Bronstad and her research team as part of her Masters Technical Report (Bronstad, 2004). All site descriptions, sampling methods and biochemical methods are transcribed, with minor editing, from her Masters Technical Report (Bronstad, 2004). Additional information is provided due to facility upgrades and changes that have subsequently taken place after the collection of the bacterial isolates on which this study is based.

3.1 Primary Site Characteristics

The Orange Water and Sewer Authority (OWASA) Mason Farm Road wastewater treatment plant (WWTP) was selected as the point around which sediment samples were collected and can be described as follows at the time of sample collection:

- Minimal agricultural impacts to the receiving waters.
- Minimal upstream point and non-point pollution sources.
- Treatment at the wastewater plant included screening, primary and secondary sedimentation, activated sludge, biological nutrient removal and disinfection with chlorine. The treatment plant had a 12 million gallons per day (MGD) capability and treated on average 8 MGD.
- The effluent of a 688-bed university hospital and a large research university was serviced by the WWTP.
- The upstream sampling site (US) was located approximately 600 meters before the effluent discharge. Additional samples were collected at points 33 meters and 965 meters after the effluent discharge and are referred to as effluent (E) and downstream (DS), respectively. The sampling locations were selected based on their location relative to the wastewater effluent discharge and their accessibility.

Additionally, sampling areas with lower flow rates were also desirable as potentially longer resident times of water, solutes and bacteria in such areas would allow for a greater accumulation of antibiotics in the sediment and higher antibiotic exposure times for bacteria.

Since the samples were collected, subsequent changes have been made to the Mason Farm Road wastewater treatment plant. These changes include a switch from chlorine to ultraviolet disinfection of the wastewater prior to discharging into Morgan Creek. OWASA has also upgraded the capacity of the treatment plant to 14.5 million gallons per day and added a reclaimed water system that will treat a portion the total plant flow and send it to the UNC campus, where it will be used initially as chiller plant water. They have also built a high capacity slow sand filter to improve the quality of the water that is being discharged on an annual basis. Figure 2 shows the approximate locations of the sampling sites.

Figure 2: Approximate Sampling Sites Around WWTP

3.2 Sample Collection

Sampling of stream sediments (bottom material below the water column or exposed at low flow) was conducted on six separate dates between April 2005 and July 2005. The US sampling site was on the upstream side of a bridge where the flow was slightly impeded. At the E sampling site, a downed log had created a pool that allowed samples to be collected at point where water had a slower flow rate. Finally, at sampling site DS, a concrete ford created a slower flow rate in front of the ford from which the samples were collected. Duplicate grab samples were collected from US, E and DS through the use of an Eckmann dredge. The top centimeter of sediment retrieved by the dredge was removed with the aid of a spatula, transferred to 125 mL sterile Nalgene HDPE (sample) bottles and placed in a cooler for transport back to laboratory; the spatula was rinsed with deionized water (Dracor, NC) between samples. The amount of sediment samples varied between approximately 30-50 grams and was enough to perform microbial analysis. In the event that the sediment sample was overlaid with water, the supernatant was pipetted out of the sample bottle before sediment homogenization by manual shaking. After the water was removed, the bottle was recapped and the sediment samples were 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 (Standard Methods buffer) and vortexed for ten seconds: This was considered to be a 10-fold dilution. For Standard Methods buffer, stock solutions of potassium dihydrogen phosphate and magnesium chloride were prepared according to the procedures specified by the method and stored in 1.25 mL- glass containers at 4°C until use. When needed, 1.25 mL of phosphate stock and 5 mL magnesium chloride stock were added to a l-L glass bottle containing 500 mL of deionized water and autoclaved (Standard Methods, 20th ed., 1998). The 10-fold sediment dilutions were prepared first and were 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 as another 10-fold dilution; ten-fold serial dilutions were prepared until the 10^{-4} dilution was reached. When created and sampled, each dilution was vortexed for five seconds to ensure homogenization.

3.3 Biochemical Testing of Bacterial Isolates

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). ADA-V agar was employed and prepared as recommended by the EPA Method 1605 for the isolation of aeromonads from drinking water. In summary, the agar was prepared according to the manufacturer's instructions, 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, molten agar. The agar was shaken slowly to uniformly distribute the antibiotics before dispensing 10-ml volumes into individual sterile 100 mm diameter Petri dishes.

A 100 µL volume of the sample dilution was evenly distributed across the surface of the agar using a flame sterilized glass "hockey stick" and a small horizontally mounted, rotating wheel as the platform on which to manually spin the plate. The agar plate was placed on the wheel and spun while the glass stick was used to spread the sample across the agar surface. ADA-V plates with the spread-plated sample were incubated for 24 +/- 2 hours at 35° C.

After incubation of the ADA-V plates, presumptive *Aeromonas* species 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 that were flame sterilized between each isolate. The TSA was prepared according to the manufacturer's recommendations in a 1-L glass bottle, with shaking to ensure homogenization and wetting of the medium upon addition to reagent water and then autoclaving.

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 composed of tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD) and glycerol (Fischer Scientific, Fair Lawn, NJ) at a final concentration of 25% (V/V). 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 autoclaved and after cooling, 1 mL aliquots were added to sterile 1.5-mL capacity microcentrifuge (Eppendorf) tubes. Using a sterilized wooden applicator stick, material recovered from one isolated colony from each TSA plate was added to the liquid of one microfuge tube and vortexed in order to homogenize the bacterial suspension. The bacterial isolates were then archived at -80°C and removed for use as needed.

The isolates were subjected to a number of additional biochemical tests in an attempt to confirm them as *Aeromonas* sp. See Table 4 for a summary of positive results associated with each of the biochemical tests used. A sterile disposable 1 mL- glass pipette was used to remove a small portion of the frozen bacterial suspension which was dotted (spotted) onto a TSA plate. Sterile wooden applicator sticks were used to triple-streak the inoculum for bacterial colony isolation and the TSA plates were incubated overnight. An individual colony was picked with a sterile wooden applicator stick and inoculated on Kliger iron agar (KIA) slants by fishtail streaking on the slant and stabbing into the butt of the agar slant. Inoculated slants were incubated both aerobically and anaerobically at 35°C. Slants were prepared by adding 5 mL of

molten agar medium to sterile glass 16 x 100mm 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 these 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 absorbent 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 with the 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 1% 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 from raw ingredients 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 screwtop 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 3 below for a summary of test descriptions and positive results for each test. A random sample of 15 isolates was subjected to a Gram stain to confirm their physiology and morphology as Gram-negative rods. Isolates were streaked on TSA and incubated overnight before staining. Reagents were supplied by Biochemical Sciences, Swedesboro, NJ, and staining was conducted according to standard procedures (MacFaddin, 2000). All biochemical results for the isolates examined can be found in Appendix A.

Table 4: Biochemical Test and Positive Results

Reagent	Test	Positive	Reagent	Test	Positive
ADA agar	Dextrin fermentation	Orange/Yellow colonies	Bile Esculin Agar	Esculin hydrolysis	Black/Brown colonies and darkening of agar
150µg 0/129 Vibriostatic	Resistance	No inhibited growth near Disk	Voges-Proskauer	Acetoin production from glucose fermentation	Pink line at interface of reagent and broth
MacConkey agar	Lactose fermentation	Pink colonies	L-Arabinose	Arabinose fermentation	Broth color change from purple to yellow
Spot Oxidase	Presence of oxidase enzyme	Blue color change after exposure to reagent	TSI agar	Glucose Fermentation with gas production	Color change from red to yellow and gas present
Spot Indole	Tryptophan oxidation	Pink color change after exposure to reagent			

3.4 Polymerase Chain Reaction (PCR) of *16S* rDNA, *gyrB*, and *rpoD*

3.4.1 PCR Primers

The literature was surveyed and three sets of primers, each for a specific genome locus were selected for this study based on the resolution that they provided in determining species within the genus *Aeromonas*. Table 4 lists the genome locus and its forward and reverse primers, the position (according to *E. coli* numbering system) and the source of the primers. All primers were ordered from Integrated DNA Technologies (Coralville, IA). They were shipped in lyophilized form and reconstituted per the manufacturer's directions.

Table 5: Genome targets and their primers used for PCR amplification and sequencing of 16S, gyrB, and rpoD

Gene of Interest	Genome Target and Primer (position)	Sequence (5' to 3')	Reference
16S rDNA	AerF *16S* (87-105)	CTACTTTTGCCGGCGAGCGG	Lee, et al, 2004
	AerR *16S* (1041-1022)	TGATTCCCGAAGGCACTCCC	Lee, et al, 2004
DNA Gyrase B Subunit	*gyrB*3F (334-354)	TCCGGCGTTCTGCACGGCGT	Yanes, et al, 2003
	*gyrB*14R (1464-1444)	TTGTCCGGGTTGTaCTCGTC	Yanes, et al, 2003

RNA polymerase σ 70 factor	*rpoD*-f (740–757)	GTCAATTCCGCCTGATGC	Soler et al, 2004
	rpoD-r (800-782)	ATCATCTCGCGCATGTTGT	Soler, et al, 2004

3.4.2 DNA Extraction and Amplification

All isolates analyzed for *16S*, *gyrB*, and *rpoD* genes by PCR were removed from the -80 °C freezer, thawed for approximately 30 minutes and 6 µl of bacterial suspension was heated for 10 min. at 99 °C to obtain crude lysates. After centrifugation at 14,000 x g for 10s, 2 µl of the isolate suspension supernatant was used in all PCR assays.

Upon completion of the PCR reaction, all amplified isolate products were analyzed based on their electrophoretic mobility on 2% agarose gels stained with ethidium bromide. All gels were made by adding 2 g of agarose to 100 mL of TE buffer and heating for two minutes in a microwave to ensure complete dissolution. The agarose was then allowed to cool for approximately five minutes before adding 4 µL of ethidium bromide, which was swirled to ensure homogenization. The agarose was poured into a mold and allowed to set for approximately one hour. Combs were placed into the mold that created wells used to load the isolate PCR product DNA. A 5 µL volume of the isolate product DNA was loaded into each well along with 1 µL of loading dye (Qiagen, Valencia, CA) and the gel was covered with 1x Tris-acetate buffer solution to ensure proper conductivity. Gels were then placed in an electric field of 110V for approximately 45 minutes to allow for appropriate separation of amplified DNA fragments. Gels for *16S* and *rpoD* were analyzed qualitatively by visualizing the gel with UV light and a gel imaging device and quantitatively for amplicon size by comparing visible bands to a 100 base pair DNA reference ladder of known size molecules. For *gyrB*, a 1000 kilobase pair DNA reference ladder was used because of the large size (1100bp) of the amplicon.

All primers referenced in Table 5 were subjected to the following PCR protocols: **1)** *16S* rDNA, 95°C for 15 minutes (*taq* polymerase activation), followed by 40 cycles of 94°C (denaturing) for one minute, 68°C (annealing) for one minute, 72°C (extension) for one minute, followed by 72°C for ten minutes, and 4°C for ten minutes; **2)** *gyrB*, 95°C for 15 minutes, followed by 40 cycles of 94°C for one minute, 55°C for one minute, 72°C for one minute, followed by 72°C for ten minutes, and 4°C for ten minutes; **3)** *rpoD*, 95°C for 15 minutes, followed by two cycles at 94°C for one minute, 63°C for one minute, 72°C for one minute, followed by two cycles at 94°C for one minute, 61°C for one minute, 72°C for one min, followed by two cycles at 94°C for one minute, 59°C for one minute, 72°C for one minute, followed by 30 cycles at 94°C for one minute, 58°C for one minute, 72°C for one minute, 72°C for ten minutes, and 4°C for ten minutes.

For each reaction 12.5 µl of Hot Start Mastermix (Qiagen, Valencia, CA) was vortexed with a 10.1 µl of RNA and DNA free water (Qiagen, Valencia, CA) and 0.2 µl of forward and reverse primer. As previously indicated, 2 µl of bacterial isolate was added to the solution after heat release of bacteria DNA. The final volume of each reaction was 25 µl. PCR amplification was then performed using the conditions described above in a thermal cycler (PTC-200, MJ Research, Inc., Watertown, MA.).

3.4.3 DNA Sequencing and Sequence Analysis.

PCR products were sequenced using the forward PCR primer after purification with a QIAquick PCR purification kit (Qiagen, Valencia, CA). Briefly, purified DNA was recovered from excess PCR product. After purification, the DNA was quantitatively analyzed for UV fluorescence to determine the proper amount of purified DNA to be included in the sample that was sent for DNA sequencing. DNA sequencing was performed at the Lineberger Sequence

facility (UNC campus, Chapel Hill, NC) using the BigDye Teminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Sequencing was done only in the forward direction as a cost saving measure. Ideally, bidirectional sequencing would have been performed, but, due to the number of isolates and loci being sequenced, it was thought to be too expensive for the limited budget of this project ($30 per isolate).

After the sequencing was complete, each sequence within a locus was edited using ContigExpress® (Invitrogen Corporation, Carlsbad, CA). The nucleotide sequences of *16S*, *gyrB* and *rpoD* were independently aligned by the CLUSTALW program (Computational Biology Service Unit, Cornell University, Ithica, NY). Genetic distances were obtained using Kimura's 2-parameter model (Kimura, 1980) and evolutionary trees were constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) with MEGA, version 3.0 (Kumar et al, 2001). Additional trees were constructed by the UPGMA method to validate the NJ method (Nei and Kumar, 2000)

3.5 MALDI-MS

All MALDI analysis was performed by Dr. Maura Donohue and her research team from US EPA-Cincinnati, OH. She analyzed 133 isolates obtained as described previously. All biochemical and molecular data was shared between our UNC lab and Dr. Donohue's lab. All methods described below are transcribed with minor editing with permission of Dr. Donohue.

3.5.1 Reagents

Tryptic soy broth (TSB) and 5% sheep blood agar were purchased from BD Biosciences (Bedford, MA). Ampicillin-dextrin agar (ADA) was purchased from BioLife (Milano, Italy). The reagents for the biochemical test were purchased from bioMerieux (Durham, NC). Protein Standard I and sinapinic acid were acquired from Bruker Daltronic (Billerica, MA). Methanol

and acetonitrile were purchased from Tedia (Fairfield, OH). Trifluoroacetic acid (TFA), vancomycin and glycerol were purchased from Sigma Aldrich (St. Louis, MO).

3.5.2 Bacterial Reference Strains

The strains used to create the library of known *Aeromonas* strains are listed in Appendix B. All strains used in this library were either type, reference, or other well-characterized isolates that have been previously identified as belonging to a specific species by both biochemical and DNA-DNA hybridization studies. The *A. salmonicida* type strain, ATCC 33658 (subspecies *salmonicida*), was not included in this study as it represented psychrophilic salmonids that grow well at lower temperature (20 °C) and are primarily considered fish pathogens. The *A. salmonicida* stains that were included in the study were motile, grew at 35 °C, and had been isolated from clinical (feces) and environmental samples (water). Based on DNA studies this group is referred to as *A. hydrophila*-like (HG3) because they belong to hybridization group 3 (*A. salmonicida*), but have phenotypic traits that resemble *A. hydrophila*.

3.5.3 Sample Preparation

All samples were inoculated onto TSA plates and incubated for 24 hours at 35 °C. Individual colonies were carefully transferred from the agar surface with a sterile loop to 5 mL of TSB and incubated for 5 hr at 35 °C. One-hundred µL of the TSB broth was spread onto 5% sheep blood agar plates and incubated at 35 °C overnight. The bacteria were harvested from the plates in 5 mL of sterile saline. The optical density of each sample was measured at 600 nm and adjusted to 0.500 ± 0.050 OD. The cells were washed three times by centrifugation and re-suspended in water. The final cell pellet was suspended in 20 µL of water. One µL of each bacterial suspension was pipetted onto the stainless steel MALDI target plate and immediately overlaid with 1.5 µL of 40% methanol, which was allowed to slowly evaporate at room

temperature. For MS analysis, the dried cells were overlaid with 1.0 μL of 10 mg/mL sinapinic acid. The sinapinic matrix solution was prepared by dissolving 10 mg in 1 mL of 70 % acetonitrile/0.1% TFA. The matrix/sample spots were crystallized by air-drying.

3.5.4 MALDI-MS Analysis

All mass spectra were acquired using a Biflex III (Bruker Daltonics, Billericia, MA, USA) MALDI-MS with a nitrogen laser (337 nm) operated in positive linear mode. The spectra were the average of four spectra and each spectrum was the average of 100 shots. The summed spectra were smoothed and externally calibrated using the standard calibrant mixture, Protein Calibration Standard I. The $[M+H]^+$ of insulin and myoglobin in this standard were used to externally calibrate the mass spectrometer. The acceleration voltage was maintained at 20 kV, the pulse voltage was maintained at 1300 V, and the extraction delay time was 225 ns. The data files were transferred to XMASS ver. 5.1.5 (Bruker Daltonics) for automated peak extraction and analysis.

3.5.5 Data Processing, Cluster Analysis and Reference Library

From the spectra, peak lists were generated using a peak picking algorithm in the XMASS software employing apex peak detection. Peak lists were checked to ensure completeness and accuracy, and stored for further analysis.

Peak lists were exported into Excel™ (Microsoft Corporation, Redmond, WA, USA) and the mass values were binned in ascending order. The ions within ± 5 Da of each other were binned together for masses less than 9000 Da. For mass values greater than 9000 Da, ions with a ± 20 Da spread were binned together. A total of 254 bins in the range of *m/z* 3000 to 26,000 were used in the cluster analysis. Peak lists were checked to ensure completeness and accuracy for Phylogenetic Analysis Using Parsimony (PAUP*) analysis. In this analysis, MALDI-MS

data were converted to presence/absence data. This was then used to calculate a distance matrix using PAUP* version 4.0b software (Swofford, 2002). In total, three algorithms were used in the analysis: Neighbor-joining (NJ), Unweighted Pair Group Method with Arithmetic mean (UPGMA), and Maximum-likelihood.

The reference library contained the *m/z* signature of five independent analyses (a total of 20 spectrums). Peak list and spectra reproducibility was assessed to set peak selection parameters in order to produce the most stable *m/z* signature for both the reference strains and unknown water isolates. Also, random reference strains were chosen for MALDI–MS analysis to determine if there were any differences between reference *m/z* signature and newly analyzed strain *m/z* signature. It should be noted that no changes were ever made to the reference *m/z* signatures. The *m/z* signatures of the unknown sediment isolates were analyzed independently up to three times. Each time their *m/z* signature was compared against the reference library.

3.5.6 Sample Identification

The identity (species) of the isolates was determined by analyzing the *m/z* signature of the isolates against the reference strains. The NJ algorithm and bootstrap values were used to determine the identity (species) of the isolates. The UPGMA algorithm was used to confirm the NJ determination. These two distance algorithms were used to determine the isolates relatedness to the reference strains, based on similarly of *m/z* signatures. The calculated bootstrap values determined the strength of the relationship that the isolate had to the genus and species (10,000 replicates). For the isolate to have a significant correlation to a specific species, the bootstrap values calculated for genus needed to be ≥ 99 and for species ≥ 50.

3.6 Statistical Analysis of Data

All statistical analysis was done with SPSS v. 15.0 (SPSS, Inc., Chicago, Ill) and GraphPad InStat softwares (GraphPad Software, Inc., San Diego, Ca). Based on the species findings from MALDI-MS and *gyrB* sequence analysis, a contingency test using the Chi-square test of independence was performed. The use of a contingency table is particularly appropriate for analyzing categorical data, while the Chi-Square statistic compares the tallies or counts of categorical responses between two (or more) independent groups. The variables being tested were sampling sites and numerical counts of the different *Aeromonas* species found in each site. Three different contingency tables were created to test: 1) Isolates from the upstream location versus the isolates from the downstream location; 2) isolates from the effluent location versus the isolates from the downstream location; and 3) the isolates from the effluent location versus the isolates from the upstream location. For this analysis the H_o was that there is no relationship between sampling site location and the species profile of the isolates collected there.

In addition to chi-square analyses, t-tests were used to evaluate the difference in the findings from MALDI-MS and *gyrB* analyses. Two t-tests were performed, one comparing the average total species identified by each method for all collection sites (downstream, upstream, and effluent), and the other comparing the average total bacteria identified by each method for all collection sites. The null hypotheses for both t-tests was that MALDI-MS and *gyrB* sequence analysis will determine the same number of species and identify the same number of bacteria. The t-test approach was chosen as opposed to a regression analysis because there were not enough sample site replicates to generate useful information using a regression based approach. According to the Odum Institute, a minimum of 50 replicate samples would be needed for a regression analysis approach to be appropriate.

4.0 Results

The following sections will explain in detail the successful and unsuccessful PCR amplifications of the three housekeeping genes included in the MLST analysis (*rpoD*, *gyrB*, and 16 rDNA). Further information will be provided about the subsequent sequence analysis done on the *gyrB* and *16S* loci. This will be followed by the results and species findings of MALDI-MS and, finally, a summary of the statistical analysis of the species data will be explained.

4.1 PCR Amplification and Sequencing

Successful amplification and eventual sequencing requires that a primer be able to anneal and amplify a single locus of interest, with a positive result being a single band for each isolate on an agarose gel. For the *16S* locus, the primers were able to anneal and amplify 113 of 133 isolates being tested with an approximate fragment size of 953bp. Of the 113 amplified isolates, 97 of the final sequences were clean and usable (Figures 3 and 4), resulting in a 73 percent recovery and characterization from the total initial number of 133 isolates..

Figure 3: An unusable sequence chromatogram for the 16S locus

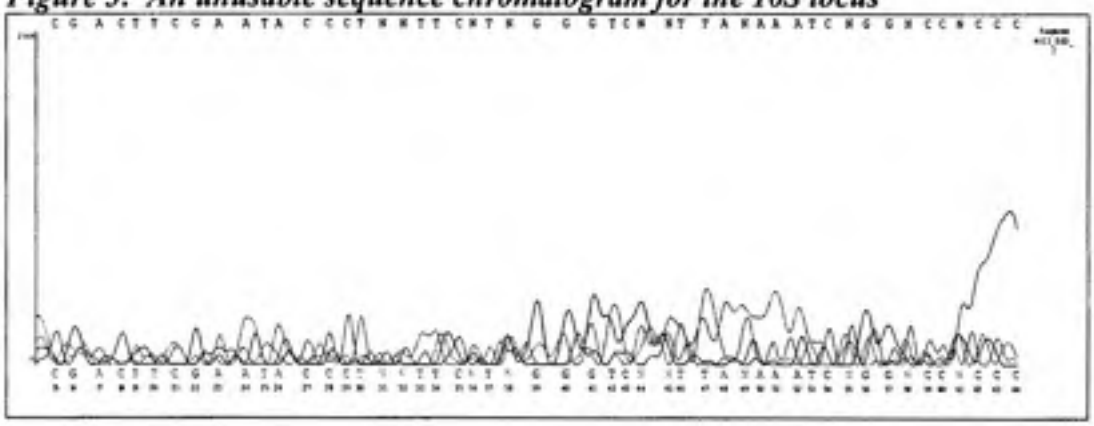
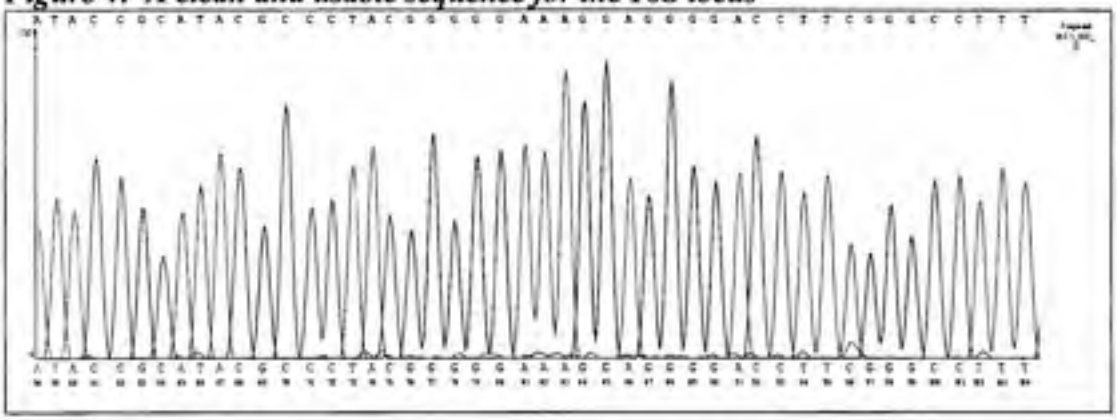


Figure 4: A clean and usable sequence for the 16S locus



The other successful primer pair was for the gyrase B locus. These primers were able to amplify 118 of 133 isolates tested with an approximate fragment size of 1100bp. Of the 118 isolates submitted for sequencing, 106 were clean and usable sequences, resulting in a final recovery of 80% of the total initial number of 133 isolates. For both successfully amplified loci, *16S* and gyrase B, the final fragment size used for the phylogenetic analysis, after editing and cropping of the usable sequences, was 594bp for *16S* and 708bp for gyrase B.

4.2 Unsuccessful Primers for Amplification of Locus *rpoD*

The initial primer set used for the amplification of the *rpoD* locus was reported by Soler and co-workers (2004) to be able to generate a PCR product fragment of 820bp. However, in this investigator's hands, the *rpoD* primer set published in the literature was unable to generate any amplified PCR product DNA for sequencing. This was despite performing the touch down PCR protocol that was published in their paper (see section 3.4.2), altering the concentration of DNA (1:10 and 1:100), and altering the thermocycler temperatures (68°C, 55°C, and 52°C). In order to confirm that the published sequence was correct, a set of *rpoD* amplified type and reference strains of *Aeromonas* were accessed from the GenBank database and aligned. Following alignment and using the position (see section 3.4.1) published in the literature, it was found that the published sequence of the primer was faulty in several of its nucleotide pairs based

on our alignment (Table 6). This resulted in no annealing of the primers to the gene of interest. From the same sequence alignment, several candidate PCR primers were extracted, but they have yet to be tested. Once they have been tested and applied to the isolate collection used in this study, an addendum to this technical report will be provided and this new information will be included in any manuscripts prepared from this research.

Table 6: Misalignment of rpoD-F and rpoD-R primers and consensus gene sequence at the corresponding gene positions

Forward Primer Sequence *rpoD*-F (740-757)	GTC AAT TCC GCC TGA TGC
Consensus Gene Sequence for *rpoD* (740-757)	CTG TCC ATC GCC CAG ATC
Reverse Primer Sequence *rpoD*-R (800-782)	ATC ATC TCG CGC ATG TTG T
Consensus Gene Sequence for *rpoD* (800-782)	CCC GGA ACC GGA GTG GCT A

4.3 *16S* rDNA Sequence Analysis

Analysis of the *16S* gene locus was carried out for the 97 clean and usable sequences from field isolates and for 26 type and reference sequences from GenBank (Appendix B), collectively representing the 17 DNA HGs . The overall nucleotide difference among all strains (n=123) is 118bp of 594bp or 19% variability. Among the 26 type and reference strains, the total nucleotide variability of the 594bp gene fragment is only 33bp or five percent variability. This indicates that there is a high level of conservation within these type and reference strains and that some but not all sequences are identical. Among the complexes for the type and reference strains, the extent of variation is quite different. *A. hydrophila* complex (n=7) has only 9bp of the 594bp that are different; the *A. caviae* complex (n=5) has 20bp difference; and the *A. sobria* complex (n=4) has 18bp difference (Table 7). The unique strains of *Aeromonas* (n=10) have a slightly higher magnitude of variability among the species with 31bp difference. For the three

outlier groups, *V. cholerae* and *E. aerogenes* were 31 nucleotides different and *P. aeruginosa* was 46 nucleotides different from all *Aeromonas* type and reference strains.

Among the field isolates collected at the three sampling sites (US, DS, and E) the nucleotide variability is also quite different. The upstream sampling site isolates (n=32) have a variability of 85 nucleotides out of 594 nucleotide fragment (14% variability). For the downstream sampling site (n=30) isolates have 64 variable nucleotides out 594 (11% variability). The isolates of the effluent sampling site (n=28) have 39 variable nucleotides out of 594 (7%varibility). Unfortunately, because the sequences used in the analysis are condensed, partial sequences, one cannot derive accurate amino acid changes based on nucleotide variability because the actual reading frame and codons are not known.

Table 7: Genetic variability among type and reference strains of Aeromonas species for the 16S locus

	***A. hydrophila* complex** (*A. hydrophila, A. bestiarum, and A. salmonicida) n=9*	***A. caviae* complex** (*A. caviae, A. media, A. eucrenophila) n=5*	***A. sobria* complex** (*A. veronii bv sobria, A. trota, A. jandaei, A. schubertii) n=4*	**Unique strains** (*A. enchelia, A. sobria, A. allosacchrophila, A. popoffii, A. spp (HG11 and HG 13) n=10*
Number of differences	9 bp	20bp	18bp	31bp
Percent variability	1.5%	3.4%	3.0%	5.2%

Phylogenetically, the *16S* rDNA locus is able differentiate to the genus level, but the species do not separate out using this locus because it is so highly conserved and similar among the aeromonad species. Figure 5 (Appendix C) shows an unrooted NJ phylogram of all the isolates tested including the type and reference strains. All of the strains have collapsed into one

cluster, with the three outlier groups still evident. This is again because of the relative homogeneity of the locus among aeromonads.

Figure 6 (Appendix C) is a consensus bootstrap (100 replicates) tree of all 123 isolates. It demonstrates the relative homogeneity of the locus, with most bootstrap values less than 50. Briefly, a bootstrap test of phylogeny is one of the most commonly used tests of the reliability of an inferred tree and is evaluated using a bootstrap resampling technique. If there are *m* sequences, each with *n* nucleotides (or codons or amino acids), a phylogenetic tree can be reconstructed using some tree building method. From each sequence, *n* nucleotides are randomly chosen with replacements, giving rise to *m* rows of *n* columns each. These now constitute a new set of sequences. A tree is then reconstructed with these new sequences using the same tree building method as before. Next the topology of this tree is compared to that of the original tree. For each interior branch of the original tree that is different from the bootstrap tree the sequences it partitions is given a score of 0; all other interior branches are given the value 1. This procedure of resampling the sites and the subsequent tree reconstruction is repeated a hundred times, and the percentage of times each interior branch is given a value of 1 is noted. This is known as the bootstrap value. Generally, a bootstrap value of 90 or greater is needed to consider a topology "significant" (Nei and Kumar, 2000). For instance, in the *16S* bootstrap consensus tree constructed using the NJ algorithm, there are bootstrap values equaling one for some internal branch groups. This means that in 100 replications, the clusters occurred only once or one percent of the time. Based on the aforementioned definition, this is not a reliable or significant pairing.

Within the *Aeromonas* genus represented in Figure 6, there are only ten clusters with bootstrap values greater than 50% and only four of the ten clusters were significant with

bootstrap values ≥90%. These were a cluster of two *A. sobria* type strains NCIMB 12065 and ATCC 43979 (99%), a cluster of *A. sp* type strains ATCC 35941 and LMG 13069 (98%), a cluster of two unknown isolates MC3 DS1 11 and MC3 US2 15 (98%), and a cluster of three consisting of one type strain (*A. popoffii* LMG 317541) and two field isolates MC4 US1 1 and MC4 US1 7 (95%). The other six groups consisted of a cluster of one type strain (*A. media* ATCC 33907) and four field isolates MC5 US1 1, MC5 E2 10, MC3 US2 1, and MC3 US1 12 (64%); a cluster of one type strain (*A. hydrophila* ATCC 7966) and two field isolates MC3 DS2 10 and MC5 DS1 14 (83%); a cluster of two type strains *A. eucrenophila* ATCC 23309 and *A. enchelia* LMG 16328 (67%); a large cluster with a bootstrap value of 69% consisting of nine field isolates subdivided into two smaller clusters MC4 E2 7 and MC5 DS 1 11 (61%), and MC3 DS1 16, MC4 E2 14, MC3 US2 5, MC4 US2 5, MC3 DS1 8, MC3 DS2 16, and MC4 US1 10 (63%); a cluster of five type and reference strains with a bootstrap value of 51% also subdivided into two smaller clusters consisting of *A. schubertii* ATCC 43700 and *A. sp HG13* CDC 2478-85 (86%), and *A. veronii bv veronii* ATCC 35624 and *A. veronii bv sobria* CDC 0437-84 (78%) with *A. jandaei* ATCC 49568 associated with the larger cluster. The final large cluster of 11 strains with an overall bootstrap value of 68% is also subdivided into two clusters. The first sub-cluster consist of one type strain (*A. eucrenophila* CECT 4199) and five field isolates MC3 DS1 4, MC4 E1 5, MC3 DS1 14, MC4 DS1 4, MC4 DS1 2 (59%). The second sub-cluster consist of five field isolates MC4 E1 4, MC5 DS2 16, MC5 DS2 10, MC4 E1 6, and MC4 E2 2 (57%). The remainder of the field and type isolates included in the analysis existed as a single large cluster (Figure 7). Figure 7 (appendix C) displays the same bootstrapped data, but as a slanted bootstrap phylogram. In this figure, one can easily detect the vast majority of

Aeromonas strains as a single, large bootstrapped cluster that is distinct from the three other genera (*V. cholera, E. aerogenes, and P. aeruginosa*) 100% of the time.

Figures 8, 9, and 10 (Appendix C) are slanted consensus bootstrap (100 replicate) phylograms for each sampling site (US, DS, E) that include field isolates and type and reference strains based on the species identified by MALDI-MS. Figure 8 is a phylogram (n=47) for the upstream site. There are seven significant clusters with bootstrap values ≥90%. The first cluster (100%) is a group of two type strains of *A. sobria* (NCIMB 12065 and ATCC S3979). The second cluster (94%) are two field isolates MC4 US1 1 and MC4 US1 7. The third cluster (100%) is also of group of two type strains for *A. sp HG11* (ATCC 35941 and LMG 13062). The fourth cluster (100%) consists of three type and reference strains of *A. veronii bv veronii* (ATCC 35624), *A. veronii bv sobria* (CDC 0437-84) and *A. sp HG 13* (CDC 2478-85). The fifth cluster (96%) consists of three field isolates MC4 US1 10, MC3 US2 5, and MC4 US2 5. The sixth cluster (100%) consists of three type and reference strains of *A. trota* ATCC 49657 and *A. caviae* (ATCC 15467 and NCIMB 13016). The final cluster (100%) consists of two field isolates MC3 US1 10 and MC5 US2 12. All of the other isolates and type and reference strains included in the analysis are grouped into a single large cluster.

Figure 9 is a phylogram (n=49) for the downstream sampling site. This tree shows five distinct clusters, four of which are significant (bootstrap value ≥90%) clusters. The first is a large cluster with a bootstrap value of 90%. This cluster consist 23 field isolates and five type and reference strains *A. media* (ATCC 33907 and CDC 0862-83), *A. hydrophila* ATCC 7966, and *A. bestiarum* (ATCC 13444 and 23211). The second cluster (97%) consist of three field isolates MC4 DS2 9, MC5 DS1 5, and MC4 DS2 6, and three type strains *A. trota* ATCC 49657 and *A. caviae* (ATCC 15406 and NCIMB 13016). Also, field isolate MC5 DS2 13 is associated

with this cluster and has a bootstrap value of 92%. The third cluster (99%) is a group of two type strains of *A. sobria* (NCIMB 12065 and ATCC $3979). The fourth cluster (100%) is also of group of two type strains for *A. sp HG11* (ATCC 35941 and LMG 13062). The remainder of the field and type/reference isolates fall into a single cluster.

Figure 10 is a phylogram (n=42) for the effluent sampling site. There are six significant (≥90%) clusters within the tree. The first is a cluster (98%)of two type strains of *A. bestiarum* (ATCC 23211 and 13444) and four field isolates MC3 E2 9, MC5 E2 13, MC3 E1 4, and MC3 E2 5. The second cluster (100%) is a group of two type strains of *A. sobria* (NCIMB 12065 and ATCC $3979). The third cluster (100%) is also of group of two type strains for *A. sp HG11* (ATCC 35941 and LMG 13062). The fourth cluster (98%) is a group of two type strains for *A. veronii bv veronii* (ATCC 35624) and *A. veronii bv sobria* (CDC 0437-84) and four field isolates MC4 E2 2, MC4 E1 4, MC4 E1 5 and MC4 E1 6. The four aforementioned clusters are included within a large fifth cluster (96%) that, in addition to the previously mentioned isolates, contains three type strains for *A. hydrophila* (ATCC 7966) and *A. media* (ATCC 33907 and CDC 0862-83) and 13 field isolates. The sixth cluster (no bootstrap value reported) consists of the remainder of the field and type isolates tested.

4.4 Gyrase B Sequence Analysis

As for the analyses of the *16S* locus, the analyses of the *gyrB* locus included the 106 isolate sequences plus 22 additional type and reference strains that were extracted from GenBank (Appendix B), all of which were included to be representative of the 17 DNA HGs of the genus. Comparing all of the isolates and type strains, the overall nucleotide difference was 229bp of 708bp or 33 percent variability. .

Among the 22 type and reference strains, the overall nucleotide difference of the 708bp fragment is 202bp or 29% variability. Table 8 shows the genetic variability within each of the complexes and among the unique species. The unique strains (n=7) show the most variability and the *A. hydrophila* complex (n=5) are the least variable. For the outlier strains included in the analysis, *V. cholera* was 245 nucleotides different and *E. aerogenes and P. aeruginosa* were 83 nucleotides different from all *Aeromonas* type and reference strains.

Among the field isolates collected from each respective sampling site (US, DS, and E), there was also a difference in the observed variability between sites. The isolates collected from the upstream sampling site (n=41) had 142 variable nucleotide sites within the 708bp partial sequence (20% variability). The field isolates collected from the downstream site (n=36) had 146 variable nucleotides (21% variability). The effluent site isolates (n=29) had the lowest amount of variable nucleotides with 133 variable sites out of the 708 nucleotide partial sequence (19%variability). As with the *16S* site no amino acid substitutions could be deduced from the condensed, partial sequences because the reading frame is unknown and the codons are arbitrarily assigned.

Table 8: Genetic variability among Aeromonas species for the gyrB locus

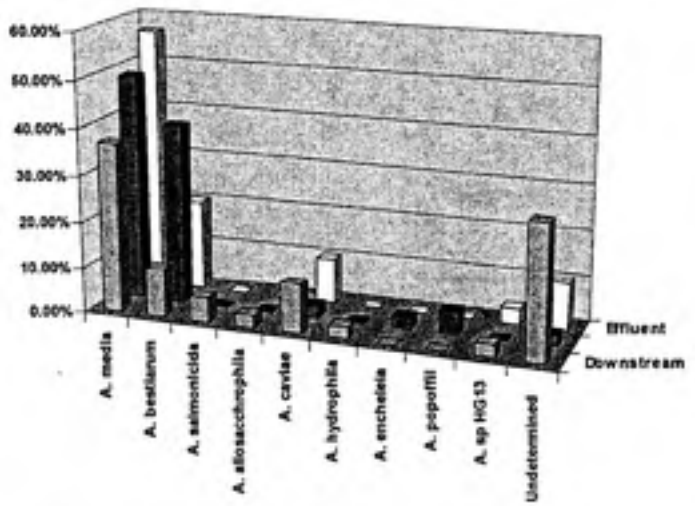
	A. hydrophila* complex** (*A. hydrophila, A. bestiarum, and A. salmonicida*) ***n=5	***A. caviae* complex** (*A. caviae, A. media, A. eucrenophila*) ***n=5***	***A. sobria* complex** (*A. veronii bv sobria, A. trota, A. jandaei, A. schubertii*) ***n=5***	**Unique strains** (*A. enchelia, A. sobria, A. allosacchrophila, A. popoffii, A. spp (HG11 and HG 13)* ***n=7***
Number of differences	73 bp	88bp	102bp	135bp
Percent variability	10%	12.5%	14.5%	19.1%

Figure 11 (Appendix C) shows the consensus NJ bootstrap (100 replicates) for the *gyrB* locus. Figure 12 (Appendix C) shows a condensed slanted consensus bootstrap (100 replicates) tree showing only significant clusters with bootstrap values ≥90%. All significant clusters mentioned above remain and the other branches with bootstrap values less than 90% are collapsed. From these two trees, there are numerous clusters that are species informative. The first is a large cluster (100%) that includes 51 field isolates from all sampling stations and two type/reference isolates of *A. media* (CECT 4234 and CECT 4232). The second cluster (100%) contains one field isolate MC5 US2 12 and two type/reference strains of *A. encheleia* (CECT 4856 and CECT 4342). The third species informative cluster (100%) contains two type strains of *A. salmonicida* (CECT 894 and CECT 4237) and two field isolates MC3 DS1 10 and MC5 DS2 3. The fourth species informative cluster (100%) contains one reference strain of *A. popoffii* and two field isolates MC4 US1 1 and MC4 US1 4. The two aforementioned clusters are included within a fifth larger cluster (100%) that contains one type strain of *A. bestiarum* (LMG 13662) and 30 field isolates from all sampling stations. The fifth species informative cluster (100%) contains field isolate MC3 DS1 14 paired with a reference strain of *A. allosacchrophila*. The sixth cluster (99%) that is species informative contains field isolates MC5 DS1 14 and MC3 DS2 10 and two type strains of *A. hydrophila* (ATCC 49140 and CECT 839). The seventh and final cluster (100%) consists of ten field isolates and two type/reference strains of *A. punctata (caviae)* (CECT 4221) and *A. sp HG13* (ATCC 43946). In this last locus, species were determined by location relative to the two type and reference strains with in the cluster. If an isolate did not associate with a cluster, it was designated as undetermined. All species determinations for this locus can be found in Appendix E.

Figure 13 shows the percentage of each species determined by gyrase B sequence analysis for each sampling location. The most prevalent species in the downstream sampling site was *A. media* (36%) followed closely by *A. bestiarum* (28%). *Aeromonas caviae* and undetermined *Aeromonas species* both made up 11% of the isolates. The remainder of the isolates were *A. salmonicida* (5%) and *A. allosacchrophila, A. hydrophila,* and *A. sp HG13* each comprising 3% percent of the total isolates (n=38).

The upstream site also had *A. media* as the most prevalent species, making up 50% of the total isolates (n=41). The next most prevalent species was *A. bestiarum* (39%) followed by *A. popoffii* with five percent. The remaining isolates were *A. encheleia* and undetermined aeromonads with two percent each.

The effluent sampling site was also dominated by *A. media,* which comprised 57% of the total isolates (n=30). *A. bestiarum* comprised 20%, with *A.* caviae and undetermined aeromonads each representing 10% of the total isolates, respectively. The remainder of the isolates was *A. sp HG13*, making up only 3% of the total isolates tested from this location.



	A. media	A. bestiarum	A. salmonicida	A. allosacchrophila	A. caviae	A. hydrophila	A. encheleia	A. popoffii	A. sp HG13	Undetermined
Downstream	36.84%	10.53%	5.26%	2.63%	10.53%	2.63%	0.00%	0.00%	2.63%	28.95%
Upstream	48.78%	39.02%	0.00%	0.00%	2.44%	0.00%	2.44%	4.88%	0.00%	2.44%
Effluent	56.67%	20.00%	0.00%	0.00%	10.00%	0.00%	0.00%	0.00%	3.33%	10.00%

Figure 13: Percentage of species by sampling site from Gyrase B analysis

Figure 14 shows the total percentage of each species for all isolates tested (n=109) using the gyrase B locus. *Aeromonas media* was the most prevalent species in all isolates tested, with 46% of the total. The next most prevalent was *A. bestiarum* comprising nearly a quarter of the isolate with 24%. This was followed by undetermined aeromonads (14%), *A. caviae* (7%), *A. salmonicida* (2%), *A. popoffii* (2%), and *A. sp HG13* (2%). The final three species were *A. hydrophila, A. encheleia,* and *A. allosacchrophila* making up one percent, respectively.

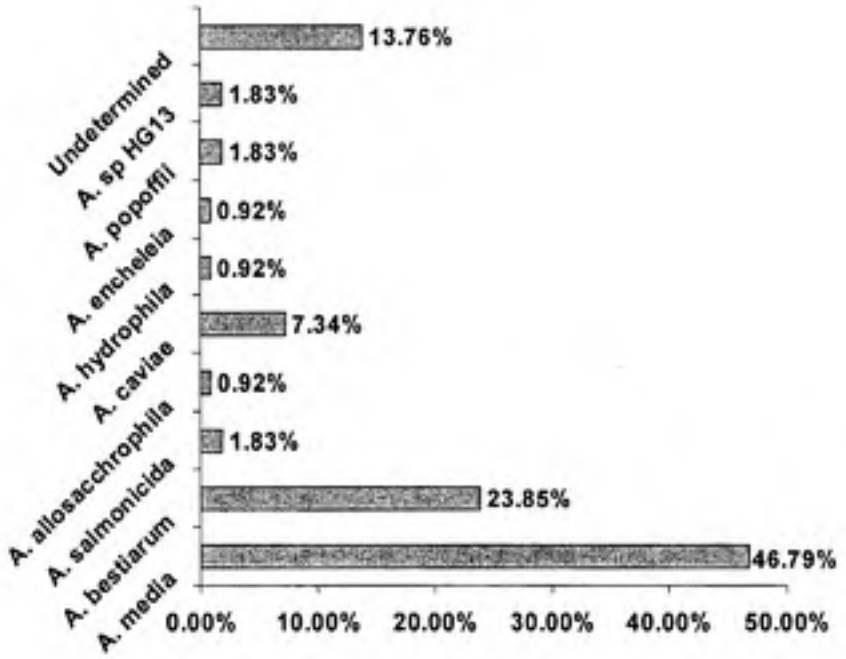


Figure 14: Percentage of total isolates from Gyrase B analysis

4.5 MALDI-MS Species Determination

Phylogenetically, MALDI-MS is able to separate species within the genus *Aeromonas* with a much higher resolution than that of *16S* and equal to that of Gyrase B sequences. Figure 15 (Appendix C) shows the phylogenetic tree generated by Dr. Maura Donohue, for the 133 isolates. The tree separates into six distinct clusters. Within each cluster, the isolates group together with positive controls, suggesting speciation.

Appendix D is a list of isolates, the complex level data from biochemical phenotyping, and species calls based on MALDI-MS m/z signatures. Among the isolates collected from the downstream collection site, a preponderance of isolates was classified as *A. media/A. caviae HG5B* (20). The remainder of the isolates collected were classified as follows: *A. sobria* (7), *A. hydrophila* (3), *A. hydrophila-like* (1), *A. bestiarum* (8), *A. caviae* (6), *A. sp HG11* (1), and *A.*

veronii bv sobria (1). In contrast to the downstream site, the upstream collection site was equally dominated by *A. media/A. caviae HG5B* (19) and *A. bestiarum* (18). The remainder of the isolates for this collection site was made up of *A. caviae* (3) and *A. bestiarum/A. hydrophila-like HG3* (1). The effluent sampling site species profile is similar to that of the downstream site. The effluent site is again dominated by *A. media/A. caviae HG5B* (23). The additional isolates are composed of the following species: *A. bestiarum* (6), *A. caviae* (2), *A. trota* (3), *A. hydrophila* (1), *A. veronii bv veronii* (1), and *A. veronii bv sobria* (1).

Figure 16 summarizes the above data as a percentage of species per sampling site. For the downstream site, *A. media/caviae HG5B* makes up 44% of the isolates. *A. bestiarum, A. sobria,* and *A. caviae* together comprise another large percentage of the isolates with 17%, 15%, and 13%, respectively. The remainder of the isolates is composed of *A. hydrophila* with 7% percent and *A. hydrophila-like HG3 and A. sp HG11* both making up 2% percent of the isolates collected from the downstream site. For the upstream collection site, *A. media/caviae HG5B* and *A. bestiarum* share nearly equal representation, making up 47% and 44% of the isolates collected, respectively. The rest of the isolates collected from the upstream site are comprised of 7% *A. caviae* and 2% percent *A. hydrophila-like HG3.* The majority of the isolates collected from the effluent sample site are *A. media/caviae HG5B* – making up 62% of the isolates. The next most abundant species collected was *A. bestiarum* comprising 16% of the isolates. The rest of the isolates collected from the effluent site were *A. trota* (8%), *A. caviae* (6%), and *A. hydrophila* and *A. veronii(bv sobria and veronii)* each making up 3% percent.

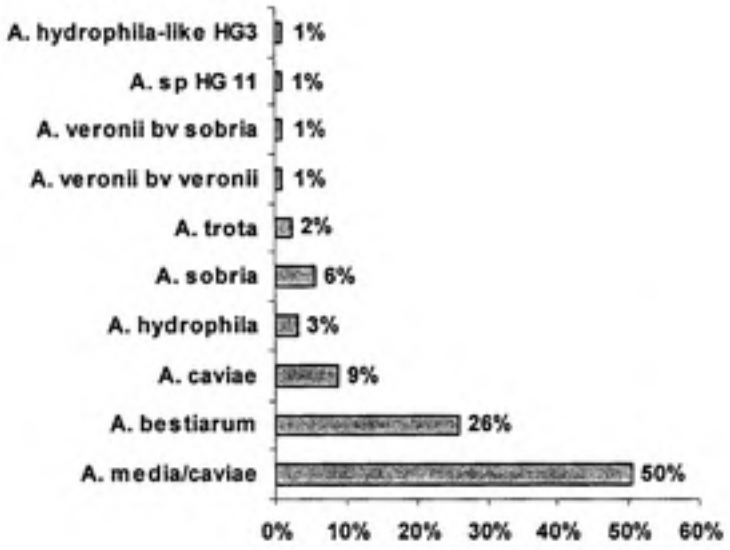


Figure 17: Percentage of total isolates from MALDI-MS analysis

4.6 Statistical Analysis of MALDI-MS and Gyrase B analysis

For the MALDI-MS and Gyrase B data, a chi-square test was used to analyze the independence of each sampling site. Three different analysis were done using a 2 x 3 contingency table, as described in the methods (see section 3.6), to compare the upstream, downstream, and effluent sampling sites. For each test the two most prevalent species in all sampling sites (*A. media* and *A. bestiarum)* were used and all other species were grouped to get one number. The H_o is that there is no relationship between sampling site location and the species profile of the isolates collected in them. Tables 9 and 10 summarize the findings for MALDI-MS and Gyrase B, respectively.

Table 9: Summary of Chi-square analysis for MALDI-MS α=0.05

All sites	X^2 value – 17.2548 Deg of Freedom – 4 Probability – 0.0017
Effluent versus Upstream	X^2 value – 7.529 Deg of Freedom – 2 Probability – 0.0232
Effluent versus Downstream	X^2 value – 3.405 Deg of Freedom – 2 Probability – 0.1822
Upstream versus Downstream	X^2 value – 12.5349 Deg of Freedom – 2 Probability – 0.0019

Table 10: Summary of Chi-square analysis for Gyrase B sequence analysis α=0.05

All sites	X^2 value – 19.932 Deg of Freedom – 4 Probability –0.0005
Effluent versus Upstream	X^2 value – 3.502 Deg of Freedom – 2 Probability – 0.1736
Effluent versus Downstream	X^2 value – 6.039 Deg of Freedom – 2 Probability – 0.0475
Upstream versus Downstream	X^2 value – 17.170 Deg of Freedom – 2 Probability – 0.0002

Tables 9 and 10 show that we can reject the null hypothesis for the effluent versus upstream and the upstream versus downstream tests because the probability values were less than an α of 0.05. This means that the species profiles were different between these sampling sites.

This does not, however, hold true to the effluent versus downstream test. Here there is an association between the two collection sites, and the species profiles are similar.

Table 11a: Mean number of bacteria identified from all sample sites using MALDI-MS and gyrB sequence analysis

Method	Number of Sampling Sites	Mean Number of Bacteria Identified	Std. Deviation	Std. Error Mean
MALDI-MS	3	41.33	4.509	2.603
GyrB	3	31.33	7.506	4.333

Table 11b: Two tailed independent samples t-test comparing the mean number of bacteria identified using MALDI-MS and gyrB sequence analysis

	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confiden Interval of th Difference	
						Upper	Lo
Equal variances assumed	1.978	4	.119	10.000	5.055	-4.036	2
Equal variances not assumed	1.978	3.277	.135	10.000	5.055	-5.345	2

Tables 11 (a and b) and 12 (a and b) show the data for each t-test performed (see section 3.6). Table 11a compares the average mean number of bacteria identified from all sampling sites. MALDI-MS was able to identify an average of 41.33 bacteria, while *gyrB* was able to identify an average of 31.33 bacteria. Table 11b compares these means statistically, but demonstrates that there is not a significant (< 0.05) difference in the mean number of bacteria identified by each method.

Table 12a: Mean number of species identified from all sample sites using MALDI-MS and gyrB sequence analysis

Method	Number of Sampling Sites	Mean Number of Species Identified	Std. Deviation	Std. Error Mean
MALDI-MS	3	6.00	1.732	1.000
GyrB	3	5.33	1.528	.882

Table 12b: Two tailed independent samples t-test comparing the mean number of species identified using MALDI-MS and gyrB sequence analysis

	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confiden Interval of th Difference	
						Upper	Lo
Equal variances assumed	.182	.692	.500	4	.643	.667	
Equal variances not assumed			.500	3.938	.644	.667	

Table 12a compares the mean number of species identified from all three sampling sites. MALDI-MS was able to identify an average of 6 species compared to a mean of 5.33 species for *gyrB* method. Table 12b statistically compares the mean number of species identified using each method, but demonstrates that there is not a significant (<0.05) difference in the number of species identified using either method.

5.0 Discussion

The taxonomy of the genus *Aeromonas* has been a challenging endeavor leading to numerous difficulties in identifying species within the genus, especially for those aeromonads that are of environmental origin. Twenty to thirty years ago most scientists working with

Aeromonas relied on numerous biochemical tests to resolve species differences. To date, the *Aerokey II* (Carnahan et al, 1991) is the most used biochemical method for species determination of clinical isolates, but has poor resolving power in determining species for environmental and veterinary isolates. As methods improved, especially with the advent of DNA/DNA hybridization, species determination became easier, but even this method lacked the ability to fully resolve all of the species. Other methods including single locus PCR (*16S*), Fatty Acid Methyl-esters analysis (FAMES), and ribotyping proved to have limited success in the resolution of species within the genus *Aeromonas* (Marinetti and Altwegg, 1992; Altwegg, 1993; Huys et al, 1994). Several new methods, including MLST and MALDI-MS, have shown promise in deciphering the complex networks of species within the genus *Aeromonas*. It is the results of these methods that are discussed in the following paragraphs.

5.1 *16S* and Gyrase B Genes

Although the intent was to perform an MLST analysis on the isolates, this was not the outcome because only two genetic loci were successfully analyzed: 16 rDNA and gyrase B. An analysis of a third locus, *rpoD*, was attempted, but unfortunately the primers used were unable to amplify any gene product. Of the genes that were analyzed, only gyrase B showed significant promise for speciation. The *16S* locus, as stated previously, is highly conserved within this genus. The overall genetic variability for the *16S* locus was 19% among all of the isolates and only 5% among the type and reference strains. This lack of variability among the type and reference strains is likely due to these being, essentially, laboratory strains maintained by various culture collections. These laboratory bacteria are maintained in pure cultures and are grown in optimal conditions. Conversely, the environmental isolates are exposed to increased selective pressures and interact in the environment with other bacteria, both of which can allow for the

accumulation of mutations and recombination events. It is this lack of genetic variability that does not allow for clear speciation of the genus *Aeromonas* using this locus. Indeed, as the phylograms indicate, there is almost no variability, resulting in the bacteria essentially residing within one large cluster. The only significant species informative cluster in the large NJ tree was the *A. popoffii* pairing with two field isolates.

As mentioned in the results section, an attempt was made to improve the resolving power by limiting the type strains to only those species identified by MALDI-MS and by grouping the isolates by collection site. This definitely improved the readability of the trees, but there were few significant (>90%) clusters. In the downstream sample, the large cluster contained numerous field isolates, but none occurred in combination with one single type/reference strain. In fact, this cluster had six different type/reference strains for three different species (*A. media, A. hydrophila, and A. media*). The other species informative cluster in the downstream sample was a cluster of three type/reference strains for two species (*A. caviae* and *A. trota*) and four field isolates. But, as with the previous cluster, it is difficult to determine in which complex these isolates belong, much less to which species they correspond. The upstream sample site tree had only one significant species informative cluster. This was a cluster of three type/reference strains for two different species (*A. caviae* and *A. trota*) grouped to a single field isolate. As with the other previously mentioned clusters, it was again difficult to determine complex or species determinations. The effluent site tree had two significant species informative clusters. The first is a single cluster of two type/reference strains of *A. bestiarum* and four field isolates. The second cluster is two type/reference strains for *A. veronii* (*bv sobria and veronii*) that grouped with four field isolates. Out of 97 field isolates included in the analysis, a total of only nine isolates could be definitively speciated.

This lack of species resolving power was expected for the *16S* gene locus, because it is well documented that there is no *16S* rDNA- based PCR that can separate the aeromonad species from one another. Simply put, the species are just too closely related genetically to each other. Several pairs of "bona fide" species by conventional biochemical characterization and DNA-DNA hybridization are only 1-3 base pairs different from one another along the entire 1500bp *16S* region (Martinez-Murcia et al, 1992; Horneman, 2006). However, the *16S* locus does have the ability to differentiate to the genus level. Included in the analysis were three outlier genera of related γ-subclass of proteobacteria. The *16S* locus was able to differentiate the outlier isolates from the field and type/reference isolates 100% of the time. Carnahan (2001), reported the same findings for the *16S* locus using similar outlier genera.

The gyrase B locus proved to have a good deal more variability, with 33% difference among all strains and 29% among the type and reference strains. This increased variability of the locus improved the resolving power compared to *16S*, and allowed for species level determinations of a majority (86%) of the isolates included in the analysis (n=109) of this locus. This is consistent with reports by several researchers (Yanez et al, 2003; Soler et al, 2004; Kupfer et al, 2006), who report a high level of resolution using the universal primers for the gyrase B region (see methods) that allow for the determination of intra-species differences, strain identifications, and species identification following a phylogenetic frame.

5.2 MALDI-MS

In contrast to the variable resolution obtained with the DNA sequences – poor for *16S* but better for gryB – MALDI-MS is able to make distinct species calls using three algorithms (see methods). This analytical method has a high level of resolution in making species calls and produces highly reproducible peaks under constant conditions. The data provided here

demonstrate some interesting trends. The isolates obtained from the sediment in and around the WWTP are relatively low in diversity encompassing only nine species. Additionally, the upstream and downstream sampling sites are unique in their species profiles. This observation is important given the profile of the effluent species. The effluent appears to have an additive effect on the downstream site, where most of the species found in the sediment around the effluent outfall are also found downstream but not upstream. The relationships in species diversity among the upstream, effluent and downstream stations is indicative of potential contamination and either persistence or re-growth (or both) in the environment of some of the species isolated at the effluent site. These findings are corroborated by the statistical analysis of the MALDI-MS data, which found that the upstream site is independent of (significantly different from) the downstream and effluent sampling sites in terms of species composition and prevalence. The statistical analysis also showed that the effluent and downstream sites are not independent (not significantly different) ($p=0.1822$) and have a similar species profile. This supports the hypothesis that the effluent is contributory to the increased species diversity found in the downstream sampling site, including several pathogenic strains.

5.3 Pathogenicity and Public Health Significance

Many of the isolates collected around the WWTP are considered human or fish pathogens. *A. bestiarum* is a known fish pathogen causing furunculosis, and it was found in great abundance in all sampling sites. Those species considered human pathogens found in low numbers among the isolates are *A. hydrophila, A. caviae, A. trota, A. veronii biovar sobria, A. veronii biovar veronii, and A. sobria.* Because these species are not found in high quantities, they may not represent a clear threat to the public health, but it is important to recognize their presence in the sediment, given that Morgan Creek is in the Jordan Lake watershed.

The water where the sediment was sampled is classified as class WS-IV water by the North Carolina Division of Water Quality. This means these waters are protected for secondary recreation, fishing, wildlife, fish and aquatic life propagation and survival, agriculture, and other uses. Secondary recreation includes wading, boating, and other uses involving human body contact with water, where such activities take place in an infrequent, unorganized, or incidental manner. Additionally, they can be used as sources of potable water. That being said, in the area around the WWTP, this water is not used for any secondary recreational purposes, though incidental contact with irrigation waters supplied from the creek is possible on the golf course adjacent to the creek.

Jordan Lake provides a source of municipal drinking water to Cary, North Carolina, and is a large primary contact recreational area for much of central North Carolina. Given the presence of these pathogenic species in the sediment of a tributary of Jordan Lake, and the potential for proliferation in the environment, these waters downstream of the wastewater treatment plant should be monitored for contamination by the pathogenic strains of aeromonads. This is especially true after a large storm event when flushing of these aeromonads downstream in large numbers could occur. Generally, when the creek is at normal to low stage, the presence of these pathogenic strains is of lower risk because the flow of the water is slow and impaired by deadfall and other debris. Therefore, these bacteria are not likely to make it downstream the approximately 20 miles to Jordan Lake. This topic will be considered in more detail in the following section.

5.4 Sediment- Versus Water-Associated Aeromonads

All of the isolates collected in this research study were sediment associated. Given this fact, only inferences can be made about which bacterial species are present in the water column. This is an important fact to consider, especially given the public health importance of the pathogenic *Aeromonas* strains found in this study. Similar gram-negative bacteria, including vibrios and pseudomonads, routinely form biofilms on sediments and other surfaces in order to persist, grow, and survive (Hall-Stoodley et al, 2004). Indeed, most bacteria tend toward association with sediments and surfaces because of the selective advantages of being in a biofilm: Decreased predation, a stable environment in which to grow, increased resistance to environmental stressors, easy acquisition of nutrients, and a safe mode of environmental dispersal (Hall-Stoodley et al, 2004). This being said, the species profile of sessile or sediment associated aeromonads could be completely different than those found as planktonic, free-living aeromonads in the water column.

5.4.1 Biofilms and Gene Transfer

Biofilms are not usually composed of a single genus of bacterium, but are often a colony of several genera of bacteria grouped together. Because of this, it is also important to consider that the association of aeromonads with other bacteria within biofilms in sediment makes the possibility of horizontal gene transfer more likely. Sorenson et al (2005) found that biofilms are uniquely suited for horizontal gene transfer because they sustain high bacterial density and metabolic activity, even in harsh environments. Some of the mobile elements that they identified were plasmids, insertion sequences, transposons, integrons, pathogenicity islands, and

bacteriophages. It is these elements that are transferred indiscriminately in biofilms that can contribute to the plasticity of the bacterial genome.

5.5 Future Research

The focus of future research should be primarily on the development of methods for the accurate and rapid identification of *Aeromonas* in the environment. As with all microbiological research of potential human health importance, most of the methods development takes place in the clinical setting. However, this is often not sufficient for reliable application to environmental samples. A focus on developing methods to identify *Aeromonas* should allow researchers to better determine which species are often the most prevalent and which ones are potentially the most virulent, including those detected in the environment and their relationship to those detected in clinical samples. Other research should focus specifically on better characterization and quantification of the virulence and pathogenicity of the aeromonads found in this study, and to determine if they are the same as or are distinctly different from clinical strains in these properties. This research should also focus on transmission pathways and mechanisms of infection and disease in humans and what routes of exposure are most effective when infecting a host.

For this sampling area, future research should focus on several aspects. A quantification and characterization of aeromonads in and around the WWTP should be undertaken. Because these isolates were collected over a short course of time during the summer, additional isolates should be collected over a yearly period to capture seasonal data and should include raw sewage influent, effluent, upstream, and numerous downstream points, including Jordan Lake. An additional area of research should focus on aeromonad survival, proliferation and gene transfer

mechanisms for antibiotic resistance and virulence traits in the environment and in WWTPs, with special attention being paid to the role of biofilms. These could either be done in conjunction with seasonal collection of isolates, or as discrete, bench-top experiments where conditions can be easily manipulated to test a range of environmental variables and their conditions. This is especially important given the renewed interest in the use of reclaimed waters by OWASA and the University of North Carolina – Chapel Hill. A third area of research should focus on the potential resistance of these aeromonads to chlorine and UV disinfection, the two disinfection methods applied to the wastewater and also widely used for drinking water. This is important for both reuse and for drinking water quality.

Other studies could also be undertaken to better characterize the ecology of aeromonads, and to determine what factors and conditions in the environment and in the various Aeromonas species allows the bacteria be able to persist and proliferate in numerous environmental media and at different environmental conditions. Along similar lines, more research should focus on the ecology of effluent discharged aeromonads. This should include efforts to determine if they are distinctly different from environmental isolates in their species, virulence and antibiotic resistance traits and their potential sources (i.e. can they be traced back to a human source, or are they part of the normal flora of a WWTP).

Other research should also be undertaken to further characterize aeromonad antimicrobial resistance properties. Past research (Bronsted, 2004) focused on only four antibiotics as determined by phenotypic resistance and on molecular characterization of quinolone resistance genes for these same isolates (Folan, 2007). This characterization (both phenotypic and

genotypic) should be greatly expanded to include resistance to several different classes of antibiotics, given the sewage contribution of a large hospital to the sewage treated by the WWTP.

Finally, one of the major shortcomings of this study was the failure of the *rpoD* PCR protocol. Given that previous studies have found this to be an informative genomic site, new primers should be selected and tested for their ability to detect and speciate the isolates used in this study and future isolates. Furthermore, attention should be given to expanding the list of other genetically and phylogenetically informative gene loci within the aeromonad genome.

6.0 Conclusions

The rapid and specific detection of infectious microorganisms in both clinical and environmental samples is a public health priority. This report describes the attempted use of multilocus sequence typing, based on the *16S* rDNA gene and two housekeeping genes *gyrB* and *rpoD*, as well as a MALDI–MS analytical chemical method applied to whole intact cells, to detect and differentiate between species of waterborne aeromonads, including the potential human pathogen *A. hydrophila*, recovered from creek sediments

The attempt to include the housekeeping gene *rpoD* in the multilocus PCR approach was unsuccessful. The published primers used to amplify the *rpoD* gene of the *Aeromonas* strains of this study were incapable of amplifying *Aeromonas* DNA in the hands of this investigator. As stated previously, the primers for the *rpoD* locus failed to anneal because the published sequence was incorrect for several nucleotides. This prevented the annealing of the primer to the target site. It should also be noted that on further inspection the primers were also found to be very close together on the genome and would not produce an amplicon that could be visualized.

The results of this study indicate that a multilocus sequenced typing approach based on PCR amplification and sequencing of two loci, *16S* rDNA and the housekeeping gene GyraseB

did provide sufficiently robust data for definitive species determination within this genus. However, only one of the two loci yielded sequence information that distinguished *Aeromonas* species. The Gyrase B locus was highly informative and was able to accurately speciate 86% of the isolates tested.

The other promising analytical method used to speciate environmental aeromonads was MALDI-MS. MALDI–MS fingerprinting of bacteria, when compared to other methods employed, offers significant advantages for the identification of aeromonads and for their differentiation at the species level. These advantages are (1) the capability to detect numerous proteins within a single sample, (2) high throughput, and (3) relatively simple sample preparation techniques. Additionally, MALDI-MS produces accurate and reproducible results within a single run, and the process is fast – taking less than five minutes per sample.

Neither of the two methods used in this study, when compared statistically using a t-test, were significantly different in the average number of species or bacteria they were able to identify. Observationally, however, the MALDI-MS method was able to identify on average more species and more bacteria than the *gyrB* locus. Because of this and because of the aforementioned reasons, MALDI-MS appears to be more ideally suited for environmental monitoring.

From the results of this study it can be concluded that:

1) MALDI-MS provided robust and internally validated data when applied to the *Aeromonas* environmental isolates of this study by resolving and identifying nine distinct species within the isolate set analyzed;
2) Multilocus sequence typing based on the *16S* rDNA and gyrase B loci investigated in this study did provide an adequate level of resolution to make species-level

identifications for 86% of the environmental isolates tested, with GryB providing definitive speciation sequence data;

3) More research needs to be done using these loci and perhaps other housekeeping gene sequences in an effort to achieve a multilocus sequence typing method that is capable of delineating with accuracy the species within the genus *Aeromonas*. This research should include further characterization of these isolates on the basis of the *rpoD* housekeeping gene locus, which was not successfully amplified in this study;
4) The sewage effluent outfall appears to be a contributor to the overall species composition and diversity of Morgan Creek. This impact was based on the presence and predominance of certain *Aeromonas* species isolates detected in Morgan Creek sediments at the effluent discharge site and at the downstream when compared to the species profile and prevalence of upstream isolate species; and
5) Potentially pathogenic species of *Aeromonas*, notably *A. hydrophila, A. caviae, A. trota, A. veronii biovar sobria, A. veronii biovar veronii, and A. sobria* were present in the effluent outfall and downstream sediments. The presence of these *Aeromonas* species creates the potential for their persistence and possible regrowth in the environment and subsequent downstream human exposures. Therefore, further work needs to be done to characterize the virulence properties of these potentially pathogenic *Aeromonas* species isolates.

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Appendix A – Biochemical Test Results (Bronsted, 2005)

Isolate	Complex	Indole	Oxidase	MAC	KIA	VP	Larab	BE
MC3 DS1 1	A. Caviae	+	+	-	*	-	+	+
MC3 DS1 2	A. Caviae	+	+	-	*	-	+	+
MC3 DS1 4	A. Caviae	+	+	-	+	-	-	+
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. Caviae	+	+	-	*	-	-	-
MC4 DS1 6	A. Caviae	+	+	-	*	-	+	+
MC4 DS1 12	A. Caviae	+	+	-	*	-	+	+
MC4 DS1 16	A. Caviae	+	+	-	*	-	+	+
MC4 DS2 1	A. (genus level only)	+	+	-			+	-
MC4 DS2 4	A. (genus level only)	-	+	-			+	+
MC4 DS2 6	A. (genus level only)	+	+	-			+	+
MC4 DS2 9	A. (genus level only)	+	+	-			+	+
MC5 DS1 4	A. Hydrophila	+	+	-	red tip g(ah)	+	+	+
MC5 DS1 5	A. Caviae	+	+	-	a/a	-	+	+
MC5 DS1 7	A. Hydrophila	+	+	-	a/a g	-	+	+
MC5 DS1 8	A. Caviae	+	+	-	a/a	-	+	+
MC5 DS1 11	A. Caviae	+	+	-	a/a	-	+	+
MC5 DS1 14	A. Hydrophila	+	+	-	a/a g	-	+	+
MC5 DS1 17	A. Caviae	+	+	-	a/a	-	+	+
MC5 DS2 1	A. Caviae	+	+	-	a/a	-	+	+
MC5 DS2 2	A. Caviae	+	+	-	a/a	-	+	+
MC5 DS2 3	A. Hydrophila	+	+	-	(a/h)	+	+	+
MC5 DS2 5	A. Hydrophila	+	+	-	(a/h)	-	+	+
MC5 DS2 6	A. Caviae	+	+	-	a/a	-	+	+
MC5 DS2 8	A. Caviae	+	+	-	a/a	-	+	+
MC5 DS2 10	A. Caviae	+	+	-		-	+	-
MC5 DS2 11	A. Caviae	-	+	-		-	+	+
MC5 DS2 12	A. Hydrophila	+	+	-	a/a g	-	+	+

Appendix A – Biochemical Test Results (Bronsted, 2005)

Isolate	Complex	Indole	Oxidase	MAC	KIA	VP	Larab	BE
MC5 DS2 13	A. Caviae	+	+	-	a/a	-	+	+
MC5 DS2 16	A. Caviae	+	+	-		-	+	-
MC5 DS2 17	A. Caviae	+	+	-	a/a	-	+	+
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	A. Caviae	+	+	-	a/a	-	+	+
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 5	A. Caviae							+
MC4 E1 6	A. Caviae							+
MC4 E1 7	A. Caviae	+	+	-	y/y	-	+	+
MC4 E1 10	A. Hydrophila	+	+	-	*	+	+	+
MC4 E1 14	A. Caviae	+	+	-	y/y	-	+	+
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. Caviae	+	+	-	*	-	-	-
MC4 E2 9	A. Caviae	+	+	-	+	+	-	-
MC4 E2 13	A. Caviae	+	+	-	*	-	+	+
MC4 E2 14	A. Caviae	+	+	-	*	-	+	+
MC4 E2 15	A. Sobria	+	+	-	+	+	-	-
MC4 E2 16	A. Caviae	+	+	-	*	-	+	+
MC5 E1 2	A. Caviae	+	+	-	a/a	-	+	+
MC5 E1 4	A. Caviae	+	+	-	a/a	-	+	+
MC5 E1 12	A. Caviae	+	+	-	a/a	-	+	+
MC5 E1 15	A. Caviae	+	+	-	a/a	-	+	+
MC5 E1 17	A. Caviae	+	+	-	a/a	-	+	+
MC5 E1 19	A. Caviae	+	+	-	a/a	-	+	+
MC5 E2 1	A. Caviae	+	+	-	a/a	-	+	+
MC5 E2 2	A. Caviae	+	+	-	a/a	-	+	+

Appendix A – Biochemical Test Results (Bronsted, 2005)

Isolate	Complex	Indole	Oxidase	MAC	KIA	VP	Larab	BE
MC5 E2 4	A. Caviae	+	+	-	a/a	-	+	+
MC5 E2 5	A. Caviae	+	+	-	a/a	-	+	+
MC5 E2 6	A. Caviae	+	+	-	a/a	-	+	+
MC5 E2 7	A. Caviae	+	+	-	a/a	-	+	+
MC5 E2 9	A. Caviae	+	+	-	a/a	-	+	+
MC5 E2 10	A. Caviae	+	+	-	a/a	-	+	+
MC5 E2 13	A. Caviae	-	+	-	a/a	-	+	+
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. Sobria	+	+	-	*	-	+	+
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	+	+	-	+	+	+	-

Appendix A – Biochemical Test Results (Bronsted, 2005)

Isolate	Complex	Indole	Oxidase	MAC	KIA	VP	Larab	BE
MC4 US2 16	A. Caviae	+	+	-	*	-	+	+
MC5 US1 1	A. Caviae	+	+	-	a/a	-	+	+
MC5 US1 3	A. Hydrophila	+	+	-	a/a g	-	+	+
MC5 US1 6	A. Caviae	+	+	-	a/a	-	+	+
MC5 US1 8	A. Caviae	+	+	-	a/a	-	+	+
MC5 US1 13	A. Hydrophila	+	+	-	red tip g (ah)	+	+	+
MC5 US1 15	A. Hydrophila	+	+	-	red tip g (ah)	+	+	+
MC5 US1 18	A. Hydrophila	+	+	-	red tip g (ah)	-	+	+
MC5 US2 5	A. Hydrophila	+	+	-	a/a	+	+	+
MC5 US2 12	A. Hydrophila	+	+	-	a/a g	-	+	+

Legend for Appendix A	
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-Prokauer
Larab	L-arabinose fermentation
BE	Bile Esculin
Complex	Grouping according to Abbott et al., 2003
+	Indicates positive result
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. hydrophilla* control)
(ah)	For "TSI": identical to *A. hydrophilla* control

Appendix B – Positive Controls

Table 7: MALDI-MS Positive Controls	
Genus Species	**Strain Designation**
Aeromonas hydrophila (HG1)	ATCC 7966, ATCC 35654
A. bestiarum (HG2)	ATCC 51108
A. salmonicida (HG3)	CDC 0434-84
A. caviae (HG4)	ATCC 15468
A. media (HG5)	ATCC 33907, ATCC 35950
A. eucrenophila (HG6)	ATCC 23309, LMG 13060, LMG 13687
A. sobria (HG7)	ATCC 35993
A. veronii bv sobria (HG8)	ATCC 9071
A. jandaei (HG9)	ATCC 49568
A. veronii bv veronii (HG10)	ATCC 35624, ATCC 35622
A. sp (HG11)	LMG 13076, LMG 13075
A. schubertii (HG12)	ATCC 43700
A. sp Group 501 (HG13)	ATCC 43946
A. trota (HG14)	ATCC 49657, ATCC 49659
A. allosacchrophila (HG15)	ATCC 51208, ATCC 35942
A. encheleia (HG16)	CECT 4342, ATCC 51930
A. popoffii (HG17)	CDC 164-78

Table 8: 16s rDNA Positive Controls	
Genus species	**Strain Designation**
Aeromonas hydrophila (HG1)	ATCC 7966
A. bestiarum (HG2)	ATCC 13444, ATCC.23211, ATCC 23213
A. salmonicida (HG3)	NCIMB 1102, NCIMB 1110, CECT 894
A. caviae (HG4)	NCIMB 13016, ATCC 15467
A. media (HG5)	ATCC 33907, CDC 0862-83
A. eucrenophila (HG6)	ATCC 23309
A. sobria (HG7)	ATCC 43979, NCIMB 12065
A. veronii bv sobria (HG8)	CDC 0437-84
A. jandaei (HG9)	ATCC 49568
A. veronii bv veronii (HG10)	ATCC 35624
A. sp (HG11)	LMG 13062, ATCC 35941
A. schubertii (HG12)	ATCC 43700
A. sp Group 501 (HG13)	CDC 2478-85
A. trota (HG14)	ATCC 49657
A. allosacchrophila (HG15)	CECT 4199
A. encheleia (HG16)	LMG 16331, LMG 16328
A. popoffii (HG17)	LMG 317541

Appendix B – Positive Controls

Table 9: Gyrase B Positive Controls	
Genus species	**Strain Designation**
Aeromonas hydrophila (HG1)	CECT 839, ATCC 49140
A. bestiarum (HG2)	LMG 13662
A. salmonicida (HG3)	CECT 4237, CECT 894
A. caviae (HG4)	CECT 4221
A. media (HG5)	CECT 4234, CECT 4232
A. eucrenophila (HG6)	CECT 4827, CECT 4224
A. sobria (HG7)	CECT 4245
A. veronii bv sobria (HG8)	CECT 4246
A. jandaei (HG9)	CECT 4228
A. veronii bv veronii (HG10)	CECT 4486
A. sp (HG11)	Could not locate
A. schubertii (HG12)	ATCC 43700
A. sp Group 501 (HG13)	ATCC 43946
A. trota (HG14)	CECT 4255, CECT 4935
A. allosacchrophila (HG15)	CECT 4199
A. encheleia (HG16)	CECT 4342, CECT 4856
A. popoffii (HG17)	LMG 317541

LMG, Culture collection of the Labratorium voor Microbiologie Gent, Gent, Belgium; CECT, Coleccion Espanola de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; ATCC, American Type and Culture Collection, Manassas, Virginia, USA; CDC, Center for Disease Control and Prevention, Atlanta, Georgia, USA

Appendix C – Phylogenetic trees

Figure 5: Unrooted phylogram for 16s gene locus

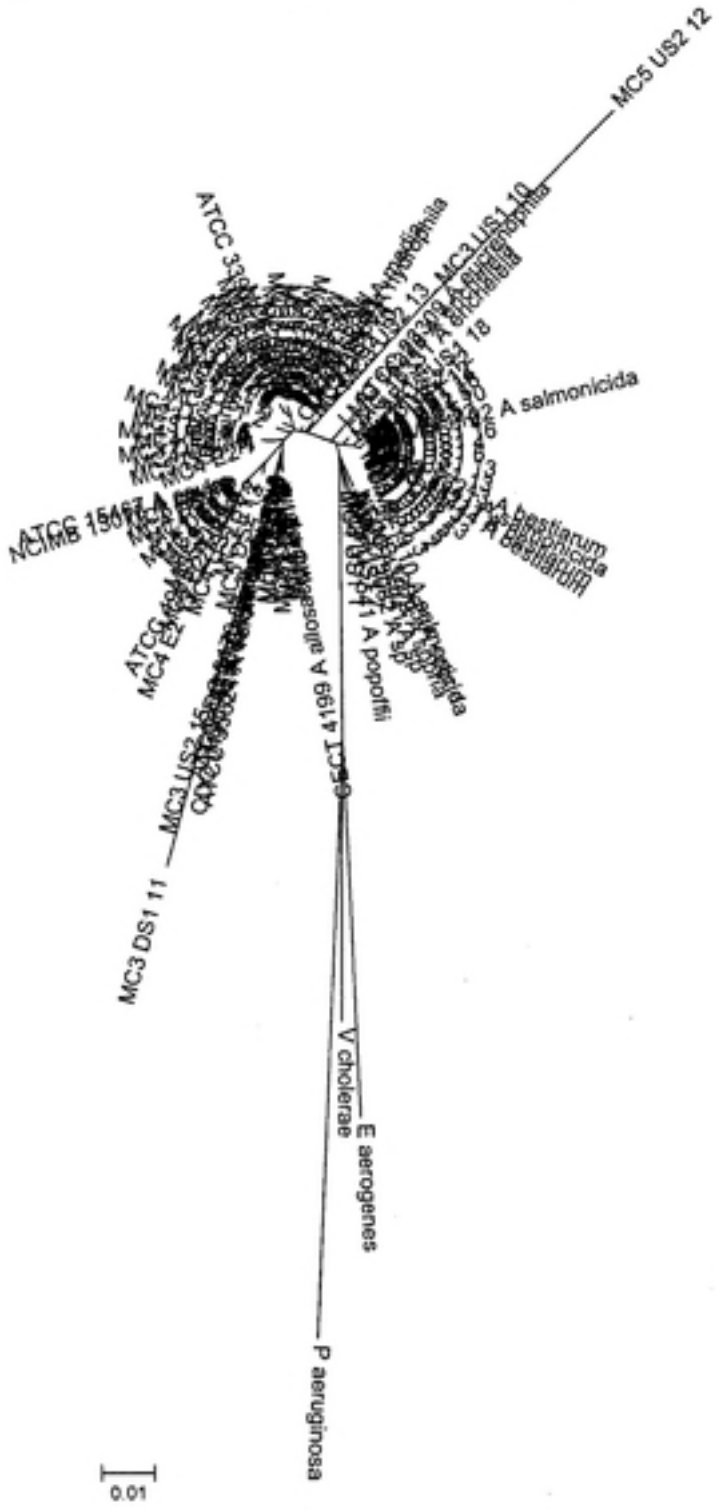


Figure 6: Neighbor joining consensus bootstrap (100 replicates) tree for 16s locus

Figure 7: Slanted consensus bootstrap (100 replicates) tree for the 16s locus

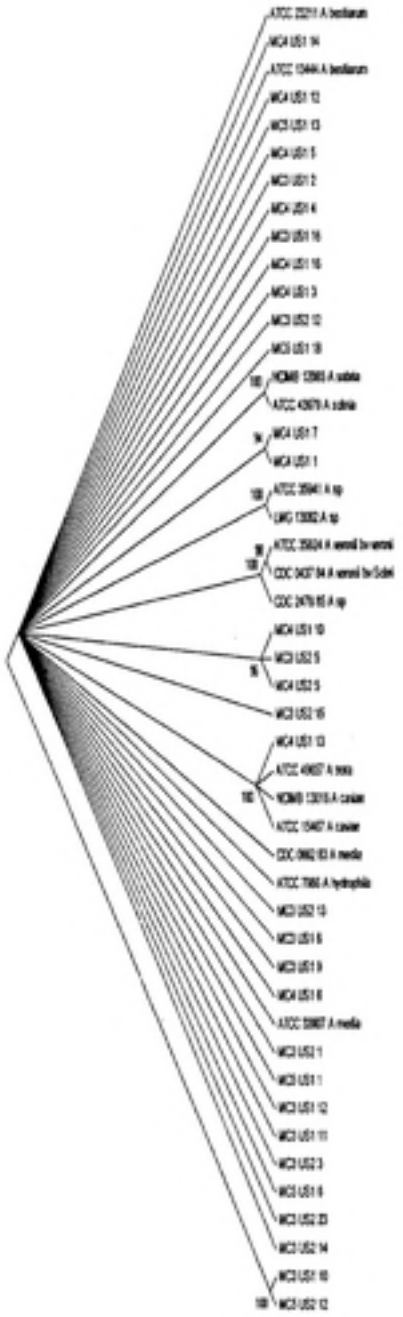


Figure 8: Slanted consensus bootstrap (100 replicates) tree for US sampling site (16s locus)

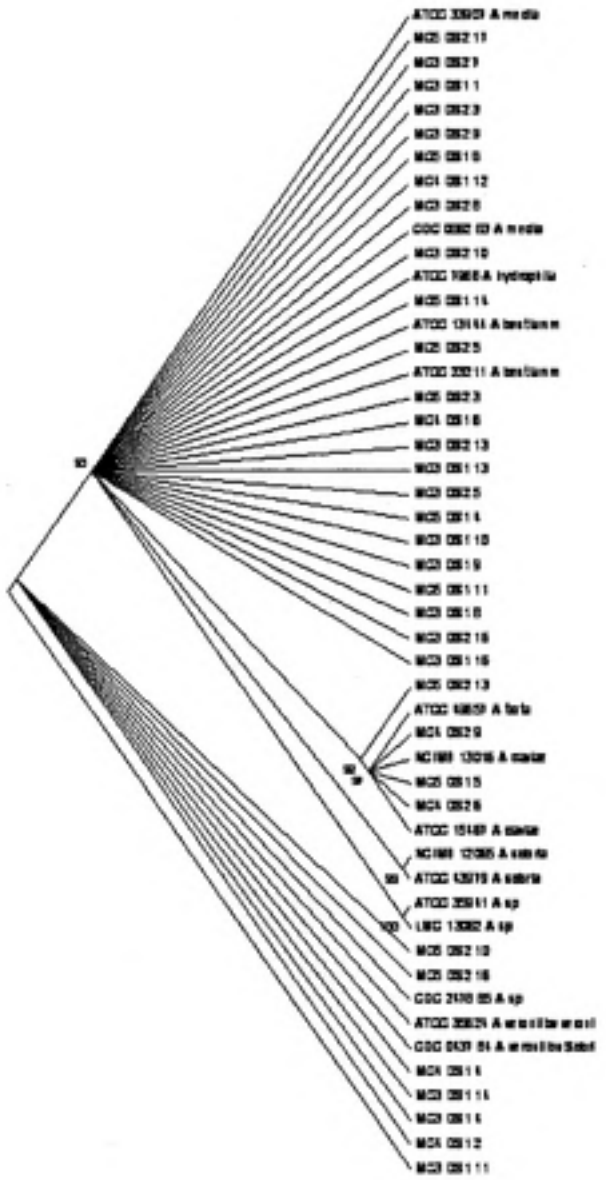


Figure 9: Slanted Consensus Bootstrap (100 replicates) for the DS sampling site (16S locus)

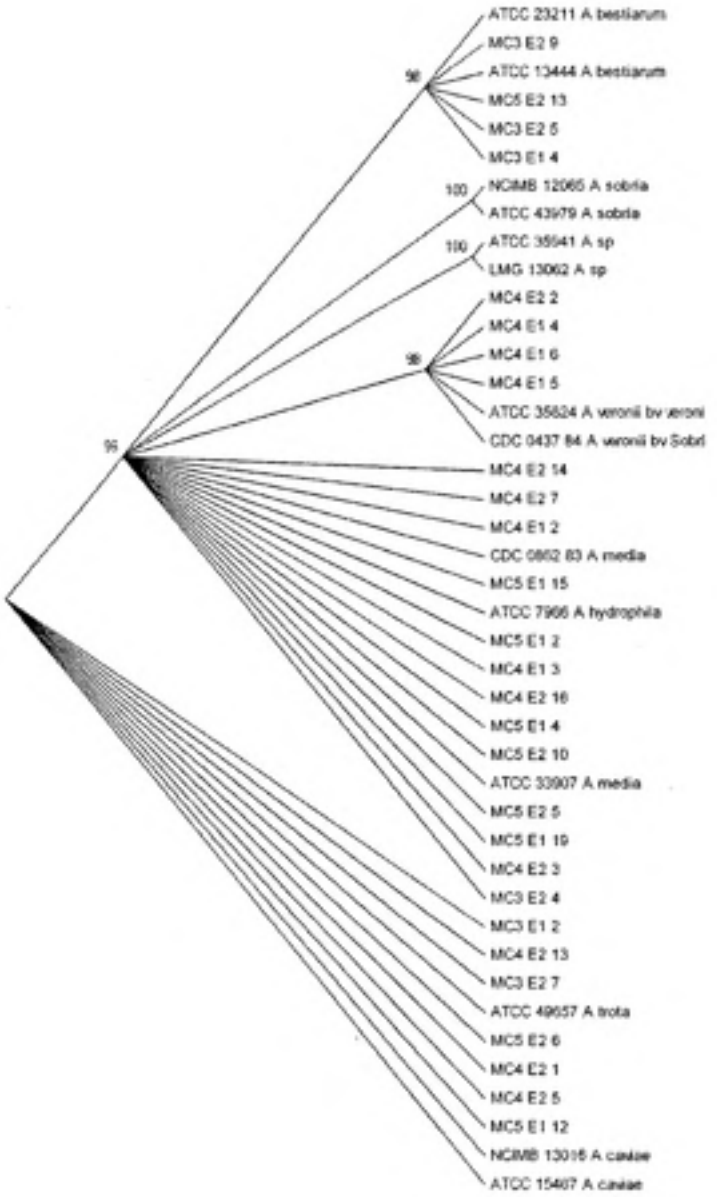
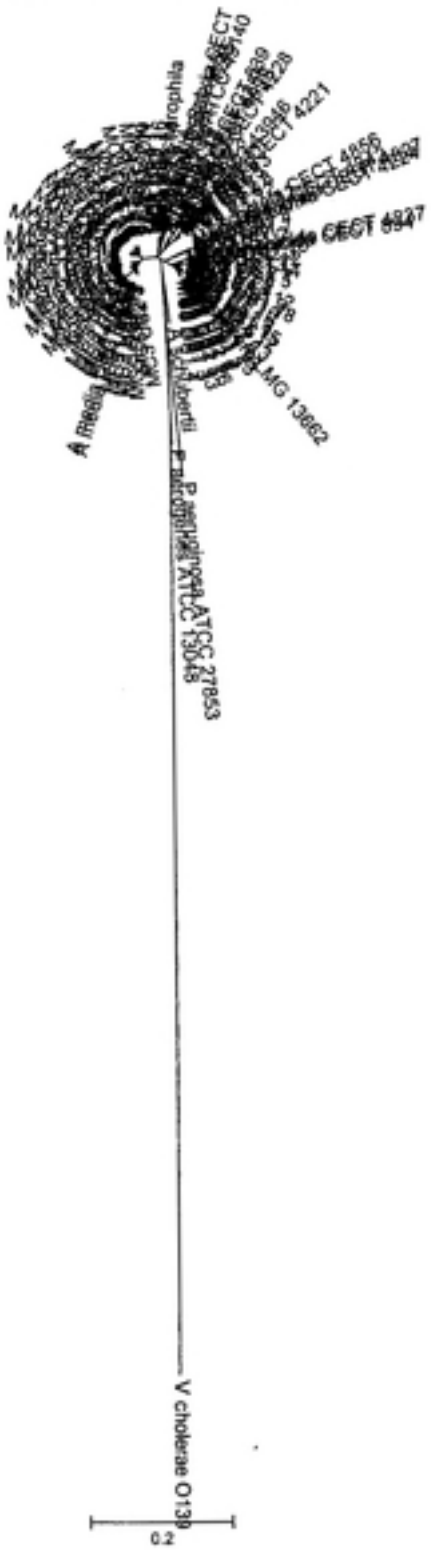


Figure 10: Slanted consensus bootstrap (100 replicates) tree for the effluent sampling site (16s loci)

Figure 11: Unrooted phylogram for Gyrase B locus



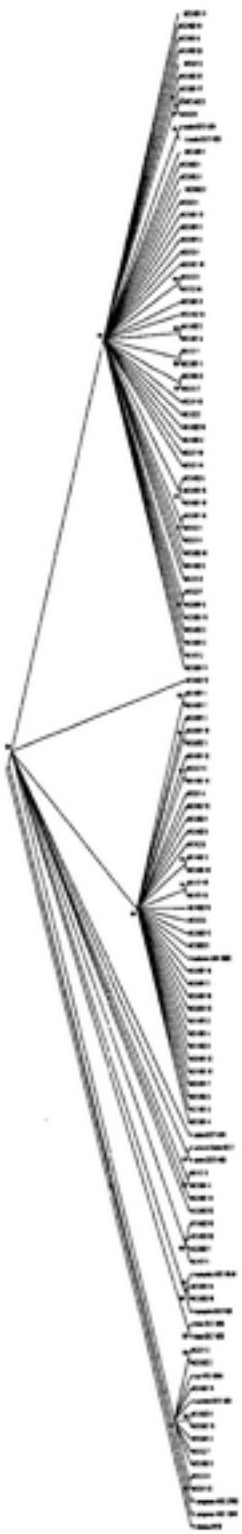


Figure 12: Slanted consensus bootstrap (100 replicates) tree for the gyrB loci

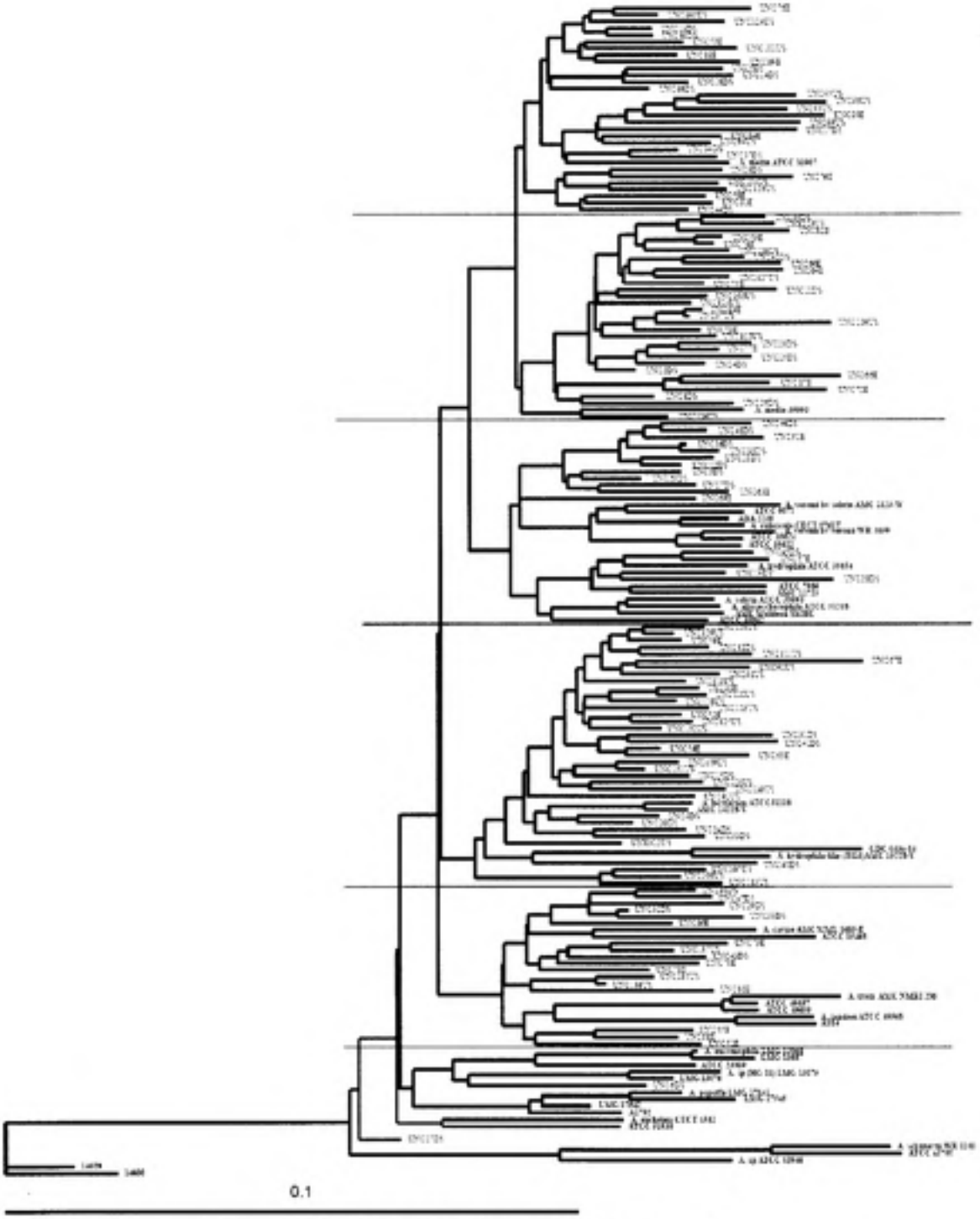


Figure 15: MALDI-MS Neighbor Joining Tree

Appendix D – MALDI-MS Species Determination and Complex Level Determinations

EPA ID	Isolate	Complex	MALDI-MS Determination
1	MC3 DS1 1	*A. Caviae*	*A.media/A.caviae HG5B*
2	MC3 DS1 2	*A. Caviae*	*A.media/A.caviae HG5B*
3	MC3 DS1 4	*A. Caviae*	*A.sobria*
4	MC3 DS1 8	*A. Caviae*	*A.media/A.caviae HG5B*
5	MC3 DS1 9	*A. Caviae*	*A.media/A.caviae HG5B*
6	MC3 DS1 10	*A. Hydrophila*	*HG11*
7	MC3 DS1 11	*A. Hydrophila/A. Caviae*	*A.veronii bv sobria*
8	MC3 DS1 12	*A. Caviae*	*A.media/A.caviae HG5B*
9	MC3 DS1 13	*A. Hydrophila*	*A.bestiarum*
10	MC3 DS1 14	*A. Hydrophila*	*A.hydrophila*
11	MC3 DS1 16	*A. Caviae*	*A.media/A.caviae HG5B*
12	MC3 DS2 1	*A. Caviae*	*A.bestriaum*
13	MC3 DS2 2	*A. Caviae*	*A.media/A.caviae HG5B*
14	MC3 DS2 3	*A. Caviae*	*A.media/A.caviae HG5B*
15	MC3 DS2 5	*A. Hydrophila*	*A.bestiarum*
16	MC3 DS2 7	*A. Caviae*	*A.media/A.caviae HG5B*
17	MC3 DS2 8	*A. Caviae*	*A.media/A.caviae HG5B*
18	MC3 DS2 9	*A. Caviae*	*A.media/A.caviae HG5B*
19	MC3 DS2 10	*A. Caviae*	*A.hydrophila*
20	MC3 DS2 13	*A. Caviae*	*A.bestiarum*
21	MC3 DS2 16	*A. Caviae*	*A.media/A.caviae HG5B*
22	MC4 DS1 2	*A. Hydrophila*	*A.sobria**
23	MC4 DS1 4	*A. Caviae*	*A.media/A.caviae HG5B*
24	MC4 DS1 6	*A. Caviae*	*A.bestiarum*
25	MC4 DS1 12	*A. Caviae*	*A.media/A.caviae HG5B*
26	MC4 DS1 16	*A. Caviae*	*A.media/A.caviae HG5B*
27	MC4 DS2 1		*Undetermined*
28	MC4 DS2 4		*A.hydrophila*
29	MC4 DS2 6		*A.caviae*
30	MC4 DS2 9		*A.caviae*
31	MC5 DS1 4	*A. Hydrophila*	*A.bestiarum*
32	MC5 DS1 5	*A. Caviae*	*A.caviae*
33	MC5 DS1 7	*A. Hydrophila*	*A.bestiarum*
34	MC5 DS1 8	*A. Caviae*	*A.media/A.caviae HG5B*
35	MC5 DS1 11	*A. Caviae*	*A.media/A.caviae HG5B*
36	MC5 DS1 14	*A. Hydrophila*	*A.sobria*
EPA ID	Isolate	Complex	MALDI-MS Determination
37	MC5 DS1 17	*A. Caviae*	*A.media/A.caviae HG5B*
38	MC5 DS2 1	*A. Caviae*	*A. sobria**
39	MC5 DS2 2	*A. Caviae*	*A.caviae*
40	MC5 DS2 3	*A. Hydrophila*	*A. hydrophila like (HG3)*
41	MC5 DS2 5	*A. Hydrophila*	*A. bestiarum*
42	MC5 DS2 6	*A. Caviae*	*A.media/A.caviae HG5B*
43	MC5 DS2 8	*A. Caviae*	*A. caviae*
44	MC5 DS2 10	*A. Caviae*	*A. sobria**

Appendix D – MALDI-MS Species Determination and Complex Level Determinations

45	MC5 DS2 11	*A. Caviae*	*A. hydrophila*
46	MC5 DS2 12	*A. Hydrophila*	*A.sobria*
47	MC5 DS2 13	*A. Caviae*	*A. caviae*
48	MC5 DS2 16	*A. Caviae*	*A.sobria*
49	MC5 DS2 17	*A. Caviae*	*A.media/A.caviae HG5B*
51	MC3 E1 2	*A. Caviae*	*A. bestiarum*
52	MC3 E1 4	*A. Caviae*	*A.media/A.caviae HG5B*
53	MC3 E2 4	*A. Caviae*	*A. bestiarum*
54	MC3 E2 5	*A. Caviae*	*A. trota*
55	MC3 E2 7	*A. Caviae*	*A. bestiarum*
56	MC3 E2 9	*A. Hydrophila*	*A.hydrophila*
57	MC3 E2 14	*A. Caviae*	*A.media/A.caviae HG5B*
58	MC4 E1 1	*A. Caviae*	*A.media/A.caviae HG5B*
59	MC4 E1 2	*A. Caviae*	*A.media/A.caviae HG5B*
60	MC4 E1 3	*A. Caviae*	*A.sobria*
61	MC4 E1 4	*A. Caviae*	*A. veronii bv sobria*
62	MC4 E1 5	*A. Caviae*	*A.sobria*
63	MC4 E1 6	*A. Caviae*	*A.media/A.caviae HG5B*
64	MC4 E1 7	*A. Caviae*	*A. bestiarum*
65	MC4 E1 10	*A. Hydrophila*	*A.media/A.caviae HG5B*
66	MC4 E1 14	*A. Caviae*	*A. bestiarum*
67	MC4 E1 16	*A. Hydrophila*	*A.trota*
68	MC4 E2 1	*A. Caviae*	*A. veronii*
69	MC4 E2 2	*A. Hydrophila*	*A.media/A.caviae HG5B*
70	MC4 E2 3	*A. Caviae*	*A. media/A.caviae HG5B*
71	MC4 E2 5	*A. Caviae*	*A.media/A.caviae HG5B*
72	MC4 E2 7	*A. Caviae*	*A.media/A.caviae HG5B*
EPA ID	Isolate	*Complex*	*MALDI-MS Determination*
73	MC4 E2 13	*A. Caviae*	*A.trota*
74	MC4 E2 14	*A. Caviae*	*A.media/A.caviae HG5B*
75	MC4 E2 16	*A. Caviae*	*A.media/A.caviae HG5B*
76	MC5 E1 2	*A. Caviae*	*A.media/A.caviae HG5B*
77	MC5 E1 4	*A. Caviae*	*A.media/A.caviae HG5B*
78	MC5 E1 12	*A. Caviae*	*A.caviae*
79	MC5 E1 15	*A. Caviae*	*A.media/A.caviae HG5B*
81	MC5 E1 19	*A. Caviae*	*A.media/A.caviae HG5B*
82	MC5 E2 1	*A. Caviae*	*A.media/A.caviae HG5B*
83	MC5 E2 2	*A. Caviae*	*A.media/A.caviae HG5B*
84	MC5 E2 4	*A. Caviae*	*A.media/A.caviae HG5B*
85	MC5 E2 5	*A. Caviae*	*A.media/A.caviae HG5B*
86	MC5 E2 6	*A. Caviae*	*A.caviae*
87	MC5 E2 7	*A. Caviae*	*A.media/A.caviae HG5B*
88	MC5 E2 9	*A. Caviae*	*A.media/A.caviae HG5B*
89	MC5 E2 10	*A. Caviae*	*A.media/A.caviae HG5B*
90	MC5 E2 13	*A. Caviae*	*A.bestiarum*
91	MC3 US1 2	*A. Hydrophila*	*A.bestiarum*
92	MC3 US1 3	*A. Hydrophila*	*A.bestiarum*

Appendix D – MALDI-MS Species Determination and Complex Level Determinations

93	MC3 US1 6	*A. Caviae*	*A.media/A.caviae HG5B*
94	MC3 US1 9	*A. Caviae*	*A.media/A.caviae HG5B*
95	MC3 US1 10	*A. Caviae*	*A.media/A.caviae HG5B*
96	MC3 US1 11	*A. Caviae*	*A.media/A.caviae HG5B*
97	MC3 US1 12	*A. Caviae*	*A.media/A.caviae HG5B*
98	MC3 US1 16	*A. Hydrophila*	*A.bestiarum*
99	MC3 US2 1	*A. Caviae*	*A.media/A.caviae HG5B*
100	MC3 US2 2	*A. Caviae*	*A.media/A.caviae HG5B*
101	MC3 US2 3	*A. Caviae*	*A.media/A.caviae HG5B*
102	MC3 US2 5	*A. Caviae*	*A.media/A.caviae HG5B*
103	MC3 US2 12	*A. Hydrophila*	*A. bestiarum*
104	MC3 US2 13	*A. Caviae*	*A.media/A.caviae HG5B*
105	MC3 US2 14	*A. Caviae*	*A.media/A.caviae HG5B*
106	MC3 US2 15	*A. Caviae*	*A.media/A.caviae HG5B*
107	MC3 US2 23	*A. Caviae*	*Undetermined*
108	MC4 US1 1	*A. Hydrophila*	*A.bestiarum/A.hydrohila-like (HG3)*
EPA ID	Isolate	*Complex*	*MALDI-MS Determination*
109	MC4 US1 3	*A. Hydrophila*	*A.bestiarum*
110	MC4 US1 4	*A. Hydrophila*	*A.bestiarum*
111	MC4 US1 5	*A. Hydrophila*	*A.bestiarum*
112	MC4 US1 6	*A. Hydrophila*	*A.media/A.caviae HG5B*
113	MC4 US1 7	*A. Hydrophila*	*A.bestiarum*
114	MC4 US1 10	*A. Caviae*	*A.media/A.caviae HG5B*
115	MC4 US1 11	*A. Caviae*	*A.caviae*
116	MC4 US1 12	*A. Hydrophila*	*A. caviae*
117	MC4 US1 13	*A. Caviae*	*A.caviae*
118	MC4 US1 14	*A. Caviae*	*A.bestiarum*
119	MC4 US1 16	*A. Hydrophila*	*A.bestiarum*
120	MC4 US2 5	*A. Caviae*	*A.media/A.caviae HG5B*
121	MC4 US2 6	*A. Caviae*	*A.bestiarum*
122	MC4 US2 12	*A. Caviae*	*A.bestiarum*
123	MC4 US2 16	*A. Caviae*	*A.media/A.caviae HG5B*
124	MC5 US1 1	*A. Caviae*	*A.media/A.caviae HG5B*
125	MC5 US1 3	*A. Hydrophila*	*A.bestiarum*
126	MC5 US1 6	*A. Caviae*	*A.media/A.caviae HG5B*
127	MC5 US1 8	*A. Caviae*	*A.media/A.caviae HG5B*
128	MC5 US1 13	*A. Hydrophila*	*A.bestiarum*
129	MC5 US1 15	*A. Hydrophila*	*A.bestiarum*
130	MC5 US1 18	*A. Hydrophila*	*A.bestiarum*
131	MC5 US2 5	*A. Hydrophila*	*A.bestiarum*
132	MC5 US2 12	*A. Hydrophila*	*A. bestiarum*

Appendix E - Species determination by gyrase B sequence analysis

EPA ID	Isolate	Complex	Gyrase B species
1	MC3 DS1 1	A. Caviae	A. media
2	MC3 DS1 2	A. Caviae	A. media
3	MC3 DS1 4	A. Caviae	Undetermined
4	MC3 DS1 8	A. Caviae	A. media
5	MC3 DS1 9	A. Caviae	A. media
6	MC3 DS1 10	A. Hydrophila	A. salmonicida
7	MC3 DS1 11	A. Hydrophila/A. Caviae	Undetermined
8	MC3 DS1 12	A. Caviae	A. media
9	MC3 DS1 13	A. Hydrophila	Undetermined
10	MC3 DS1 14	A. Hydrophila	A. allosaccharophila
11	MC3 DS1 16	A. Caviae	A. media
12	MC3 DS2 1	A. Caviae	Undetermined
13	MC3 DS2 2	A. Caviae	A. media
14	MC3 DS2 3	A. Caviae	A. media
15	MC3 DS2 5	A. Hydrophila	A. bestiarum
16	MC3 DS2 7	A. Caviae	Undetermined
17	MC3 DS2 8	A. Caviae	Undetermined
18	MC3 DS2 9	A. Caviae	A. media
19	MC3 DS2 10	A. Caviae	A. hydrophila
20	MC3 DS2 13	A. Caviae	A. bestiarum
21	MC3 DS2 16	A. Caviae	A. media
31	MC5 DS1 4	A. Hydrophila	Undetermined
32	MC5 DS1 5	A. Caviae	A. caviae
33	MC5 DS1 7	A. Hydrophila	A. bestiarum
34	MC5 DS1 8	A. Caviae	A. media
35	MC5 DS1 11	A. Caviae	A. media
36	MC5 DS1 14	A. Hydrophila	A. hydrophila
37	MC5 DS1 17	A. Caviae	A. media
38	MC5 DS2 1	A. Caviae	Undetermined
39	MC5 DS2 2	A. Caviae	A. sp HG13
40	MC5 DS2 3	A. Hydrophila	A. salmonicida
41	MC5 DS2 5	A. Hydrophila	A. bestiarum
42	MC5 DS2 6	A. Caviae	A. caviae
43	MC5 DS2 8	A. Caviae	A. caviae
44	MC5 DS2 10	A. Caviae	Undetermined
45	MC5 DS2 11	A. Caviae	Undetermined
46	MC5 DS2 12	A. Hydrophila	Undetermined
47	MC5 DS2 13	A. Caviae	A. caviae
48	MC5 DS2 16	A. Caviae	Undetermined
49	MC5 DS2 17	A. Caviae	A. media
51	MC3 E1 2	A. Caviae	A. sp HG13
52	MC3 E1 4	A. Caviae	A. bestiarum
53	MC3 E2 4	A. Caviae	A. media
54	MC3 E2 5	A. Caviae	A. bestiarum

Appendix E - Species determination by gyrase B sequence analysis

EPA ID	Isolate	Complex	Gyrase B species
55	MC3 E2 7	A. Caviae	A.caviae
56	MC3 E2 9	A. Hydrophila	A. bestiarum
57	MC3 E2 14	A. Caviae	Undetermined
58	MC4 E1 1	A. Caviae	A. media
59	MC4 E1 2	A. Caviae	A. media
60	MC4 E1 3	A. Caviae	A. media
61	MC4 E1 4	A. Caviae	Undetermined
63	MC4 E1 6	A. Caviae	Undetermined
64	MC4 E1 7	A. Caviae	A. media
65	MC4 E1 10	A. Hydrophila	A. bestiarum
66	MC4 E1 14	A. Caviae	A. media
67	MC4 E1 16	A. Hydrophila	A. bestiarum
76	MC5 E1 2	A. Caviae	A. media
77	MC5 E1 4	A. Caviae	A. media
78	MC5 E1 12	A. Caviae	A. caviae
79	MC5 E1 15	A. Caviae	A. media
81	MC5 E1 19	A. Caviae	A. media
82	MC5 E2 1	A. Caviae	A. media
83	MC5 E2 2	A. Caviae	A. media
84	MC5 E2 4	A. Caviae	A. media
85	MC5 E2 5	A. Caviae	A. media
86	MC5 E2 6	A. Caviae	A. caviae
87	MC5 E2 7	A. Caviae	A. media
88	MC5 E2 9	A. Caviae	A. media
89	MC5 E2 10	A. Caviae	A. media
90	MC5 E2 13	A. Caviae	A. bestiarum
91	MC3 US1 2	A. Hydrophila	A. bestiarum
92	MC3 US1 3	A. Hydrophila	A. bestiarum
93	MC3 US1 6	A. Caviae	A. media
94	MC3 US1 9	A. Caviae	A. media
95	MC3 US1 10	A. Caviae	A. media
96	MC3 US1 11	A. Caviae	A. media
97	MC3 US1 12	A. Caviae	A. media
98	MC3 US1 16	A. Hydrophila	A. bestiarum
99	MC3 US2 1	A. Caviae	A. media
100	MC3 US2 2	A. Caviae	A. media
101	MC3 US2 3	A. Caviae	A. media
102	MC3 US2 5	A. Caviae	A. media
103	MC3 US2 12	A. Hydrophila	A. bestiarum
104	MC3 US2 13	A. Caviae	A. media
105	MC3 US2 14	A. Caviae	A. media
106	MC3 US2 15	A. Caviae	A. media
107	MC3 US2 23	A. Caviae	A. media
108	MC4 US1 1	A. Hydrophila	A. popoffii
109	MC4 US1 3	A. Hydrophila	Undetermined

Appendix E - Species determination by gyrase B sequence analysis

EPA ID	Isolate	Complex	Gyrase B species
110	MC4 US1 4	A. Hydrophila	A. bestiarum
111	MC4 US1 5	A. Hydrophila	A. bestiarum
112	MC4 US1 6	A. Hydrophila	A.media
113	MC4 US1 7	A. Hydrophila	A. popoffii
114	MC4 US1 10	A. Caviae	A. media
115	MC4 US1 11	A. Caviae	A.caviae
116	MC4 US1 12	A. Hydrophila	A. bestiarum
118	MC4 US1 14	A. Caviae	A. bestiarum
119	MC4 US1 16	A. Hydrophila	A. bestiarum
120	MC4 US2 5	A. Caviae	A. media
121	MC4 US2 6	A. Caviae	A. bestiarum
122	MC4 US2 12	A. Caviae	A. bestiarum
123	MC4 US2 16	A. Caviae	A. media
124	MC5 US1 1	A. Caviae	A. media
125	MC5 US1 3	A. Hydrophila	A. bestiarum
126	MC5 US1 6	A. Caviae	A. media
127	MC5 US1 8	A. Caviae	A. media
128	MC5 US1 13	A. Hydrophila	A. bestiarum
129	MC5 US1 15	A. Hydrophila	A. bestiarum
130	MC5 US1 18	A. Hydrophila	A. bestiarum
131	MC5 US2 5	A. Hydrophila	A. bestiarum
132	MC5 US2 12	A. Hydrophila	A. encheleia