Evaluation of Fecal Contamination by Seagulls in an Urban Estuarine Environment Using Microbiological and Molecular Approaches

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ABSTRACT

JENNIFER LEIGH MURPHY: Evaluation of Fecal Contamination by Seagulls in an Urban Estuarine Environment Using Microbiological and Molecular Approaches
(Under the direction of Mark D. Sobsey, Ph.D.)

Wild birds are important non-point sources of fecal contamination of surface waters, as they have been reported to excrete large amounts of fecal indicator bacteria and occasionally harbor enteric pathogens, such as *Campylobacter* and *Salmonella*. Hundreds of waterfowl frequent the sandflats within Talbert Marsh, a constructed wetland in Orange County, CA, on a daily basis for nesting and feeding purposes. The focus of this study was on the role of seagull fecal contamination as a potentially important contributor to the non-point sources of fecal contamination into Talbert Marsh waters, which eventually flow into the recreational surf-zone at Huntington Beach. Using traditional microbiological methods, *Salmonella*, *Campylobacter*, F+ coliphages, and bacterial indicators were isolated from Talbert Marsh seagull feces and/or estuarine waters. Genotypic methods, including PCR, RT-PCR, reverse line blot hybridization, PFGE, and nucleotide and amino acid sequencing, were employed on subsets of frozen suspensions of isolates for further characterization and to determine genetic relatedness. There is some limited evidence supporting the idea that seagull feces deposited on the sandflats of Talbert Marsh did impact the surrounding estuarine waters. A subset of isolates were relatively similar in both nucleotide and amino acid sequence and therefore may have come from the same population. Overall however, it is unlikely that pathogens from seagull feces in Talbert Marsh pose a significant health risk to swimmers in the ocean

waters at Huntington Beach. Multiple adverse health outcomes have been linked to exposure to marine recreational water of poor microbiologic quality and identifying sources of fecal contamination as human or animal in origin is becoming a more important tool in decision making to manage fecal contamination of these waters.

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CHAPTER 1

Introduction

Introduction to Study

Ocean waters along the U.S. coastline receive pollutants from both point sources and non-point sources. Over the last 30 years, there has been a focus on identifying and controlling pollutants from point sources, such as those in effluent discharges into coastal receiving waters by industries and sewage treatment plants. As a result, concentrations of these pollutants have decreased significantly. However, water quality in and near heavily urbanized areas remains poor due to non-point sources of pollution (National Research Council 1993). Non-point source pollution arises when water in the form of street cleaning, "rainfall, snowmelt, or irrigation runs over land or through the ground, picks up pollutants, and deposits them into rivers, lakes, and coastal waters or introduces them into ground water (U.S. Environmental Protection Agency 2004)." The United States Environmental Protection Agency (US EPA) states that non-point source pollution is the leading reason that 40 percent or more of our Nation's waters are not clean enough for basic uses such as fishing or swimming (U.S. Environmental Protection Agency 2004).

In contrast to the gradual introduction of water runoff from natural landscapes into receiving waters, runoff from nonporous urban landscapes remains above the surface, accumulates, and runs off in large amounts. In addition, urban storm sewer systems channel this runoff resulting in large volumes of quickly flowing runoff being deposited into

receiving waters. Recent reports state that urban runoff is the principal source of impairments to estuarine systems and rapid growth in coastal towns and cities will increasingly degrade coastal receiving waters (U.S. Environmental Protection Agency 2004). Urban runoff is not only responsible for pollutants such as increased sediments and nutrients, but it is also an important source of microbial pollution in nearshore regions, wetlands, marshes, and estuaries (Sanders, Horne et al. 1999; U.S. Environmental Protection Agency 2004). Improperly-managed waste and broken or decaying sewage pipes may release large quantities of bacteria, viruses, and protozoans into the surrounding environment. Urban runoff can have great influence on both the fate and transport of these microbes into the water cycle and can adversely affect both the health of coastal ecosystems and human populations found in these environments (Sanders, Horne et al. 1999).

A collaborative study among researchers at the University of California, Irvine (UC-Irvine), the University of California, Berkeley (UC-Berkley), and the University of North Carolina, Chapel Hill (UNC-Chapel Hill) was conducted within a southern California watershed directly landward of Huntington State and City Beaches. Talbert Watershed, in Orange County, CA, receives large inputs of urban runoff, especially during storm events within the wet season, and includes three tidally influenced flood control channels and a wetland known as Talbert Marsh located at the outlet of the watershed (Saunders, Horne et al. 1999). During flood tides, water flows from the Pacific Ocean through Talbert Marsh and inland along the channel network; during ebb tides, water flows out of the channel network through Talbert Marsh and into the Pacific Ocean (Grant, Sanders et al. 2001). Talbert Marsh is a reconstructed saltwater marsh that was completed in 1989 to not only provide control of

urban runoff but, among a number of other purposes, to create a wildlife habitat (Santa Ana Watershed Project Authority 2004).

Vegetation and open water in wetlands provides food, rearing areas, and cover for waterfowl and shorebirds (Water Education Foundation 2000). As a result, Talbert Marsh has become an area that hundreds of waterfowl frequent on a daily basis for nesting and feeding purposes. Wild birds have long been considered important non-point sources of fecal contamination to surface waters, excreting fecal indicator bacteria, such as enterococci, and enteric pathogens (Mundt 1962; Geldreich and Kenner 1969; Mitchell and Ridgwell 1971; Hussong, Damare et al. 1979; Standridge, Delfino et al. 1979; Benoit, Brousseau et al. 1993; Alderisio and DeLuca 1999). Recent studies conducted at Huntington State and City Beaches found elevated levels of enterococci, the State regulated bacterial indicator of fecal pollution, in the surf zone of the beaches. The elevated enterococci levels led to beach closures and to increased efforts to determine the sources of these bacteria for their better control. These studies also found that enterococci bacteria were present at high concentrations in bird feces, marsh sediments, and on marine vegetation in Talbert Marsh. Studies concluded that the marsh was the net source of enterococci based on comparing concentrations of these bacteria at different locations near the marsh and at different tidal cycles. Overall, researchers determined that the marsh enterococci were a significant cause of poor surf zone water quality at Huntington State and City Beaches (Grant, Sanders et al. 2001). These findings prompted research to look more closely at not only the potential sources of microbial contamination within Talbert Marsh, but at the types of bacterial pathogens that also could be present in the marsh.

Tidally influenced constructed wetlands, such as Talbert Marsh, can be designed and managed to optimize pathogen inactivation. Slow flow velocities (<0.7 m/s) and long residence times of water (10 h) are conditions in which freshwater wetlands have been found to operate most effectively (Grant, Sanders et al. 2001). In marine wetlands, these conditions may further minimize or prevent contamination because of longer exposure to salt water and UV light, both of which have activity against bacteria, viruses, and protozoan parasites. However, Talbert Marsh is highly dynamic system whose water flow is dominated by semidiurnal, unequal tides, so water velocities and residence times greatly vary. Not only are there four daily tidal extremes, but the period of tidal range fluctuation from maximal to minimal and back to maximal (also know as the spring-neap-spring transition) oscillates over 14-15 days. Furthermore, pump stations determine whether inland runoff is intermittently discharged into drainage channels, diverted into the regional sanitary sewer system, or stored in pump station forebays (Grant, Sanders et al. 2001).

The joint study by UC-Irvine, UC-Berkley, and UNC-Chapel Hill focused on the runoff from Talbert Marsh for several reasons. The region's wetlands not only house rare and endangered species, but its public beaches, including Huntington State and City Beaches, receive an estimated 20 million visitors per year. Long-term monitoring has shown that Talbert Marsh and the adjacent coastline have the highest concentrations of total and fecal coliforms along the Orange County coastline (Sanders, Horne et al. 1999). Multiple adverse health outcomes, such as gastroenteritis, eye, skin, ear, nose, and throat infections, and respiratory illness, have been linked to exposure to marine recreational water of poor microbiologic quality (Haile, Witte et al. 1999). Epidemiological studies have concluded that the rates of several of these disease symptoms were increased in swimmers compared

with non-swimmers (Pruss 1998). Significant beach usage and surf zone pollution along this coastline implies that many people may acquire gastrointestinal or other types of illness from swimming and surfing in this area each year (Kim, Grant et al. 2004). Furthermore, numerous beach closures have led to a large loss of revenue to resident businesses and public outcry over the loss of popular bathing areas (Sanders, Horne et al. 1999).

The focus of this study was on the role of seagull fecal contamination as a potentially important contributor to the non-point sources of fecal contamination and in particular *Campylobacter* species and *Salmonella* species as bacterial pathogens potentially present in this system. As discussed in Chapter II, two common human bacterial pathogens harbored by birds are *Salmonella* and *Campylobacter,* both of which can cause gastrointestinal illness. Additionally, the presence of fecal indicator microbes, such as fecal coliforms, *Escherichia coli*, enterococci, and F+ coliphages, was assessed. These microbes are commonly found in the feces of warm-blooded animals and have similar structure and persistence in the environment as many human pathogenic enteric bacteria and viruses, respectively (Gerba 1987). Finally, microbial source tracking methods were employed in this study. Identifying sources of fecal contamination as human or animal in origin is becoming a more important tool in decision making to manage fecal contamination of water as the analytical methods for such source tracking improve and become further validated.

Overall, this study was intended to characterize the sources, transport, and fate of fecal contamination in the marsh and its nearby ocean waters, to help "develop a novel strategy to control the impact of urban runoff on the microbial water quality of coastal wetlands and beaches during non-storm periods, and to develop a multiple-objective decision model to aid stakeholders in selecting strategies to mitigate microbial pollution problems in coastal waters

(Sanders, Horne et al. 1999)." A key aspect of the novel strategy was to manage the water flow of the marsh and its adjacent surface waters in ways that would minimize impacts on the microbial quality of adjacent ocean beach water. However, potential control measures based on manipulating the marsh system and its release of fecally contaminated water must first be based on reliable identification and quantification of fecal contamination and its sources and how and when this contamination is introduced into the system. In addition to determining presence and concentrations of these microbes in the feces of waterfowl, microbial source tracking methods must be used to determine the source of fecal pathogens in the surrounding marsh waters.

Overall Research Questions

Do the feces of seagulls in Talbert Marsh contain the pathogens *Salmonella* and *Campylobacter* and/or specific groups of F+ coliphages which serve as indicators of fecal contamination?

Do the waters surrounding the sandflats at which seagulls and other waterfowl congregate contain the pathogens *Salmonella* and *Campylobacter* and/or the same specific groups of F+ coliphages which serve as indicators of fecal contamination?

Is it likely that the seagull population in Talbert Marsh contributes pathogens and fecal indicators into the estuarine waters of lower Talbert Marsh that ultimately flow into the Huntington Beach surfzone? Can microbial source tracking methods, including strain-typing and genetic analysis, be used to better understand the impact waterfowl have on the Talbert Marsh environment?

Aims of Study

To identify key bacterial pathogens and selected indicators of fecal contamination in the feces of seagulls present in Talbert Marsh

- To use conventional phenotypic and newer genotypic methods to identify and quantify *Salmonella* and *Campylobacter* species in the feces of seagulls.

- To use conventional phenotypic and genotypic methods to identify and quantify indicators of fecal contamination, specifically F+ coliphages, in the feces of seagulls.

To identify key bacterial pathogens and indicators of fecal contamination in the estuarine waters of Talbert Marsh

- To use conventional phenotypic and newer genotypic methods to identify and quantify *Salmonella* and *Campylobacter* species in the estuarine waters of Talbert Marsh.

- To use conventional phenotypic and genotypic methods to identify and quantify indicators of fecal contamination, specifically F+ coliphages and fecal indicator bacteria, in the estuarine waters of Talbert Marsh.

To use microbial source tracking methods to characterize the presence and impacts of fecal contamination from seagulls in Talbert Marsh and its potential transport to and presence in the nearby estuarine waters

Overall Approaches

Individual, freshly-deposited seagull feces and estuarine water samples from Talbert Marsh were shipped at 4^{o}C overnight to UNC-Chapel Hill laboratories so that processing could begin within 24 hours of collection. The samples were accompanied by forms providing information on samples and environmental conditions that was to be used in data analysis.

To identify and quantify *Salmonella* and *Campylobacter* in the feces of seagulls present in Talbert Marsh, known volumes of seagull feces were submitted to a series of enrichment culture steps to resuscitate injured cells and increase target cell concentrations. Enrichments were then subcultured onto appropriate selective agars for isolation of distinct colonies and subsequent biochemical testing to phenotypically identify the bacterial complexes or species present.

To identify and quantify F+ coliphages in the feces of segaulls present in Talbert Marsh, a known volume of freshly-deposited seagull feces was submitted to a Double

Agar Layer plaque assay in which *E. coli* Famp host cell infection by F+ coliphages present in the feces was visualized and quantified.

To identify and quantify *Salmonella* and *Campylobacter* in Talbert Marsh estuarine waters, known volumes of estuarine water were submitted to a series of enrichment culture steps, subcultured onto appropriate selective agars, and subsequently submitted to biochemical testing. Additionally, water samples were analyzed for fecal indicator bacteria, including fecal coliforms, *Escherichia coli*, and enterococci, using Colilert and Enterolert defined substrate quantal assays, respectively.

To identify and quantify F+ coliphages in known volumes of estuarine water, two different US EPA methods were employed. Because phage concentration in the estuarine waters was unknown, one method (EPA 1601) first included an enrichment step for low concentrations of coliphages, while the other method (EPA 1602), designed for higher concentrations of coliphages, was a Single Agar Layer plaque assay method. Both methods use *E. coli* Famp host cell infection by F+ coliphages for detection.

Bacteria and F+ coliphages isolated in these studies were further analyzed and characterized using genotypic methods to provide both species confirmation and microbial source tracking information.

- *Salmonella* isolates were subjected to antimicrobial resistance analysis and pulsed-field gel electrophoresis (PFGE) for strain-typing and source tracking information.

- Stored *Campylobacter* isolates were genetically confirmed using a series of polymerase chain reaction (PCR) assays and isolates were also further characterized using nucleotide sequence analysis.

- Finally, F+ coliphage isolates were analyzed using RT-PCR, PCR, Reverse Line Blot nucleic acid hybridization, and nucleotide sequence analysis to identify genogroup and for microbial source tracking purposes.

Introduction to Study Site

Orange County, California

Population

Southern California is one of the fastest growing regions in the United States in terms of numeric population growth. In terms of population, Orange County is the 2nd largest county

in California and the 5th largest county in the country. Its population in January 2004 was slightly over 3,000,000 people and the population growth is expected to continue, with projections of nearly 3.1 million in 2005 and 3.6 million by 2030. Orange County is also one of the most densely populated areas in the United States; in January 2004, its population density was estimated at 3,822 persons per square mile, making it denser than Los Angeles County. As of 2003, the average Orange County household contained 3.0 persons, higher than both the California and United States averages (Orange County Executive Office 2004).

Climate

Southern California has a warm, dry Mediterranean climate and coastal areas have a moderate climate with frequent fog in the summer. Spring and fall months are the mildest seasons; Orange County averages 328 days of sunshine per year with an average daytime temperature of 73°F. Most of the yearly precipitation is in the form of rain during the winter months (California Tourism Commission). The Santa Ana region of southern California receives on average 15 inches of rainfall per year (California Environmental Protection Agency State Water Board 2003).

Land Use

Orange County is made up of 798 square miles of land, including 42 miles of coastline. In 2004, 28% of the land was used for residential housing, while 24% of the county was classified as uncommitted or vacant. Another 22% of the land was dedicated to open space and recreation facilities, including nine beaches, three harbors, and regional parks. The County's cities and state agencies are also accounted for in the open space and local park

category. Twelve percent of the county's total land area was used for commercial, industrial, and public institutional uses and 10% was used for transportation. Only 4% of land in Orange County was used for agriculture (Orange County Executive Office 2004).

In 2003, the County's value of agricultural production was $311.6 million. Nursery stock and cut flowers, strawberries, avocados, peppers, and green beans are the County's top agricultural products (Orange County Executive Office 2004). The 2002 USDA Census of Agriculture indicates that Orange County had 348 farms encompassing slightly over 68,000 acres of land, with over 10,000 acres devoted to harvested cropland. The inventory of livestock within the County indicates 793 cattle and calves, 392 beef cows, 42 hogs and pigs, 67 sheep and lamb, and 195 broilers and chickens. Data on numbers of milk cows and egg-layers (20 weeks or older) was withheld to avoid disclosing data for individual farms. The numbers of all of the types of livestock were lower than that reported during the previous three censuses (United States Department of Agriculture National Agricultural Statistics Service 2002).

Pollution Sources

Urban runoff in Orange County is comprised of some rainwater seasonally and a variety of pollutants including "hydrocarbons from autos and gas stations; Styrofoam from fast-food restaurants; animal droppings; improperly disposed-of human waste; detergents from car-washing; lawn-mowings, fertilizers, and pesticides; all sorts of paper and plastic goods; medical waste; copper from brake shoes, etc (Orange County Health Care Agency 1999)." These contaminants generally wash off the streets and lawns and into storm drains that ultimately flow into the ocean (Mehta 2002). Potential sources of fecal indicator bacteria in

this region include "the offshore discharge of partially treated sewage effluent, the offshore discharge of power plant cooling water that contains fecal indicator bacteria from plant wash-down and other activities, bather shedding, the accumulation of bird droppings along the shoreline and offshore, the exfiltration of sewage-contaminated groundwater, and contributions from watershed outlets located north and south of the study area including the Los Angeles River, the San Gabriel River, and outlets for Huntington Harbor and Newport Bay (Kim, Grant et al. 2004)."

Pollution Regulation – The Ocean Plan, AB 411, and NPDES

The California Ocean Plan was adopted by the California State Water Resources Control Board (SWRCB) in 1972 and was amended in 1978, 1983, 1988, 1990, and 1997 (State Water Resources Control Board 2005). The Ocean Plan sets bacterial water quality standards for ocean waters to ensure the protection of water contact recreation and shellfish harvesting. These water quality objectives are incorporated into discharge permits issued by Regional Water Quality Control Boards (RWQCBs). The first Ocean Plan contained total and fecal coliform standards and in 1990, an enterococci monitoring requirement was added at the US EPA's insistence that enterococci are a superior indicator of human health effects in marine waters (O'Connell and Palmer 2002). California's current bacterial standards for contact recreation in ocean and bay waters are as follows (National Resources Defense Council 2005):

> Geometric mean of 35 enterococci (ENT)/100ml in five samples in a 30-day period or 104/100ml in a single sample (for beaches meeting California's AB 411 thresholds).
>
> Most probable number (MPN) of total coliform (TC) organisms less than 1,000/100ml, provided that not more than 20 percent of the samples at any station, in any 30-day period, shall exceed 1,000/100ml, and provided further that no single

sample, when verified by a repeat sample taken within 48 hours, shall exceed 10,000/100ml.

Geometric mean of 200 fecal coliform (FC)/100ml in five samples in a 30-day period or 400/100ml in a single sample used by counties that choose to test for fecal coliform.

Ratio of TC to FC may not exceed 5 when TC is 5,000 or greater.

In October 1997, California passed Assembly Bill (AB) 411 (or the Right to Know Bill), and since 1999 all beaches with more than 50,000 annual visitors or beaches located adjacent to stormdrains that flow during the summer must be monitored weekly from April to October. The legislation required more protective standards and uniform bacterial monitoring programs. Water samples must be tested for three bacterial indicator organisms (TC, FC, and ENT) and beaches failing to meet the state's criteria for any one of these three microbes must post conspicuous warning signs to notify the public of health risks associated with swimming in these areas (Orange County Sanitation District 1999; National Resources Defense Council 2005). These new regulations "require a beach posting if single sample thresholds for any of the three indicator bacteria are exceeded and a beach closure if there is a known sewage spill affecting the beach (Orange County Sanitation District 1999)."

Most of southern California has separate stormwater and sanitary sewer systems, so both dry and wet weather runoff flows to the ocean without treatment (Grant, Sanders et al. 2001). Flood-control channels, catch basins, and storm drains, often assisted by pump stations, aid rivers and streams in directing the accumulated waters to an appropriate receiving water body, such as a marsh, wetland, harbor, or the ocean (Orange County Health Care Agency 1999). However, since 1990 Orange County stormwater systems operators have been required to develop a management program to prevent pollutants from being dumped or

washed by stormwater runoff into the stormwater systems and ultimately into local waterbodies (Watershed & Coastal Resources Division). In 1987, amendments to the federal Clean Water Act (CWA) added Section 402(p), which defined stormwater discharges from industrial and municipal systems as point sources subject to the National Pollutant Discharge Elimination System (NPDES) Permit Program. As directed by the CWA, the US EPA published regulations to define stormwater discharges subject to NPDES permits and established a regulatory framework for these discharges. Regulations require that Municipal Separate Storm Sewer Systems (MS4s) subject to NPDES permits implement best management practices (BMPs), including both source controls and treatment measures, to reduce pollutants in stormwater discharges to the maximum extent practicable (MEP). In California, MS4 permits also require permittees to reduce pollutant discharge so water quality standards are met (Currier, Jones et al. 2005).

In Orange County, the NPDES permit bases bacteriological compliance limits on the California Ocean Plan and the RWQCB Santa Ana Region Basin Plan. These limits place the surfzone (beach) and recreational water of the Pacific Ocean (three-mile limit) under the jurisdiction of the State of California. In accordance with the NPDES permit, the Orange County Sanitation District (OCSD) (discussed below) must perform beach sampling for total coliforms, fecal coliforms, and enterococci up to five days per week at 17 surfzone stations along approximately 15 miles of beach, including Huntington State and City Beaches. Additionally, monitoring is performed at a station in Talbert Channel and in the Santa Ana River (Orange County Sanitation District 1999). There is no compliance limit for bacteria in the plant or for the final effluent discharged by the plant five miles offshore (Orange County Sanitation District 2005).

Sewage Treatment and Stormwater Control

The OCSD has two operating facilities in Orange County that cover a service area of 471 square miles and provide service for 2.4 million residents. Wastewater Treatment Plant #2 is located in the northwest portion of the County, directly shoreward of Talbert Marsh and slightly downshore of the Huntington Beach surf zone. On a daily basis, this plant receives an average of 151 million gallons of residential, industrial, and commercial wastewater and releases approximately 10^3 m^3 of blended primary and secondary treated water into the ocean though a 120-inch diameter pipeline that extends five miles from shore and discharges approximately 200 feet below the ocean surface. An additional emergency standby 78-inch diameter pipeline stretches one-mile from shore. The second operating facility, Reclamation Plant #1, is located in central Orange County. It receives an average of 87 million gallons of wastewater per day. About 10 million gallons of secondary effluent water is sent to the OCSD for further processing (advanced treatment by water reclamation processes) and used for landscape irrigation and other purposes. The remaining effluent is piped back to Sewage Treatment Plant #2 and discharged through the ocean outfall (Orange County Sanitation District 1999; Boehm, Sanders et al. 2002; McGee 2005; Orange County Sanitation District 2005).

The OCSD treatment plant uses preliminary treatment, advanced primary treatment, and secondary treatment to treat wastewater. In preliminary treatment, wastewater passes through filtering screens and then into grit chambers that remove large materials which are then sent to a landfill. For advanced primary treatment, wastewater is pumped into large settling basins. Chemicals are added to reduce hydrogen sulfide, control odors, and to encourage flocculation. As heavier solids settle, the lighter material is skimmed off the top

of the basins. Secondary treatment uses microbes to feed on remaining organic material following advanced primary treatment. OCSD uses both trickling filters and activated sludge to conduct secondary treatment. Furthermore, chlorine disinfection of all wastewater was implemented as a tertiary treatment in 2002 (Orange County Sanitation District 2005).

Solids that settle out during advanced primary treatment are further processed to reduce disease-causing bacteria, viruses, and parasites, are pressed to reduce water content, and are then considered biosolids. OCDS supports 100% recycling of its biosolids; Class A biosolids, which have no detectable pathogens in 4-gram quantities of dry solids, are land applied in surrounding California counties and Class B biosolids, which have significantly reduced levels of pathogens, are land applied in Arizona. OCSD biosolids are also composted or landfilled in both California and Arizona (Orange County Sanitation District 2005).

In 1999, the City of Huntington Beach and the County of Orange began diverting dry weather runoff that was collecting in the Talbert Marsh forebays. This runoff was sent to the sanitary sewer system for treatment and disposal in an attempt to reduce runoff impact. During the stormy season, generally from November to March, the diversions are terminated to prevent overburdening the treatment facilities (Reeves, Grant et al. 2004).

Although OCSD has no compliance limit for bacteria in the plant or for the final effluent discharged by the plant five miles offshore, the District has set its own daily operational goals for chlorine residual and bacterial level within the final effluent. Daily bacterial goals for the final effluent, which are diluted 100:1 by an outfall diffuser, are <100,000 MPN/100 ml for TC, <20,000 MPN/100 ml for FC, and <3,500 MPN/100 ml for ENT (Orange County Sanitation District 2005). Ocean outfall dye studies conducted by OCSD have shown that

neither the 120-inch pipeline nor the out-of-service 78-inch pipeline leak and all water quality tests performed offshore indicate that the discharge plume is not moving onshore (Orange County Sanitation District 1999). One study (Boehm, Sanders et al. 2002) did present evidence that the OCSD's sewage outfall might be a source of fecal contamination in the surf zone at Huntington Beach by internal wave driven cross-short transport. Follow-up studies have largely disputed the outfall as being a source of pollution, as the water quality problem in the surf zone persisted even after dramatic reductions in fecal indicator bacteria concentrations were found following OCSD's implementation of partial disinfection of sewage outfall (Noble and Xu 2004).

Recent History of Huntington State Beach

Beach Closures

Beginning in April 1999, monitoring data reveals elevated concentrations of fecal indicator bacteria in the Huntington State Beach surf and on June 27, TC concentrations exceeded 10,000 MPN or CFU/100 ml. A sewage spill was initially suspected and on July 1 approximately 5000 feet along Huntington State Beach were closed to the public. Over the next several weeks, the bacterial plume spread, closing a total of more than four and one-half miles of recreational beach extending from the mouth of the Santa Ana River northward past Huntington Beach Pier (see Figure 1.1) (Orange County Sanitation District 1999; KOMEX H2O Science Incorporated 2003). No significant sewage leaks were located (Grant, Sanders et al. 2001) and by August of 1999, enterococci levels remained high, even after coliform concentrations declined. Because of the large area of closed beach, especially during peak summer usage, these events together represent one of the largest known losses in the U.S. to

recreational activities and revenues due to bacterial contamination. Over $20 million has been spent in attempt to identify the source of pollution (Noble 2005).

Studies

The beach closures at Huntington State and City Beaches prompted numerous investigations by local agencies, including OCSD, Orange County Health Care Agency, State Department of Parks and Recreation, City of Huntington Beach, and Santa Ana RWQCB. The three primary sources of contamination investigated by these agencies were 1) onshore sewage pipes, 2) offshore sewage outfall, and 3) stormwater drainage systems. Drainage from land-based sources was determined to be the most likely source of contamination at Huntington Beach based on the following four lines of evidence: "1) The high bacterial concentrations that were found in the stormwater forebays and which were subsequently pumped into the conveyance system; 2) The citrus studies that tracked the transport of Talbert Channel discharge water onto the contaminated beach areas; 3) The tidal signature of the beach contamination was consistent with tidally influenced drainage from land based sources; and 4) Diversion of stormwater from pump stations upstream of Talbert Marsh into the sewage system mitigated almost all of the beach contamination (Lemus and Weisberg 2000)." None of there initial studies considered the influence of waterfowl in Talbert Marsh as a source of the contamination at Huntington Beach.

A number of independent studies (Grant, Sanders et al. 2001; Boehm, Grant et al. 2002; Boehm, Sanders et al. 2002; Choi, Chu et al. 2003; Boehm, Shellenbarger et al. 2004; Kim and Grant 2004; Kim, Grant et al. 2004; Noblet, Young et al. 2004; Reeves, Grant et al. 2004; Turbow, Lin et al. 2004; Sanders, Arega et al. 2005) by researches at local universities,

namely UC-Irvine and California State University, San Bernardino, were also conducted following the Huntington Beach closures. These studies focused on a number of issues, including variability in surf zone water quality (Boehm, Grant et al. 2002), mass budget analysis of fecal indicators bacteria (Kim, Grant et al. 2004), generation of enterococci in the marsh (Grant, Sanders et al. 2001) and their antibiotic resistance patterns (Choi, Chu et al. 2003), groundwater discharge (Boehm, Shellenbarger et al. 2004), the impact of stormwater runoff from the Santa Ana River (Ahn, Grant et al. 2005), and surf zone entrainment and along-short transport of pollution from the Santa Ana River and Talbert Marsh outlets (Ahn, Grant et al. 2005). Furthermore, another study developed and applied a model to "predict the dry-weather tidal cycling of FIB [fecal indicators bacteria] in Talbert Marsh, an estuarine, intertidal wetland in Huntington Beach, California, in response to loads from urban runoff, bird feces, and resuspended sediments (Sanders, Arega et al. 2005)." This model predicts that surface water concentrations of TC, EC (*Escherichia coli*), and ENT in the wetland are driven by loads from urban runoff and resuspended wetland sediments on which the waterfowl forage daily (Sanders, Arega et al. 2005). All of these studies aid in understanding the dynamics of fecal contamination at Huntington Beach, although more studies are needed to fully assess its sources and effects.

The Impact of Stricter Regulations

AB 411 and the amended California Ocean Plan regulations, including beach postings in the event of single sample threshold exceedence and the introduction of enterococci, are more protective of public health than previous regulations. Analysis of 1998 monitoring data using the standards set by Assembly Bill 411 shows 143 exceedences of fecal indicator

bacteria, versus 12 occurrences when reporting under the previously-used standards (Orange County Sanitation District 1999). In addition, between July 26, 1999 and September 5, 2000, the Orange County Health Care Agency reported 99 postings at Huntington State and City Beaches. Of these, 72% were triggered by ENT single-sample violations and 25% by ENT geometric mean standard violations (Grant, Sanders et al. 2001). These postings would not have occurred prior to AB 411 or under the pre-amended California Ocean Plan. Due to these new, stricter regulations, there has been an increase in the number of days that the beaches throughout the State, especially around freshwater outlets (storm drains, rivers, and creeks), exceed the health criteria. (Orange County Sanitation District 1999). It is important to note, however, that the completion of Talbert Marsh, and subsequent influx of waterfowl to the area, and the stricter enterococci water quality standard addition to the California Ocean Plan occurred during the same year. This provides further reason to look more closely at pathogens in the marsh area than to try and interpret the human health risks based on different bacterial indicators and their specific action levels.

Talbert Watershed Geography

A watershed is a "geographic area draining into a river system, ocean, or other body of water through a single outlet and includes the receiving waters. Watersheds are usually bordered, and separated from other watersheds, by mountain ridges or other naturally elevated areas (Watershed & Coastal Resources Division)." In Orange County, CA, there are 13 watersheds. Talbert Watershed is located in the northern part of Orange County about 40 miles south of Los Angeles. The watershed includes portions of the cities of Costa Mesa, Fountain Valley, Huntington Beach, Newport Beach, and Santa Ana (Watershed & Coastal

Resources Division). Talbert watershed receives an average of 29 cm of rainfall annually, over 90% of which occurs between November and April. Daily high/low temperatures average 23/17^{o}C in September and 17/8^{o}C in January (Sanders, Arega et al. 2005).

Talbert Watershed, covering 3,400 hectares and standing only a few feet above sea level, drains the urbanized areas of Huntington Beach and Fountain Valley, CA. The surrounding urbanized areas consist of residential and commercial developments, plant nurseries, and light industry. On the western side of the Watershed, street gutters direct urban runoff into inlets and, because Talbert Watershed is nearly flat, a system of underground stormwater pipelines connect the inlets to a network of three flood control channels through transfer (or pump) stations. Each transfer station is made up of a forebay, where water can be stored, and several pumps that pump runoff into the flood control channels that converge near the ocean at a constructed wetland called Talbert Marsh (Grant, Sanders et al. 2001). Talbert Channel is the main of the three flood control channels. Huntington Beach Channel begins 2 km inland from the mouth and branches west for 5 km while Fountain Valley Channel begins 8 km from the mouth and branches east (see Figure 1.1) (Sanders, Arega et al. 2005). The channel beds consist "of beach sand and silts near the outlet and within a flood delta that penetrates a short distance into the marsh. Further inland, the marsh and channel bed consists of organic rich silts and muds, except the upper reaches of Talbert Channel and Fountain Valley Channel where the bed is lined with concrete (Watershed & Coastal Resources Division; Sanders, Arega et al. 2005)."

Talbert Marsh used to be a widespread saltwater wetland and dune system, however only a 10 hectare remnant is all that remains (see Figure 1.2). It has become a "typical south California tidal saltwater marsh with open water, wetland, and upland habitats (Grant,

Sanders et al. 2001)." Pickle weed is abundant and several bird species have begun utilizing the area (Grant, Sanders et al. 2001). Over the past century, the wetland has been drained and filled for agriculture and development, including Pacific Coast Highway construction and flood control channelization. A habitat restoration effort in 1990 created a new tidal inlet and tidal flushing now occurs. During high tides, ocean water floods the marsh and marsh sediments, Talbert Channel to the Fountain Valley Channel junction, and the length of the Huntington Beach channel to depths of roughly 1 meter. During ebb tides, a brackish mixture of ocean water and runoff drains from the system into the Pacific Ocean. In the poorly mixed inland zone, water residence times are at least one week, while the well-mixed zone near the mouth of the marsh is flushed during each tidal cycle (Grant, Sanders et al. 2001; Sanders, Arega et al. 2005). A recent tracer dye study conducted at Talbert Marsh found that the water residence time in the marsh during a weak spring tide is less than 40 minutes (Grant, Sanders et al. 2001). In this case, it is likely that the marsh is not appreciably inactivating pathogens, but is distributing them to ocean receiving waters.

Birds in Talbert Marsh

Several bird surveys have been conducted in Talbert Marsh. Surveys conducted monthly and bi-monthly at both high and low tide between 1990 and 1994 identified 64 species of water birds and 24 species of land birds. Special status species include Belding's Savannah sparrow, California least tern, California brown pelican, common loon, double-crested cormorant, long-billed curlew, elegant tern, least tern, black skimmer, and snowy plover (California Environmental Resources Evaluation System 2005). Another study found that gulls and elegant terns constituted 80% of birds visible in Talbert Marsh, with an average of

228 birds present during the day and the largest congregation of birds consisting of 1180 individuals at one time (Grant, Sanders et al. 2001).

Wild birds, including gulls, geese, and ducks, have long been considered important non-point sources of fecal contamination to surface waters (Mundt 1962; Geldreich and Kenner 1969; Mitchell and Ridgwell 1971; Hussong, Damare et al. 1979; Standridge, Delfino et al. 1979; Benoit, Brousseau et al. 1993; Alderisio and DeLuca 1999). Feces of gulls and geese may contain up to 10^7 and 10^4 colony forming units (CFU) of fecal coliforms per gram (wet weight) of feces, respectively (Hussong, Damare et al. 1979; Benoit, Brousseau et al. 1993; Ricca and Cooney 1998; Alderisio and DeLuca 1999). In geese and swans, the amount of enterococci is reported to be lower than fecal coliforms and dependent on diet. Furthermore, in some shallow aquatic environments contaminated by certain bird populations, fecal coliforms have been reported in the range of 10^2 to 10^3 CFU per 100 ml of surface water and 10^4 CFU per 100 ml of sediment (Hussong, Damare et al. 1979).

On average, the deposition rate of birds is approximately one feces per bird every three hours (Grant, Sanders et al. 2001). In one study, the wet weight of gull feces was measured over a two year period and ranged from 0.01 g to an unusually high 2.49 g, with a mean wet weight of 0.48 g per gull fecal sample (Alderisio and DeLuca 1999). The microbial content, including both fecal indicator microbes and pathogenic bacteria, of gull feces has been well documented. A range of mean concentrations of fecal coliform bacteria per gram of gull feces has been reported, from low concentrations of 22.0 (Fenlon 1981) and 39.7 (Girdwood, Fricker et al. 1985) CFU/g to up to 10^7 (Gould and Fletcher 1978; Benoit, Brousseau et al. 1993; Ricca and Cooney 1998) CFU/g. Ricca reported over 80% of herring gull fecal samples studied carried enterococci at concentrations up to 10^8 CFU/g. Three-quarters of

these fecal samples also contained somatic coliphages and 9.4% contained F+ coliphages at concentrations of 10^0 to 10^2 plaque forming units (PFU)/g (Ricca and Cooney 1998). Studies have also reported both Vero cytotoxin-producing *Escherichia coli* O157 and Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains from gull feces in Japan and the UK (Wallace, Cheasty et al. 1997; Makino, Kobori et al. 2000).

Other studies have focused on the presence of *Salmonella* (Williams, Richards et al. 1976; Fenlon 1981; Rosef 1981; Butterfield, Coulson et al. 1983; Fenlon 1983; Kapperud and Rosef 1983; Fricker 1984; Girdwood, Fricker et al. 1985; Monaghan, Shedden et al. 1985; Kirkpatrick 1986; Refsum, Handeland et al. 2002) and *Campylobacter* (Fenlon 1981; Rosef 1981; Kapperud and Rosef 1983; Whelan, Monaghan et al. 1988; Moore, Gilpin et al. 2002; Refsum, Handeland et al. 2002) in seagull feces. A researcher in Canada found 8.71 and 15.91% of the ring-billed gull population in the Montreal area were carriers of *Salmonella* spp. and *Campylobacter* spp., respectively (Benoit, Brousseau et al. 1993). In Northern Ireland, 13.7% of gull fecal samples tested were positive for *Campylobacter* spp. Of these, 75% belonged to the urease-positive thermophilic *Campylobacter* (UPTC) taxon, 17.9% were *C. lari*, and 7.1% were *C. jejuni* (Moore, Gilpin et al. 2002). An intense study focused on a number of different gull species found in both urban and rural environments. A majority (63.2%) of the tested fecal samples of the urban herring gull (*Larus argentatus*) were positive for *Campylobacter* as opposed to only 4.2% of tested fecal samples of its rural counterpart. The urban black headed gull (*L. ridibundus*) feces also contained *Campylobacter* (42.9%) and *Salmonella* (5.7%). Fecal samples of the rural species tested, including the common gull (*L. canus*) and the black-headed gull (*L. ridibundus*), were 18.9 and 13.2% positive for *Campylobacter*, respectively, while no *Campylobacter* was detected

in the feces of the lesser black-backed gull (*L. fuscus*) or the great black-backed gull (*L. marinus*) (Kapperud and Rosef 1983).

Introduction to Microbes

Salmonella and *Campylobacter* were the human bacterial pathogens focused on in this study. As discussed in Chapter II, both are commonly found in the gastrointestinal tracts of birds and both can cause gastrointestinal illness in humans. Additionally, the presence of the FC, *Escherichia coli* (ET), ENT, and F+ coliphages, was assessed. These fecal indicator microbes are commonly found in the feces of warm-blooded animals and have similar structure and persistence in the environment as many human pathogenic enteric bacteria and viruses, respectively (Gerba 1987).

Salmonella

Incidence

Incidence and severity of human salmonellosis has risen dramatically over the last 10 - 15 years. Several European countries have undergone a 20-fold increase in incidence since 1980. Antibiotic-resistant strains of *S. typhimurium* have also arisen at an alarming rate since the early 1990s, substantially adding to the public health burden. In developed countries, incidence is high in children and the elderly and more than 80% of cases occur individually as opposed to outbreaks. At least 2501 known serotypes of *Salmonella* (World Health Organization 2005) occur within only two species, *S. enterica* and *S. bongori*. Most isolates from humans and warm-blooded animals belong to subspecies I: Salmonella enterica subspecies enterica. Serotyping of somatic (O), flagellar (H), and capsular (K) antigens is

used to identify differences among subspecies. Two serotypes of *S. enterica* subsp. *enterica*, denoted *S.* Typhimurium and *S.* Enteritidis, are known to cause the majority of zoonotic cases of salmonellosis in many countries (Lake, Hudson et al. 2002).

Symptoms

Symptoms of salmonellosis occur 12 to 72 hours after infection, last four to seven days, and include "acute onset of fever, abdominal pain, diarrhea, nausea and sometimes vomiting" (World Health Organization 2005). Symptoms can become more severe in young, elderly, and immunocompromised patients, leading to dehydration and even death (World Health Organization 2005). The median period for fecal shedding is five weeks (Lake, Hudson et al. 2002). Antibiotic treatment is not generally necessary, however the emergence of multi-drug resistant *Salmonella* strains threatens to limit the use of this form of treatment (World Health Organization 2005).

Reservoirs

Poultry and pigs are considered major reservoirs of *Salmonella*, but they are also found in fish, terrapins, frogs, and birds (Lake, Hudson et al. 2002). Some studies suggest *Salmonella* incidence is probably low for most wild bird species, however other studies suggest a sometimes high prevalence of *Salmonella* in gulls and corvids due to nesting near and feeding on human waste and garbage (Hernandez, Bonnedahl et al. 2003).

Transmission

Transmission of salmonellae in humans is primarily through the consumption of raw or undercooked meat, poultry, poultry-derived products, or other contaminated foods or through direct contact with contaminated animals. Human to human transmission is uncommon in developed countries, however less is understood about transmission in developing countries (World Health Organization 2005).

Survival

Survival of salmonellae in dry environments is a characteristic of these organisms, and they have been shown to survive for months in soil. Studies have also shown that *Salmonella* have a prolonged survival potential in estuarine environments and the potential for survival and growth in warm temperature conditions (Rhodes and Kator 1988). In one seawater study (Jamieson, Madri et al. 1976), researchers added known concentrations of *S. typhi* to sterilized seawater of different salinities and temperatures and found survival was inversely proportional to salinity and temperature. Maximum survival was for seven days (t_{90} = 23 hours) at 4 C and 0.5% salinity and minimum survival was for five days (t_{90} = 17 hours) at 37 C and 3.5% salinity. In another study, samples of water were taken from the open sea and others at a distance of 50 - 250 m from the mouth of a sewer in the sea and each was inoculated with different species of *Salmonella*. Survival time in open sea samples ranged from 12 - 23 days, while samples collected near the coast survived 3 - 12 days (Mitscherlich and Marth 1984). It is important to note that these data are taken from a secondary source that does not report inactivation rates or changes in concentration (from initial to final) over time. Finally, *in situ* studies at two depths of seawater found t_{90} values ranging from 1.4 to

3.7 days and significant differences between microbial inactivation rate and temperature were not consistently observed. In addition, microbial reductions occurred more rapidly under field conditions than at similar temperatures in laboratory seeded seawater experiments (Wait and Sobsey 2001).

A number of studies have assessed the survival of *Salmonella* spp. in feces. As reported in Mitscherlich and Marth (Mitscherlich and Marth 1984), *S. senftenberg* survived for 14 days at 8°C, 8 days at 20°C, and >8 days at 37°C in pig slurry. *Salmonella enterica* Typhimurium was not detectable after 19 days at 37°C, but survived for at least 60 days at 4 and 20°C in cattle slurry and 48 days at 37°C in cattle manure. Analysis of decimal reduction times (DRT; times require for 90% reduction) in this study found that *Salmonella* survived less well in liquid manure than solid manure at 20 and 37°C and the opposite at 4°C. In addition, greater survival occurred at 20 and 37°C in fresh manure slurry than old manure slurry, indicating that "manure stored at warm temperatures for a period of time is an unfavorable environment for survival of pathogens (Mitscherlich and Marth 1984; Guan and Holley 2003)." Again, these data are taken from a secondary source that does not report inactivation rates or changes in concentration (from initial to final) over time. In the early 1970s, a study reported that *S. typhimurium* survived 6 – 18 weeks (t_{90} = 150 – 450 hours) in sheep feces in New Zealand. Survival was longer in shaded versus exposed sites, and in summer versus winter months (Tannock and Smith 1972). Additionally, Berkowitz et al. (Berkowitz, Kraft et al. 1974) studied the survival of various *Salmonella* serotypes inoculated into wet poultry feces. The t_{90} value, or the time it takes to eliminate 90% of the microorganisms present in the sample, averaged 184 hours at 9 – 12 C, 112 hours at 18 – 20 C, and 40 hours at 30 C. When samples were allowed to dry, the t_{90} value dropped to 21 hours at 20 C. As listed in

the appendices of Feachem et al. (Feachem, Bradley et al. 1980) , t_{90} values for *Salmonella* in feces or night soil in tropical climates ranged from 40 – 100 hours.

Campylobacter

Incidence

Campylobacter is considered the most common bacterial cause of human gastroenteritis worldwide, with as many as 400 million cases annually (Haberberger and Walker 1994). Isolation rates of *Campylobacter* from diarrheal specimens in developing countries is 5 to 20%; Table 1.1 shows examples of these rates in diarrhea specimens from children in developing countries that are less than five years old. Incidence data of *Campylobacter* infection in developing countries has substantial gaps due to the lack of national surveillance programs and incidence values in terms of number of cases for a population do not exist. Case-control studies based in communities in developing countries do however provide incidence estimates of 40,000 to 60,000/100,000 for children less than five years of age. In developed countries, this estimate is significantly lower, at an estimated 300/100,000 (Coker, Isokpehi et al. 2002). In the developed world, both children and adults are at risk for infections while *Campylobacter* is endemic in the developing world and infection is usually limited to children (Rao, Naficy et al. 2001). In the United States, most cases occur as isolated, sporadic events and active surveillance through the CDC's FoodNet indicates about 15/100,000 diagnosed each year. However, as many cases go undiagnosed or unreported, campylobacteriosis is estimated to affect over 1 million US citizens each year (Centers for Disease Control Division of Bacterial and Mycotic Diseases 2003). Of the estimated 16 species and six subspecies, *C. jejuni* and *C. coli* most commonly cause human disease in

developing and developed countries. *C. upsaliensis*, *C. concisus*, and aerotolerant *Campylobacter* may also be of pathogenic importance (Coker, Isokpehi et al. 2002).

Symptoms

Symptoms of campylobacteriosis generally occur two to five days post infection, last three to six days, and include diarrhea (often accompanied by blood), abdominal pain, fever, headache, nausea, and/or vomiting (World Health Organization 2000). Patients may excrete *Campylobacter* organisms in the range of 10^6 to 10^8 bacteria/g feces (Taylor, Perlman et al. 1993). In the developed world, the organism is shed for an average of 2-3 weeks after cessation of diarrheal symptoms, while in the developing world, shedding occurs for as short as one week in children post diarrheal symptoms (Rao, Naficy et al. 2001). More severe complications of campylobacteriosis include reactive arthritis, Guillain-Barre syndrome, or death, especially among the young, elderly, and immunocompromised patients (World Health Organization 2000). In the US, approximately 1 in 1000 diagnosed campylobacteriosis infections lead to Guillain-Barre syndrome and an estimated 124 fatal cases occur each year (Centers for Disease Control Division of Bacterial and Mycotic Diseases 2003). Treatment is rarely administered, but may include electrolyte replacement, rehydration, and antimicrobial treatment (World Health Organization 2000).

Reservoirs

C. jejuni and *C. coli* have a minimum growth temperature requirement of approximately 30^{o}C, so warm-blooded animals serve as ideal reservoirs (Baker, Ball et al. 2002). *Campylobacter* has a wide distribution in domestic and food production animals such as

poultry, cattle, pigs, sheep, ostriches, shellfish, cats, and dogs (World Health Organization 2000). Wild birds such as ducks and seagulls have also been shown to be ideal reservoirs of *Campylobacter* and aid in transmission through the environment, mainly due to their internal temperature of 42^{o}C and ease of mobility (Skirrow 1990; Jones 2001). Fallacara et al (2001) reported 40% prevalence of *C. jejuni* in mallard ducks.

Transmission

In developed countries, transmission of *Campylobacter* to humans is mostly foodborne, such as by ingestion of undercooked meats and meat products and contaminated milk (Centers for Disease Control Division of Bacterial and Mycotic Diseases 2003). However, an estimated 20% of cases of illness caused by *Campylobacter* are due to vehicles of infection other than food, including water (Mead, Slutsker et al. 1999). The presence of *Campylobacter* in streams, lakes, ponds, and other source drinking waters has been implicated in a number of outbreaks in developed nations, including the U.S., New Zealand, Finland, England, Wales, Australia, and Canada. These cause of most, if not all, of these outbreaks was untreated surface water (Clark, Price et al. 2003). Waterborne outbreaks of Campylobacter generally occur in spring and early fall months, "an association attributed to seasonality of surface water contamination and infection in cattle herds (Clark, Price et al. 2003)." Campylobacteriosis in the U.S. has been estimated to affect 7 million people per year, causing up to 500 deaths and costing between $1.2 and $6 billion (Todd 1997). In developing countries, poor hygiene behaviors, especially by mothers, and suboptimal water sanitation are often linked to childhood diarrheal illness (Rao, Naficy et al. 2001). The contribution of each of these transmission routes toward the overall burden of disease is

poorly documented and understood, as reports are made sporadically, there is a wide reported occurrence of campylobacters, and the sample volumes, methods, and conditions of their occurrence have varied among different studies (World Health Organization 2000).

Survival

Because their metabolic activity does not completely cease at temperatures lower than 30°C, *Campylobacter* cells can potentially adapt and survive in transit between hosts. Although *Campylobacter* is considered a fragile organism in dry and aerated conditions, studies of its complex physiology (Kelly 2001) and its possible formation of viable but non-culturable cells have demonstrated its resilience under some environmental conditions. In water, *Campylobacter* has shown to survive eight to 28 days at 4°C and 16°C, but survival time was greatly shortened to 43 hours as temperature increased to 22°C and above. Furthermore, stream water at 4°C has shown to sustain significant numbers of VBNC *C. jejuni* for more than 4 months (Guan and Holley 2003). It is important to note that these data are taken from a secondary source that does not report inactivation rates or changes in concentration (from initial to final) over time.

Few studies have demonstrated the survival of *Campylobacter* in feces. One study reported survival for 3 days in cattle feces and liquid cattle manure and for 2 days in liquid swine manure at room temperature. *C. jejuni* has also been recovered from 12 to 21 days in human feces stored at 4°C. Another study found that *C. jejuni* was more resistant than *Salmonella enterica* Typhimurium and *Yersinia enterolitica* to the anaerobic digestion of cattle slurry at 28°C. *Campylobacter* has also been found to generally survive in the soil for up to one month after application to both the sandy arable and clay loam grassland soils

(Guan and Holley 2003). Again, these data are taken from a secondary source that does not report inactivation rates or changes in concentration (from initial to final) over time. To date, no studies have been published on the survival of *Campylobacter* in gull feces. Determining *Campylobacter* sources, presence and survival in water and feces is pivotal in understanding transmission of the organism between animals, humans, and the presence of it in the environment (Baker, Ball et al. 2002).

F+ Coliphages

General Information

Bacteriophages (or "phages") are viruses that infect and replicate in bacterial cells. Their formal classification is based mainly on capsid morphology and the single- or double-stranded nature of the nucleic acid. There are six major families (or morphological groups) of bacteriophages found in sewage and sewage-polluted waters. The term coliphage refers to bacteriophage that infect coliforms, including *Escherichia coli* and possibly other Enterobacteriaceae, found in the intestinal tracts of all warm-blooded animals (Leclerc, Edberg et al. 2000). The three groups of bacteriophages considered most useful as indicators of fecal contamination and enteric viruses are somatic coliphages, F+ coliphages, and phages that infect *Bacteriodes fragilis*.

Somatic coliphages are a heterogeneous group; they represent different virus families, are of different sizes, have diverse genomes consisting of single- or double-stranded DNA, and possess one of several helical tail types or lack a tail completely (Leclerc, Edberg et al. 2000). A common characteristic of somatic coliphages is infection via a basic cell wall receptor site on an F- *E. coli* host strain (IAWPRC 1991). The F+ coliphages are comprised

of two families, F+RNA coliphages (*Leviviridae*) and F+DNA coliphages (*Inoviridae*), which consist of single-stranded genetic material and do not contain tails (Leclerc, Edberg et al. 2000). Both types infect host bacteria via the F+ pilus; F+DNA coliphages infect through the tip of the pilus whereas F+RNA coliphages infect through its sides (Duckworth 1987). The F+ pilus is encoded for by the F-plasmid classically found in *E. coli* K-12, but it has also been introduced into other hosts in the laboratory to eliminate detection of somatic coliphages (IAWPRC Study Group on Health Related Water Microbiology 1991). The F+RNA family contains two genera: Levivirus, which contains Groups I and II phages, and Allolevivirus, which contains Groups III and IV phages. The F-DNA family, or filamentous phages, are noneveloped, flexible, rod-shaped with circular single-stranded DNA (Vinje, Oudejans et al. 2004). *Bacteriodes fragilis* phages, particularly strain HSP40, infect the anaerobe *B. fragilis* with a high degree of host-strain specificity and have the highest recovery rate from individual people (Leclerc, Edberg et al. 2000).

Coliphages as Indicators

Detection of bacteriophages offers many useful applications to environmental health as they are indicators of a variety of contaminants including feces, domestic sewage, bacterial and viral pathogens, and host organisms such as fecal coliforms. They also can be used to assess water and wastewater treatment efficiency, environmental fate of enteric viruses, and water movement in surface waters and groundwaters (Gerba 1987). However the use of somatic coliphages and F-RNA coliphages as indicators of fecal contamination has been greatly debated. Although the natural environment of bacteriophage would seem to be the same as its host, its occurrence is variable in the feces of warm-blooded animals and rare in

the feces of humans. It has been suggested that these low concentrations in feces may be a limitation of the bacterial host while in the gut due to microflora, diet, or physiological state of the animal. Conversely, high concentrations of bacteriophages are consistently found in raw sewage. Several studies have cited a correlation between the presence of coliforms, somatic coliphages, and enteric viruses in contaminated waters; other studies have been unable to verify this correlation (Gerba 1987).

More recent studies have focused on the use of F+ coliphages as enteric viral indicators. With over 140 enteric viruses identified in human feces and sewage (generally in low concentrations) and expensive, technically demanding, and time-consuming viral detection methods, the use of these phages as viral indicators is attractive. Kott (1981) has detailed a basis for this use, including phage abundance in wastewater and polluted water, inability to reproduce outside the host organism, greater resistance to inactivation than enteroviruses, and detection by quick, simple, and cheap methods. In addition, F+ coliphages have similar structure and persistence in the environment as some of the enteric viruses. There is evidence that F-RNA phages, and possibly somatic coliphages, are likely to be positively correlated with counts of enteroviruses in domestic effluents and receiving waters (Gerba 1987). Serological classification of F-RNA phages yields four general RNA phage groups that may provide some insight into the origin of fecal contamination.

High numbers of F+ coliphages are not generally isolated from the feces of domestic animals such as cows, pigs, and horses (Dhillon, Dhillon et al. 1976; Osawa, Furuse et al. 1981), but several studies have found that many avian species, including seagulls, may be constant sources of these microbes. One study found that bay seagulls released an estimated $4.4 * 10^5$ pfu F+ bacteriophages into the environment per day (Calci, Burkhardt et al. 1998).

Another study found F+ bacteriophages in 20% of seagull feces samples assayed (Grabow, Neubrech et al. 1995). Muniesa et al reported F+ bacteriophages in 39% of samples with concentrations ranging from 4 to 20 pfu/g (Muniesa, Jofre et al. 1999).

Although generally detected in environmental waters along with F-RNA coliphages, the sanitary significance of F-DNA coliphages has yet to be adequately assessed. However, in a study in Mission Bay, San Diego bathing waters, F-DNA coliphages were the predominate F+ coliphages isolated. F-DNA coliphages best predicted risks of gastrointestinal illness in these bathers and therefore provide some evidence that these colipages have some predictive ability for human health effects associated with bathing in fecally contaminated water (Sobsey 2005). In contrast, F-RNA coliphages have been widely shown to be associated with fecal contamination in water. Based on current published data, groups II and III F-RNA phages are mainly found in environments impacted by human waste, while groups I and IV are mainly found in environments impacted with animal pollution (Furuse 1987; Schaper, Jofre et al. 2002; Scott, Rose et al. 2002; Cole, Long et al. 2003).

Bacteriophages infecting species of genus *Bacteroides* and specifically *B. fragilis* phages infecting some strains of the bacterium are apparently human-specific, at least in some parts of the world. However, *B. fragilis* phages of fecal sources other than humans have been detected on some *B. fragilis* host bacteria and the apparent human-specificity of certain hosts for these phages may not be true in all parts of the world. Also, *B. fragilis* phages are only found in relatively low concentrations in sewage and other fecal waste effluents in fecally contaminated waters. This lack of detectability limits their usefulness as a fecal indicator. Furthermore, the need for strict anaerobic condition in culturing the bacterial host for detecting them can be difficult for routine laboratory analysis (Sinton, Finlay et al. 1998;

Sobsey 2005). It is important to note that of the many bacteriophage studies done, a wide variation in results has been reported. Much of this variation is likely due to the lack of a standardized method (Leclerc, Edberg et al. 2000). Standardization of methods is now being established by the USEPA (U.S. Environmental Protection Agency 2001; U.S. Environmental Protection Agency 2001), by the European Union (Mooijman, Ghameshlou et al. 2005), and by the International Standards Organization (Mooijman, Bahar et al. 2001; Mooijman, Bahar et al. 2002).

Fecal Coliforms & Enterococci

Concentrations of fecal indicator bacteria have been monitored for many decades (mainly since the 1950s) to assess recreational water quality. *Escherichia coli* and enterococci have been established by the US EPA as the preferred indicators of fecal contamination in recreational waters. Recent studies along the southern California shoreline have shown an abundance of fecal bacterial indicators in both dry and wet weather, near stormwater inputs, and along the beach (Noble, Moore et al. 2003).

Members of the total coliform group occur in human feces, but are also present in the feces of other animals and even in soils and wood, and therefore are not recommended as fecal indicators. Fecal coliforms are a subset of total coliforms and are generally more fecal-specific. However, they also include the genus *Klebsiella* which is often not of fecal origin. *Escherichia coli*, a species within the fecal coliform group, is specific to fecal material from humans and other warm-blooded animals and is regarded as the preferred coliform indicator. However, in tropical and sub-tropical climates *E. coli* may have environmental reservoirs and may not be feces-specific (Bermudez and Hazen 1988; Byappanahalli and Fujioka 2004).

Enterococci are a subgroup within the fecal streptococcus group that are generally more feces-specific than the other streptococci. It has even been suggested that certain species of enterococci, such as *E. faecium* and *E. faecalis*, are human-specific. However, evidence for this is limited. Futhermore, enterococci other than *E. faecalis* and *E. faecium* have been found in human feces and in sewage, including enterococci thought to be of specific animal sources, such as *E. bovis* of cattle. Hence, despite the apparent animal host specificity of some enterococci, there is a lack of strict specificity in linking *Enterococcus* spp. to specific animal sources. Furthermore, some enterococci have environmental reservoirs, such as terrestrial vegetation and seaweed, and therefore they are not feces-specific. Enterococci are noted for their ability to survive in salt water and other enterococci are thought to provide good information on fecal presence and, in this way, better information on pathogen presence and persistence in the environment (U.S. Environmental Protection Agency 2004; Sobsey 2005).

Introduction to Methods Used In This Study

To initially detect and enumerate *Salmonella*, *Campylobacter*, and fecal indicators from both seagull feces and estuarine waters, conventional microbiological methods were employed. These methods are relatively easy to execute, inexpensive, and are routinely performed in many microbiological laboratories. Genotypic methods were then used to genetically confirm and further characterize microbes isolated from the two sources. These methods often provide more reliable and specific analysis and recent advancements in the field of molecular biology have resulted in more routine employment of these types of methods.

Conventional Phenotypic Methods

Culture techniques combined with a series of physiological and biochemical tests have been the classic approach used to quantify and classify bacteria in environmental samples for decades. These methods include: growth in selective enrichment broth and/or on selective media; biochemical reactions, such as response to oxidase, catalase, or urease; antibiotic susceptibility testing against a panel of antimicrobial agents; and bacteriophage or bacteriocin (bactericidal peptides) susceptibility testing (Theron and Cloete 2000; Zaidi, Konstantinou et al. 2003).

Although these methods generally provide genus and species level identification of many species of bacteria and can be useful as a starting point when attempting to identify an unknown isolate, they do not always provide accurate identification and can present many negative challenges. Bacteria tend to vary in growth conditions, growth phase, and mutation, likely allowing deviation from the physiology and genotypic mix of the population found in nature. For example, the selective pressure of a certain antibiotic may allow a subgroup within a species to rapidly evolve and become dominant. Differences between each of these subgroups may be very subtle and difficult to distinguish by conventional methods alone. These methods also rely on the culturability of cells, tend to underestimate the bacterial densities in environmental samples, are labor-intensive, and are slow. Furthermore, conventional phenotypic methods only reflect a very small fragment of the entire genome, limiting the amount of information necessary for specific identification (Theron and Cloete 2000; Simpson, Santo Domingo et al. 2002; Zaidi, Konstantinou et al. 2003).

Molecular Genotypic Methods

Infectious diseases outbreaks are often caused by clonally related organisms, which are members of the same species that have common virulence factors, biochemical traits, and genomic characteristics. However, there is enough diversity at the species level due to source, geographical location, and temporal differences that organisms may be divided into subspecies or strains. The ability to strain type is very important for epidemiological surveillance and to determine fecal contamination sources. Usually phenotypic methods are unable to provide information regarding strain and in the last decade a series of genotypic molecular methods have been developed for this purpose. Many of these methods differ in their analytical approach, but all rely on the polymerase chain reaction (PCR) and DNA sequencing for determination of bacterial strain (Olive and Bean 1999).

Genotypic methods analyze bacteria at the genome level, thereby providing comprehensive and genome-specific identification of genus and species. In prokaryotes, genes for the three ribosomal RNA (rRNA) molecules (16S, 23S, and 5S) are generally linked together and co-transcribed in a single operon (Clementino, de Filippis et al. 2001). Many genotypic methods rely on specific genes encoded within the bacterial 23S rDNA for differentiation at the genus and even species levels. An average bacterial 16S rRNA molecule is approximately 1500 nucleotides long, with sufficient sequence differences to also allow differentiation (Theron and Cloete 2000). Furthermore, the internal spacer regions (ITS), which are series of genes that separate the 16S and 23S genes, exhibit a large degree of sequence and length variation. They are useful for genus and species differentiation because they vary considerably between species due to the presence of several functional units within them such as tRNA genes (Clementino, de Filippis et al. 2001). In general, most

molecular genotypic methods involve PCR amplification and sequencing of these regions. Data is compared to both known standards and other environmental isolates on large databases available through the Internet. Genotypic methods are highly sensitive, rapid, and allow direct detection of unique sequences to identify organisms.

Genetic methods can sometimes be applied directly to bacteria and other microbes in environmental samples without the need to culture. The nucleic acid of the target microbes in the sample can be extracted and directly PCR amplified. Limitations of this approach are the low concentrations of the target microbes in the environmental samples, often requiring prior concentration of the microbes, and the presence of inhibitors and other interfering materials that prevent efficient microbe recovery, nucleic acid extraction and/or amplification by PCR. These limitations are being overcome with improved methods.

Polymerase Chain Reaction

Many genotypic molecular methods rely on amplification of portions of the genome using the polymerase chain reaction (PCR). Briefly, target DNA is copied by a thermostable DNA polymerase enzyme in the presence of nucleotides and oligonucleotide primers designed to hybridize specifically to the target sequence. Multiple cycles of heating and cooling in a thermal cycler produce rounds of DNA denaturation, primer annealing, and primer extension. Target DNA is multiplied exponentially, producing billions of copies of target DNA from a single original copy, generally in three hours or less. There are numerous variations involved in PCR analysis, including multiplex PCR, RT-PCR, and real-time PCR. In RT-PCR, RNA targets are first reverse transcribed into cDNA using a reverse transcriptase. Multiple

primers target a series of sequences in multiplex PCR. Real-time PCR allows quantitative analysis of PCR amplification.

Sequencing

DNA sequencing is also often employed in genotypic molecular methods. Sequencing is the enumeration of individual nucleotide base pairs along a linear segment of DNA. Currently, Sanger dideoxy sequencing is the most often used automated method. In this method, four separate *in vitro* DNA synthesis reactions are performed using a synthetic 5'-end-labeled oligodeoxynucleotide as a primer and the ssDNA to be sequenced as the template strand. Each reaction also contains a low concentration of one of four 2',3'-dideoxynucleoside triphosphates, or ddNTPs, which lack a 3'hydroxyl group, and a higher concentration of the normal deoxynucleoside triphosphates (dNTPs). In each reaction, ddNTP is randomly incorporated at a position of the corresponding dNTP. Because the ddNTP lacks the 3' hydroxyl group, it prevents addition of the next nucleotide and terminates polymerization. The mixtures of terminated fragments, representing all positions of each indicated base, from each of the four reactions are subjected to gel electorphoresis and autoradiography in parallel. Finally, the sequence of the original DNA template strand can be determined directly from the autoradiogram (Lodish, Berk et al. 2000).

Pulsed-field Gel Electorphoresis (PFGE)

PFGE is considered the "gold standard" of molecular typing methods. In this technique, pure culture bacterial cells are placed in agarose plugs and the entire DNA genome is digested using a series of rare-cutting restriction enzymes. Digested plugs are then

embedded into hollow wells within an electrophoresis gel and large fragments are separated as the gel is subjected to alternately-pulsed, perpendicular electric fields. This process allows clear separation of large DNA fragments ranging from 10 – 800 kilobases and produces distinct banding patterns which can be photographed and stored digitally. Advantages of this method are that it is straightforward, extremely sensitive to minute genetic differences, highly reproducible, and it allows creating of databanks for all organisms. Disadvantages are that it is time-consuming, allows limited simultaneous processing, requires a database, and may be too sensitive to broadly discriminate source (Olive and Bean 1999; Simpson, Santo Domingo et al. 2002; Meays, Broersma et al. 2004).

Nucleic Acid Hybridization

Nucleic acid hybridization is a technique that "involves mixing single strands of two sources of nucleic acids, a probe which typically consists of a homogeneous populations of identified molecules (e.g. cloned DNA or chemically synthesized oligonucleotides) and a target which typically consists of complex, heterogeneous population of nucleic acid molecules (Strachan and Read 1999; Sanders, Arega et al. 2005)." Initially, double-stranded nucleic acids must first be separated by either heating or alkaline treatment, then single stranded molecules are mixed and allowed to reassociate. Annealing of a probe DNA strand and a complementary target DNA strand will form a labeled probe-target heteroduplex, allowing identification of fragments that are related in sequence to the probe within a complex population. One disadvantage of this technique is the formation of homoduplexes, which occurs when complementary probe or target DNA strands anneal (Strachan and Read 1999).

The Reverse Line Blot Hybridization method (RLB) is a type of nucleic acid hybridization method that allows the simultaneous screening of relatively many DNA samples against relative many genetic probes. Oligonucleotide probes are covalently linked to the carboxyl groups of a negatively charged and activated membrane in parallel lines using a miniblotter. Excess oligonucleotide solution is aspirated and the blot is inactivated and washed. The blot is placed back in the miniblotter, but rotated 90° so that the slots of the miniblotter now are perpendicular to the lines containing the probes. Individual slots are filled with heat-denatured biotin-labeled PCR products and hybridization takes place. After several washing steps, the blot is incubated with a streptavidin-peroxidase conjugate, which interacts with the biotin of the PCR product. Again following washing steps, chemiluminescent detection is visualized by exposure of the blot to an X-ray film. This method is not only sensitive and specific, but reproducible, easy to perform, and rapid (Kaufhold, Podbielski et al. 1994).

Microbial Source Tracking

Microbial source tracking is the concept that the "origin of fecal pollution can be traced using microbiological [i.e. fecal coliform/streptococcus ratio, presence of human enteric viruses], genotypic [i.e. pulsed-field gel electrophoresis, ribotyping], phenotypic [i.e. multiple antibiotic resistance (MAR) analysis, biochemical tests], and chemical methods [i.e. presence of caffeine or coprostanol] (Scott, Rose et al. 2002)." Because waters contaminated with human feces are likely to contain human-specific enteric pathogens, they pose a great risk to human health. Indicator organisms have been traditionally used to predict the presence of fecal pollution in water, however most of these organisms also exist in the intestinal tracts of warm-blooded animals, thereby limiting their ability to differentiate

human from animal sources of contamination. Microbial source tracking allows detection of subtle differences between these organisms, so that the host or environment from which they were derived can be identified. Knowing the origins of fecal pollution allows better assessment of health risks and the development of actions necessary to prevent further contamination (Scott, Rose et al. 2002).

Two main approaches used in bacterial typing for microbial source tracking applications are phenotypic methods and genotypic methods. Phenotypic methods are based on the analysis of bacterial phenotype, or the expression of a bacterial genome through translation into structural proteins and enzymes for specific properties such as antimicrobial resistance. There are a number of both non-molecular and molecular-based phenotypic methods employed in bacterial detection. These methods are useful in providing an indication of fecal sources and their pollution levels in water, generally down to the species present; however, they have many limitations, including the inability to determine strain types within these species and non-uniformity due to insufficient resolution or consistency of properties. In addition, these methods tend to be time-consuming and introduce biases that because they only reflect organisms that can be readily isolated from the environment. Alternatively, genotypic methods are based on the analysis of the bacterial genome itself. These molecular-based methods can provide information on an organism's genomic composition, on specific genetic traits, and on the exact nucleotide sequences within a genetic locus, allowing much more discrimination and detail of characteristics such as strain types, and also enable tracking of fecal contamination sources (Simpson, Santo Domingo et al. 2002; Zaidi, Konstantinou et al. 2003).

Summary

Objectives of this study were to determine if seagulls in Talbert Marsh are reservoirs of *Salmonella*, thermophilic *Campylobacter*, and fecal indicators, including F+ coliphages, and if they are shedding detectable levels of these microbes into Talbert Marsh and nearby ocean waters. It is possible that these microbes, and other associated microbes, pose a threat of infectivity to humans using the swimmable nearshore waters at Huntington Beach that receive output from Talbert Marsh. Both conventional microbiological methods and more-recently developed genotypic molecular methods were employed to detect, enumerate, and genetically confirm the presence of both pathogenic and fecal indicator microbes in both seagull feces and water samples. Microbial source tracking methods, including PCR, PFGE, and nucleic acid hybridization techniques, provide a means to better track and understand the impact seagull feces have on this estuarine environment and its waters that flow into the Huntington Beach surf zone.

Overall, results of these studies serve to aid in the process of identifying urban wetland fecal contamination and developing appropriate methods for controlling urban runoff to mitigate its harmful effects on surf zone water quality. Furthermore, results also may also aid in identifying measures to improve the efficiency of constructed wetlands, ultimately allowing the removal, not the accumulation, of fecal contamination containing potentially harmful human pathogens. Information from these studies may be applied to similar estuarine environments in an attempt to improve fecal contamination impacts by waterfowl on surrounding water quality.

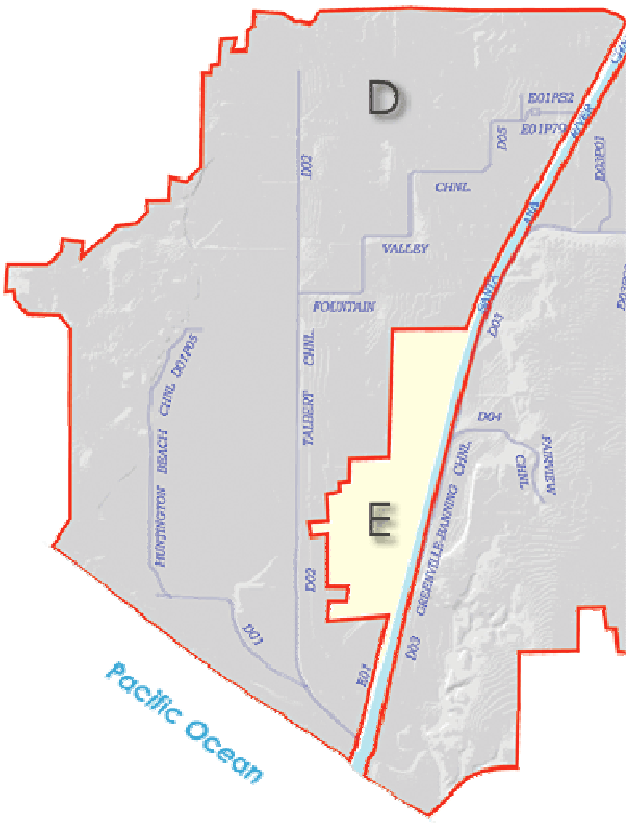

Figure 1.1: Talbert Watershed boundaries (D) and schematic of 3 flood control channels, Huntington Beach Channel, Talbert Channel, and Fountain Valley Channel (Watershed & Coastal Resources Division)

Figure 1.2: Talbert Marsh and surrounding environment

WHO region and country	Isolation rate (%)
Africa	
Algeria	17.7
Cameroon	7.7
Ethiopia	13.8
Nigeria	16.5
Tanzania	18.0
Zimbabwe	9.3
Americas	
Brazil	9.9
Guatemala	12.1
Eastern Mediterranean	
Egypt	9.0
Jordan	5.5
Southeast Asia	
Bangladesh	17.4
Thailand	13.0
Western Pacific	
Laos	12.1

Table 1.1: Isolation rates of *Campylobacter* from diarrhea specimens from <5-year-olds in selected developing countries (Coker, Isokpehi et al. 2002)

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CHAPTER II

Evaluation of Fecal Contamination by Seagulls in an Urban Estuarine Environment Using Microbiological Approaches

Introduction

Fecal contamination of coastal environments by human and animal sources is an important environmental and public health impact that can degrade environmental quality and cause waterborne infectious disease risks to exposed humans and animals. Despite regulations and management systems to address these impacts, there is inadequate and uncertain information on them in many coastal environmental settings. Highly urbanized coastal areas are especially vulnerable to potential human health risks from fecal contamination of their coastal waters due to high populations, multiple sources of both point and non-point fecal contamination, and the prevalent practice of using these waters for primary contact recreation.

Talbert Marsh in Orange County, California, is a tidally-influenced constructed wetland that receives urban runoff from Talbert Watershed and drains into the surf zone at Huntington State and City Beaches. Hundreds of wild birds, specifically seagulls and terns, take daily refuge on the sandflats of the marsh. Recent studies have found that enterococci bacteria are present at high concentrations in bird feces, marsh sediments, and on marine vegetation in Talbert Marsh and concluded that the marsh enterococci are a significant cause of poor surf zone water quality at Huntington State and City Beaches (Grant, Sanders et al. 2001). These beaches receive several million visitors annually. Multiple adverse health

outcomes, including gastroenteritis, have been linked to exposure to marine recreational water of poor microbiologic quality throughout the United States and worldwide. The recent findings in Talbert Marsh prompted this research to examine the relationships between the types of enteric bacterial pathogens and microbial indicators present in the feces of marsh seagulls and the types and sources of the same microbes within the marsh waters potentially impacted by these seagulls.

Background

Wild Birds and Infectious Diseases

Wild birds harbor and shed a number of microorganisms that are infectious to human populations and the spread of these pathogens by wild birds is a persistent public health burden. In North America, birds have been implicated in the spread of diseases such as viral encephalitis, Lyme disease, influenza, salmonellosis, mycobacteriosis, and chlamydiosis. Recent studies have also found that multi-drug resistant bacteria, including *Salmonella spp*. and *Campylobacter spp*., have also been isolated from wild birds. Birds may serve as hosts to these disease-causing organisms or they may carry infected arthropod vectors that may be dispersed over vast distances. An even greater public health burden exists when these birds harbor organisms responsible for emerging infectious diseases, or diseases that appear and rapidly increase in incidence in previously unaffected populations or regions as epidemics and pandemics. The migratory nature of birds may establish new endemic foci of disease far from where infection was acquired. The global spread of disease by birds has been likened to the spread of disease by humans following the advent of aircraft travel (Reed, Meece et al. 2003).

Waterfowl

Waterfowl is a collective term for over 140 species of swans, geese, and ducks within the class Aves, order Anseriformes, and family *Anatidae* (Wikipedia 2005). Waterfowl are most well known as reservoirs for all influenza A viruses, which are spread by fecal-oral transmission in untreated water (Webster 1997). The emergence of avian influenza A H5N1 is a recent example of how birds and especially waterfowl have contributed to the spread of a potentially zoonotic pathogen, including waterborne transmission among birds.

A number of studies have analyzed the bacteriological composition of waterfowl feces and its impact on surrounding natural water environments. Among the many species of free-living waterfowl, these studies have generally focused on Canada Geese, whistling ducks, mallard ducks, and swans. These birds live within natural wetland environments, including lakes, streams, rivers, and estuarine and coastal regions. Several studies have also determined the impact of waterfowl feces on man-made water environments such as private amenity ponds (Abulreesh, Paget et al. 2004), lakes within public parks (Feare, Sanders et al. 1999), and within zoological settings (Fallacara, Monahan et al. 2004). Bacteria studied include *Escherichia coli* (*E. coli*) (Hussong, Damare et al. 1979; Fallacara, Monahan et al. 2001; Fallacara, Monahan et al. 2004; Middleton and Ambrose 2005), *Campylobacter jejuni* (*C. jejuni*) (Hill and Grimes 1984; Fallacara, Monahan et al. 2001; Fallacara, Monahan et al. 2004), *Salmonella* spp. (Fallacara, Monahan et al. 2001; Alley, Connolly et al. 2002; Fallacara, Monahan et al. 2004), *Yersinia* spp. (Kawaoka, Otsuki et al. 1984), fecal coliforms (Hussong, Damare et al. 1979), and fecal streptococci (Hussong, Damare et al. 1979; Middleton and Ambrose 2005).

Gulls

Gulls are within the class *Aves* and also live in and around natural water environments, but are categorized within the order Charadriiformes and family *Larida*. There are over 50 species of these seabirds, most belonging to the large genus *Larus*. Gulls are typically found in coastal and inland regions and coexist successfully with humans. They are generally ground-nesting omnivores, taking live food, such as crabs and small fish, or scavenging (Wikipedia 2005). Gulls are known to be attracted to untreated sewage, sewage treatment facilities, garbage dumps, solid waste landfills, manure management systems, and other sources of enteric pathogens. They often congregate in large numbers within these areas and ingest a variety of microbes before flying many miles to other food sources or to roosting areas, where they rest, preen, and defecate (Fenlon 1981; Butterfield, Coulson et al. 1983; Reed, Meece et al. 2003). Marked gulls have been observed to travel 25 miles to feed at dumps on a daily basis (Snoeyenbos, Morin et al. 1967).

The microbial content of gull feces, including both fecal indicator microbes and pathogenic bacteria, has been well documented. Specific information on bacteria and fecal indicators that are found in gull feces that were analyzed in this study, including *Campylobacter*, *Salmonella*, fecal coliforms, and F+ coliphages, are described below. Other microbes studied in gull feces include, but are not limited to, *Shigella* (Karaguzel, Koksal et al. 1993), *Helicobacter* (Seymour, Lewis et al. 1994), *Aeromonas* (Benoit, Brousseau et al. 1993), *Listeria* (Quessy and Messier 1992), *Edwardsiella* (Berg and Anderson 1972), drug-resistant *E. coli* (Makino, Kobori et al. 2000), and pathogenic marine vibrios (Badley, Phillips et al. 1990).

Salmonella

The presence of *Salmonella* in gulls is very well documented (Berg and Anderson 1972; Fenlon 1981; Rosef 1981; Butterfield, Coulson et al. 1983; Fenlon 1983; Kapperud and Rosef 1983; Fricker 1984; Girdwood, Fricker et al. 1985; Selbitz, Lorenz et al. 1991; Quessy and Messier 1992; Benoit, Brousseau et al. 1993; Palmgren, Sellin et al. 1997; Sixl, Karpiskova et al. 1997; Refsum, Handeland et al. 2002; Wahlstrom, Tysen et al. 2003; Palmgren, Aspan et al. 2005). Although class *Aves* is one of the largest reservoirs of *Salmonella* (Berg and Anderson 1972), the reported carriage rates of *Salmonella* in wild gulls is generally under 10% (see Table 2.1) and the numbers of *Salmonella* found in positive gull feces samples are also generally low (fewer than 200/g) (Girdwood, Fricker et al. 1985). In an extensive study of the incidence of *Salmonella* carriage by gulls in Scotland, Girdwood et al. (Girdwood, Fricker et al. 1985) found marked differences in geographical and seasonal carriage rates. These researchers found that higher carriage rates are found in areas associated with relatively higher human population densities, more refuse tips, and a greater amount of sewage. They also found that variation in carriage rates at different times of the year broadly reflects the variations seen in the number of reported human cases. They surmised this was a circular condition: low carriage rate in humans means low incidence of environmental contamination with *Salmonella* and less opportunity for gulls to feed on *Salmonella*-contaminated sources.

At least 2501 known serotypes of *Salmonella* (World Health Organization 2005) occur within only two species, *S. enterica* and *S. bongori*. Most isolates from humans and warm-blooded animals belong to subspecies I: *Salmonella enterica* subspecies *enterica*.

Serotyping of somatic (O), flagellar (H), and capsular (K) antigens is used to identify differences among subspecies. Two serotypes of *S. enterica* subsp. *enterica*, denoted *S. typhimurium* and *S. enteritidis*, are known to cause the majority of zoonotic cases of salmonellosis in many countries (Lake, Hudson et al. 2002). Serotyping of the *Salmonella* isolated from gulls finds serotypes of similar range and frequency to those found in both the human population and in sewage. This suggests that gulls likely ingest *Salmonella* while feeding on contaminated sewage sludge (Berg and Anderson 1972; Fenlon 1981; Butterfield, Coulson et al. 1983; Fenlon 1983; Kapperud and Rosef 1983; Fricker 1984; Reed, Meece et al. 2003).

Fenlon (Fenlon 1981) found the numbers of *Salmonella* in seagull feces to be similar to numbers found in sewage and suggests that gulls may carry infected material without actually being infected themselves. Similarly, by using a re-capture and re-sampling method, Palmgren et al. (Palmgren, Aspan et al. 2005) found that *Salmonella* infection in gulls was of short duration, and that infection was predominately expressed as carriage without disease. Others suggest that young birds may exhibit septicemia and enteritis while adult gulls suffer from a chronic form of *Salmonella* infection, involving lesions in a number of internal organs. Berg et al. (Berg and Anderson 1972) believe this implies that gulls excrete *Salmonella* for extended periods of time. However, a maximum duration of four days for *Salmonella* excretion in laboratory-maintained gulls leads others to assert that gulls are not important vectors in the spread of salmonellosis in humans (Girdwood, Fricker et al. 1985).

Drug-resistance in *Salmonella* isolated from gull feces has also been assessed in several studies. In 1981, Fenlon (Fenlon 1981) tested *Salmonella* isolates from seagull feces to the following antibiotics: sulfamethoxazole/trimethoprim (25µg), chloramphenicol (10 µg),

streptomycin 10 µg), tetracycline (10 µg), neomycin (10 µg), ampicillin (10 µg), and furzaolidone (15 µg). He found that 13% of isolates were resistant to one or more of the following: chloramphenicol, streptomycin, tetracycline, neomycin, and ampicillin. A later study found that 33% of *S. typhimurium* isolates collected from black-headed gulls in the Czech Republic were sensitive to all drugs assayed. Proportions of the strains resistant to sulfomethoxazol-trimethoprim, tetracyclin, and streptomycin were 58%, 16%, and 8%, respectively (Sixl, Karpiskova et al. 1997). Finally, among 50 gulls examined, researchers in Sweden found 2 isolates of *S. typhimurium* with multiple antibiotic resistance (Palmgren, Sellin et al. 1997).

Pulsed-field gel electrophoresis (PFGE) has allowed *Salmonella* isolates from seagulls, other animals, humans, and the environment to be compared by molecular epidemiology approaches. A study in Norway used PFGE and computerized numerical analysis to compare 142 isolates of *S. typhimurium* from avian wildlife, including 26 gulls, domestic animals, and the environment. Results suggested that gulls represent only a minor source of human *S. typhimurium* infections and that gulls may also constitute a source of infection in domestic animals and animal feed production factories or vice versa (Refsum, Heir et al. 2002). Another study used PFGE to compare three serovars of *Salmonella* from wild-living gulls, fish-meal factories, animal feed production factories, humans, and domestic animals in Norway. Identical PFGE profiles indicated potential epidemiological links between different factors as well as between gulls and factories (Nesse, Refsum et al. 2005). Birds are an important reservoir of *Salmonella* and because they live in such close proximity to humans, these and other studies have been useful for assessing avian impacts on surrounding environments.

Campylobacter

The presence of *Campylobacter* spp. in seagull feces has been investigated in several studies (Rosef 1981; Kapperud and Rosef 1983; Glunder and Petermann 1989; Quessy and Messier 1992; Palmgren, Sellin et al. 1997; Sixl, Karpiskova et al. 1997; Broman, Palmgren et al. 2002; Moore, Gilpin et al. 2002; Wahlstrom, Tysen et al. 2003). As seen in Table 2.2, documented carriage rates vary, with percent positive samples ranging from 13.7% (Moore, Gilpin et al. 2002) to 63.0% (Sixl, Karpiskova et al. 1997). However Palmgren et al. (Palmgren, Sellin et al. 1997) analyzed 50 gull feces samples and was unable to isolate any *Campylobacter* spp. A study by Kapperud et al. (Kapperud and Rosef 1983) analyzed the carriage rates in urban versus rural seagulls. Twelve percent of rural gull feces samples (n = 125) were positive for *Campylobacter* spp., while 50% of urban gull feces samples (n = 54) were positive for *Campylobacter* spp. One study found a pronounced seasonal variation in *C. jejuni* carriage, with the highest rates found in late autumn (Broman, Palmgren et al. 2002).

Two studies describe antimicrobial resistance of *Campylobacter* spp. isolated from seagull feces. In one study, all 26 isolates tested were resistant to at least three agents (penicillin, tetracycline, and sulfomethoxazol-trimethoprim), while all were sensitive to augmentan, cefotaxim, ciprofloxacin, erythromycin, nitrofurantoin, and cephazidine. Four of the 26 isolates were resistant to ampicillin and nalidixic acid (Sixl, Karpiskova et al. 1997). Stanley et al. (Stanley and Jones 1998) found that 100% of *C. jejuni* isolates from seagulls (n = 20) were metronidazole-resistant. Overall, avian isolates in the study had a higher average minimum inhibitory concentration (MIC) value than cattle, lambs, and clinical isolates.

Most PFGE studies involving *Campylobacter* spp. isolated from avian species focus on chickens (Michaud, Menard et al. 2005; Ronner, Borch et al. 2005), turkeys (Borck and Pedersen 2005), and migrating birds other than gulls (Broman, Waldenstrom et al. 2004). PFGE has been used to analyze three strains of urease-positive thermophilic *Campylobacter* isolated from seagulls and this study found that the three PFGE profiles were indistinguishable (Kaneko, Matsuda et al. 1999). Another study used macrorestriction profiling (MRP) by PFGE to analyze *C. jejuni* isolates from gulls, and to compare a subset of gull isolates to isolates from broiler chickens and humans originating from the same geographical area. Although the MRPs most prevalent in gull isolates did not occur among isolates from humans and broilers, identical MRPs were found in two gull isolates and one human isolate, suggesting colonization by the same *C. jejuni* clone (Broman, Palmgren et al. 2002).

Fecal Indicator Microbes

Few studies have been done on the presence of fecal indicator microbes, including fecal coliforms, enterococci, and F+ coliphages, in seagull feces. In a study of 23 gull fecal samples, Muniesa et al. (Muniesa, Jofre et al. 1999) found total coliforms, fecal coliforms, and fecal streptococci in 100% of samples analyzed with geometric means of 1.7×10^7, 1.4×10^7, and 2.2×10^5 CFU/g of feces, respectively. The majority of samples contained between 10^7 and 10^9 CFU of total coliforms and fecal coliforms per gram of feces, while fecal streptococci numbers were more widely distributed. Another study found an average concentration of fecal coliform bacteria per gram of feces of 3.68×10^8 in a study of 249 ring-billed gulls. In this study, seasonal averages were all within this same order of

magnitude (Alderisio and DeLuca 1999). Concentrations of fecal coliforms (CFU/g of feces) in a study of 484 seagull feces samples ranged from 1.1 x 10^6 to 2.4 x 10^7 (Benoit, Brousseau et al. 1993)

The mean density of F+ coliphages from seagull feces in one study was 281 pfu/g of feces, which was the highest of the 11 animal feces types analyzed. Researchers estimated that the daily per capita F+ coliphages (pfu/day) released into the environment were 4.4 x 10^5 for bay seagulls and 5.9 x 10^6 for landfill seagulls (Calci, Burkhardt et al. 1998). In a study of 23 gull fecal samples, Muniesa et al. (Muniesa, Jofre et al. 1999) found F+ coliphages in 39% of the samples with a mean value of 3.4 pfu/g of feces and values ranging from 4 to 20 pfu/g feces. Cole et al. (Cole, Long et al. 2003) found F+ coliphages in 2 of 2 gull feces samples tested, with a mean phage density of 7.5 x 10^4 pfu/g and a range of 200 to 1.5 x 10^5 pfu/g of feces.

Impact of Bird Feces on Recreational Waters

Wild birds are important non-point sources of fecal contamination of surface waters, as they have been reported to excrete large amounts of fecal indicator bacteria and occasionally harbor enteric pathogens (Kirschner, Zechmeister et al. 2004). Input of fecal material from wild birds into recreational waters constitutes a serious public health concern, as humans who come into contact with these microbes may develop illness. A number of studies have implicated waterfowl and other wild birds as sources of both fecal indicator bacteria and enteric pathogens in water environments; these studies are described below.

O'Keefe (O'Keefe, D'Arcy et al. 2005) reviewed several early case studies performed in Scotland and remarked that urban non-human diffuse sources, such as domestic pets and bird roosts in urban areas, were of significant concern in bathing water quality.

Abulreesh et al. (Abulreesh, Paget et al. 2004) took the approach of collecting water and sediment samples from amenity ponds in England with and without resident ducks and geese. Samples were analyzed for fecal indicators and pathogenic bacteria. Results from this study suggest that the quality of amenity ponds might be adversely affected by waterfowl.

Hussong et al. (Hussong, Damare et al. 1979) conducted a study to obtain qualitative and quantitative measures of the bacterial flora of two waterfowl species (Canada geese and whistling swans) and to determine the fate of these bacterial released by the birds into the waters of the Chesapeake Bay. Fecal material and cloacal swabs and surface waters and sediments were analyzed for a number of fecal indicator and pathogenic bacteria. Results indicated that concentrations of waterfowl can cause an elevation in fecal coliforms counts of surface water and sediment and the study provided a method for quantitative prediction of fecal coliform loading arising from migratory waterfowl populations.

In Lake Onalaska, Wisconsin, Hill et al. (Hill and Grimes 1984) analyzed water and sediment samples for *C. jejuni* and standard fecal indicators. Cecal contents of transient waterfowl representing species present during fall migration were also assayed for *C. jejuni*. Although these researchers were not successful in isolating *C.*

jejuni from any of the samples, fecal indicator counts from the water and sediment reflected the influx of approximately 619,000 ducks and geese during fall migration.

A study by Meyer et al. (Meyer, Appletoft et al. 2005) was conducted to determine the sources of fecal contamination in a small recreational lake in Iowa. Multiple samples of lake water, well water, and known fecal sources were analyzed for *E. coli* by phenotypic methods. Moderate to high levels of *E. coli* were found in lake water samples from the swimming area and the both human and goose fecal material were concluded as the likely sources of contamination.

In 1978 in Madison, Wisconsin, a public swimming beach was closed due to intermittent high fecal coliform counts during summer and fall months. Standridge et al. (Standridge, Delfino et al. 1979) determined that a combination of meteorological events and waterfowl wastes were the cause of the high bacterial counts. Results showed that fecal material, deposited by the 100-200 resident mallard ducks, contained fecal coliform bacteria which multiplied in the beach sands and were subsequently transported into the recreational lake waters.

Obiri-Danso et al. (Obiri-Danso and Jones 1999) monitored two freshwater bathing sites in Northwest England for two years. Analysis of various inputs showed that bacterial indicators and *Campylobacter* originated from a mixture of sources including a sewage treatment plant, agricultural runoff, streams, and mallard ducks.

Wither et al. (Wither, Rehfisch et al. 2005) conducted a study in Northwest England to quantify the fecal indicator load from starlings and assess its significance on surrounding water quality. Surveys of bird numbers and locations were conducted and the daily fecal organism production by the birds was estimated. The spatial

distribution of fecal organisms from the starling population was then statistically linked to synoptic water quality data. Researchers confirmed a statistically significant link between the bird population and water quality data.

Although a number of studies have focused on the microbial content and impact of waterfowl feces, little research has looked at the impact that microbes released specifically by seagulls may have on surrounding water environments. Levesque et al. (Benoit, Brousseau et al. 1993) published a study that documents the influence of gull droppings on indicator bacteria in water and documents the concentrations of fecal indicators and two pathogenic bacteria in gull fecal matter. Researchers collected seagull droppings at a ring-billed gull colony in Quebec three times over a one-month period and analyzed the droppings for fecal coliforms, *Salmonella* spp., and *Aeromonas* spp. In a second study, they collected water samples at three depths within a spring-fed lake in the city and analyzed each for fecal coliforms prior to and after attracting seagulls to the beach with food. Using ratios of *Salmonella* spp. and *Aeromonas* spp. to fecal coliforms previously measured in gull feces, they were able to estimate the concentrations of both bacteria in the water. The researchers concluded that gulls can contribute to the bacteriological degradation of recreational water.

To date, no studies have thoroughly assessed the impact of seagulls on marine water quality. Genthner et al. (Genthner, James et al. 2005) used a library-based approach to study a marine beach environment and concluded that enterococci isolated from the beach were of seagull origin. Similarly, in a study that looked at environmental effects on *Campylobacter* survival in surface waters, Obiri-Danso et al. (Obiri-Danso, Paul et al. 2001) found that *Campylobacter lari* and urease-positive thermophilic campylobacters (UPTC) from birds

were the predominant campylobacters in bathing waters of an urban coastal beach. While these studies did find evidence of microbes of seagull origin, they did not focus on assessing the microbial impact of sea birds on marine coastal water quality.

Another aspect of fecal contamination of recreational waters that must be addressed is the presence of microbes in sand. Wheeler et al. (Wheeler Alm, Burke et al. 2003) found that wet freshwater beach sand is a reservoir of fecal indicator bacteria, and that enteric pathogens may also be present in beach sand. Similarly, Bolton et al. (Bolton, Surman et al. 1999) found that both *Salmonella* and *Campylobacter*, including the strains prevalent in seagulls and other migratory birds, were present in beach sands.

Although studies have thoroughly assess the microbial content of seagull feces and concentrations, seasonal fluctuations, antimicrobial resistance patterns, and genetic relatedness of isolates, the contribution and impacts of seagull feces into recreational waters is difficult to establish. This is especially true in coastal and estuarine environments where seagulls frequent because these are dynamic systems. Changes in water levels due to tides and water flow caused by currents dilute concentrations of bacteria contributed by birds, depending on the time of day or year. Salinity, UV radiation, and availability of nutrients may also unpredictably affect the survival of fecal indicators and pathogenic bacteria. Furthermore, seagulls, which are non-migratory birds that feed in various locations throughout the day before roosting in these coastal environments, may contribute various numbers and types of microbes to a system in an unpredictable manner.

Talbert Marsh as a Potential Source of Fecal Contamination

As previously discussed, recent studies conducted at Huntington State and City Beaches in southern California found elevated levels of enterococci bacteria, the State regulated indicator of fecal pollution, in the surf zone of the beaches. The elevated enterococci levels led to beach closures and to increased efforts to determine the sources of these bacteria for their better control. These studies also found that enterococci bacteria were present at high concentrations in bird feces, marsh sediments, and on marine vegetation in Talbert Marsh. Studies concluded that the marsh was the net source of enterococci based on comparing concentrations of these bacteria at different locations near the marsh and at different tidal cycles. Overall, researchers determined that the marsh enterococci were a significant cause of poor surf zone water quality at Huntington State and City Beaches (Grant, Sanders et al. 2001). These findings prompted research to look more closely at not only the potential sources of microbial contamination within Talbert Marsh, but at the types of bacterial pathogens that also could be present in the marsh.

The focus of this present study was on the role of waterfowl fecal contamination as a potentially important contributor to the non-point sources of fecal contamination and in particular *Campylobacter* species and *Salmonella* species as bacterial pathogens potentially present in this system. As noted above, two common human bacterial pathogens harbored by birds are *Salmonella* and *Campylobacter* (Fenlon 1981; Rosef 1981; Kapperud and Rosef 1983; Whelan, Monaghan et al. 1988; Moore, Gilpin et al. 2002; Refsum, Handeland et al. 2002), both of which can cause gastrointestinal illness. Also of interest in this study were fecal indicator microbes, such as fecal coliforms, *Escherichia coli*, enterococci, and F+ coliphages, which are commonly found in the feces of warm-blooded animals and have

similar structure and persistence in the environment as many human pathogenic enteric bacteria and viruses, respectively (Gerba 1987). Identifying sources of fecal contamination as human or animal in origin is becoming a more important tool in decision making to manage fecal contamination of water as the analytical methods for such source tracking improve and become further validated.

Objectives

The objectives of this study are as follows:

To detect and identify key bacterial pathogens and selected indicators of fecal contamination in the feces of seagulls present in Talbert Marsh

- To use conventional microbiological methods to identify and quantify *Salmonella* and *Campylobacter* species in the feces of seagulls.
- To use conventional microbiological methods to identify and quantify indicators of fecal contamination, specifically F+ coliphages, in the feces of seagulls.

To detect and identify key bacterial pathogens and indicators of fecal contamination in the estuarine waters of Talbert Marsh

- To use conventional microbiological methods to identify and quantify *Salmonella* and *Campylobacter* species in the estuarine waters of Talbert Marsh.
- To use conventional microbiological methods to detect, identify, and quantify indicators of fecal contamination, specifically F+ coliphages, in the estuarine waters of Talbert Marsh.

To use several genotypic microbial source tracking methods, including antibiotic resistance analysis and pulsed-field gel electrophoresis, to characterize the presence and impacts of fecal contamination from seagulls in Talbert Marsh and its potential transport and presence into the nearby estuarine waters.

Approaches

Individual, freshly-deposited seagull fecal specimens from the sandflats where seagulls take daily refuge within Talbert Marsh and estuarine water samples from several areas within Talbert Marsh were collected (see Figure 2.1). Water samples collected upmarsh were intended to assess the microbial presence in the waters prior to contact with the sandflats or the impact of incoming tides and currents that could carry contaminants picked up from the sandflats, while waters collected adjacent to and downmarsh of the sandflats were intended to assess microbial presence in the waters following contact with the sandflats and associated with outgoing tides and currents. All samples were shipped at 4^{o}C overnight to UNC-Chapel Hill laboratories so that processing could begin within 24 hours of collection.

Enteric Bacterial Pathogens

To identify and quantify *Salmonella* and *Campylobacter*, known volumes of seagulls feces and known volumes of estuarine water were submitted to a series of enrichment culture steps to resuscitate injured cells and increase target cell concentrations. Enrichments were then subcultured onto appropriate selective agars for isolation of distinct colonies and subsequent biochemical testing to phenotypically identify the bacterial complexes or species present. A number of positive isolates were subjected to additional analyses, including antimicrobial resistance testing and pulsed-field gel electorphoresis (PFGE), for further identification of strain types present.

Microbial Indicators

To identify and quantify F+ coliphages in the feces of seagulls present in Talbert Marsh, a known volume of freshly-deposited waterfowl feces was submitted to a Double Agar Layer plaque assay in which *E. coli* Famp host cell infection by F+ coliphages present in the feces was visualized and quantified. Two different US EPA methods were employed to identify and quantify F+ coliphages in known volumes of estuarine water. Because phage concentration in the estuarine waters was unknown, one method (EPA 1601) first included an enrichment step for low concentrations of coliphages, while the other method (EPA 1602), designed for higher concentrations of coliphages, was a Single Agar Layer plaque assay method. Both methods use *E. coli* Famp host cell for detection of infection by F+ coliphages. Additionally, water samples were analyzed for fecal indicator bacteria, including fecal coliforms, *Escherichia coli*, and enterococci, using Colilert and Enterolert defined-substrate quantal assays, respectively.

Methods

Seagull Feces Samples

Sample Collection and Preparation

The intention and expectation of UNC researchers was for UCI researchers to collect bird feces from the sandflats within Talbert Marsh (see Figure 2.1) directly following a visual confirmation of seagull defecation and for collectors to avoid collecting other debris such as sand grains and vegetation. Per sampling expedition, ten fecal samples were collected using separate, sterile wooden applicator sticks and placed into separate, sterile, pre-labeled, and pre-weighed 50-ml conical tubes. A chain of custody form was completed for each sampling

trip, indicating the following: date, time, bird species or common name, sites or locations of collection, air temperature, current weather conditions, current or recent storm events, sample collector, sample storage prior to shipment, and shipping information. Conical tubes and the chain of custody forms were shipped overnight in small coolers to UNC at 4 C.

Upon arrival in the lab, conical tubes containing fecal specimens were weighed separately. The weight of the empty conical tube (determined before samples were collected) was subtracted from this weight to determine to wet weight of each fecal sample. Dulbecco's phosphate buffered saline (DPBS) was added up to the 35 ml mark on the conical tube. DPBS is a buffered saline solution that is compatible with the physiology and integrity of both eukaryotic and prokaryotic cells. Fecal samples were then homogenized with DPBS using a magnetic stir bar and plate.

Salmonella *Microbiological Analyses*

Approximately fifteen milliliters, or about 43%, of the fecal homogenate was submitted to a series of enrichment steps in a 3-dilution and 3-replicates-per-dilution (3 X 3) MPN format.

Pre-Enrichment

A pre-enrichment step was carried out in buffered peptone water (BPW), which contains nutrients to facilitate resuscitation of sub-lethally injured bacteria and to prevent bacterial damage due to changes in pH. Fecal homogenate volumes of 4.5 milliliters, as well as volumes of 0.5 and 0.05 ml were employed in enrichments to represent undiluted, and approximately 10-fold and 100-fold diluted samples, respectively. The ratio of fecal homogenate volume to diluent (BPW) volume was 1:10 for optimal enrichment with 4.5 ml

of fecal homogenate added to 40.5 ml in triplicate to serve as "undilute," 1.5 ml of fecal homogenate added to 13.5 ml of BPW to serve as "10^{-1}," and 1.5 ml of the 10^{-1} dilution added to 13.5 ml of BPW to serve as "10^{-2}." The 10^{-1} and 10^{-2} dilutions were then separated into triplicate enrichment volumes of 4.5 ml, each containing 0.5 ml and 0.05 ml of fecal homogenate, respectively. Dilutions were incubated on a shaker platform rotating at about 100 rotations per minute (RPM) at 37 C overnight.

Selective Enrichment

After overnight incubation, pre-enrichment tubes were inverted five times to mix and sterile wooden applicator sticks were used to transfer several microliters of each pre-enrichment broth into 10 ml of Rappaport-Vassiliadis (RV) broth for selective enrichment of *Salmonella spp*. RV broth contains Malachite Green Oxalate and has a low pH, both of which are inhibitory to most organisms other than *Salmonella spp*. Enrichments were incubated at 43 C for 24 hours to help select for bacteria such as *Salmonella* that are able to survive this elevated temperature.

Subculture

RV enrichments were subcultured for isolation onto Salmonella-Shigella (SS) agar plates that were then incubated in an inverted position at 37 C overnight. SS agar is a moderately selective medium for the isolation of pathogenic enteric bacilli, especially those belonging to the *Salmonella* genus. It contains brilliant green, bile salts, and citrates that inhibit most gram-positive microorganisms. Sodium thiosulfate and ferric citrate enable the detection of hydrogen sulfide production, which cause the centers of *Salmonella* colonies to turn black.

Tan colored colonies with black centers were considered presumptive positive *Salmonella* and an isolated colony was re-subcultured for biochemical testing and storage.

Controls

A *Salmonella typhimurium* positive control (ATCC strain 14028 obtained from the Sobsey laboratory collection) and a negative control (DPBS) were used in each set of experiments.

Biochemical Analysis by Enterotube® II

A presumptive positive *Salmonella* colony was submitted to Enterotube® II, a commercial product for the biochemical identification of Gram-negative intestinal bacilli. The Enterotube® II consists of twelve small compartments, each containing appropriate media for the following tests: glucose, lysine, ornithine, H_2S/indole, adonitol, lactose, arabinose, sorbitol, Voges-Proskauer, Dulcitol/PA, urea, and citrate. A sterile wire was used to obtain growth from an isolated colony and was then pushed through the twelve compartments, inoculating each test. The Enterotube® II was incubated at 37 C overnight. Positive results from each compartment were recorded (Kovac's reagent is added to the indole test compartment), resulting in a 5-digit code that was located in a coding booklet provided to identify the organism.

Storage

Presumptive positive *Salmonella* colonies were suspended in 0.2 ml of tryptic soy broth (TSB) containing 25% glycerol and stored at -80 C.

Antimicrobial Susceptibility Testing

A subset of *Salmonella spp*. isolates representing all positive seagull feces samples was tested for antimicrobial resistance, expressed as a minimum inhibitory concentration (MIC) against the broad range of antimicrobials shown in Table 2.3. A sterile wooden stick was used to transfer several *Salmonella* colonies to sterile deionized water in a glass tube. The tubes were mixed well and, if necessary, adjusted to a turbidity standard of 0.5 McFarland units. Growth from more colonies were added to match the standard, if necessary. Ten microliters of the cell suspension were added to a Mueller Hinton broth tube which was mixed ten times and then poured into a plastic tray. A multiple pipettor was used to transfer 50 µl of this suspension into each well of dehydrated antimicrobials. Antimicrobials were oriented from low to high concentrations horizontally within well trays. A plastic adhesive cover was secured to the top of the well tray to prevent desiccation and trays were incubated overnight at 35 C. The well with the lowest antibiotic concentration in which there was growth was recorded for each antimicrobial. These concentrations were compared to susceptible, intermediately-resistant, and resistant standard concentrations set by the NCCLS.

PFGE

A subset of *Salmonella spp*. isolates representing all positive seagull feces samples was genotypically characterized using PFGE for strain-typing and to assess the genetic variation among isolates. Briefly, freshly grown colonies were suspended in 500 µl of 0.9% NaCl, vortexed, and centrifuged at 25,000 rcf (Eppendorf centrifuge 5417C) for 30 seconds to pellet cells. The cell suspension volume was adjusted so that the pellet size was between three and four millimeters in diameter in order to harvest the appropriate number of cells.

Supernatant was removed and the cell pellets were resuspended in 150 µl TEN buffer (100 mM EDTA, 100 mM Tris, 150 mM NaCl; pH 7.5). Then 150 µl of molten 2% FMC InCert® Agarose (Cambrex, Rockland, ME) was added to the suspensions and mixed, and this was added to BioRad gel molds to make the plugs. Plugs were digested with proteinase K (20 mg/ml) in ES buffer (400 mM EDTA, 1.0% Sarkosyl; pH 9.3) overnight at 50 C. Plugs were then washed several times with gentle shaking in CHEF TE buffer (100 mM Tris,100 mM EDTA; pH 7.5). Total genomic DNA was digested by placing thin slices from the respective plug in 300 µl DNS buffer (100 mM Tris, 5 mM $MgCl_2$; pH 8.0), washing four times, and adding 20 U of *Xba*I in D buffer BSA (Promega, Madison, WI) and 1% to the wells at 37 C overnight.

PFGE was performed with the BioRad CHEF-DR® II System in 0.5x Tris-borate-EDTA. DNA macrorestriction fragments were resolved on 1% (w/v) SeaKem GTG® agarose (BMA, Rockland, ME) gels. Pulse Marker 50 to 1000 kb (New England Biolabs, MA) consisting of concatemers of lambda DNA were used as size standards. Gels were run for 19 hours at 6 V/cm with pulse times ramped at 2.2 to 63.8 seconds. Gels were stained by immersion in ethidium bromide (5 µg/ml), destained, and visualized under UV light.

Interpretation of macrorestriction fragment patterns were performed following the criteria of Tenover et al. to determine the category of genetic relatedness of strains. Basically, isolates are designated as indistinguishable if their restriction patterns have the same numbers of bands and the corresponding bands are the same apparent size. If isolate patterns differ by changes consistent with a single genetic event such as a point mutation or an insertion or deletion of DNA, which typically results in two to three band differences, the isolates are considered to be closely related. Isolates are considered to be possibly related if the PFGE

patterns differ by changes consistent with two independent genetic events, which typically results in four to six band differences. These isolates are generally unlikely to be related epidemiologically. Finally, isolates are considered unrelated if PFGE patterns differ by changes consistent with three or more independent genetic events, typically seven or more band differences (Tenover, Arbeit et al. 1995).

Campylobacter *Microbiological Analyses*

Approximately fifteen milliliters, or about 43%, of the initial fecal homogenate in DPBS was submitted to *Campylobacter* enrichment in a 3-dilution and 3-replicates-per-dilution (3 X 3) MPN format.

Selective Enrichment

Enrichment was in Preston Enrichment (PE) broth, a medium low in carbohydrates because campylobacters do not ferment carbohydrates. PE broth is composed of a nutrient base of beef extract (1% w/v), sodium chloride (NaCl) (0.5% w/v), and peptones (1% w/v) to which laked horse blood (5% v/v) was added. Laked blood is blood that has been hemolyzed, or whose red blood cells have been destroyed in such a way that hemoglobin is liberated into the medium in which the cells are suspended, by alternate freezing and thawing. This process does not use chemicals for hemolysis and therefore is more gentle on fragile *Campylobacter* cells. Blood is included to quench toxic oxygen compounds, such as hydrogen peroxide, that are often formed when media are exposed to light and also neutralizes trimethoprim antagonists (Donnison 2002). Antibiotics included in PE broth

were rifampicin (0.001% w/v), cycloheximide (0.01% w/v), trimethoprim lactate (0.001% w/v), and polymyxin B (0.000066% w/v).

Dilutions were made as for *Salmonella* enrichment, with volumes of 4.5, 0.5, and 0.05 ml for undiluted, and approximately 10-fold and 100-fold sample volumes. For each dilution, the ratio of fecal homogenate volume to diluent volume was 1:10 for optimal enrichment with 4.5 ml of fecal homogenate added to 40.5 ml of PE broth in triplicate to serve as "undilute," 1.5 ml of fecal homogenate added to 13.5 ml of PE broth to serve as "10^{-1}," and 1.5 ml of the 10^{-1} dilution added to 13.5 ml of PE broth to serve as "10^{-2}." The 10^{-1} and 10^{-2} volumes were then separated into triplicate enrichment volumes of 4.5 ml, each containing 0.5 ml and 0.05 ml of fecal homogenate, respectively. Dilutions were incubated microaerophilically, initially at 37 C for 4 hours to resuscitate injured cells, then transferred to 42 C for 44 hours for selective enrichment of thermotolerant *Campylobacter*.

Subculture

Following incubation, 50 µl of each PE enrichment were subcultured for isolation onto CBA plates. CBA plates were inverted and incubated microaerophilically at 42 C for 48 hours. CBA plates are Brucella agar supplemented with sheep blood; vancomycin, which inhibits Gram-positive bacterial growth; trimethoprim, a broad spectrum antibiotic; and cephalothin, an antibiotic that inhibits the growth of streptococci. Colonies that were small, round, whitish, and encircled with a thin, clear halo were considered presumptive culture-positive thermotolerant campylobacters. Occasionally, other colonies not specifically fitting this description were also considered presumptive culture-positive thermotolerant campylobacters.

Storage

Presumptive culture-positive *Campylobacter* colonies were suspended in 0.2 ml of TSB containing 25% glycerol and stored at -80 C for subsequent regrowth for biochemical testing and future molecular analyses.

Controls

A *Campylobacter jejuni* positive control (obtained from the UNC Hospitals clinical laboratory) and a negative control (DPBS) were used in each set of experiments.

Biochemical Tests

When able to be regrown, presumptive culture-positive thermotolerant *Campylobacter* colonies were submitted to two biochemical tests, the catalase and oxidase tests, for further identification.

1. Catalase Test

Catalase is an enzyme that catalyzes the breakdown of hydrogen peroxide with the release of free oxygen. Hydrogen peroxide is a byproduct of respiration and is toxic if it accumulates in the cell. Some bacteria possess catalase as a defense mechanism to minimize the harm done by hydrogen peroxide by splitting it into water and oxygen (MacFaddin 2000). To test for catalase activity of bacteria isolated in this study, a drop of 3% hydrogen peroxide was added to a bacterial colony. The production of bubbles, or oxygen gas, indicated a positive reaction.

2. Oxidase Test

Oxidases are enzymes found in aerobic bacteria that activate oxidation of reduced cytochrome by molecular oxygen, which in turn acts as an electron acceptor in the terminal stage of the electron transfer system. The oxidase test reagent dyes are artificial electron acceptors and the final oxidase reaction yields a colored product (MacFaddin 2000). In this study, a dry, sterile paper disc was moistened with *p*-phenylenediamine dihydrochloride (Difco 261181) and a bacterial colony was then smeared onto the disc. A positive result was indicated by a color change to purple within several seconds. A negative result was indicated by no change in color or the development of color after 60 seconds.

Gram Stain

Oxidase- and catalase-positive colonies were submitted to a Gram stain and then viewed under 100X microscopy using a Leitz Orthoplan 2 microscope. The gram stain is a differential staining technique that differentiates between Gram-positive and Gram-negative bacterial cells based on uptake and retention of Crystal Violet dye. Briefly, a bacterial colony was smeared onto a sterile water droplet on a slide and allowed to air dry. The slide was then heat fixed by passing it through a flame quickly five times. Crystal violet stain was added to the slide, allowed to set for 1-2 minutes, then rinsed with deionized water. Both Gram-positive and Gram-negative bacteria become stained and appear purple at this step. Gram's iodine was then added to the slide for 1 minute and rinsed with deionized water. This allows the stain to be better retained by forming an insoluble crystal violet-iodine complex. With the slide at an angle, Gram's decolorizer, a mixture of ethyl alcohol and acetone, was added drop by drop. This causes the dehydration of the peptidoglycan in the

gram-positive cell wall, trapping the crystal violet-iodine complex within the cell. In gram-negative bacteria, the decolorizer dissolves the outer membrane of the cell wall and the crystal violet-iodine complex is unable to be retained within the cell. Finally, safranin counterstain is added to the slide, allowed to set for 1 minute, and rinsed with deionized water. Since gram-positive bacteria are already stained purple, they are not affected by the counterstain, but gram-negative bacteria that are now colorless become directly stained by the safranin and appear pink. *Campylobacter* cells are Gram-negative, curved rods. They may also appear S-shaped and gull-wing-shaped when two cells form short chains. Cells in old cultures may form coccoid bodies.

Microscopy

After three years of cell storage at -80 C and several freeze-thaws, stored cell suspensions were studied using two different microscopy techniques to assess cell concentration and cell viability for future studies.

1. Cell Counting

Ten microliters of bacterial suspension were placed under a coverslip of known area and viewed under a 60X oil-immersion objective of a Nikon FXA microscope. Cells were counted for ten separate fields within the coverslip area. Photographs were taken with an Optronics DEI 750 camera and were analyzed using Scion Image software on a Mac G3 computer. The number of fields per coverslip was determined and the average number of cells per field was multiplied by the total number of fields. This number was then divided by

the volume of bacterial suspension placed under the coverslip (10 µl) to determine the number of cells per microliter in the original bacterial suspension (see Figure 2.2).

2. Live/Dead ("Viability") Assay

The Live/Dead® *Bac*Light™ Bacterial Viability assay uses mixtures of the green-fluorescent nucleic acid stain SYTO® 9 and the red-fluorescent nucleic acid stain propidium iodide. When used alone, SYTO® 9 labels all bacteria in a population, both those with intact membranes and those with damaged ones. Propidium iodide however only penetrates bacteria with damaged membranes and causes a reduction in the SYTO® 9 fluorescence when both dyes are present. When a correct mixture of the two dyes is applied to bacteria, bacteria with intact cell membranes stain fluorescent green and bacteria with damaged membranes stain fluorescent red.

A subset of presumptive culture-positive *Campylobacter* isolates stored in TSB with 25% glycerol were subjected to the Live/Dead® *Bac*Light™ Bacterial Viability assay. Briefly, equal volumes of Component A and Component B, which are solutions of SYTO® 9 and propidium iodide mixed in two different proportions provided in the kit, were mixed in a microfuge tube. Three microliters of the dye mixture were added for each milliliter aliquot of bacterial suspension. Because bacterial suspension volumes were low, 0.15 µl of the dye mixture was added to 50 µl aliquots of bacterial suspension. These were mixed well and incubated at room temperature in the dark for 15 minutes. Five microliters of the stained bacterial suspension was trapped between a slide and an 18 millimeter square coverslip. The live (green fluorescent) and dead (red fluorescent) cells were observed separately with fluorescein and Texas Red bandpass filter sets under 100X magnification using a Leitz

Orthoplan 2 microscope. A digital camera and Scion software were used to capture microscope images for subsequent qualitative analysis.

F+ Coliphage Microbiological Analyses

Double Agar Layer (DAL) Plaque Assay

Five milliliters, or about 14%, of the fecal homogenate in DPBS was submitted to a double agar layer plaque assay method for visualization and quantification of F+ coliphages. The fecal homogenate was micro-centrifuged (Eppendorf centrifuge 5415C) at 735 x g at 4 C for 20 minutes. One milliliter of the supernatant was placed in a sterile 15 ml conical tube and 0.5 ml of log phage *E. coli* Famp broth culture was added. Tubes were incubated at 37 C for five minutes to allow phage infection and replication to begin. For top agar, the following constituents were added to molten agarose (1% w/v) at the indicated final concentrations: glucose (0.1% w/v), a growth nutrient for *E. coli*; $CaCl_2$ (0.03% w/v) and $MgSO_4$ (0.015% w/v), to aid in phage attachment to bacterial pili; ampicillin and streptomycin (0.0015% w/v each) antibiotics, to prevent the growth of all bacteria except *E. coli* Famp; and 3 ml of log phage *E. coli* Famp broth culture, for F+ coliphage infection and replication. Six milliliters of the molten top agar were added to each tube of supernatant/*E. coli* Famp mixture, the tubes were swirled to mix, and poured onto tryptic soy bottom agar plates. After solidifying, the plates were inverted and incubated at 37 C overnight. Plaques, or circular clearing zones in the bacterial lawn that arise from discrete infectious phages, were counted for each sample and concentrations were expressed as plaque forming units per milliliter (PFU/ml).

Controls

An MS2 F+ coliphage positive control (obtained from the Sobsey laboratory collection) and a negative control (DPBS) were used in each set of experiments.

Storage

A pipet tip on a micropipettor was used to recover plaque material from the top layer of agar of a subset of well-isolated plaques. This phage "plug" was broken up and suspended in 200 µl of PBS with 25% glycerol and stored at -80 C for future molecular analyses.

Estuarine Water Samples

Sample Collection and Preparation

The intention of UNC researchers was for UCI researchers to collect each set of three 4 liter water samples consisting of one sample collected upmarsh of the sandflats, one sample collected in an area directly surrounding the sandflats, and one sample collected downmarsh of the sandflats (see Figure 2.1). Per sampling expedition, three water samples were collected into separate, sterile cubitainers. A chain of custody form was completed for each sampling trip, indicating the following: date, time, sites or locations of collection, sample volume, water conditions including temperature, salinity, turbidity, pH, and total dissolved solids, air temperature, current weather conditions, current or past storm events, sample collector, sample storage prior to shipment, and shipping information. Coolers containing cubitainers and the chain of custody forms and chilled to a target temperature of 4 C were shipped overnight by air express carrier to UNC. Upon arrival, sterile stir bars were added to

each sample. A 50 ml aliquot of well-mixed sample was transferred into a sterile conical tube for salinity measurement.

Salmonella *Microbiological Analyses*

Approximately one liter of each water sample was submitted to a series of enrichment steps in a 3-dilution and 3-replicates-per-dilution (3 X 3) MPN format using methods and materials similar to those used for bird fecal samples. Pre-enrichment was carried out in BPW using three 10-fold different volumes of 250, 25, and 2.5 ml in triplicate and a ratio of water sample volume to diluent volume of 1:1 for optimal enrichment. Enrichments were incubated on a shaker platform rotating at about 100 RPM at 37 C overnight. Pre-enrichment bottles were inverted five times to mix and 100 µl of each pre-enrichment broth was transferred into 10 ml of RV broth for selective enrichment of *Salmonella* spp. by incubating at 43 C for 24 hours. RV enrichments were subcultured for isolation onto SS agar plates that were inverted and incubated at 37 C overnight. Presumptive positive *Salmonella* colonies were re-subcultured for biochemical testing and storage, as described previously.

Campylobacter *Microbiological Analyses*

Approximately one liter of each water sample was submitted to a series of enrichment steps in a 3-dilution and 3-replicates-per-dilution (3 X 3) MPN format using methods and materials similar to those used for bird fecal samples. Enrichment was carried out in PE broth using three 10-fold different volumes of 250, 25, and 2.5 ml in triplicate and a ratio of water sample volume to diluent volume of 1:1 for optimal enrichment. Enrichments were

incubated microaerophilically, initially at 37 C for 4 hours to resuscitate injured cells, then transferred to 42 C for 44 hours under microaerophilic conditions. Then 50 µl volumes of PE enrichment was subcultured onto triplicate CBA plates, which were inverted and incubated microaerophilically at 42 C for 48 hours. Suspect *Campylobacter* colonies were submitted to catalase and oxidase tests and Gram stain for further identification. Presumptive culture-positive *Campylobacter* colonies were suspended in 0.2 ml of TSB containing 25% glycerol and stored at -80 C for future molecular analyses. After three years of cells storage at -80 C and several freeze-thaws, a subset of *Campylobacter* water isolate cell suspensions were studied using a Live/Dead® *Bac*Light™ Bacterial Viability assay.

F+ Coliphage Microbiological Analyses

Because the concentration of F+ coliphages in water samples was unknown, two USEPA methods were used for detecting F+ coliphages. Method 1601: F+ (F+) and Somatic Coliphage in Water by Two-step Enrichment Procedure (U.S. Environmental Protection Agency 2001) is designed for lower concentrations of coliphages and involves an enrichment step. Method 1602: F+ (F+) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure (U.S. Environmental Protection Agency 2001) is designed for higher concentrations and employs direct detection of plaques in agar medium instead of broth enrichment. The MS2 F+ coliphage positive control was obtained from the Sobsey laboratory collection.

Method 1601

Method 1601 was done according to the standard protocol with modification of a 3 volume x 3 replicates per volume MPN assay. One day one, one liter of well-mixed sample was added to a four liter bottle containing 12.5 ml $MgCl_2$, to aid in phage attachment to host cells; 50 ml 10X TSB and 10 ml ampicillin and streptomycin stock concentrates (0.0015% w/v each). Five milliliters of log-phage *E. coli* Famp broth culture were added, the bottle was capped and inverted five times to mix, and triplicate volumes of 300 ml, 30 ml, and 3 ml were quickly dispensed into sterile containers, beginning with the smallest volume. A positive control of 100 ml of MS2-spiked deionized water was added to 1.25 ml $MgCl_2$, 5 ml 10X TSB, 1 ml ampicillin and streptomycin, and 0.5 ml log-phase *E. coli* Famp broth culture. A negative control of 100 ml deionized water was also included. All samples were incubated at 37 C for 16-24 hours. After overnight incubation, enrichment bottles were mixed by inverting 25 times, 0.6 ml of the enrichments were transferred into sterile Eppendorf tubes, and 0.3 ml of chloroform were added to each tube and vortexed for one minute. Tubes were centrifuged (Eppendorf centrifuge 5415C) at 735 x g for 1 minute. Ten microliters of the supernatant were spot-plated onto prepared *E. coli* Famp spot plates containing *E. coli* in tryptic soy agar (TSA), allowed to absorb, inverted, and incubated at 37 C overnight. Spot plates were checked for lysis zone formation and results were recorded. A pipet tip was used to remove a subset of well-isolated lysis zones from the top layer of agar for each sample. This phage "plug" was broken up and suspended in 200 µl of PBS with 25% glycerol and stored at -80 C for future molecular analyses.

Method 1602

Method 1602 was done according to the standard protocol, with minor modification. Briefly, 100 milliliters of well-mixed samples were added into a 500 ml bottle containing 0.5 ml $MgCl_2$, placed into a 37 C water bath for five minutes, and 10 ml of log-phage *E. coli* Famp broth culture was added to the bottle. Ten milliliters of deionized water was added to the temperature control bottle. Both bottles were placed into a 45 - 48 C water bath and when the temperature control bottle reached ~43 C, the samples were removed from the water bath, 100 ml of 2X TSA containing 2 ml ampillicin and streptomycin stock solution (0.003% w/v each) were added, bottles were inverted five times to mix, and approximately 33 ml was poured into each of six 150 millimeter Petri dishes. This procedure was repeated for controls, using 100 ml MS2-spiked deionized water for a positive control and 100 ml deionized water for a negative control. Plates were allowed to solidify, were inverted, and were incubated at 37 C for 16-24 hours. Plates were checked for lysis zone formation and results were recorded. A pipet tip on a micropipettor was used to remove a subset of well-isolated lysis zones from the top layer of agar for each sample and this phage "plug" was broken up and suspended in 200 µl of PBS with 25% glycerol and stored at -80 C for future molecular analyses.

Fecal Indicator Bacteria Analyses

Water samples were also analyzed for fecal coliforms, *E. coli*, and enterococci, using the commercial defined substrate multiple-well culture methods of Colilert®-18 and Enterolert® by IDEXX, respectively, in Quanti-trays; MPNs were calculated. For Colilert®-18, 100 milliliters of well-mixed sample were transferred into a sterile bottle containing sodium

thiosulfate. Positive control bottles of *Klebsiella pneumoniae* and *E. coli* were likewise spiked into 100 ml deionized water and a negative control of 100 ml deionized water were also used. An Colilert®-18 medium packet was added to each bottle, the bottles were shaken to dissolve the reagent, and each bottle was then poured into a Quanti-tray®/2000 that was flicked to remove air bubbles and sealed using a Quanti-tray® Sealer. Quanti-trays were incubated at 37 C for 4 hours, then transferred to 44.5 C for 18 hours. Following incubation, Quanti-trays were then read for fecal coliforms by comparing yellow intensity to positive controls and for *E. coli* by viewing the trays under a long wavelength UV light and comparing intensity of fluorescence to positive controls. For both coliforms and *E. coli*, the number positive out of the 49 larger wells and the number positive out of the 48 smaller wells were recorded and the Quanti-tray®/2000 MPN table was used to compute MPNs.

For Enterolert®, the steps of the procedure and materials were similar to those for Colilert®-18, except the selective medium for enterococci was different. Briefly, 100 milliliters of well-mixed sample were transferred into a bottle containing sodium thiosulfate. A positive control of *Enterobacteriacae* was likewise spiked into 100 ml deionized water and a negative control of 100 ml deionized water were also used. An Enterolert® medium packet was added to each bottle, the bottles were shaken to dissolve the reagent, and each bottle was then poured into a Quanti-tray®/2000 that was flicked to remove air bubbles, sealed using a Quanti-tray® Sealer, and incubated at 41.5 C for 24 hours. Quanti-trays were read for enterococci by viewing them under a long wavelength UV light and comparing intensity of fluorescence to positive controls. The number positive out of the 49 larger wells and the number positive out of the 48 smaller wells were recorded and the Quanti-tray®/2000 MPN table was used to compute MPNs.

Data Management and Statistical Analysis

3X3 MPN

Due to the 3X3 MPN set-up used in a number of these experiments, each sample analyzed had the potential of producing up to nine positive outcomes (indicated by bacterial growth or coliphage plaquing): three for the undilute, three for the 10^{-1} dilution, and three for the 10^{-2} dilution. When applicable, each of these positive outcomes found by sample analysis was isolated and purified and will be referred to as an "isolate" from that sample.

MPN Calculations

Seagull Feces

MPN/g for *Salmonella* and *Campylobacter* in seagull feces was calculated using the Thomas Equation:

$$\frac{MPN}{g} = \frac{P}{[N * T]^{1/2}}$$

where

P = number of positive tubes
N = grams of feces in negative tubes
T = total grams of feces analyzed

PFU/g for F+ coliphages in seagull feces were calculated by dividing the number of plaques counted by the total grams of feces submitted to the DAL assay.

Water Samples

MPN/ml for *Salmonella* and *Campylobacter* in water samples was calculated using the Thomas Equation:

$$\frac{MPN}{ml} = \frac{P}{[N * T]^{1/2}}$$

where

P = number of positive tubes
N = milliliters of water in negative tubes
T = total milliliters of water analyzed

Statistical Analyses

When possible, statistical analyses were employed to assess the significance of variation in results obtained. Graphpad's Instat Version 3.06 was used for Chi-squared analyses, to compare means between two groups, for nonparametric ANOVA analyses, and for correlation analyses. Stata software was used to create box and whisker plots.

Results

Seagull Feces Samples

From November 2001 through October 2002, 160 samples were collected on the exposed sandflats of Talbert Marsh by UCI researchers on a roughly bi-monthly basis. Ten additional samples were collected from Newport Beach, approximately eight miles down-coast of Talbert Marsh. The intention of UNC researchers was for seagull feces to be collected directly following a visual confirmation of seagull defecation and for collectors to avoid collecting other debris such as sand grains and vegetation. According to chain of custody

forms and/or visual inspection of the samples upon arrival at UNC, this was not always the case. A number of samples were described as possibly originating from terns. This was unconfirmed and for the purposes of this study these samples will be deemed as originating from seagulls, which are the dominant bird species on the sandflats within Talbert Marsh (Griffin, Stokes et al. 2000). Furthermore, fecal samples were occasionally not fresh, but appeared dried-out and aged, and samples were often accompanied by sand grains and vegetation. One sample appeared to only be composed of seaweed (S6), while another shipment of ten samples (M1-M10) appeared to only be sand. Wet weights of all samples ranged from 0.28 g to 25.68 g, with an arithmetic mean wet weight of 5.39 g. Excluding the seaweed and sand samples, a total of 149 seagull feces samples from the sandflats within Talbert Marsh were analyzed in this study and the arithmetic mean wet weight of seagull feces was 4.8 g. See Table 2.4 for seagull feces sample collection data.

Salmonella

Of the 149 seagull feces samples from the sandflats within Talbert Marsh that were received for analysis, twenty samples (ten each from J and K samples) were unable to be scored for *Salmonella* because negative controls were positive and four samples (H3, H5, H6, and H9) were unassayable and were not counted among samples analyzed. Of the 125 samples analyzed, ten samples (8.0%) were confirmed as *Salmonella spp*. by the Enterotube® II test, with MPN/g concentrations ranging from 0.4 to $>7.4 \times 10^2$ (see Table 2.5). These data are also graphically presented in Figure 2.3. The maximum discrete values were used for greater-than values in this and all further analyses. Positive and negative controls run in parallel with these samples were positive and negative, respectively.

Twenty-nine of the thirty-seven isolates from the 10 confirmed positive *Salmonella spp.* seagull fecal samples collected from the sandflats within Talbert Marsh were submitted to an MIC analysis. All isolates were susceptible to five of the nine antimicrobials (see Table 2.6 and Figure 2.4). As shown in Table 2.7, one isolate from sample U1 was intermediately susceptible to chlortetracycline, chloramphenicol, and tetracycline, and resistant to ampicillin. Four separate isolates, representing three different seagull fecal samples, were intermediately susceptible to chlortetracycline. One isolate from sample U8 was resistant to ampicillin. Originally, every replicate in a sample was not deemed necessary for MIC analysis, however analysis of the remaining eight isolates does appear necessary for a complete understanding of the diversity of *Salmonella* within Talbert Marsh seagulls.

Table 2.8 provides a summary of both PFGE and MIC results for *Salmonella* isolates analyzed in this study. *Salmonella* isolates considered indistinguishable by PFGE analysis were each assigned a letter value. Additionally, an isolate determined to be closely related to or derived from another isolate(s) was designated by that letter followed by a "*." The positive control *Salmonella typhimurium* strain ATCC 14028 was not related to any of the strains isolated from Talbert Marsh seagull feces. With a few exceptions, isolates between samples (i.e. F2, U8) were quite different. However, all four isolates from sample F2 were indistinguishable from each other. This was also the case in seven of the eight U1 isolates, two of the four I9 isolates, two of the nine U8 isolates, and two of the five V3 isolates. The majority of isolates (7/9) from sample U8 appear to be different strains. In three cases, isolates from samples collected on the same collection day but from different fecal specimens, were identical. Two isolates (I9-3 and V3-3) were determined to be closely related to, or derived from, other strains: I9-3 was derived from the group designated "C"

and V3-3 was derived from the group designated "R." Upon several restriction attempts, one isolate (I9-2) was unable to produce PFGE banding patterns. This isolate requires further analysis, as it may likely have been incorrectly identified by the culture and biochemical testing methods used.

It is also interesting to note the similarities between PFGE and MIC data for isolates (see Table 2.8). As expected, isolates with identical PFGE patterns responded similarly to the antimicrobials to which they were exposed. For example, all "B" and all "G" types were susceptible to antimicrobials tested against. There were some exceptions however. Three of the four "C" types were intermediately-resistant to CTET, while the derivative isolate was susceptible to all antimicrobials. Differences between PFGE patterns and MIC response were also found within samples. Samples U1, U8, and V3 each had one isolate that displayed a unique PFGE banding pattern when compared to the other isolates within the sample. These unique isolates also reacted differently to antimicrobial analysis, generally being intermediately-resistant or resistant to some antimicrobials, while all of the other isolates within the sample were found to be susceptible to antimicrobials tested.

Campylobacter

Of the 149 seagull feces samples from the sandflats within Talbert Marsh that were received, one sample (O2) was unassayable due to a methodological error. Of the 148 samples analyzed, all isolates within each of 46 samples (31.1%) were considered presumptive culture-positive for thermotolerant *Campylobacter* by biochemical testing and Gram staining. As detailed in Chapter III, a number of these isolates were confirmed as *Campylobacter* positive by the polymerase chain reaction. Thirty-eight additional samples

produced colonies following enrichment, but not every isolate within each positive sample was able to be regrown for biochemical testing and Gram-staining. These samples were therefore considered incompletely analyzed. Of these 38 samples, 23 samples were later as confirmed as *Campylobacter* positive by the polymerase chain reaction (see Chapter III). Subsets of isolates within another six samples (G2, G8, Q3, Q10, T1, and T7) were unable to be confirmed via biochemical testing, Gram-staining, or by the polymerase chain reaction. Partial biochemical testing and Gram-staining results within each of these samples were used to calculate MPN values; the values described likely underestimate the true concentrations as confirmation of some growth was unable to be achieved. The remaining nine samples (G6, G9, H6, I10, S4, S5, S10, T9, and V6) that produced colonies after enrichment, but which none of the isolates were able to be determined as campylobacters using either biochemical or molecular tests, were identified as non-detects. Therefore, using biochemical tests and/or molecular tests, a total of 75 (50.7% of total analyzed) seagull feces samples from the sandflats within Talbert Marsh were identified as thermotolerant *Campylobacter*, with MPN/g concentrations ranging from 0.2 to $>5.2 \times 10^2$ (see Table 2.5). These data are also graphically presented in Figure 2.3. The maximum discrete values were used for greater-than values in this and all further analyses.

Positive controls were often problematic. Positive and negative controls were run in parallel with each of the 15 samples sets (groups of ten samples per set). Of the 11 sample sets that contained positive samples, seven sets were positive and negative for positive and negative controls, respectively. The remaining four sets had positive controls that were negative, although presumptive culture-positive thermotolerant *Campylobacter* were isolated from samples within the sets. Of the four sets of samples that did not contain positive

samples, only one set was positive and negative for positive and negative controls, respectively. The remaining three sets had positive controls that were negative. Occurrence of both positives and non-detects were described regardless of the outcomes of positive controls.

To assess cell concentration and cell viability for future studies, stored cell suspensions were studied using two different microscopy techniques. Cell counts were performed on a subset of presumptive culture-positive thermotolerant *Campylobacter* isolates stored at -80 C in TSB with 25% glycerol. Ten stored cell suspensions from 10 separate seagull feces samples collected over the length of the study were chosen as represent samples for the entire collection of stored cell suspensions. Cell morphologies were rod-like or coccoid (images not shown) and cell counts ranged from 1.87 x 10^5 cells/µl to 1.17 x 10^6 cells/µl, with an arithmetic mean of 7.47 x 10^5 cells/µl (see Table 2.9). These data indicate that a high number of cells were still present within stored suspensions despite freeze-thaw events. This information on cell concentration is important for future studies performed on these stored suspensions.

In addition, several cell suspensions were submitted to a viability assay to determine whether cells present were intact, damaged, or no longer alive. Presumptive culture-positive thermotolerant *Campylobacter* isolates (four isolated from seagull feces and one from a water sample, described below) which had been stored at -80 C in TSB with 25% glycerol and had undergone multiple freeze-thaw events, were chosen for analysis and cell morphologies and staining features of these isolates were compared. Cells were stained with both SYTO® 9 green fluorescent nucleic acid stain and propidium iodide red fluorescent nucleic acid stain and then viewed under 100X magnification in a fluorescence microscope equipped with

fluorescein and Texas Red bandpass filter sets. The cell morphology and staining features of isolates analyzed varied slightly, but all isolates showed evidence of live/undamaged cells (images not shown). Generally cells were rod-like, but often S-shaped and gull-wing-shaped cells were visible. This data provides evidence that a fraction of cells within the stored cell suspensions are viable despite freeze-thaw events. Again, this information on viability is important for future studies performed on these stored cell suspensions.

A number of attempts were made to resuscitate freeze-thawed *Campylobacter* cell suspensions, including incubation in PE broth, Bolton broth, half-strength Exeter broth (including Oxoid *Campylobacter* Growth Supplement) and/or subculture on CBA. Variations were made in type of antibiotics added, with the elimination of Rifampicin and Cycloheximide, which are known to be toxic to stressed *Campylobacter* cells, and the addition of Cefoperazone and Amphotericin B. Variations were also made by increasing the length of incubation at 37 C for cell recovery and delaying the addition of antibiotics until after 24 hours of initial incubation. Despite these attempts, adequate cell recovery did not occur, therefore eliminating the ability to perform PFGE on *Campylobacter* isolates for further comparison.

F+ coliphages

The analysis of seagull feces for F+ coliphages was added after sampling had already begun, therefore fewer seagull feces were assayed for F+ coliphages than for the other microbes. Of the 109 seagull feces samples from the sandflats within Talbert Marsh that were analyzed, 31 (28.4%) were considered presumptive positive for F+ coliphages, with PFU/g ranging from 1.9 to >2.0 x 10^4 (see Table 2.5). These data are also graphically

presented in Figure 2.3. The maximum discrete values were used for greater-than values in this and all further analyses. Positive and negative controls run in parallel with each set of samples were positive and negative, respectively. Further molecular analyses on these isolates are presented in Chapter 3.

Seasonality

Tables 2.10, 2.11, and 2.12 present data for each microbe isolated from seagull feces from Talbert Marsh based on season (the two Autumn seasons during which samples were collected were combined). Chi-squared analyses of the percent-positivity of *Salmonella* and F+ coliphages based on season were considered, but the numbers of positive samples were too low for valid and meaningful analysis. However, as indicated in Tables 2.10 and 2.12, the observed values were higher than the expected values during the Summer and Autumn months for both microbes. Observed values of *Salmonella* were lower than expected in the Spring months and as expected in the Winter months, while the reverse was true of F+ coliphages. These results suggest that the number of positive samples likely varied between seasons for both of the microbes. However, too few samples were analyzed to document significantly different seasonal occurrence.

For *Campylobacter*, as shown in Table 2.11, Autumn and Winter months had considerably lower observed values than expected values, while Spring and Summer months had considerably higher observed values than expected values. A Chi-squared analysis was performed on the *Campylobacter* data set because it contained more positive samples. The null hypothesis was that there was no difference between the numbers of positive samples among seasons, and this null hypothesis would be true if there was no difference between

observed and expected values. The Chi-squared results indicate that significant differences in *Campylobacter* did occur among seasons (P = 0.0003). For complete details of statistical analyses, please refer to the Appendix.

Seasonal data for concentrations of each microbe in seagull feces were analyzed using the Kruskal-Wallis Test (nonparametric ANOVA) and Dunn's Multiple Comparisons Test (post-test). For each microbe, ANOVA analyses indicated that variations between concentration medians for each season were significantly greater than expected by chance alone, with P values of 0.0282, <0.0001, and 0.0006 for *Salmonella*, *Campylobacter*, and F+ coliphages, respectively. The post-tests conducted indicated whether or not pairs of seasons differed and the extent of the difference (see Table 2.13). For *Salmonella*, significant difference (at the 5% level) in concentrations occurred between the months of Spring and Summer. For *Campylobacter*, a significant difference (at the 5% level) in concentrations occurred between the months of Autumn and Spring, Autumn and Summer, Winter and Spring, and Winter and Summer. For F+ coliphages, a significant difference (at the 5% level) occurred between the months of Winter and Summer and between Spring and Summer. For complete details of statistical analyses, please refer to the Appendix.

Co-occurrence of Microbes in Seagull Feces

Tables 2.14, 2.15, and 2.16 present data for the co-occurrence of microbes in seagull feces from Talbert Marsh. In samples analyzed for both *Salmonella* and *Campylobacter*, eight samples (6.5%) were positive for both microbes. Similarly, in samples analyzed for both F+ coliphages and *Salmonella*, six samples (6.3%) were positive for both microbes. Co-occurrence was highest (21.3%) in samples analyzed for both *Campylobacter* and F+

coliphages. In the first two cases, co-occurrence of two microbes was much less frequent than the absence of both microbes, which dominated the findings. In the case of *Campylobacter* and F+ coliphages, co-occurrence was much less frequent than the absence of both microbes or the presence of just *Campylobacter*.

The majority of *Salmonella*-positive samples also contained *Campylobacter* (80.0%) or F+ coliphages (66.7%), when assayed for each respectively. The opposite effect was seen in *Campylobacter*-positive samples: 12.1% of the *Campylobacter*-positive samples also contained *Salmonella* and 39.7% of the *Campylobacter*-positive samples also contained F+ coliphages, when assayed for each respectively. A majority of F+ coliphage-positive samples also contained *Campylobacter* (74.2%), while 21.4% of F+ coliphage-positive samples also contained *Salmonella*, when assayed for each respectively.

Correlation analyses were performed on samples analyzed for each pair of microbes. As seen in Figures 2.5, 2.6, and 2.7, the majority of data points for each pair are focused in the lower left corner of the plot (0,0), indicating the high occurrence of non-detects in at least one of the microbes. Positive weak correlations were found between *Salmonella* and *Campylobacter* (Spearman r = 0.2292) and between F+ coliphages and *Campylobacter* (Spearman r = 0.2754). A moderately positive correlation was found between *Salmonella* and F+ coliphages (Spearman r = 0.3597). Tests that Spearman correlation values were significantly different than zero were all significant, with P values for *Salmonella* versus *Campylobacter*, F+ coliphages versus *Campylobacter*, and *Salmonella* versus F+ coliphages of 0.0104, 0.0039, and 0.0003, respectively. Although these results do indicate some degree of positive correlation, data may be skewed by the low numbers of positive samples and the dominance of one or a few outlying values, as seen in the corresponding plots.

Other Solid Samples

Although intended to be, twenty-one additional samples that were analyzed were actually not seagull feces samples collected from the sandflats within Talbert Marsh. These samples consisted of ten seagull fecal samples from Newport Beach, ten sand samples from Talbert Marsh, probably containing seagull feces, and a seaweed sample also from Talbert Marsh and possibly containing seagull feces. Of the ten seagull feces samples collected from Newport Beach (N1 – N10), two samples were confirmed positive for *C. jejuni* using molecular methods (see Chapter III), with concentrations of 2.4 and 3.2 MPN/g, respectively. A separate sample was considered presumptive positive for F+ coliphages with a concentration of 0.9 PFU/g. None of the Newport Beach samples were positive for *Salmonella*. Of the ten sand samples (M1 – M10) collected from Talbert Marsh, three samples were positive for thermotolerant *Campylobacter* by biochemical tests and Gram-staining, with concentrations of 0.1, 0.2, and 0.4 MPN/g, respectively. Four sand samples from Talbert Marsh were presumptive positive for F+ coliphages, with concentrations of 0.4, 0.4, 0.5, and 0.6 PFU/g respectively. *Salmonella* were not detected in any of the sand samples. None of the microbes analyzed were isolated from the one seaweed sample (S6).

Water Samples

From August 2002 through November 2002, 24 4-liter Talbert Marsh water samples were collected by UCI researchers from five different areas surrounding the sandflats within the marsh (see Figure 2.1 and Table 2.17). Samples were collected on a roughly bi-monthly basis and generally during low tides when the sandflats were exposed. Tidal data is from the closest reference station at Newport Bay Entrance in Corona del Mar, about eight miles south

of Talbert Marsh's outlet to the ocean. Although each sample set (except one) includes one sample from an area directly surrounding the sandflats, the other two samples per set often varied in collection sites, occasionally both being collected upmarsh of the sandflats. Chain of custody forms were often sent incomplete.

Salmonella

Of the 24 water samples received, 23 were analyzed for *Salmonella*. One sample (W) leaked during shipment so this assay was omitted. No *Salmonella* were found in any of the 23 1-liter sample volumes. Positive and negative controls run in parallel with each set of water samples (groups of three samples per set) were positive and negative, respectively.

Campylobacter

Of the 24 1-liter water samples analyzed, seven samples (29.2%) were considered presumptive culture-positive for thermotolerant *Campylobacter* by biochemical testing and Gram staining, with MPN/L concentrations ranging from 1.4 – 11.5. These data are presented in Table 2.18 and Figure 2.8. Positive and negative controls were run in parallel with each of the eight sample sets (groups of three samples per set). Of the five sample sets that contained positive samples, two sets were positive and negative for positive and negative controls, respectively. The remaining three sets had positive controls that were negative, although presumptive culture-positive thermotolerant *Campylobacter* were isolated from samples within the sets. These positives samples were included in the data compilation. Of the three sets of samples that did not contain positive samples, all sets were positive and negative for positive and negative controls, respectively.

As noted above, a number of attempts were made to resuscitate freeze-thawed *Campylobacter* cell suspensions, including incubation in PE broth, Bolton broth, half-strength Exeter broth (including Oxoid *Campylobacter* Growth Supplement) and/or subculture on CBA. Variations were made on type of antibiotics added, with the elimination of Rifampicin and Cycloheximide, which are known to be toxic to stressed *Campylobacter* cells, and the addition of Cefoperazone and Amphotericin B. Variations were also made by increasing the length of incubation at 37 C for cell recovery and delaying the addition of antibiotics until after 24 hours of initial incubation. Despite these attempts, adequate cell recovery did not occur, therefore eliminating the ability to perform PFGE on *Campylobacter* isolates for further comparison.

F+ coliphages

Data for F+ coliphages isolated using Methods 1601 and 1602 is limited due to persistent problems with bacterial host culture growth. New host culture stocks were restarted several times throughout the experimental time period, however, these cultures were often contaminated with bacteria that resulted in a "slit-like" appearance in the agar (likely due to gas production). Other problems included overgrowth of host bacteria that covered up potential plaques or the complete absence of host bacteria growth on both experimental and control plates. Chloroform extractions were often spot-plated a number of times due to problems with host bacteria growth on spot plates and often these results were ambiguous. In some cases, molecular analyses detected F+ coliphages that Methods 1601 and 1602 were unable to detect (see Chapter 3). These data are detailed below and the differences are shown in Table 2.19.

Method 1601

Of the 24 water samples received, 18 were analyzed for F+ coliphages using Method 1601. One sample (W) leaked during shipment so this assay was omitted. Five additional water samples (S, T, U, V, and X) were unable to be scored for plaques, as a bacterial lawn would not grow upon several attempts. Ten of 18 (55.6%) samples produced plaques, with MPN/L ranging from 1.0 to >146 (see Table 2.18). Positive and negative controls run in parallel with each set of samples were positive and negative, respectively.

Chloroform extracts for four negative samples (K, L, N, and O) and two unassayed samples (V and X) were stored at -80 C. Molecular analyses (see chapter 3) confirmed that samples N, Y, and X were positive. Chloroform extracts for the remaining negative and unassayed samples were not archived and therefore were not able to undergo molecular analyses to provide further information on presence. In total, 13 water samples (65% of water samples analyzed) were positive for F+ coliphages using Method 1601.

Method 1602

A total of 20 water samples were assayed for F+ coliphages using Method 1602. Three additional samples were unable to be scored for plaques as a bacterial lawn would not grow upon several attempts and one sample leaked during shipment so this assay was omitted. Positive and negative controls were run in parallel with each of the seven sample sets (groups of three samples per set). Each sample set contained positive samples and all but one set were positive and negative for positive and negative controls, respectively. Ten samples (50.0%) were determined to be presumptive positive for F+ coliphages, with PFU/L ranging from 10 to 630 (see Table 2.18).

Fecal coliforms and E. coli

Of the 24 water samples sent, 20 were analyzed for fecal coliforms and *E. coli*. Three water samples were not analyzed due to lack of Coliliert®-18 materials, and one sample leaked during shipment so this assay was omitted. Of the 20 samples analyzed, 19 (95.0%) were positive for fecal coliforms, with MPN/L ranging from 10 to 3873, and 9 (45.0%) were positive for *E. coli*, with MPN/L ranging from 10 to 266. These data are presented in Table 2.18 and Figure 2.8. Positive and negative controls were run in parallel with each of the eight sample sets (groups of three samples per set). The *Klebsiella pneumoniae* positive control did not produce expected results in four of the seven sample sets, likely due to strain contamination. The *E. coli* positive control produced expected results in six of the seven sample sets; no *E. coli* was detected in the one sample set containing a negative *E. coli* positive control. The negative controls included were always negative.

Enterococci

Of the 24 water samples sent, 23 were analyzed for enterococci. One sample leaked during shipment so this assay was omitted. Of the 23 samples analyzed, all (100%) were considered presumptive positive, with MPN/100 ml ranging from 391 to 12997. These data are presented in Table 2.18 and Figure 2.8. Positive and negative controls were run in parallel with each of the eight sample sets (groups of three samples per set) and all were positive and negative, respectively.

Seasonality

Tables 2.20, 2.21, and 2.22 present data for *Campylobacter*, F+ coliphages, and *E. coli* isolated from Talbert Marsh water samples based on season. Chi-squared analyses of the percent-positivity of these microbes based on season were considered, but the total numbers of samples analyzed were too low for valid and meaningful analysis. However, as indicated in Table 2.20, the observed values for *Campylobacter* were lower than the expected values in the Summer months and higher than the expected values in the Autumn months. The opposite was true for F+ coliphages and *E. coli*, with observed values being higher than the expected value in the Summer months and lower than the expected value in the Autumn months (see Tables 2.21 and 2.22). These data suggest that the number of positive samples did vary between seasons for each microbe. Analyses of seasonal differences in percent-positivity were omitted for fecal coliforms and enterococci, as they were present in 100% and 95% of samples, respectively.

For Summer and Autumn months, concentration differences of each microbe in Talbert Marsh water samples were analyzed using the Mann-Whitney Test. For *Campylobacter*, fecal coliforms, *E. coli*, and enterococci, analyses indicated that variations between concentration medians for each season were not significantly different at the 5% level, with P values of 0.1802, 0.2014, 0.2999 and 0.2300, respectively. However, for F+ coliphages, significant difference (at the 5% level) in concentrations occurred between the two seasons. A significantly higher mean concentration of F+ coliphages was observed in the Summer months (114 MPN/L) than the Autumn months (4 MPN/L). For complete details of statistical analyses, please refer to the Appendix.

Co-occurrence of Microbes

Tables 2.23, 2.24, and 2.25 present data for the co-occurrence of microbes in water samples from Talbert Marsh. Due to the low number of samples (n= 24), statistical analyses on these data are limited. In water samples analyzed for both *Campylobacter* and *E. coli*, three samples (15.0%) were positive for both microbes. Similarly, in water samples analyzed for both F+ coliphages and *Campylobacter*, three samples (13.0%) were positive for both microbes. Co-occurrence was highest (35.0%) in samples analyzed for both *E. coli* and F+ coliphages. In the first two cases, co-occurrence of two microbes was less frequent than the absence of both microbes. In the case of *E. coli* and F+ coliphages, co-occurrence was more frequent than the absence of both microbes, but less frequent than the presence of just F+ coliphages.

In only two cases did the majority of samples positive for one microbe also contain another microbe, when assayed for both. Sixty percent of *Campylobacter*-positive samples also contained *E. coli* and 78% of *E. coli*-positive samples also contained F+ coliphages. In all other pairings, less than 50% of the samples positive for one microbe were also positive for another microbe.

Correlation analyses were performed on samples analyzed for each pair of microbes (see Figures 2.9, 2.10, and 2.11). A positive weak correlation was found between *E. coli* and F+ coliphages (Spearman r = 0.1256), while a moderately negative correlation was found between *Campylobacter* and F+ coliphages (Spearman r = -0.3747). No correlation was found between *Campylobacter* and *E. coli* (Spearman r = 0.05862). No other tests of Spearman correlation values were significantly different than zero, with P values for *E. coli* versus F+ coliphages, F+ coliphages versus *Campylobacter*, and *E. coli* versus

Campylobacter of 0.5978, 0.0712, and 0.8061, respectively. Although some results do indicate limited degrees of correlation, none of the correlations were significant and data may be skewed by the low numbers of samples and the dominance of one or a few outlying values, as seen in the corresponding plots.

Water Collection Site

Presence or absence of each microbe based on sample location is shown in Table 2.26. Tables 2.27, 2.28, and 2.29 present data for each microbe isolated from Talbert Marsh water samples based on sample location (sites 1 and 2 represent "upmarsh," site 3 represents "adjacent," and sites 4 and 5 represent "downmarsh;" see Figure 2.1). Statistical analyses of the *Campylobacter* presence or concentration data were considered, but the number of samples was too low for valid and meaningful analysis. Therefore, the number of positive samples were divided into the total number of samples analyzed. Using this fraction, the expected number of positive samples per location could be calculated under the null hypothesis that no difference in the numbers of positive samples existed between sample locations. As indicated in Table 2.27, the observed values of positive samples both upmarsh and downmarsh of the sandflats were lower than expected values, while the observed values adjacent to the sandflats were slightly higher than the expected values. These results suggest that the number of positive samples did vary between sample locations and was higher than expected at the location adjacent to the sandflats.

Similarly, statistical analyses of the F+ coliphage data were considered, but the numbers of samples was too low for valid and meaningful analysis. However, as indicated in Table 2.28, the observed value upmarsh was lower than the expected value, while the observed value

downmarsh was higher than the expected value. The observed value for F+ coliphage presence adjacent to the sandflats was within 5% of its expected value if no variation occurred. These results suggest that the number of positive samples did vary between sample locations and was higher than expected at the location downmarsh of the sandflats.

Finally, statistical analyses of the *E. coli* data were considered, but again the number of samples was too low for valid and meaningful analysis. However, as indicated in Table 2.29, the observed value upmarsh was higher than the expected value, while the observed values both adjacent to the sandflats and downmarsh were lower than the expected values. These results suggest that the number of positive samples did vary between sample locations and was higher than expected at the location upmarsh of the sandflats.

Discussion

Seagulls

Several bird surveys have been conducted in Talbert Marsh. Surveys conducted monthly and bi-monthly at both high and low tide between 1990 and 1994 identified 64 species of water birds and 24 species of land birds (California Environmental Resources Evaluation System 2005). Although a gull-specific census has not been conducted at Talbert Marsh, one study found an average of 228 birds present during the day and the largest congregation of birds consisting of 1180 individuals at one time. Gulls and elegant terns constituted 80% of birds visible in Talbert Marsh. Furthermore, the study found that the deposition rate of seagulls in Talbert Marsh was one feces per bird every three hours. This rate also agrees with deposition rates of seagulls in captivity (Grant, Sanders et al. 2001). Based on the above information, and the average wet weight of seagull feces from Talbert Marsh found in

the present study (4.8 grams), it can be estimated that approximately 7004 grams (15.4 pounds) of seagull feces are deposited in Talbert Marsh on a daily basis, with a worst case of 36,250 grams (79.9 pounds). Data for the numbers of seagulls present only on the sandflats within Talbert Marsh are not available. However, the sandflats provide a wide, solid surface for seagulls to congregate, suggesting that a large portion of the seagull feces are deposited in this area within the marsh.

No formal counts of gulls visiting the sewage treatment plant (Orange County Sanitation District (OCDS)) directly adjacent to Talbert Marsh have been conducted (McGee September 2005), however it can be assumed that Talbert Marsh gulls frequent this treatment plant for feeding purposes based on several studies detailing this phenomena (Fenlon 1981; Butterfield, Coulson et al. 1983; Reed, Meece et al. 2003).

Salmonella

The carriage rate of *Salmonella* in seagulls of 8.0% found in this study falls within the range of carriage rates found in the literature. Of the studies detailed in Table 2.1, the median carriage rate is 7.8%, with a minimum rate of 2.1% and a maximum rate of 55.0%. The rate described in this study may also be underestimated for several reasons. Single fecal samples, and not entire cloacal washings, were collected only from a subset of seagulls, and may not reflect representative sampling from the entire population present in Talbert Marsh. Furthermore, because samples were collected post-defecation and analyzed 24 hours post-collection, temperature fluctuations, exposure to UV light, and exposure to low pH due to uric acid in the fecal samples and other environmental stressors could have likely decreased

numbers of *Salmonella* present. No *Salmonella* were isolated from the ten Talbert Marsh sand samples, as was reported in another study (Bolton, Surman et al. 1999).

The arithmetic average MPN/g of positive *Salmonella* samples of 82 falls within the reported concentration ranges found in the literature of 0.81 – 191 MPN per gram of feces (Fenlon 1981) and <200 MPN per gram of feces (Fenlon 1981; Girdwood, Fricker et al. 1985). Because carriage rates and concentrations of *Salmonella* in individual seagull feces are relatively low, coupled with anticipated short carriage and excretion periods (average of four days), it seems unlikely that gull feces could have a consistently large *Salmonella* impact on the microbiological quality of environmental waters. However, large numbers of gulls often congregate near waters and the collective contribution of gulls, especially ones who have previously fed at sewage or dump sites such as OCDS, may potentially have a great impact on water quality, at least periodically (Girdwood, Fricker et al. 1985). Based on numbers calculated above, an estimated average possible daily load of *Salmonella* by marsh seagull feces is 5.7×10^5 organisms, with an estimated worst case of 2.7×10^7 organisms.

Data indicate that there were likely differences in the presence of *Salmonella* in seagull feces based on season, with Summer and Autumn months having a higher observed number of positive samples than what would be expected if no variation between seasons existed. Furthermore, concentrations of *Salmonella* in seagull feces were significantly different between Spring and Summer months. Conversely, in Scotland, Monaghan et al. (Monaghan, Shedden et al. 1985) found higher *Salmonella* carriage rates by seagulls in the wintering/non-breeding months of October through February (15.5%) than during the breeding months of April through July (9.1%). They also found that females had higher carriage rates than males during the non-breeding season, likely due to feeding differences in months preceeding

mating. Differences between observed rates in the Monoghan study and in the present study could be due to a number of factors, including numbers of seagulls present in Talbert Marsh, the dominant sex of seagulls present in Talbert Marsh, exposure of seagulls to contaminated sources, differences in seagull behaviors (ie. mating) between species in the UK and the US, or various other environmental conditions.

No *Salmonella* were detected in water samples in this study, even though water samples were collected during the time period when the highest numbers of *Salmonella* were isolated from gull feces. In total, 24 liters of water were analyzed for *Salmonella*; 50% of samples (12 liters) were collected upmarsh of the sandflats where seagulls congregate, 33.3% of samples (eight liters) were collected adjacent to the sandflats, and 16.7% of samples (four liters) were collected downstream of the sandflats. The eight liters of water collected adjacent to the sandflats may simply not have been a large enough volume for the detection of *Salmonella* or may not have been collected at a tidal stage at which *Salmonella* were present in the water at the sample site. The daily flushing of Talbert Marsh waters likely influences both the concentrations as well as the locations of bacteria of seagull feces origin in the marsh. The same applies for the four liters of water collected downstream of the sandflats. It is also possible that *Salmonella* in these water samples did not survive environmental conditions, such as higher salinity and longer exposure to UV light, during travel to the end of the marsh. Furthermore, feces deposited on the sandflats were likely subject to desiccation and UV light, injuring or killing *Salmonella* cells present, prior to entering the waters of Talbert Marsh.

Although most of the *Salmonella* isolates from gull feces in this study were susceptible to antibiotics tested, approximately 3.5% of *Salmonella* isolates from gull feces were resistant

to ampicillin. No samples showed multiple antibiotic resistance according to clinical criteria, as in previous studies (Fenlon 1981; Palmgren, Sellin et al. 1997; Sixl, Karpiskova et al. 1997), although one isolate was intermediately susceptible to chlortetracycline, chloramphenicol, and tetracycline, and resistant to ampicillin. This isolate and the majority of other isolates that were found to exhibit intermediate susceptibility to chlortetracycline and ampicillin, were collected during the Autumn months of 2002. One other isolate found to be intermediately susceptible to chlortetracycline was collected during the Summer months of 2002. Chlortetracycline, chloramphenicol, and tetracycline are approved for use in animal feed and ampicillin is approved for use in injectibles, suggesting that the microbes intermediately susceptible or resistant to these antibiotics may have originated from agriculture sources. Furthermore, these antibiotics are used in human medicine, suggesting that the microbes may have originated from human sources. Given that Talbert Marsh is adjacent to the large OCSD sewage treatment plant, and that these birds have access to the plant, it is possible that acquisition of antimicrobial resistant bacteria could have occurred from this source. Further genetic analysis may be necessary to establish such associations and links to specific fecal sources.

Additionally, PFGE analysis of the *Salmonella* isolates from seagull feces provides important strain type information. It is obvious that there is wide variation in the strains isolated between samples. Identical strains were only found within a sample, or among samples collected on the same day. Furthermore, within a sample, derivatives of strains and strains altogether unrelated were present. The above results indicate that there is a wide variety in the strain types of *Salmonella* found in the seagull feces from Talbert Marsh. Similarities of PFGE and MIC results found between and among samples are also of interest.

It is important to analyze both *Salmonella* isolated from the sewage treatment plant where seagulls feeds and from other animal sources in the area to better understand the variation present in the Talbert Marsh environment. Additionally, *Salmonella* analysis needs to be performed on larger volumes and a greater number of water samples in attempt to isolate the bacteria. More thorough PFGE analysis, MIC analysis, and molecular-based genotyping of all of these isolates would provide a better understanding of the impacts of *Salmonella* from seagulls in Talbert Marsh into the esturarine waters.

Campylobacter

The carriage rate of *Campylobacter* in seagulls of 37.2% found in this study falls within the range of carriage rates found in the literature. Of the studies in detailed in Table 2.2, the median carriage rate is 23.5%, with a minimum rate of 0.0% and a maximum rate of 63.0%. Again, the rates described in this study may also be slightly underestimated for the same reasons described for *Salmonella* above. Additionally, three (30.0%) of the ten Talbert Marsh sand samples were positive for *Campylobacter*. Although concentrations were low, these findings support a previous study (Bolton, Surman et al. 1999) and indicate that these bacteria can survive outside of an animal host in deposited fecal matter and in a material such as sand that is likely to come into daily contact with marsh waters.

Data for concentrations of thermophilic *Campylobacter* in waterfowl or gull feces are not available in the published literature, however limited data on the concentrations of *Campylobacter* are available for commercial broiler chickens. Two studies describe the average concentration of *Campylobacter* in broiler chicken feces as 1.5×10^6 CFU/g and 10^7 CFU/g, respectively (Pearson, Greenwood et al. 1993; Stern, Reiersen et al. 2005). These

data are not comparable to those of wild birds because of the differences in feeding, roosting, and other environmental conditions. The arithmetic average MPN/g of positive thermophilic *Campylobacter* samples of gull feces in this study was 48, well below the concentrations found in broiler chickens. Again, however, if large numbers of gulls congregate near the waters of Talbert Marsh, their feces may collectively have a great impact on water quality. Based on numbers calculated above, an estimated average possible daily load of *Campylobacter* by marsh seagull feces is 3.4×10^5 organisms, with an estimated worst case of 1.9×10^7 organisms.

Statistical tests show that differences in the presence of *Campylobacter* in seagull feces based on season were significant, with Spring and Summer months having a higher percentage of positive samples than Autumn and Winter months. This finding contradicts results by a Swedish study (Broman, Palmgren et al. 2002) that found the highest rates of *C. jejuni* carriage in the late autumn. Again, differences between observed rates in the Swedish study and in the present study could be due to a number of factors, including numbers of seagulls present in Talbert Marsh, the dominant sex of seagulls present in Talbert Marsh, exposure of seagulls to contaminated sources, differences in seagull behaviors (ie. mating) between species in Sweden and the US, or various other environmental conditions. Differences in concentrations of *Campylobacter* in seagull feces also followed this seasonal pattern. No statistically significant differences were found between Autumn and Winter months or between Spring and Summer months. However, statistically significant differences were found between all other combinations of months.

Approximately 29% of water samples analyzed in the study were positive for *Campylobacter* with MPN/L ranging from 1.4 to 11.4. Although unable to be verified

statistically, the presence of *Campylobacter* in water samples does appear to vary during the seasons in which samples were taken. In contrast to seagull feces data although, higher than expected numbers of positive samples were found during the Autumn months than the Summer months. These findings are somewhat supported by a study of bird-impacted waters (Obiri-Danso and Jones 1999) that found higher counts of campylobacters in Winter months. There were no statistically significant differences in concentrations of *Campylobacter* from Talbert Marsh waters based on season. However, a complete evaluation of seasonal variation in Talbert Marsh requires year-round sampling.

Due to the low number of water samples analyzed, statistical analyses on the data were limited. Almost 43% of *Campylobacter*-positive samples were collected upmarsh of the sandflats, possibly suggesting that marsh water impacted by seagull feces from the sandflats was moved upmarsh by incoming tides. These results may also indicate potential sources of *Campylobacter* in Talbert Marsh other than seagull feces on the sandflats. The majority of samples positive for *Campylobacter* (57.1%) were from the eight liters of water collected adjacent to the sandflats. Furthermore, the observed number of positive samples in this region of the marsh was higher than what was expected if there was no variation in the number of positive samples due to sample location. These results suggest that the seagull feces on the sandflats may be a source of *Campylobacter* to the adjacent waters.

Campylobacter was not isolated from the four liters of water downstream of the sandflats. Additionally, the observed number of positive samples in this region of the marsh was lower than what was expected if there was no variation in the number of positive samples due to sample location. The low volume of water collected from this area may simply not have been a large enough volume for the detection of *Campylobacter*, especially due to the daily

flushing of Talbert Marsh waters. It is also likely that *Campylobacter* in these water samples did not survive environmental conditions, such as higher salinity and longer exposure to UV light, during travel to the outlet end of the marsh. Furthermore, feces deposited on the sandflats were likely subject to desiccation and UV light, injuring or killing *Campylobacter* cells present, prior to submersion into the waters of Talbert Marsh.

It is important to note that occurrence of negative positive controls for three sets of water samples, even though thermophilic *Campylobacter* were isolated from all three sets of samples, limits the reliability of the enrichment and subculture procedure for these samples. Because known positive controls were not detected, it is likely that the procedures underestimated the species and concentrations of *Campylobacter* present in these samples.

Both cell counting by microscopy and the Live/Dead® *Bac*Light™ Bacterial Viability assay microscopy insured the presence of high numbers of potential *Campylobacter* cells for re-culturing and use in subsequent analyses. Unfortunately, despite attempts, *Campylobacter* cells were not able to be recovered for further analysis by PFGE. This type of analysis would have provided valuable information on the strain types found in both seagull feces and water and would have allowed comparison of these types for microbial source tracking purposes.

Fecal Indicators

F+ coliphages

The carriage rate of F+ coliphages in seagull feces in this study was found to be 27.7%, which is lower than the rates of 39% and 100% found in the literature (Muniesa, Jofre et al. 1999; Cole, Long et al. 2003). Again, the rates described in this study may also be slightly underestimated for the same reasons described for *Salmonella* and *Campylobacter* above.

Additionally, four (40.0%) of the ten Talbert Marsh sand samples were positive for F+ coliphages. Although concentrations were low, these findings indicate that these viruses can survive outside of a host environment and in a material that is likely to come into daily contact with marsh waters.

The arithmetic average PFU/g of positive F+ coliphage seagull samples of 1.4×10^3 was within the range of one reported study another (200 – 1.5×10^5 pfg/g) (Cole, Long et al. 2003), but substantially higher than another (4 to 20 pfu/g) (Muniesa, Jofre et al. 1999). Because carriage rates and concentrations of F+ coliphages in individual seagull feces are relatively low, coupled with anticipated short carriage and excretion periods (average of four days), it seems unlikely that gull feces could have a consistently large F+ coliphage impact on the microbiological quality of environmental waters. However, large numbers of gulls often congregate near waters and the collective contribution of gulls, especially ones who have previously fed at sewage or dump sites such as OCDS, may potentially have a great impact on water quality, at least periodically (Girdwood, Fricker et al. 1985). Based on numbers calculated above, an estimated average possible daily load of F+ coliphages by marsh seagull feces is 9.8×10^6 viral particles, with an estimated worst case of 7.2×10^8 viral particles.

Data indicate that there were likely differences in the presence of F+ coliphages in seagull feces based on season, with Summer and Autumn months having a higher observed number of positive samples than what would be expected if no variation between seasons existed. Furthermore, concentrations of F+ coliphages in seagull feces were significantly different between Winter and Summer months and between Spring and Summer months. Seasonal distribution information for F+ coliphages in seagull feces is not available in the literature.

The two methods used for F+ coliphage detection and enumeration in Talbert Marsh water samples provided different results. Both methods simultaneously detected the presence of F+ coliphages in seven of the sixteen positive samples. Overall, Method 1602 detected F+ coliphages in a greater number of samples, and detected higher concentrations of F+ coliphages in these samples. These results are unexpected, as Method 1601 contains an enrichment step meant to increase concentrations of F+ coliphages in samples.

Eighty percent of water samples analyzed in the study were positive for F+ coliphages, with PFU/L ranging from 10 – 630. Although unable to be verified statistically, the presence of F+ coliphages in Talbert Marsh water samples appears to vary during the seasons in which samples were taken. Similar to seagull feces data, higher than expected numbers of positive samples were found during the Summer months; conversely, lower than expected numbers of positive samples were found during the Autumn months. However, statistically significant differences were discovered in concentrations of F+ coliphages from Talbert Marsh waters based on season, with a significantly higher mean concentration observed in the Summer months than the Autumn months. Again, however, a complete evaluation of seasonal variation in Talbert Marsh requires year-round sampling.

Due to the low number of water samples analyzed, statistical analyses on the data were limited. More than 31% of samples positive for F+ coliphages were from the water collected adjacent to the sandflats. These results suggest that the seagull feces on the sandflats may be a source of F+ coliphages to the adjacent waters. The majority of F+ coliphage-positive samples were collected upmarsh of the sandflats, possibly suggesting that marsh water impacted by seagull feces from the sandflats was moved upmarsh by incoming tides. These results may also indicate potential sources of F+ coliphages in Talbert Marsh other than

seagull feces on the sandflats. Additionally, F+ coliphages were also isolated from all samples (four liters) of water collected downstream of the sandflats, possibly suggesting that marsh water impacted by seagull feces from the sandflats was moved downmarsh by outgoing tides.

Bacterial Indicators

All Talbert Marsh water samples analyzed were positive for enterococci and all but one of the samples analyzed were positive for fecal coliforms. The range of concentrations of fecal coliforms was 0 – 398 MPN/100 ml, with a median concentration of 25 MPN/100 ml. All of these concentrations fall under California's current fecal coliform standard for contact recreation in ocean and bay waters of 400 MPN/100ml in a single sample. The range of concentrations of enterococci was 39 – 1300 MPN/100 ml, with a median concentration of 108 MPN/100 ml. Almost 57% of the samples analyzed had concentrations greater than California's current enterococci standard for contact recreation in ocean and bay waters of 104/100ml in a single sample. Less than one-half of the samples analyzed were positive for *E. coli* and concentrations of these were relatively low, ranging from 0 – 27 MPN/100 ml. California's current standards for contact recreation in ocean and bay waters do not include *E. coli*. The concentrations of the above bacterial fecal indicators did not vary significantly between the two seasons analyzed.

Seagull feces were not assayed for these bacteria, but their presence in water samples also suggests the presence of fecal contamination in the waters. Although the highest concentrations of fecal coliforms, *E. coli*, and enterococci were found in samples collected upmarsh of the sandflats, these microbes were also found in samples adjacent to the sandflats

suggesting the presence of fecal contamination. The lowest concentrations of both *E. coli* and enterococci occurred downmarsh of the sandflats. The lower concentrations found both adjacent to the sandflats and downmarsh of the sandflats may be due to a lower number of samples analyzed from theses areas and may also be due to the daily flushing of Talbert Marsh waters and dilution from tidal flow. It is also likely that fecal indicator bacteria in these water samples did not completely survive environmental conditions, such as higher salinity and longer exposure to UV light, during travel to the outlet end of the marsh.

Co-Occurrence of Microbes

Co-occurrence of any two microbes in either seagull feces or water samples was generally low. Among seagull feces samples, the presence of *Salmonella* best predicted the presence of *Campylobacter* and to a lesser extent, also predicted the presence of F+ coliphages. Furthermore, the presence of F+ coliphages also predicted the presence of *Campylobacter*. However, these microbes were found less commonly than was *Campylobacter*, and therefore are not good indicators of the overall presence of *Campylobacter* within seagull feces. Among water samples, the presence of *Campylobacter* best predicted *E. coli* and the presence of *E. coli* best predicted the presence of F+ coliphages. Correlation analyses often indicated some degree of correlation. However, the analyses mostly reflected the high numbers of non-detects (seagull feces), the dominance of one or a few outlying values, and the low number of samples analyzed (water samples) and are generally inconclusive.

Risks Associated with Seagull Feces Contamination

The number of infecting cells of *Salmonella* generally required to cause disease in humans can be as few as 15-20 cells, depending up age and health of host and strain differences among members of the genus (U.S. Food & Drug Administration Center for Food Safety & Applied Nutrition 2003). Acute symptoms include nausea, vomiting, abdominal cramping, diarrhea, fever, and headache. These symptoms occur six to 48 hours post infection and can last one to two days depending on host characteristics. Chronic consequences, including arthritic symptoms may develop three to four weeks post infection (U.S. Food & Drug Administration Center for Food Safety & Applied Nutrition 2003).

C. jejuni is the leading bacterial cause of diarrhea in the United States (World Health Organization 2000). A human feeding study suggested that the number of *Campylobacter* cells generally required to cause disease in humans may be 400 to 500 bacteria (U.S. Food & Drug Administration Center for Food Safety & Applied Nutrition 2003), however, more recently, another study has found that significantly fewer cells may be required for infection (15.2 CFU/L) (Teunis, Van den Brandhof et al. 2005). Numbers of bacteria required to cause infection vary in individuals and studies suggest that host susceptibility may also regulate infectious dose. Symptoms include diarrhea, often containing blood, fever, abdominal pain, nausea, headache, and muscle pain. These symptoms usually occur two to five day post ingestion and can last as long as seven to ten days. In about 25% of cases, relapses of campylobacteriosis occur. Subsequent sequelae are relatively rare and include reactive arthritis, hemolytic uremic syndrome, and septicemia. In very few cases, sequelae such as recurrent colitis, acute cholecystitis, and Guillain-Barré syndrome have occurred (U.S. Food & Drug Administration Center for Food Safety & Applied Nutrition 2003).

Although fecal indicator microbes, such as fecal coliforms, enterococci, and F+ coliphages, themselves do not pose a threat to human health, their presence in water samples also suggests the presence of fecal contamination, and therefore the possible presence of bacterial and viral pathogens, in the waters.

Conclusion

In this study, we asked if bacterial pathogens, specifically thermophilic *Campylobacter* spp. and *Salmonella* spp., and fecal indicators, specifically F+ coliphages, were present in the feces of seagulls that congregate on the sandflats of Talbert Marsh and/or in the surrounding estuarine waters. Using both conventional microbiological and molecular methods (see Chapters III and IV), we were able to determine that these microbes were present in the feces of seagulls and in water samples, suggesting that the feces of these seagulls may likely impact the surrounding waters. To the author's knowledge, this is the first report on concentrations of *Campylobacter* found in seagull feces. In addition, we isolated fecal coliforms, including *E. coli*, and enterococci, the US EPA's choice microbes for assessing the microbiological quality of recreational waters, from Talbert Marsh waters. Although these were not analyzed in bird feces samples, the presence of these bacteria further suggest fecal contamination of these waters.

Large numbers of seagulls often congregate near coastal waters and the collective contribution of seagulls, especially ones who have previously fed at sewage or dump sites such as OCDS, may potentially have a great impact on water quality, at least periodically. In this study, average estimated daily loads into Talbert Marsh for *Salmonella*, *Campylobacter*, and F+ coliphages were determined to be 5.7×10^5, 3.4×10^5, and 9.8×10^6, respectively.

Using highest bird counts and microbe concentrations recorded in Talbert Marsh, these numbers increased by three to four logs. Sewage feeding among seagulls that frequent Talbert Marsh is suggested by the *Salmonella* antibiotic resistance and possibly by PFGE patterns and further analysis of this phenomenon will allow us to better understand potential sources and impacts of contamination.

Seasonal differences of presence and concentrations of microbes were noted in this study. Summer and Autumn months had higher observed numbers of positive *Salmonella* seagull fecal samples than what was expected if no variation between seasons existed and concentrations of *Salmonella* significantly differed between Spring and Summer months. These results contradict previous reports that found higher prevalence of *Salmonella* in the Winter months. Statistically significant seasonal differences at the 5% level were found in *Campylobacter* prevalence in seagull feces, with Spring and Summer months having higher percentages of positive samples than Autumn and Winter months. Concentrations of *Campylobacter* in seagull feces also followed this seasonal pattern. These results contradict previous reports that found higher prevalence of *Campylobacter* in seagull feces in late autumn. Conversely, the presence of *Campylobacter* in Talbert Marsh waters appeared to be higher in the Autumn than the Summer months, although this could not be shown as statistically significant. Finally, higher observed numbers of positive F+ coliphage samples were found in Summer and Autumn months than what was expected if no variation between seasons existed and concentrations significantly differed between Spring and Summer months and Winter and Summer months. In water samples, the presence of F+ coliphages appeared to be higher in the Summer than the Autumn months, although, again, these results could not be shown as statistically significant. Seasonal differences in the presence or

concentrations of microbes isolated from water samples were limited due to the low number of samples collected during only two seasonal periods. Larger sample sizes and sampling throughout all seasons of the year are required to generate more statistically significant results.

Water sample location was also assessed in this study, although statistical analyses were unable to be performed, as numbers of samples was too low for valid and meaningful analysis. All Talbert Marsh water samples analyzed were positive for enterococci and all but one of the samples analyzed were positive for fecal coliforms. Overall, results suggest that the number of positive samples for *Campylobacter*, F+ coliphages, and *E. coli* did vary by location. A higher number of observed than expected positive *Campylobacter* samples were found adjacent to the sandflats (although positive samples were also found upmarsh of the sandflats), while a higher number of observed than expected positive *E. coli* samples were found upmarsh of the sandflats. Neither *Campylobacter* nor *E. coli* were detected in samples taken from downmarsh of the sandflats. Finally, a higher number of observed than expected positive F+ coliphage samples were found downmarsh of the sandflats, although positive samples were also found both upmarsh and adjacent to the sandflats. Results suggest that marsh water was likely impacted by seagull feces from the sandflats and may have also been moved upmarsh by incoming tides or downmarsh by outgoing tides. It is also important to note that factors including low sample volume, dilution, tidal stage, and environmental conditions all may have had an impact on prevalence and concentrations of microbes detected in water samples.

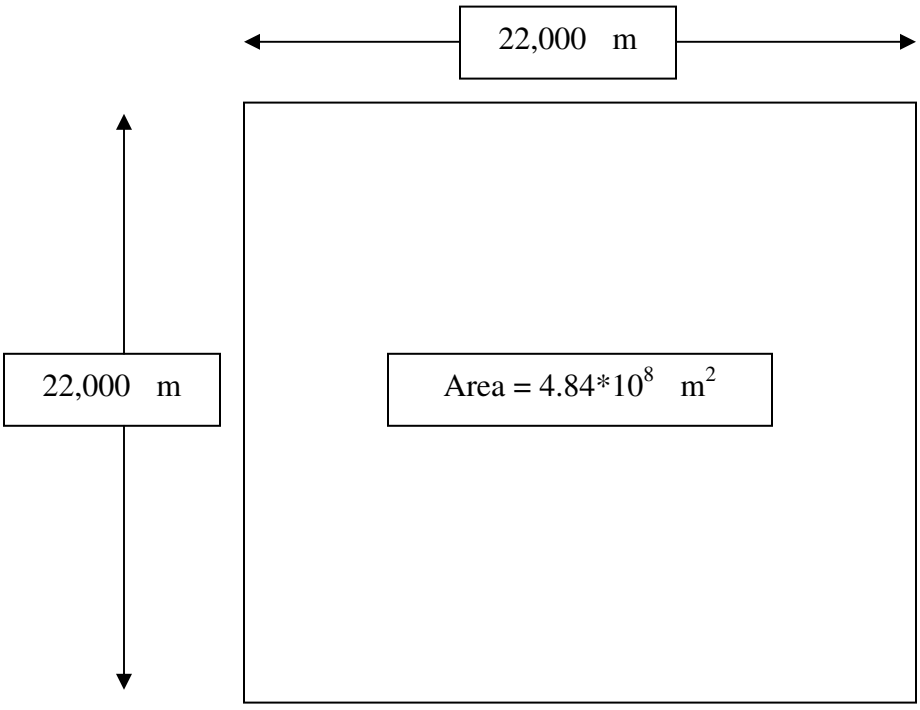
Environmental conditions, sampling or methodological inaccuracies, and limited statistical capabilities may have resulted in marked differences between this study's data and published

data in elements such as seasonality or microbial concentrations. Assessing all of these potential elements is beyond the scope of this study. However, the fact remains that thermophilic *Campylobacter* and *Salmonella* were isolated from seagull feces on the sandflats of Talbert Marsh and there are potential risks when the Talbert Marsh waters contaminated with these feces come in contact with humans, such as through recreational exposures. Further studies involving better estimates of fecal, *Salmonella*, and *Campylobacter* loads, feces dilution, water flow into and out of the marsh, and microbial die-off, need to be conducted to better understand the actual risks this fecal contamination poses to humans in the surf zone at Huntington Beach.

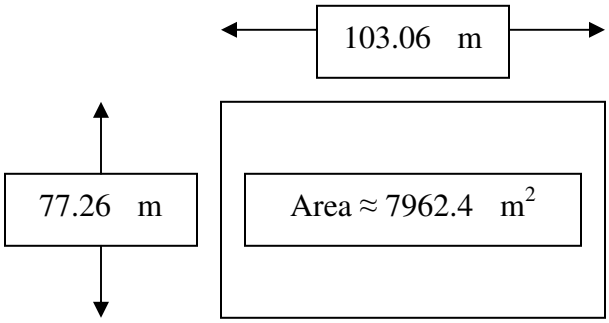
Our results are significant for a number of reasons. Talbert Marsh is a tidally influenced constructed wetland whose water depths vary as the tide moves water in and out of the marsh. Water levels also vary seasonally, with a rainy winter season during which excess water is controlled by a system of flood-controlled channels within a network of urban runoff management. Due to these fluctuations in water levels in the marsh, feces deposited on the sandflats of the marsh come into daily contact with rising marsh waters. As supported in the literature, both *Campylobacter* and F+ coliphages were also detected in Talbert Marsh sand samples. Therefore, both fecal material and sands containing pathogens are likely to be submerged within the waters and ultimately distributed both upmarsh of the sandflats and downmarsh where the marsh empties into the surf zone at Huntington Beach. Further work to genetically characterize and compare microbial isolates from Talbert Marsh seagulls and surrounding waters is necessary to further determine if there are associations between bacterial and coliphages in the seagull feces and those in water.

Figure 2.1: Sample locations in Talbert Marsh

Coverslip:



Field:



$$\#\ \text{Fields / coverslip} = 4.84*10^8 / 7962.4 \approx 60{,}786\ \text{fields}$$

Per sample: (<u>average # cells / field) * (60,786 fields / coverslip)</u> ≈ cell / µl

(10 µl sample / coverslip)

Figure 2.2: Schematic of cell counting analysis

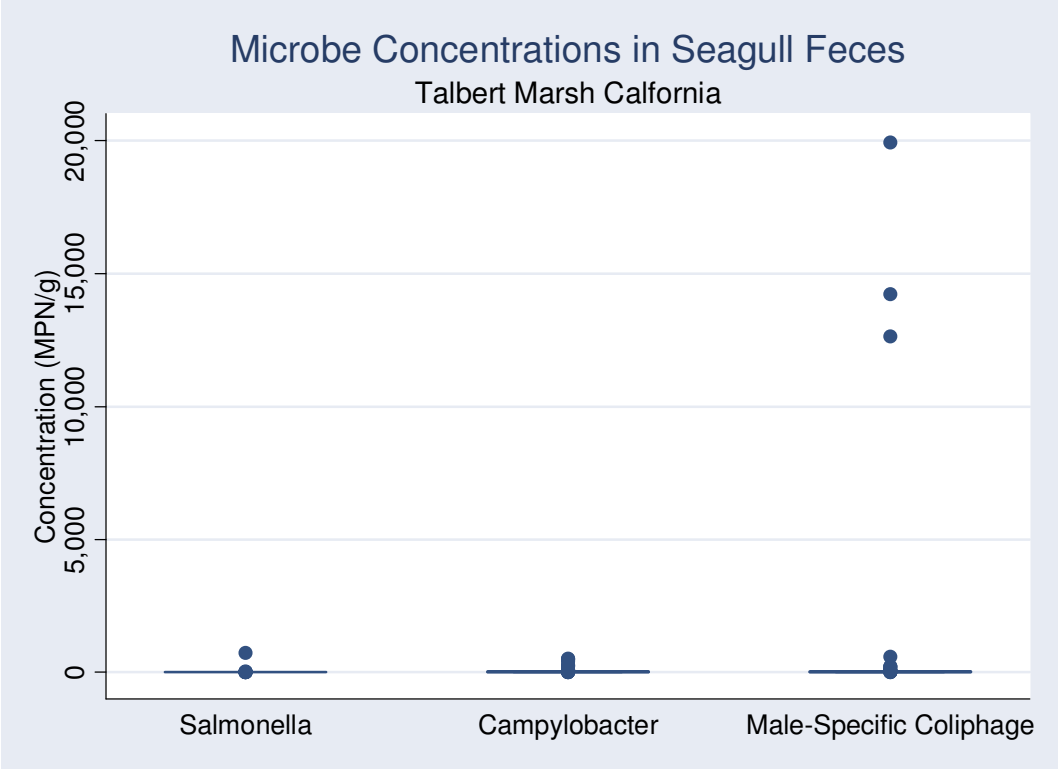


Figure 2.3: ***Salmonella*****,** ***Campylobacter*****, and F+ coliphages in Talbert Marsh seagull feces**

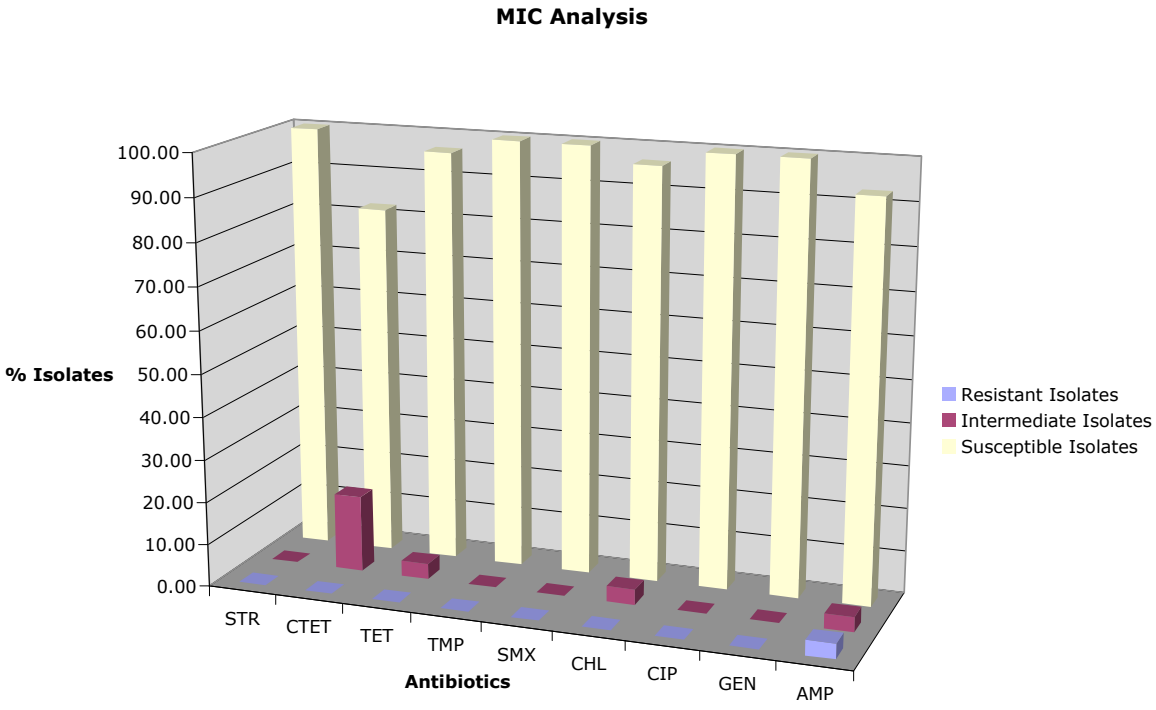
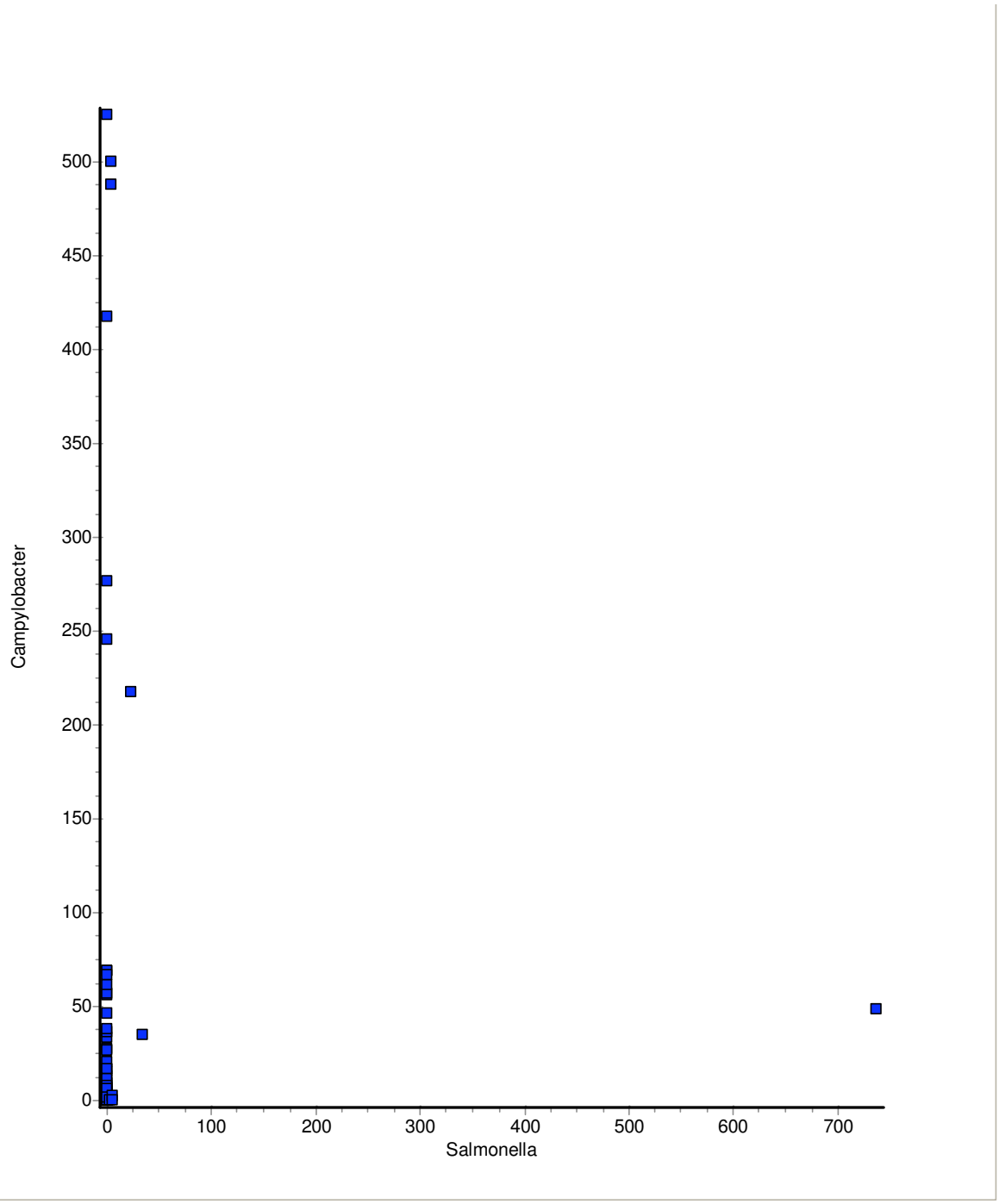


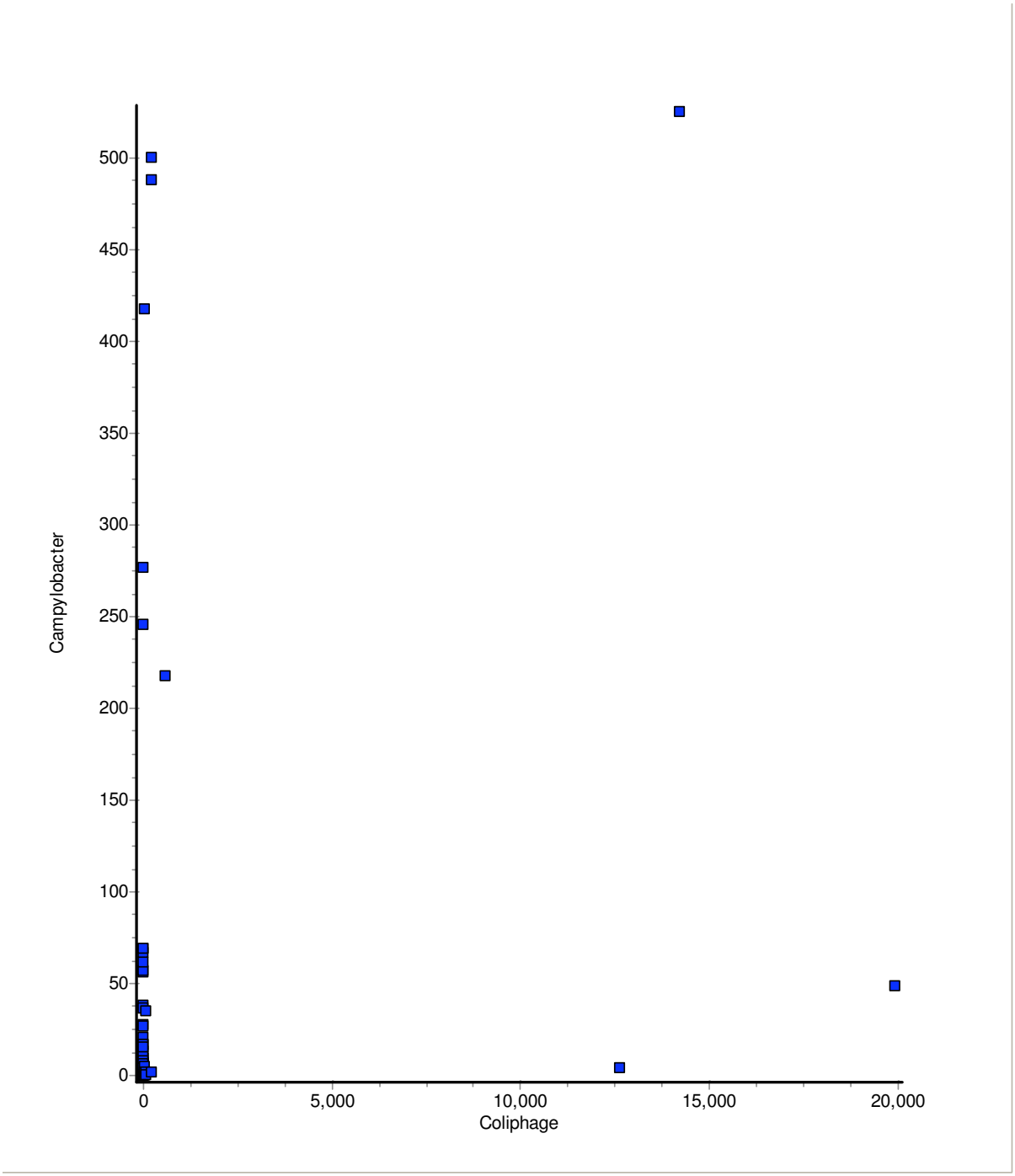
Figure 2.4: *Salmonella* antimicrobial susceptibility testing results



Spearman r = 0.2292 (corrected for ties)

95% confidence interval: 0.04983 to 0.3943

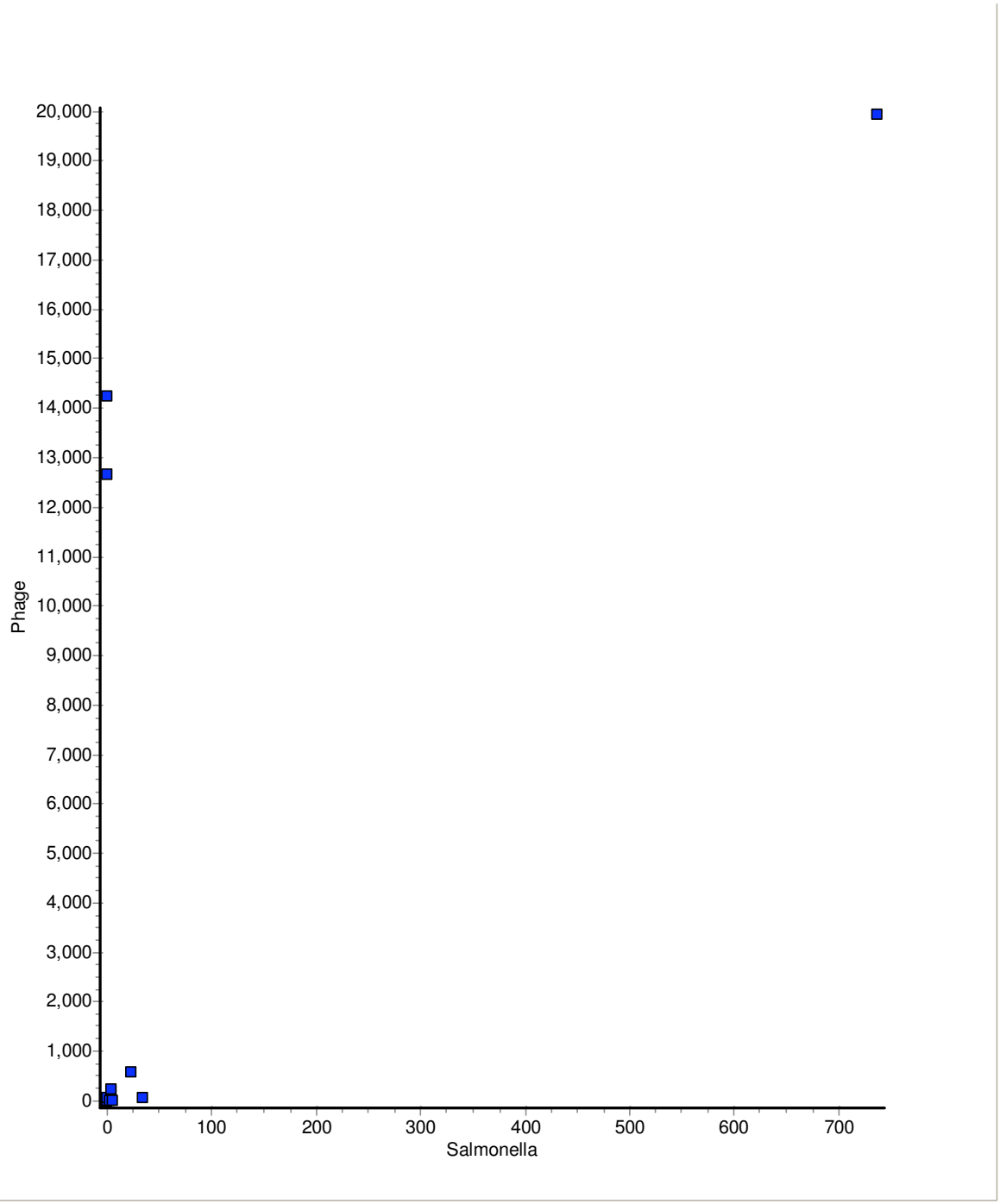
Figure 2.5: *Salmonella* versus *Campylobacter* concentrations in Talbert Marsh seagull feces (MPN/g)



Spearman r = 0.2754 (corrected for ties)

95% confidence interval: 0.08548 to 0.4460

Figure 2.6: F+ coliphage versus *Campylobacter* concentrations in Talbert Marsh seagull feces (MPN/g)



Spearman r = 0.3597 (corrected for ties)

95% confidence interval: 0.1646 to 0.5277

Figure 2.7: *Salmonella* versus F+ coliphage concentrations in Talbert Marsh seagull feces (MPN/g)

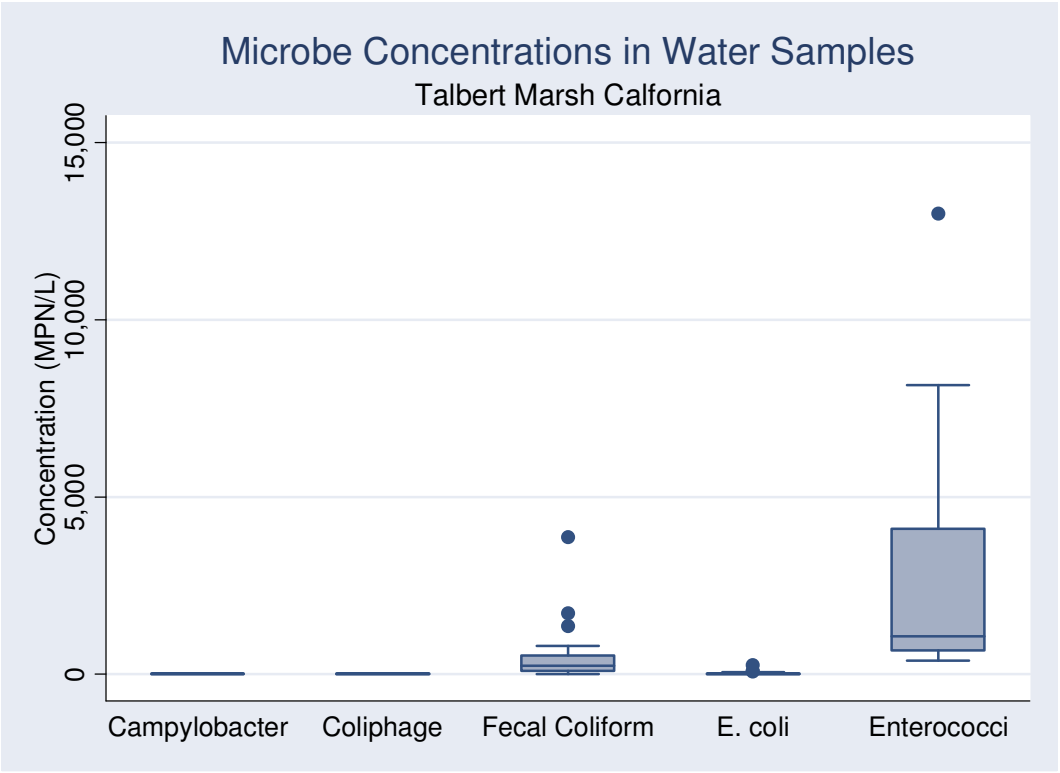
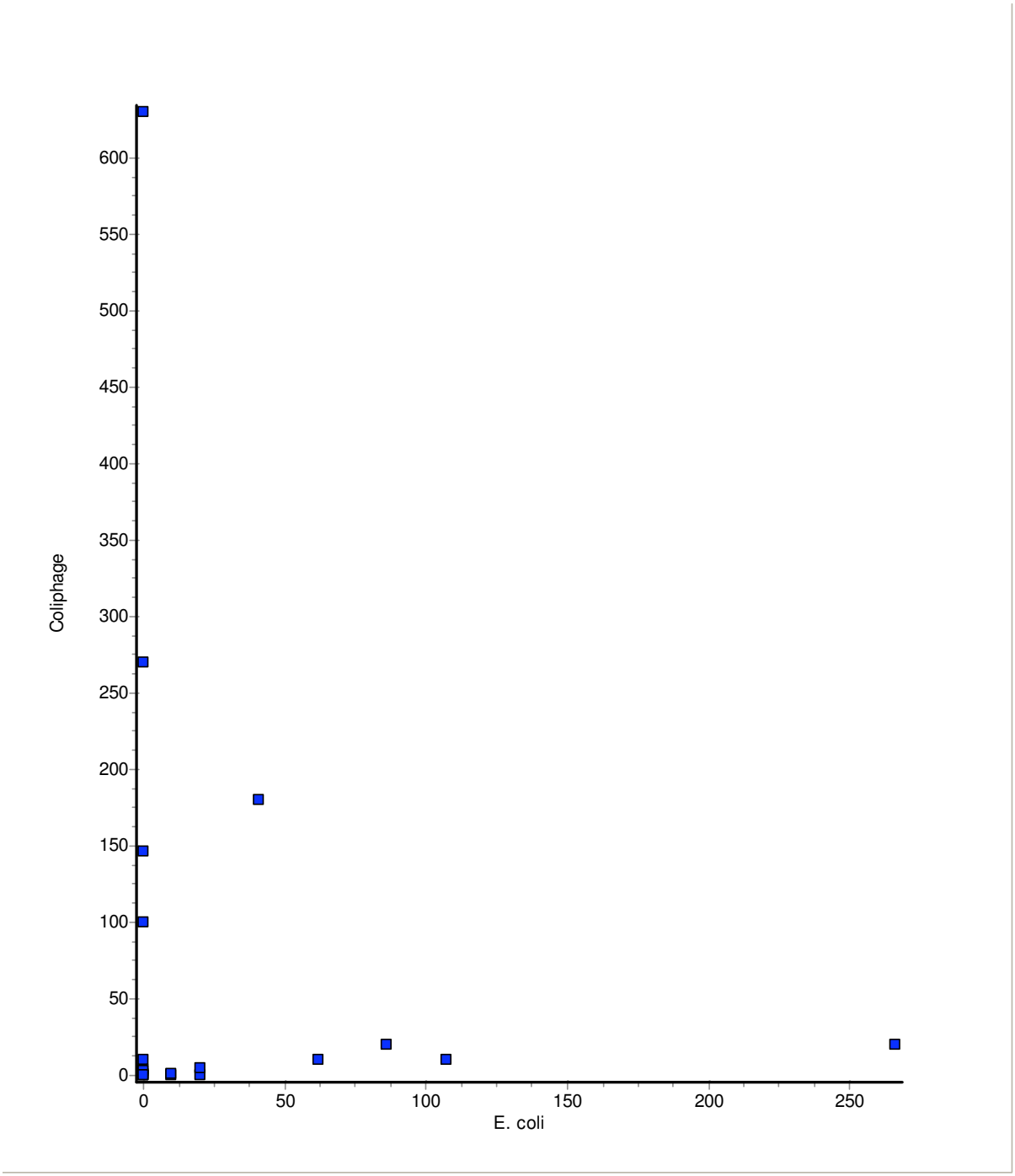


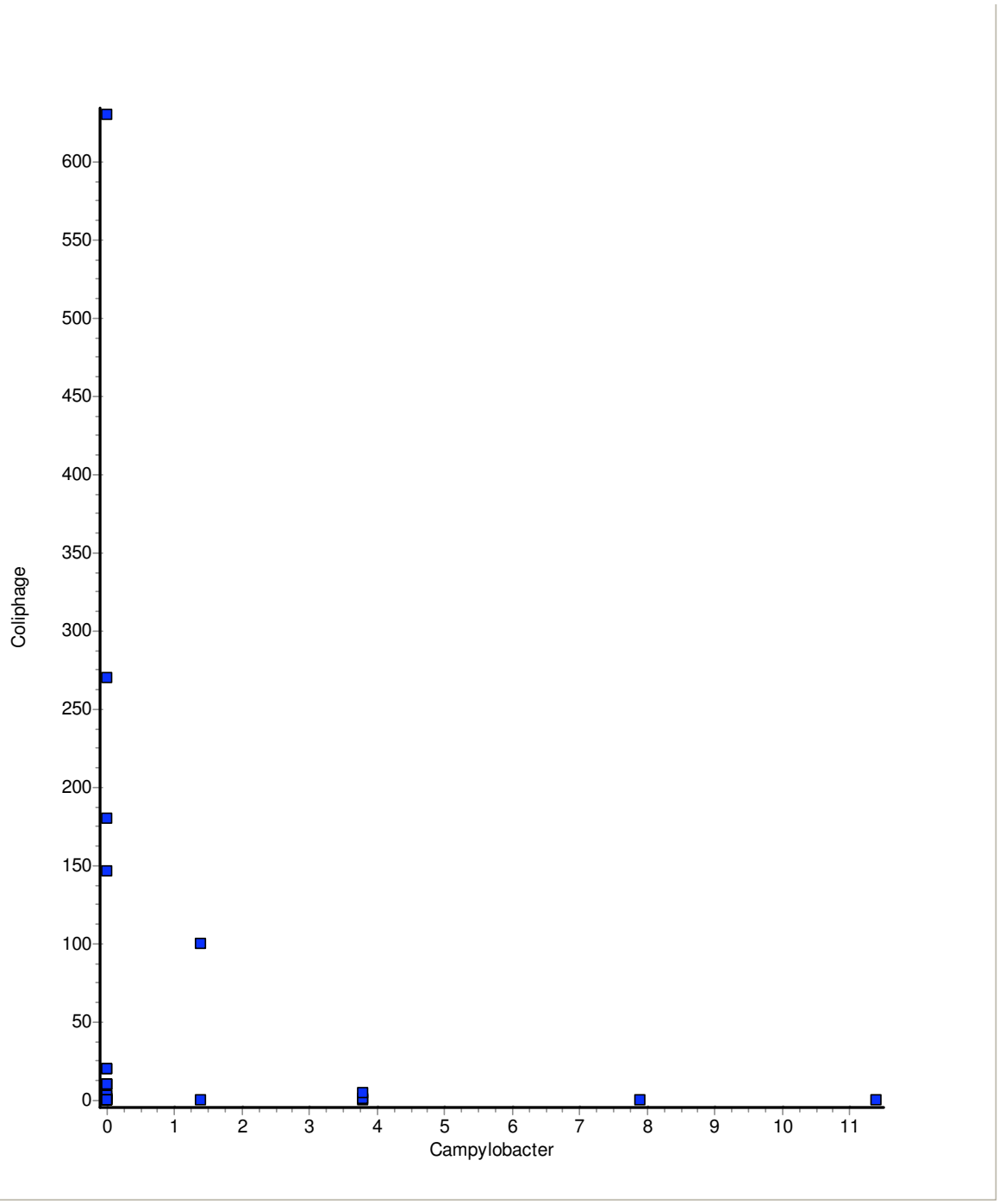
Figure 2.8: ***Campylobacter*****, F+ coliphages, fecal coliforms,** ***E. coli*****, and enterococci in Talbert Marsh water samples**



Spearman r = 0.1256 (corrected for ties)

95% confidence interval: -0.3481 to 0.548

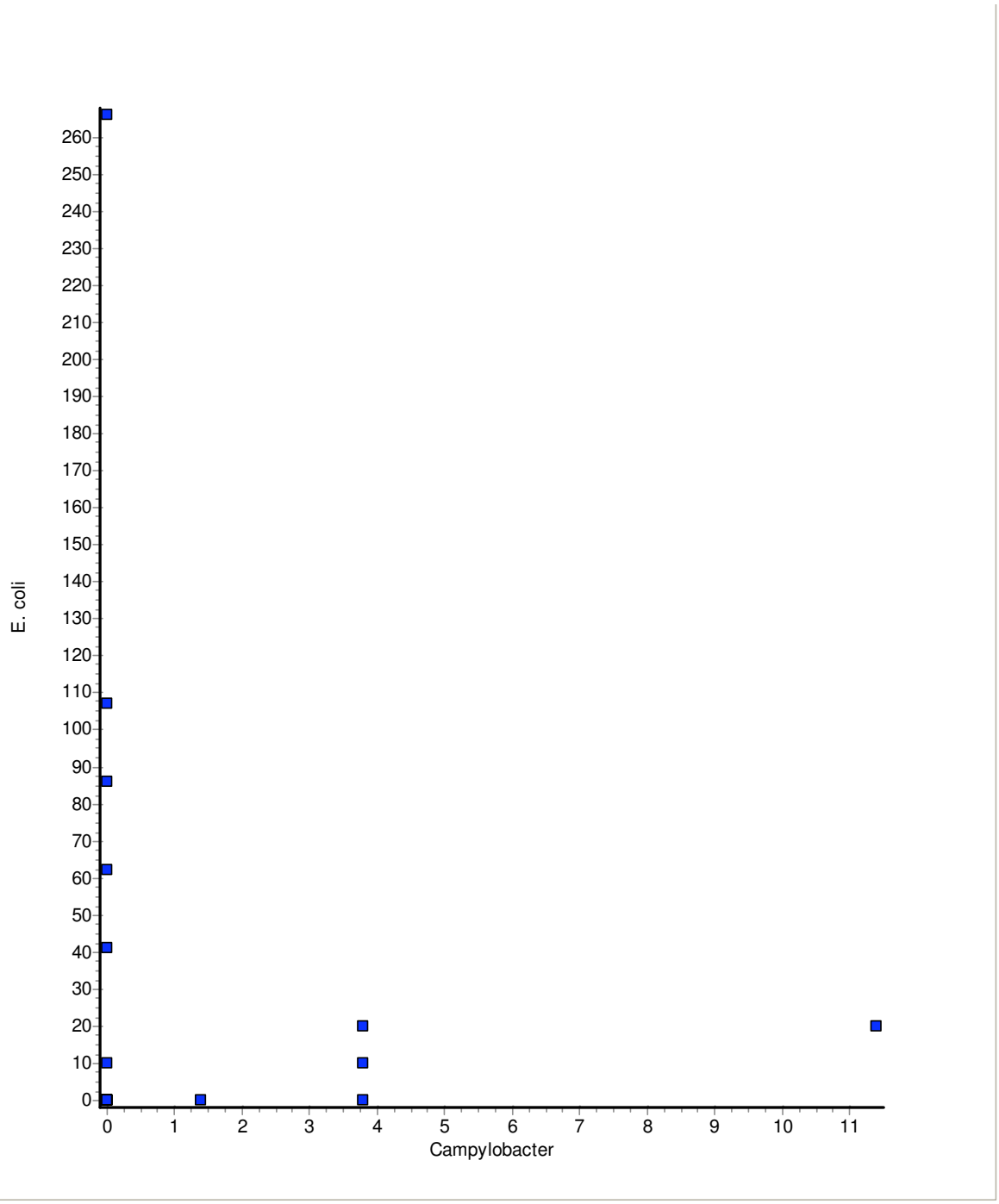
Figure 2.9: *E. coli* versus F+ coliphage concentrations in Talbert Marsh water samples (MPN/L)



Spearman r = -0.3747 (corrected for ties)

95% confidence interval: -0.6828 to 0.04649

Figure 2.10: *Campylobacter* versus F+ coliphage concentrations in Talbert Marsh water samples (MPN/L)



Spearman r = 0.05862 (corrected for ties)

95% confidence interval: -0.4060 to 0.4992

Figure 2.11: *Campylobacter* versus *E. coli* concentrations in Talbert Marsh water samples (MPN/L)

Author	Year	Location	No. Samples	*Salmonella* Carriage Rate
Berg et al.	1972	US (Oregon)	521	2.1%
Fenlon	1981	UK	1241	12.9%
Rosef et al.	1981	Norway	54	3.7%
Kapperud et al.	1983	Norway	179	2.2%
Butterfield et al.	1983	UK	2786	3.4%
Fenlon	1983	UK	20	55.0%
Fricker	1984	UK	1080	17.7%
Girdwood et al.	1985	UK	5888	7.8%
Monaghan et al.	1985	UK	2985	9.5%
Quessy et al.	1992	Canada	264	8.7%
Sixl et al.	1997	Czech Republic	41	51.0%
Wahlstrom et al.	2003	Sweden	111	4.0%
Palmgren et al.	2005	Sweden	1047	2.7%

Table 2.1: Reported *Salmonella* carriage rates in seagull feces

Author	Year	Location	No. Samples	*Campylobacter* Carriage Rate
Rosef	1981	Norway	54	50.0%
Glunder et al.	1989	Germany	65	24.6%
Quessy et al.	1992	Canada	264	15.9%
Kapperud et al.	1993	Norway	179	23.5%
Palmgren et al.	1997	Sweden	50	0.0%
Sixl et al.	1997	Czech Republic	41	63.0%
Broman et al.	2002	Sweden	786	31.8%
Moore et al.	2002	UK	205	13.7%
Wahlstrom et al.	2003	Sweden	104	22.0%

Table 2.2: Reported *Campylobacter* carriage rates in seagull feces

Antimicrobial	Abbreviation	Dilution Range (mg/mL)	MIC Breakpoints
Streptomycin*	STR	32 - 1024	R ≥ 64; S ≥ 32
Vancomycin	VAN	8 - 256	R ≥ 32
Chlortetracycline*	CTET	2 - 16	R ≥ 16; S ≥ 4
Tetracycline*	TET	0.5 - 64	R ≥ 16; S ≥ 4
Trimethoprim	TMP	2 - 64	R ≥ 4; S ≥ 2
Sulfamethoxazole*	SMX	32 - 1024	R ≥ 512; S ≥ 256
Chloramphenicol	CHL	4 - 128	R ≥ 32; S ≥ 8
Tiamulin*	TIA	8 - 64	R ≥ 32
Erythromycin**	ERY	4 - 32	R ≥ 8
Enrofloxacin	ENRO	1 - 16	R ≥ 4
Ciprofloxacin	CIP	1 - 16	R ≥ 4; S ≥ 1
Neomycin*	NEO	16 - 128	R ≥ 32
Gentamicin**	GEN	4 - 256	R ≥ 16; S ≥ 4
Ampicillin**	AMP	8 - 128	R ≥ 32; S ≥ 8
Florfenicol	FFN	4 - 128	R ≥ 16
Tylosin Base*	TYLB	5 - 80	R ≥ 16
Clindamycin	CLI	1 - 32	R ≥ 4

*Approved for use in feed (growth promoters)

**Approved for use as injectibles/water soluble (disease treatment)

Table 2.3: Antimicrobials, dilution ranges, and MIC breakpoints (Anderson and Sobsey 2002)

Sample	Date	Time	Tide[1]	Air Temp	Current Weather	Past Storm?
E	11/13/01	10:20	H/L	65	Very sunny, clear sky	rain 11-12-01
F	11/26/01	13:00	L	66	partly cloudy/sunny	rain 11-25-01
G	01/15/02	9:30	H	63	overcast, windy	no
J	01/21/02	9:00	H	57	sunny, clear	no
H	02/04/02	10:00	H/L	68	sunny, clear	no
I	02/11/02	14:35	H/L	69	clear, sunny, breezy	no
K	02/27/02	12:45	H	67	cloudy	no
M[2]	04/01/02	8:00	L/H	55	cloudy, foggy	no
N[3]	04/08/02	11:40	H/L	67	mostly cloudy	no
O	04/16/02	9:20	H/L	60	partly cloudy	rain 4-15-02
P	04/22/02	10:13	L	67	clear	no
Q	05/06/02	NR	NR	NR	NR	NR
R	05/20/02	9:30	L/H	55	cloudy	no
S[4]	05/28/02	10:00	H	65	cloudy	no
T	06/03/02	11:35	L	64	overcast, cloudy	no
U	09/09/02	15:00	L	ND	clear	no
V	10/21/02	15:05	L	19	cloudy	no

[1]Tidal data from closest reference station at Newport Bay Enterance, Corona del Mar;
L: low tide; H: high tide; B: between tides
[2]All samples were sand
[3]All samples were collected from Newport Beach
[4]One sample was seaweed
NR: not recorded

Table 2.4: Seagull feces sample collection data

	No. Samples Tested	No. Positive Samples (%)	Positive Range MPN/g
Campylobacter	148	75 (50.7)	0.2 - >524.9
Salmonella	125	10 (8.0)	0.4 - >737.0
F+ coliphages	109	31 (28.4)	1.9 - >19931.7

Table 2.5: Microbes isolated from Talbert Marsh seagull feces samples

Antibiotic	No. Isolates Tested	% Susceptible	% Intermediate Resistant	% Resistant
STR	28	**100.0%**	0.0%	0.0%
CTET	28	**82.1%**	**17.9%**	0.0%
TET	28	**96.4%**	**3.6%**	0.0%
TMP	28	**100.0%**	0.0%	0.0%
SMX	28	**100.0%**	0.0%	0.0%
CHL	28	**96.4%**	**3.6%**	**0.0%**
CIP	28	**100.0%**	0.0%	0.0%
GEN	28	**100.0%**	0.0%	0.0%
AMP	28	**92.8%**	**3.6%**	**3.6%**

Table 2.6: Antimicrobial susceptibility testing of *Salmonella* isolate

Isolate	Antibiotic	Response
I9-4	CTET	Intermediate
I10-1	CTET	Intermediate
I10-2	CTET	Intermediate
U1-1	CTET	Intermediate
	CHL	Intermediate
	TET	Intermediate
	AMP	Resistant
U8-9	AMP	Resistant
V3-2	CTET	Intermediate

Table 2.7: Intermediately-resistant and resistant *Salmonella* isolates

Sample ID	PFGE Strain	MIC Results
ATCC 14028	A	all S
F2-1	B	all S
F2-2	B	all S
F2-3	B	all S
F2-4	B	all S
I9-1	C	all S
I9-2	ND	all S
I9-3	C*	all S
I9-4	C	all S except I for CTET (8)
I10-1	C	all S except I for CTET (8)
I10-2	C	all S except I for CTET (8)
P2	D	all S
Q3	E	all S
U1-1	F	all S except I for CTET (8), I for CHL (16), R for AMP (128)
U1-2	G	all S
U1-3	G	all S
U1-4	G	all S
U1-5	G	all S
U1-6	G	ND
U1-7	G	ND
U1-8	G	all S
U7	G	all S
U8-1	H	all S
U8-2	I	all S
U8-3	J	all S
U8-4	K	all S
U8-5	L	ND
U8-6	M	ND
U8-7	N	ND
U8-8	O	ND
U8-9	O	all S except R for AMP (32)
V3-1	P	all S
V3-2	Q	all S except I for CTET (8)
V3-3	R*	ND
V3-4	R	all S
V3-5	R	ND
V4-1	Q	all S

S: susceptible to selected antimicrobials (Table 2.6)
I: intermediately-resistant to selected antimicrobials (Table 2.6)
R: resistant to selected antimicrobials (Table 2.6)
*: strain derived from that of same number designation

Table 2.8: PFGE and MIC results for *Salmonella* isolates

Sample	Estimated Cells / µl	Morphology
H4	794,473.02	rod-like
J3	935,496.54	rod-like
N10	1,168,914.78	coccoid
P3	1,168,914.78	coccoid
Q10	337,970.16	rod-like
R1	458,326.44	rod-like
S1	776,845.08	rod-like
T2	186,613.02	rod-like
U5	899,632.80	rod-like

Table 2.9: Estimated stored *Campylobacter* cell counts

Season	No. Analyzed	No. Positive	Fraction Positive	Expected Value
Autumn	30	3	0.10	2.4
Winter	26	2	0.08	2.08
Spring	59	2	0.03	4.72
Summer	10	3	0.30	0.8
Total	**125**	**10**	**0.08**	

Table 2.10: *Salmonella* in Talbert Marsh seagull feces, by season

Season	No. Analyzed	No. Positive	Fraction Positive	Expected Value
Autumn	30	11	0.37	15.20
Winter	50	16	0.32	25.34
Spring	58	41	0.71	29.39
Summer	10	7	0.70	5.07
Total	**148**	**75**	**0.51**	

Table 2.11: *Campylobacter* in Talbert Marsh seagull feces, by season

Season	No. Analyzed	No. Positive	Fraction Positive	Expected Value
Autumn	10	4	0.40	2.84
Winter	30	4	0.13	8.53
Spring	59	16	0.27	16.78
Summer	10	7	0.70	2.84
Total	**109**	**31**	**0.28**	

Table 2.12: F+ coliphages in Talbert Marsh seagull feces, by season

Microbe	Months Compared	P value
Salmonella	Autumn vs Winter	>0.05
	Autumn vs. Summer	>0.05
	Winter vs. Spring	>0.05
	Winter vs. Summer	>0.05
	Spring vs. Summer	<0.05*
Campylobacter	Autumn vs Winter	>0.05
	Autumn vs. Summer	<0.05*
	Winter vs. Spring	<0.05*
	Winter vs. Summer	<0.05*
	Spring vs. Summer	>0.05
F+ coliphages	Autumn vs Winter	>0.05
	Autumn vs. Summer	>0.05
	Winter vs. Spring	>0.05
	Winter vs. Summer	<0.001*
	Spring vs. Summer	<0.01*

*significance at the 5% level

Table 2.13: Dunn's post-test on microbial concentrations in seagull feces, by season

		Campylobacter +	***Campylobacter*** -
Salmonella	+	8	2
	-	58	56

Table 2.14: Occurrence of *Salmonella* and *Campylobacter* in seagull feces

		Campylobacter +	***Campylobacter*** -
F+ coliphages	+	23	8
	-	35	42

Table 2.15: Occurrence of *Campylobacter* and F+ coliphages in seagull feces

		F+ Coliphages +	F+ Coliphages -
Salmonella	+	6	3
	-	22	64

Table 2.16: Occurrence of F+ coliphages and *Salmonella* in seagull feces

Sample	Site	Date	Time	Tide*	Water Temp	Air Temp	Salinity	Weather	Past Storm?
A	4	08/19/02	13:45	L	21.2	71	21.1	cloudy	no
B	3	08/19/02	13:55	L	21.2	71	20.6	cloudy	no
C	1	08/19/02	14:05	L	21.2	71	20.5	cloudy	no
D	1	08/26/02	14:15	B	20.2	ND	20.9	sunny	no
E	1	08/26/02	14:21	B	20.6	ND	21.5	sunny	no
F	1	08/26/02	14:27	B	20.4	ND	20.8	sunny	no
G	1	09/09/02	14:20	B	22.2	ND	21.0	clear	no
H	3	09/09/02	14:28	B	22.2	ND	21.1	clear	no
I	3	09/09/02	14:40	B	18.8	ND	21.3	clear	no
J	1	09/16/02	12:20	L	19.6	72	21.1	clear	no
K	4	09/16/02	12:38	L	19.8	*72*	21.0	clear	no
L	3	09/16/02	12:28	L	19.4	72	21.2	clear	no
M	1	09/23/02	14:25	L	21.2	88	21.5	clear	no
N	3	09/23/02	14:31	L	20.8	88	21.4	clear	no
O	4	09/23/02	14:35	L	21	88	21.4	clear	no
P	5	10/21/02	15:00	L	17.2	19	21.7	cloudy	no
Q	3	10/21/02	15:06	L	17.6	19	21.2	cloudy	no
R	1	10/21/02	15:10	L	17.2	19	21.1	cloudy	no
S	1	10/28/02	15:43	H	ND	21	21.1	clear	no
T	2	10/28/02	15:57	H	ND	21	21.0	clear	no
U	3	10/28/02	16:00	H	ND	21	21.3	clear	no
V	1	11/18/02	14:45	L	18.8	32	21.3	clear	no
W	2	11/18/02	14:47	L	18.2	32	21.0	clear	no
X	3	11/18/02	14:53	L	17.8	32	21.0	clear	no

*Tidal data from closest reference station at Newport Bay Entrance, Corona del Mar;
L: low tide; H: high tide; B: between tides

Table 2.17: Water sample collection data

	No. Samples Tested	No. Positive Samples (%)	Positive Range MPN/L
Campylobacter	24	7 (29.2)	1.4 - 11.4
Salmonella	23	0 (0.0)	NA
F+ coliphages			
1601	20	13 (65.0)	1.0 - >146.1
1602	20	10 (50.0)	10.0 - 630.0
fecal coliforms	20	19 (95.0)	10 - 3873
Escherichia coli	20	9 (45.0)	10 - 266
enterococci	23	23 (100.0)	391 - 12997

Table 2.18: Microbes isolated from Talbert Marsh water samples

	1601	1602
Sample	**PFU/1 L**	**PFU/1 L**
A	1.2*	-
D	1.2	10*
E	1.2	10*
F	9.5	20*
H	-	270*
I	3.2	180*
J	146.1*	60
K	31.6	630*
L	12.2	100*
M	3.7*	-
N	1.0*	-
O	2.4*	-
P	-	10*
T	-	20*
V	1.2*	-
X	4.9*	-

*Concentrations used in statistical analyses

Table 2.19: F+ coliphage concentrations in positive water samples

Season	No. Analyzed	Positive	Fraction Positive	Expected
Summer	12	2	0.17	3.50
Autumn	12	5	0.42	3.50
Total	**24**	**7**	**0.29**	

Table 2.20: *Campylobacter* in Talbert Marsh water samples, by season

Season	No. Analyzed	Positive	Fraction Positive	Expected
Summer	12	9	0.75	8.00
Autumn	12	7	0.58	8.00
Total	**24**	**16**	**0.67**	

Table 2.21: F+ coliphages in Talbert Marsh water samples, by season

Season	No. Analyzed	Positive	Fraction Positive	Expected
Summer	9	5	0.56	3.79
Autumn	10	3	0.30	4.21
Total	**19**	**8**	**0.42**	

Table 2.22: *E. coli* in Talbert Marsh water samples, by season

		Campylobacter +	***Campylobacter*** -
E. coli	+	3	6
	-	2	9

Table 2.23: Occurrence of *E. coli* and *Campylobacter* in water samples

		Campylobacter +	***Campylobacter*** -
F+ coliphages	+	3	12
	-	4	4

Table 2.24: Occurrence of F+ coliphages and *Campylobacter* in water samples

		F+ coliphages +	F+ coliphages -
E. coli	+	7	2
	-	8	3

Table 2.25: Occurrence of *E. coli* and F+ coliphages in water samples

Water Station	Sample	Campy	Salm	Phage	FC	EC	ENT
1	C	-	-	-	NA	NA	+
	D	-	-	+	+	+	+
	E	-	-	+	+	+	+
	F	-	-	+	+	+	+
	G	-	-	-	+	+	+
	J	-	-	+	+	-	+
	M	-	-	+	+	-	+
	R	+	-	-	+	+	+
	S	-	-	-	+	-	+
	V	+	-	+	+	+	+
2	T	-	-	+	+	+	+
	W	+	NA	-	NA	NA	NA
3	B	+	-	-	NA	NA	+
	H	-	-	+	+	-	+
	I	-	-	+	+	+	+
	L	+	-	+	+	-	+
	N	-	-	+	+	-	+
	Q	-	-	-	+	-	+
	U	+	-	-	+	-	+
	X	+	-	+	+	+	+
4	A	-	-	+	NA	NA	+
	K	-	-	+	+	-	+
	O	-	-	+	+	-	+
5	P	-	-	+	+	-	+

Campy = *Campylobacter*
Salm = *Salmonella*
Phage = F+ coliphages
FC = fecal coliforms
EC = *E. coli*
ENT = enterococci
NA = not assayed

Table 2.26: Presence or absence of each microbe in Talbert Marsh water samples

	No. Analyzed	Positive	Fraction Positive	Expected
Upmarsh	12	3	0.25	3.50
Adjacent	8	4	0.5	2.33
Downmarsh	4	0	0	1.17
Total	**24**	**7**	**0.29**	

Table 2.27: *Campylobacter* in Talbert Marsh water samples, by location

	No. Analyzed	Positive	Fraction Positive	Expected
Upmarsh	12	7	0.58	8.00
Adjacent	8	5	0.63	5.33
Downmarsh	4	4	1.00	2.67
Total	**24**	**16**	**0.67**	

Table 2.28: F+ coliphages in Talbert Marsh water samples, by location

	No. Analyzed	Positive	Fraction Positive	Expected
Upmarsh	10	7	0.70	4.50
Adjacent	7	2	0.29	3.15
Downmarsh	3	0	0.00	1.35
Total	**20**	**9**	**0.45**	

Table 2.29: *E. coli* in Talbert Marsh water samples, by location

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CHAPTER III

Molecular Analysis of Stored *Campylobacter* Isolates Originating from Seagull Feces and Water of an Estuarine Environment

Introduction

Previously, presumptive culture-positive thermotolerant *Campylobacter* spp. were isolated from seagull feces, Talbert Marsh water samples, and other solid samples from Talbert Marsh using conventional microbiological methods and isolates were stored at -80 C in TSB with 25% glycerol (v/v) for at least one year. After freezing, these isolates were subjected to a number of freeze-thaw events in attempt to resuscitate cells for species confirmation and further molecular analyses to determine the genetic relatedness between isolates from seagull feces and those from water samples. There were repeated attempts to recover viable and culturable cells from the frozen state because of the relative lack of success in accomplishing this critical step in the intended processes aimed at further analysis and characterization. Resuscitation attempts included both general and selective enrichments and subculturing onto Campy blood agar, which was used in the original isolation. While a number of the initial resuscitation attempts were successful for biochemical testing and Gram-staining, most of the subsequent resuscitation attempts for molecular-based testing failed to produce colonies or produced colonies that did not exhibit *Campylobacter* morphology.

Although cells within the stored suspensions were no longer alive, were injured, or were in a viable but non-culturable (VBNC) state, the authors believed that cellular DNA was still present and likely intact. Several molecular-based genotypic methods, modified for use on

the frozen cell suspensions in TSB with 25% glycerol, were performed. In an attempt to confirm isolates as thermotolerant *Campylobacter* spp., cell suspensions were subjected to a multiplex PCR method developed in New Zealand for the detection of thermotolerant *Campylobacter* and for the differentiation between *C. jejuni* and *C. coli* in enriched samples. A sensitivity assay was performed to determine the efficiency of this multiplex method when performed on cell suspensions stored as they were in this work. Finally, three other PCR methods and nucleotide sequencing were also performed on a subset of the cell suspensions for further isolate characterization.

Background

Cryopreservation of Bacteria and Other Microbes

Cryopreservation is a process in which cells (or whole tissues or organisms) are preserved by cooling suspensions to sub-zero temperatures (typically -80 C or -196 C) so that any biological activity, including biochemical reactions that lead to cell death, are halted. The fact that water is a major component of living cells and necessary for cellular processes is well understood and the deleterious effects of ice on cell viability is well documented. Although the freezing process in cells has been studied, its mechanism and harmful effects on cells are still not completely understood (Simione 1998; Wikipedia 2005).

The greatest challenge to cells undergoing cryopreservation is not survival for long periods of time at low temperatures, but survival during intermediate zones of temperature (~15 C to ~60 C) that cells experience during freezing, and again during thawing. Often during freezing, the process of supercooling, or the cooling of a liquid below its freezing point without the formation of ice crystals, occurs within the cells. As ice begins to form in the

external media, the cell contents often remain unfrozen and supercooled. This is likely due to prevention of ice crystal growth in the cytoplasm by the cellular plasma membranes. Therefore, during freezing, cells are exposed to an increasingly hypertonic solution medium and intracellular water then moves out of the cell across the cell membrane to restore osmotic balance (Ozkavukcu and Erdemli 2002).

The rate of cooling has dramatic effects on cells. During slow cooling, the cells lose intracellular water rapidly and become dehydrated. Although slow cooling results in the formation of less intracellular ice, the increased solute concentrations and long period of exposure to hypertonic environments within the cell can be detrimental. In dilute aqueous solutions such as growth media, the increase in ionic composition following extracellular ice formation is approximately three molar by the time the solution reaches -10 C. Alternatively, during rapid cooling, there is less time for the intracellular water to be removed from the cell. This water eventually forms small intracellular ice crystals that are likely to enlarge during warming. It has been speculated that recrystallizing ice crystals exert enough force to rupture plasma membranes, inevitably causing cell death (Mazur 1970; Simione 1998; Ozkavukcu and Erdemli 2002).

Several factors influence the cooling rates of bacterial cells. Cell permeability has been shown to affect the rate of cellular water loss; more permeable cells are better able to tolerate rapid cooling. These cells dehydrate faster during the cooling process than less permeable cells, resulting in less intracellular ice formation. Furthermore, cells grown under aerated conditions are likely to have greater cell permeability and demonstrate a greater resistance to the harmful effects of cooling and freezing than non-aerated cells. Late log to early

stationary cells have also been shown to have greater resistance to freezing processes (Simione 1998).

Cryoprotective additives (CPAs) can be used to protect cells by reducing cellular damage due to freezing and thawing. CPAs generally have a low molecular weight, are non-toxic, and are able to permeate cells (Simione 1998). CPAs are believed to act by reducing the electrolyte concentration in the residual unfrozen solution in and around a cell, therefore protecting on a molar basis (Mazur 1970). The most commonly used CPAs for bacterial freezing are dimethyl sulfoxide (DMSO) and glycerol (Simione 1998). Other less commonly used CPAs include blood serum or serum albumin, skimmed milk, peptone, yeast extract, sorbitol, saccharose, glucose, methanol, polyvinylpyrolidone (PVP), and malt extract. The best CPA or combination of CPAs and optimum concentrations must be determined empirically for particular microorganisms (Hubalek 2003).

Equilibration is the time between mixing the CPA and cell suspension and the beginning of the cooling process, during which CPAs penetrate the cells. This period should range from 15 to 60 minutes at ambient temperature, depending on the type of bacterial cells being equilibrated. An optimum cooling rate of 1 C per minute has been documented for most bacterial cells. These cells can tolerate a less-than-ideal cooling rate, while fastidious cells require a more uniform rate. Maintaining cells at the proper temperature during storage and using appropriate thawing techniques will minimize damage. Reconstitution, or thawing, should occur as rapidly as possible until the sample is completely thawed. This may be done in a 37 C water bath with very gentle agitation and thawed material should be quickly transferred to fresh growth material to minimize the toxicity of CPAs. In general, ~10^7 bacterial cells/ml are required for adequate recovery (Simione 1998).

Cryopreservation and *Campylobacter*

While short-term preservation techniques for *Campylobacter* have been widely discussed in the literature, long-term preservation techniques for these bacteria have proven to be more problematic (Gorman and Adley 2004). *Campylobacter* species are fastidious organisms, even within their ideal growing conditions of 5 – 7% oxygen and temperatures of 37 – 42 C. Conventional laboratory freezing at -20 C and -80 C, in the presence of atmospheric oxygen, represent conditions well below the ideal. Previous work has shown that freezing *Campylobacter* cultures results in a rapid decrease in cell viability (Humphrey and Cruickshank 1985; Humphrey 1986; Chan, Le Tran et al. 2001). The formation of intracellular ice and cell dehydration are considered two of the main factors involved in cell injury and death during the processes of freezing and thawing (Mazur 1970).

In recent studies, another important aspect in the survivability of *Campylobacter* cells during freezing and thawing has been discovered. It has been predicted that an oxidative burst occurs during the thawing process and this oxidative stress has been shown to cause injury in yeast cells (Park, Grant et al. 1998). Superoxide dismutase (SOD) is an enzyme that converts harmful oxygen free radicals to H_2O_2 and O_2, followed by a catalase enzyme that converts H_2O_2 to H_2O and O_2. SOD plays an important role as a first line of defense in oxygen toxicity, especially in *C. jejuni* and *C. coli*, which contain only one type of this enzyme, known as iron superoxide dismutase (Fe SOD), unlike *E. coli* which contains three distinct types (Stead and Park 2000). Furthermore, *C. fetus* has been found to be more sensitive to exogenous superoxide anions and H_2O_2 than aerotolerant bacteria, despite the activity of SOD and catalase (Humphrey 1988). A 2002 study by Stead et al. (Stead and Park 2000) found that SOD-deficient *C. coli* isolates were more sensitive to freezing and

thawing than wild-type isolates and that this sensitivity was reduced when isolates were frozen in the absence of oxygen. Researchers concluded that superoxide radicals are likely generated during the freeze-thaw process and that SOD is important in the resistance of the *C. coli* cells to these radicals.

Another aspect in cryopreservation of *Campylobacter* is the possibility of the viable but non-culturable (VBNC) state of cells (Gorman and Adley 2004). VBNC bacterial cells have been exposed to some form(s) of environmental stress such as nutrient deprivation, temperature change, or osmotic fluctuation. While the cells are still viable and show some level of metabolic activity, they do not produce colony-forming units or other growth by conventional microbiological methodology, thereby appearing to be absent in tested samples in which they are present. Furthermore, these bacteria pose risks to public health because they often can regain infectious capability once introduced into more favorable environmental conditions, such as those found in host intestinal tracts. However, this position has been debated (Medema, Schets et al. 1992; Stern, Jones et al. 1994). VBNC phenomena in *Campylobacter* cells was first described by Rollins et al. (Rollins and Colwell 1986), who found that *C. jejuni* cells remained viable but non-culturable for greater than four months in a 4 C sterile stream-water microcosm system. Other studies have documented the occurrence of VBNC in *Campylobacter* at various temperatures (Jones, Sutcliffe et al. 1991; Medema, Schets et al. 1992), although the occurrence of a VBNC state among *Campylobacter* cells subjected to sub-zero temperatures has not been documented. It is likely that *Campylobacter* cells subjected to freeze-thaw events enter a VBNC state due to nutrient deprivation in storage media, drastic temperature changes, and variation in osmotic conditions both intracellularly and extracellularly.

A number of studies have analyzed the survivability of *Campylobacter* on meats and meat products derived from food animals under freezing conditions. In these studies, known concentrations of *Campylobacter* were inoculated onto either ground chicken, chicken skin, chicken wings, or ground beef. The meat products were then frozen and *Campylobacter* concentrations were determined after set intervals of time. Overall, short-term studies reported recovery of *Campylobacter* post-freezing, but a marked decline from original concentrations (Stern and Kotula 1982; Yogasundram and Shane 1986; Moorhead and Dykes 2002; Zhao, Ezeike et al. 2003; Bhaduri and Cottrell 2004). In a 52-week long-term freezing study, *C. jejuni* reductions on chicken wings held at -20 and -86 C were ca. 4 and 0.5 $\log_{10}$ CFU/g, respectively (Zhao, Ezeike et al. 2003). Furthermore, Stern et al. (Stern and Kotula 1982) reported 25 times greater recovery of *C. jejuni* in ground beef when frozen with 10% DMSO or glycerol as was recovered from peptone-diluted ground beef.

Fewer studies have reported the recovery of *Campylobacter* from different storage media. Most methods for long-term preservation of *Campylobacter* described in the literature involve liquid drying, liquid nitrogen, and freeze-drying, all of which require equipment not readily available in laboratories. Using Brucella Albimi broth and 10% glycerol, Mills and Gherna (Mills and Gherna 1988) found liquid nitrogen was the most effective means of long-term preservation of *Campylobacter*, followed by slow rate freeze-drying, and lastly conventional freezing at -65 C. Another study found that phosphate buffered saline supplemented with charcoal, FBP (ferrous sulphate, sodium metabisulphate, and sodium pyruvate), L-cysteine, and 10% glycerol supported the survival of *Campylobacter* strains for 135 days at -10 C. This was followed by George's medium, brucella broth with 15% glycerol, fecal calf serum with 50% tryplicase soy yeast-broth, and glycerol transport broth,

respectively (Saha and Sanyal 1991). In 2004, Gorman and Adley (Gorman and Adley 2004) published a study that aimed to identify simple and inexpensive *Campylobacter* preservation techniques that allowed quick and reliable recovery after long-term storage at conventional freezing temperatures of -20 and -85 C. They found that FBP medium was the most successful, with 100 and 80% *Campylobacter* recovery after one year at -85 and -20 C, respectively. They also found that the commercial Cryobank microbial preservation system using horse blood and glass beads or nutrient broth no. 2 culture with 15% glycerol were also very successful for storage at -85 C.

Campylobacter Detection and Characterization by PCR

Thermotolerant members of the *Campylobacter* genus, specifically *C. jejuni*, *C. coli*, and to some extent *C. lari* and *C. upsaliensis*, are major causes of acute gastroenteritis in both developing and developed nations (Eyers, Chapelle et al. 1993). Conventional microbiological isolation and identification of *Campylobacter* species are difficult, mainly because of their "complex taxonomy, biochemical inertness, and fastidious growth requirements (On and Jordan 2003)." While *C. jejuni* is principally found in poultry and *C. coli* in pigs, genetically similar isolates of both species have been found in humans, wild animals, and agricultural animals. For surveillance and risk assessment purposes, it is important to not only have dependable *Campylobacter* isolation methods, but also to have methods able to reliably differentiate between *C. jejuni* and *C. coli*. Traditionally, the two species have been differentiated by the hippurate hydrolysis biochemical test. While *C. jejuni* typically gives a positive result, negative strains within the species and positive non-*C. jejuni* species have also been described (On and Jordan 2003).

C. lari was easily distinguishable from the other thermotolerant *Campylobacter* species based on its resistance to nalixidic acid, until the 1990s when nalidixic acid-resistant *C. jejuni* and *C. coli* strains emerged. *C. lari* was initially only isolated from seagulls, but now has been isolated from a variety of environmental and animal sources. A number of *C. lari* variants have emerged, including NASC (nalidixic-acid-susceptible) stains, UPTC (urease-producing) strains, and urease producing nalidixic-acid susceptible strains (Duim, Wagenaar et al. 2004). Furthermore, recent evidence suggests that mammals and birds harbor previously undescribed campylobacter-like organisms (CLOs) (Linton, Owen et al. 1996).

Due to the phenotypic and genotypic diversity of *Campylobacter* species and variants, fast and reliable molecular identification methods are important tools for the protection of public health (On and Jordan 2003). Although DNA probes methods have been described for a number of *Campylobacter* species, they have limited application and are relatively laborious (Linton, Owen et al. 1996). The polymerase chain reaction (PCR) allows amplification of specific regions of DNA and has proven useful in the identification of *Campylobacter*. Briefly, target DNA is copied by a thermostable DNA polymerase enzyme in the presence of nucleotides and oligonucleotide primers that hybridize specifically to the target sequence. These oligonucleotide primers are designed with the aid of 16S and 23S rRNA gene sequence data that has become widely available through international databases accessible via the Internet. Multiple cycles of heating and cooling in a thermal cycler produce rounds of DNA denaturation, primer annealing, and primer extension. Target DNA is multiplied exponentially, producing billions of copies of target DNA from a single original copy.

An important problem in the bacterial screening of both environmental and clinical samples is the presence of organic and inorganic compounds that inhibit the amplification of

nucleic acids by PCR. Generally PCR inhibitors are most abundant in complex samples such as animal fluids, foods, organic soils, or samples with high bacterial concentrations. Compounds such as polysaccharides, urea, humic acids, metallic salts, and metallo-organic complexes such as hemoglobin demonstrate similar solubility to DNA, are often not completely removed during extraction protocols, and can lead to different levels of attenuation or to complete inhibition of the PCR reaction. Several methods, such as dialysis, centrifugation, ion-exchange columns, glass bead extraction, and affinity or ligand-binding spin columns, have been developed to overcome such contaminations. Disadvantages of employing these methods include loss of original sample, cross-contamination, laborious sample processing steps, and additional expense (Moreira 1998). Besides sources of PCR inhibition from environmental or clinical samples, the effects of storage additives including TSB and glycerol on the successful amplification of *Campylobacter* have not been discussed in the available literature. However, it is important to note that glycerol is present in the *Taq* enzyme used for DNA amplification and this has not been reported to have a negative effect on *Campylobacter* PCR effectiveness. Furthermore, work suggests that in some cases glycerol has been found to improve the PCR process (Smith, Long et al. 1990).

To date, a large number of PCR methods have been developed for the identification of *Campylobacter* species. While each is useful to understand, two particular studies provide a comprehensive examination of a number of published PCR assays for detection of thermotolerant *Campylobacter*. On et al. (On and Jordan 2003) evaluated 11 PCR assays for species-level identification of *C. jejuni* and *C. coli* targeting genes including 23S rRNA, *mapA*, *ceuE*, *hipO*, and random sequences. The researchers found that tests varied considerably in their sensitivity and specificity and they concluded that a polyphasic strategy

for PCR-based identification of *C. jejuni* and *C. coli* strains should be used. Similarly, Lubeck et al. (Lubeck, Wolffs et al. 2003) evaluated combinations of 15 published and unpublished PCR primers targeting the 16S rRNA gene and two published primers targeting the 23S rRNA gene for an assay that included all strains of the three predominate food-borne *Campylobacter* species (*C. jejuni*, *C. coli*, and *C. lari*), but excluded all other species. Researchers found that the published PCR assays evaluated lacked the desired selectivity and only one primer pair studied showed adequate selectivity. A comprehensive discussion of the advantages and disadvantages of the large number of *Campylobacter* molecular-based assays for both detection and speciation available in the literature is beyond the scope of this paper. The following will focus on the specific molecular methods for thermotolerant *Campylobacter* speciation used in this study.

Multiplex PCR

The multiplex PCR method used in this study was developed in New Zealand for the simultaneous detection and identification of *C. jejuni*, *C. coli*, and thermotolerant *Campylobacter* spp (Wong, Devane et al. 2004). The multiplex method involves detection of all thermotolerant *Campylobacter* by amplification of a fragment of the 23S rRNA gene involved in protein synthesis. Discrimination to the species levels of *C. jejuni* targets the *lpxA* gene that encodes the enzyme acyltransferase. This enzyme catalyzes the initial biosynthesis of lipid A, an essential molecule in lipopolysaccharides (LPS) which make up the outer membrane of gram-negative bacteria. Discrimination to the species level of *C. coli* targets the *ceuE* or *Campylobacter* enterochelin uptake gene that encodes the lipoprotein

component of a protein-binding transport system involved in iron acquisition (Institute of Environmental Science and Research Ltd).

To design the primers, sequences for each gene were downloaded from EMBL and Genbank databases for all available *Campylobacter* species and sequences were aligned using Megalign from DNAstar and MacMolly from Soft gene Gmbh version 1.2.1. Primer Express (PE Biosystems) was used to design the primers, using the following criteria (Institute of Environmental Science and Research Ltd):

> Melting temperature (T_m) near 60 C to increase specificity of DNA binding and to reduce the likelihood of mismatches
>
> G-C content within 20 – 50%
>
> Few runs of an identical nucleotide to prevent primer-dimer formation
>
> Variation in amplicon lengths to provide good visual separation between products when run on an electrophoresis gel

Primer sequences were run on the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/) to determine if the chosen sequences aligned with other non-target organisms. BLAST uses statistical theory to produce a bit score and an expect value (E-value) for every alignment. These numbers allow one to determine if similarity observed is due to a possible biological relationship or if it is attributable to chance alone. Bit scores indicate how good an alignment is and they are normalized so different alignments can be compared. In general, the higher the bit score, the better the alignment. The E-value indicates the statistical significance of an alignment and is a reflection of the database size and scoring system used. In general, the lower the E-value, the more significant the alignment (Information). As the BLAST database is continually updated, a

recent query of the primers (see figure 3.1) found that each of the primers still significantly aligns with the target organisms.

The multiplex PCR method was originally described in a study that examined the presence of *Campylobacter* in 50 retail packs of fresh chilled poultry and offal packs purchased in supermarkets in the Christchurch area. Each package was rinsed with a recovery diluent for two minutes. One portion of the rinse was used to set up a 3 number MPN in m-Exeter broth, while another portion was aseptically filtered and the filter then immersed into m-Exeter broth. Following microaerophilic incubation at 37 C for 4 hours and 42 C for 44 hours, filter enrichment broth and MPN tubes were streaked onto m-Exeter agar and incubated microaerophilically at 42 C for 48 hours. Colonies were again sub-cultured into m-Exeter broth and incubated at 42 C for 24 hours. Cells within the broth were submitted to several washing steps and DNA was released by heat-treatment at 100 C for 12 minutes. Following centrifugation to pellet cell debris, the supernatant was then subjected to the multiplex PCR reaction (described below).

The presence of thermotolerant *Campylobacter* in a sample was determined by the visualization of a "Therm" amplicon (246 bp) on an agarose gel. To confirm the presence of *C. jejuni* in a sample, both an "LpxA" amplicon (99 bp) and a Therm amplicon (246 bp) had to be visualized on an agarose gel. To confirm the presence of *C. coli* in a sample, both a "Ceu" amplicon (695 bp) and a Therm amplicon (246 bp) had to be visualized on an agarose gel. All enrichment samples tested positive for *C. jejuni* by PCR and by conventional phenotypic methods; no *C. coli* was detected by PCR or conventional methods. The sensitivity of the PCR assay was determined to be the simultaneous detection of 57 cells of *C. jejuni* and 69 cells of *C. coli*. DNA was extracted using phenol/chloroform and using 1 µl

C. jejuni standard (100 ng/µl) and 1 µl *C. coli* standard (100 ng/µl) in a total reaction volume of 50 µl.

C. lari PCR

The *C. lari* PCR used in this study was described by Eyers et al (Eyers, Chapelle et al. 1993; Eyers, Chapelle et al. 1994). Nucleotide sequences of the 23S rRNA fragments between helices 43 and 69 are between 875 and 975 base pairs in size and represent one of the most variable regions of the 23S rRNA. The 23S rRNA sequence data of 23 *Campylobacter* strains were aligned using the Dedicated Comparative Sequence Editor (DCSE) computer program. From this alignment, the researchers developed five primer pairs, one for the selective detection of thermotolerant *Campylobacter* species, and the four others for discrimination between *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis*. As the BLAST database is continually updated, a recent query of the thermotolerant *Campylobacter* spp. and *C. lari* primers (see figure 3.2) found that each of the primers still significantly aligns with the target organisms.

The PCR reactions were carried out successfully on both bacterial reference strains and a large number of clinical diarrheic stool specimens from the St. Pieters University Hospital in Belgium. Primers Therm 1 and Therm 2 amplified thermotolerant *Campylobacter* spp. and their presence was detected by visualization of a 290 bp amplicon on an agarose gel. All non-thermotolerant *Campylobacter* strains, various non-*Campylobacter* strains, and negative controls failed to produce an amplification product. Similarly, primers Therm 1 and Lari amplified *C. lari* strains and their presence was detected by visualization of a 260 bp amplicon on an agarose gel. Again, all non-*C. lari* strains and negative controls failed to

produce an amplification product. The sensitivity of the PCR assays was determined to be detection of approximately 12 cells (0.062 pg of DNA). The DNA sample volume and total volume of the reaction mixture were not described in publication (Eyers, Chapelle et al. 1993).

Fermer PCR

The Fermer PCR used in this study is part of a larger study that describes a sensitive and simple method for both the detection and differentiation of thermotolerant *Campylobacter* spp. Detection is achieved using Eyers' Therm 1 primer and a newly developed reverse primer named Therm 4. For species differentiation, the researchers used two restriction enzymes to digest the PCR product, resulting in specific restriction fragments for *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* (Fermer and Engvall 1999). A recent query of the Therm 4 primer in the BLAST database found that it still significantly aligns with the target organisms (see figure 3.3).

The PCR reaction was carried out successfully on bacterial reference strains and a large number of both human and veterinary isolates. Primers Therm 1 and Therm 4 amplified thermotolerant *Campylobacter* spp. and their presence was detected by visualization of a 491 bp amplicon on an agarose gel. The primer pair demonstrated high specificity, producing a PCR product of the correct size for all thermotolerant *Campylobacter* spp., but not for non-thermotolerant *Campylobacter* spp. or other bacteria. The sensitivity of the PCR assay was determined to be detection of as few as one to five cells or chromosomes. Again, DNA sample volume and total volume of the reaction mixtures were not described in publication. Authors note that the detection of such a low number of cells is probably enhanced by the

occurrence of three copies of the 23S rRNA genes on the *Campylobacter* chromosome (Fermer and Engvall 1999).

Linton PCR

Linton et al. (Linton, Owen et al. 1996) compared 16S rRNA sequence data for 33 species compromising the subdivision of Proteobacteria containing the *Campylobacter*, *Helicobacter*, *Arcobacter*, and *Wolinella* genera. Regions were identified which were conserved between all *Campylobacter* species, but differed from other genera. Two of these regions occurred between nucleotides 412 – 430 and nucleotides 1128 – 1211 and these sequences were used to design a primer pair specific for the *Campylobacter* genus. Primer pairs for five *Campylobacter* species important in human and veterinary medicine were also designed. As the BLAST database is continually updated, a recent query of the *Campylobacter* genus primers (see figure 3.4) found that each of the primers still significantly align with the target organisms.

The *Campylobacter* genus primers were successfully tested against a full range of strains and amplified an 816-bp amplicon solely from defined *Campylobacter* species, a urease-positive thermotolerant campylobacter (UPTC) strain, and *[B.] ureolyticus*. The latter is one of two *Bacteroides* strains recently reclassified as *Campylobacter*, although its proteolytic metabolism and fatty acid composition deviates from the rest of the genus. The authors suggest that this assay is useful for initial phylogenetic assignment of previously undescribed campylobacter-like organisms (CLOs), since their morphology and non-fermentative metabolism do not usually provide adequate grounds for phenotypic assignment. The sensitivity of the *Campylobacter* genus primers was not provided.

Objectives

The objective of this aspect of the overall study was to use molecular-based genotypic methods to confirm the presence of and differentiate between thermotolerant *Campylobacter* spp. in presumptive culture-positive cell suspensions isolated from seagull feces, Talbert Marsh water samples, and other solid samples from Talbert Marsh and subsequently stored in TSB with 25% glycerol (v/v) at -80 C.

Approaches

As described in Chapter II and below, seagull feces samples, Talbert Marsh water samples, and other solid samples from Talbert Marsh were enriched and subcultured for isolation of presumptive culture-positive *Campylobacter*. Colonies presumed to be *Campylobacter* were gently scraped from agar plates, suspended in TSB with 25% glycerol and stored at -80 C. Suspensions were regrown for subsequent biochemical testing, when possible. In an attempt to confirm stored cell suspensions as thermotolerant *Campylobacter* spp., isolates were subjected to a multiplex PCR method developed in New Zealand for the detection of thermotolerant *Campylobacter* and for the differentiation between *C. jejuni* and *C. coli* in enriched samples. A sensitivity assay was performed to determine the efficiency of this multiplex method when performed on isolates also stored as described below. Finally, three other PCR methods and nucleotide sequencing were also performed on a subset of the isolates for further characterization.

Methods

Bacterial strains

Presumptive culture-positive *Campylobacter* isolates were isolated from 148 seagull fecal samples from Talbert Marsh sand flats, ten seagull fecal samples from Newport Beach, ten Talbert Marsh sand samples, and 24 estuarine waters surrounding the Talbert Marsh sandflats where the seagulls take daily refuge (see Table 3.1 for details). As described in Chapter II, approximately 15 ml of pre-weighed seagull feces (or sand) homogenized in Dulbecco's PBS and one liter of water samples were submitted to *Campylobacter* enrichment in a 3-dilution and 3-replicates-per-dilution (3X3) MPN format. Enrichment was achieved with microaerophilic incubation in Preston's broth at 37^{o}C for 4 hours, then 42^{o}C for 44 hours. Enrichments were then subcultured in triplicate onto Campy blood agar and incubated under microaerophilic conditions at 42 C for 48 hours. Colonies that were small, round, whitish, and encircled with a thin, clear halo were considered presumptive culture-positive thermotolerant campylobacters. Occasionally, other colonies not specifically fitting this description were also considered presumptive culture-positive thermotolerant campylobacters.

After subculturing in triplicate, cultures were considered to be pure, and a number of colonies presumed to be *Campylobacter* were gently scraped from agar plates. Heavy inoculations were suspended in approximately 200 µl of TSB with 25% glycerol (v/v) and isolates were stored at -80 C. A number of cell suspensions were thawed and cells were initially able to be regrown on Campy blood agar for subsequent biochemical testing (oxidase and catalase) and were viewed by microscopy under 100X magnification following

Gram staining. Subsequent attempts to resuscitate freeze-thawed cells using both general and selective enrichments and subculturing onto Campy blood agar proved unsuccessful.

Preparation of DNA for PCR Analyses

As described in Chapter II, microscopy techniques employed on a representative subset of stored cell suspensions found that a fraction of cells within the stored cell suspensions were viable despite freeze-thaw events and cell counts ranged from 1.87 x 10^5 cells/µl to 1.17 x 10^6 cells/µl, with an arithmetic mean of 7.47 x 10^5 cells/µl. Therefore, an average of 7.47 x 10^3 cells/µl can be estimated within each 1:10 cell suspension dilution. Whether 2, 2.5, or 4 µl volumes of diluted cell suspensions (see below) were used per PCR reaction, it can be assumed that at least some viable cells were present, despite freeze-thaw events.

Initially, different volumes and dilutions of a number of thawed presumptive culture-positive *Campylobacter* cell suspensions were tested with the multiplex PCR and *C. lari* PCR assays to assess which combinations produced the best results. For each of the PCR analyses performed on the isolates (described below), cells suspensions were thawed and ten microliters of cell suspension were diluted 1:10 in plain phosphate buffered saline (PBS). Bacterial DNA was released by incubation at 99^{o}C for 12 minutes. Dilutions were then centrifuged at 12,000 x g for 10 minutes at 4^{o}C to pellet cell debris, leaving released DNA in the supernatant. The extraction protocol outlined by Wong et al. (Wong, Devane et al. 2004) was explicitly used; no other types of DNA extraction techniques were employed on cell suspensions.

Multiplex PCR

A modified version of the multiplex PCR assay described by Wong et al. (Wong, Devane et al. 2004) was used for thermotolerant *Campylobacter* detection and speciation. The multiplex assay consisted of six primer pairs targeting portions of the 23S ribosomal RNA (rRNA): one set targeted a portion specific to thermotolerant *Campylobacter* species, while the other two sets were species-specific and targeted the *lpxA* gene of *C. jejuni* and the *ceuE* gene of *C. coli*. The primers were (all sequences are 5' – 3'): Therm 1M Forward AAA TTG GTT AAT ATT CCA ATA CCA ACA TTA G and Therm 2M Reverse GGT TTA CGG TAC GGG CAA CAT TAG for the detection of thermotolerant *Campylobacter* species; LpxA Forward CCG AGC TTA AAG CTA TGA TAG TGG AT and LpxA Reverse TCT ACT ACA ACA TCG TCA CCA AGT TGT for the detection of *C. jejuni*; and CeuE Forward CAT GCC CTA AGA CTT AAC GAT AAA GTT and CeuE Reverse GAT TCT AAG CCA TTG CCA CTT GCT AG for the detection of *C. coli*. Primers were purchased from Invitrogen (Carlsbad, CA, USA).

A heavy inoculation of presumptive culture-positive *Campylobacter* colonies were stored at -80 C in approximately 200 µl of TSB/25% glycerol. Ten microliters of the cell suspension were diluted 1:10 in plain phosphate buffered saline (PBS) and bacterial DNA was released by incubation at 99^{o}C for 12 minutes. Dilutions were then centrifuged at 12,000 x g for 10 minutes at 4^{o}C to pellet cell debris, leaving released DNA in the supernatant. The multiplex PCR was performed on 2 µl aliquots of this material in a 50 µl reaction containing 1X PCR buffer (0.050 mol/l KCl, 0.010 mol/l Tris, pH 8.3) (Applied Biosystems), 0.1 pmol/µl each of primers LpxA forward and reverse, 0.1 pmol/µl each of Therm 1M forward and Therm 2M reverse, 0.2 pmol/µl each of CeuE forward and reverse,

0.025 U/µl AmpliTaq (Applied Biosystems), 0.25 mM each dNTP (Invitrogen), 4 mM $MgCl_2$, and 0.2 mg/ml bovine serum albumin (BSA). For each PCR run, positive (2 µl mixture of *C. jejuni* NCTC 11351 DNA and *C. coli* NCTC 11366 DNA) and negative (2 µl of molecular biology grade water) controls were included.

Thermal cycling conditions for the MJ Research Gradient Thermal Cycler were: an initial denaturation at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 74°C for 1 minute, with a final extension at 74°C for 8 minutes. PCR products were visualized by agarose gel electrophoresis stained with ethidium bromide. *C. jejuni* isolates were taken to be samples that contained an LpxA amplicon (99 bp) and a thermotolerant *Campylobacter* amplicon (246 bp). *C. coli* isolates were taken to be samples that contained a CeuE (695 bp) amplicon and a thermotolerant *Campylobacter* amplicon (246 bp). Thermotolerant *Campylobacter* species other than *C. jejuni* and *C. coli* were taken to be those that contained a thermotolerent *Campylobacter* amplicon (246 bp) only.

Multiplex PCR Sensitivity Assay

An analysis was performed to determine the sensitivity of the multiplex assay on colonies stored in TSB/25% glycerol. Pure strains of *C. jejuni doyleii*, *C. coli*, and *C. lari* (provided by the CDC) were each grown on Campy blood agar plates. Because cultures were considered pure, a number of colonies of each strain were gently scraped from agar plates and added to separate tubes containing 1.25 ml of TSB/25% glycerol to serve as the undiluted sample. Suspensions were then diluted in TSB/25% glycerol up to 10^{-8}. One hundred microliters of suspensions (undilute through 10^{-6}) were spread in duplicate on

Campy blood agar plates. Undiluted suspensions of strains of *C. upsaliensis* and *Campylobacter* UPTC (also provided by the CDC) in TSB/25% glycerol were also included. Plates were incubated under microaerophilic conditions at 37^{o}C for 4 hours, then at 42 C for 44 hours and average CFU for each dilution were noted. Colony counts of the countable serial dilutions were used to estimate concentrations of culturable cells, allowing cell concentrations for each dilution subjected to the multiplex PCR to be calculated. The number of cells within the highest dilution that produced a PCR product could then be determined.

As was done with the experimental presumptive culture-positive *Campylobacter* isolates stored in TSB/25% glycerol, the undiluted suspensions of each of the three positive control *Campylobacter* strains described above and each dilution (10^{-1} through 10^{-8}) of each strain were diluted 1:10 in PBS (10 µl into 90 µl PBS). These dilutions, along with TSB/25% glycerol-only negative controls, were heat shocked at 99^{o}C for 10 minutes and iced for two minutes prior to PCR analysis. The multiplex assay was performed on all dilutions and two positive control DNA suspensions (*C. jejuni* and *C. coli* strains provided by ESR, Christchurch NZ), as described above. PCR products were visualized by agarose gel electrophoresis stained with ethidium bromide. *C. jejuni* isolates contained an LpxA amplicon (99 bp) and a thermotolerant *Campylobacter* amplicon (246 bp), while *C. coli* isolates contained a CeuE amplicon (695 bp) and a thermotolerant *Campylobacter* amplicon (246 bp). *C. lari* isolates contained only a thermotolerant *Campylobacter* amplicon (246 bp). The sensitivity of the multiplex assay when performed on cells stored in TSB with 25% glycerol could be determined based on stock concentration and which serial dilutions of material harvested from colonies did or did not produce PCR products.

***C. lari* PCR**

A modified version of a PCR assay described by Eyers et al (Eyers, Chapelle et al. 1993; Eyers, Chapelle et al. 1994) was used for *Campylobacter lari* detection on isolates producing only a thermotolerant *Campylobacter* product when assayed using the multiplex PCR. The *C. lari* PCR assay consisted of three primers targeting portions of the 23S rRNA: two primers targeted a portion specific to thermotolerant *Campylobacter* species, while the third primer, along with one of the thermotolerant *Campylobacter* species primers, was species-specific and targeted a portion of the 23S rRNA specific to *Campylobacter lari*. The primers were (all sequences are 5' – 3'): Therm 1 Forward TAT TCC AAT ACC AAC ATT AGT; THERM 2 Reverse CGG TAC GGG CAA CAT TAG; and Lari Reverse ACG GCA TCA GCA ATT CTC. Primers were purchased from Invitrogen (Carlsbad, CA, USA).

As described above, a heavy inoculation of presumptive culture-positive *Campylobacter* colonies were stored at -80 C in approximately 200 µl of TSB/25% glycerol. Ten microliters of the cell suspension were diluted 1:10 in plain phosphate buffered saline (PBS) and bacterial DNA was released by incubation at 99^{o}C for 12 minutes. Dilutions were then centrifuged at 12,000 x g for 10 minutes at 4^{o}C to pellet cell debris, leaving released DNA in the supernatant. The *C. lari* PCR was performed on 4 µl aliquots of this material in a 50 µl reaction containing 1X PCR buffer (0.050 mol/l KCl, 0.010 mol/l Tris, pH 8.3) (Applied Biosystems), 0.1 pmol/µl of Therm 2 forward, 0.05 pmol/µl of Therm 2M reverse, 0.05 pmol/µl of Lari reverse, 0.025 U/µl AmpliTaq (Applied Biosystems), 0.2 mM each dNTP (Invitrogen), 2 mM $MgCl_2$, and 0.2 mg/ml bovine serum albumin (BSA). For each PCR run,

positive (4 µl of *C. lari* DNA) and negative (4 µl of molecular biology grade water) controls were included.

Thermal cycling conditions for the MJ Research Gradient Thermal Cycler were: an initial denaturation at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, and extension at 74°C for 1 minute, with a final extension at 74°C for 8 minutes. PCR products were visualized by agarose gel electrophoresis stained with ethidium bromide. *C. lari* isolates were taken to be samples that contained a 177-bp amplicon (Lari) and a 222-bp amplicon (thermotolerant *Campylobacter*).

Fermer PCR and Sequencing

A PCR assay described by Fermer et al (Fermer and Engvall 1999) was used to amplify a portion of the 23S rRNA gene of a subset of isolates only producing a thermotolerant *Campylobacter* product using both the multiplex PCR and the *C. lari* PCR. The Fermer PCR uses a primer pair, Therm 1 (developed by Eyers et al. (Eyers, Chapelle et al. 1993; Eyers, Chapelle et al. 1994)) and Therm 4, to amplify a 491 bp region of the 23S rRNA gene specific only to thermotolerant *Campylobacter* species. This primer pair is highly-specific and produces a product size that is conducive for sequence comparison of isolates. The Therm 4 primer is (5' to 3'): CTT CGC TAA TGC TAA CCC.

As described above, a heavy inoculation of presumptive culture-positive *Campylobacter* colonies were stored at -80 C in approximately 200 µl of TSB/25% glycerol. Ten microliters of the cell suspension were diluted 1:10 in plain phosphate buffered saline (PBS) and bacterial DNA was released by incubation at 99°C for 12 minutes. Dilutions were then centrifuged at 12,000 x g for 10 minutes at 4°C to pellet cell debris, leaving released DNA in

the supernatant. The Fermer PCR was performed on 2 µl aliquots of this material in a 50 µl reaction containing 1X PCR buffer (0.050 mol/l KCl, 0.010 mol/l Tris, pH 8.3) (Applied Biosystems), 0.05 pmol/µl of Therm 1 forward, 0.05 pmol/µl of Therm 4 reverse, 0.025 U/µl AmpliTaq (Applied Biosystems), 0.2 mM each dNTP (Invitrogen), 2 mM $MgCl_2$, and 0.2 mg/ml bovine serum albumin (BSA). For each PCR run, positive (2 µl mixture of *C. jejuni* NCTC 11351 DNA and *C. coli* NCTC 11366 DNA) and negative (2 µl of molecular biology grade water) controls were included.

Thermal cycling conditions for the MJ Research Gradient Thermal Cycler were: an initial denaturation at 94°C for 3 minutes, followed by 45 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, and extension at 72°C for 1 minute, with a final extension at 72°C for 5 minutes. PCR products were visualized by agarose gel electrophoresis stained with ethidium bromide. Thermotolerant *Campylobacter* isolates contained a 491-bp amplicon.

Products from the Fermer PCR amplified reaction mixture were purified using a QIA-quick PCR purification kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions Purified products were re-run on a 2% agarose gel to verify band intensity was sufficient for sequencing. Twenty microliters of each of the purified products and 50 µl of 3.2 pmol/µl Therm 1 primer stock were sent to another facility for sequencing using a BigDye Terminator Cycle Sequencing Ready Reaction kit. Negative controls were run in parallel with samples. Sequences received were then edited, entered into the BLAST database, and top hit sequences were downloaded. Multiple sequence alignments were generated using Clustal W 1.4 software and imported into TreeCon (v 1.3b). A phylogenetic tree was drawn using a neighbor-joining method and the confidence values of the internal

nodes were calculated by performing 100 bootstrap analyses. Distances between clusters were determined using pair-wise alignment analysis in the BioEdit Sequence Alignment Editor program (v 7.0.5.2).

Linton PCR

As suggested by the Chief of the CDC's National *Campylobacter* and *Helicobacter* Reference Laboratory (Fitzgerald 2005), *Campylobacter* genus PCR assay designed by Linton et al. (Linton, Owen et al. 1996) was performed on a subset of isolates to determine if they were indeed *Campylobacter* spp. not detected by the other PCR methods. The assay was performed on isolates from both Talbert Marsh seagull fecal samples and Talbert Marsh water samples that were negative for all other PCR assays. As an internal control, the assay was also performed on isolates that were PCR-positive using the multiplex and/or *C. lari* PCR assays. Positive controls included DNA for *C. jejuni doyleii*, *C. coli*, *C. lari*, *C. upsaliensis*, and a UPTC strain of *Campylobacter* (provided by the CDC). The PCR primer pair was designed using 16S rRNA gene sequences specific for the *Campylobacter* genus. The primers are (all sequences are 5' – 3'): C412 Forward GGA TGA CAC TTT TCG GAG C and C1288 Reverse CAT TGT AGC ACG TGT GTC (provided by the CDC).

Cells stored in TSB/25% glycerol and 1:10 dilutions of these suspensions in phosphate buffered saline (PBS) were incubated at 99 C for 12 minutes to release bacterial DNA. A modified version of the Linton PCR was performed on 2.5 µl aliquots of both undiluted and diluted material in a 25 µl reaction using 2X HotStarTaq mastermix (HotStarTaq DNA polymerase, PCR Buffer with 3mM $MgCl_2$, and 400 µM each dNTP) (QIAGEN, Valencia, CA) and 0.6 µM of each C412F and C1288R primers. Two positive controls (2.5 µl of *C.*

jejuni DNA and 2.5 µl *C. coli* DNA; strains provided by ESR, Christchurch NZ) and a negative control (2.5 µl of molecular biology grade water) were included.

Thermal cycling conditions for the MJ Research Gradient Thermal Cycler were: heat activation of the *Taq* polymerase at 95°C for 15 minutes, followed by 40 cycles of denaturation at 94°C for 1 min, 55°C annealing for 1 min, 72°C extension for 1 min, with a final 5 min extension step at 72°C. PCR products were visualized by agarose gel electrophoresis stained with ethidium bromide. Positive *Campylobacter* genus isolates contained an 816-bp amplicon.

Results

Multiplex PCR

As described in Chapter II and above, following initial *Campylobacter*-specific enrichment of seagull feces, Talbert Marsh water samples, and other solid samples, colony growth presumed to be of the genus *Campylobacter* was archived. All isolates were archived at -80 C in TSB with 25% glycerol (v/v) for at least one year and archived isolates were regrown, when possible, for biochemical testing. Isolates experienced a number of freeze-thaw events prior to molecular analysis. Stored cell suspensions themselves were subjected to a multiplex PCR method to confirm isolates as thermotolerant *Campylobacter* spp. and to differentiate between species. Below, multiplex PCR results obtained from each of the samples types are described separately.

Seagull Fecal Samples

Table 3.2 describes multiplex PCR results obtained from analysis of Talbert Marsh seagull feces, including numbers of samples and isolates analyzed, numbers of PCR positive samples and isolates, and outcomes of the multiplex PCR analysis. As described in Chapter II, 148 seagull fecal samples were homogenized in Dulbecco's PBS and 15 ml of fecal homogenate were subjected to *Campylobacter*-specific enrichment and subculture analyses. Eighty-four separate Talbert Marsh seagull fecal samples produced colonies presumed to be *Campylobacter*. Of these, a total of 195 isolates were archived from the 46 Talbert Marsh seagull fecal samples that were considered presumptive culture-positive for thermotolerant *Campylobacter* following regrowth, biochemical testing, and Gram-staining. Fifteen of these isolates (7.7%), representing five samples, produced amplicons when subjected to the multiplex PCR. Fourteen of these isolates (93.3%), representing four samples, produced only a thermotolerant *Campylobacter* amplicon, while one isolate (6.7%), representing one sample, produced both a thermotolerant *Campylobacter* amplicon and an LpxA amplicon, indicating the presence of *C. jejuni*. One-hundred sixty-nine isolates, representing 35 seagull fecal samples, that were considered presumptive culture-positive for thermotolerant *Campylobacter* following regrowth, biochemical testing, and Gram-staining, did not produce amplicons when subjected to the multiplex PCR. Eleven additional isolates, representing six samples, were unable to be located post-isolation and therefore were not submitted to the multiplex PCR (these are omitted from Table 3.2).

Thirty-eight additional samples produced colonies following enrichment, but not all isolates within each sample were able to be regrown for biochemical testing and Gram-staining. Of the 184 isolates archived from these 38 samples, 87 isolates (47.3%), representing 23 samples, produced amplicons when subjected to the multiplex PCR. Forty-

seven of these isolates (53.4%), representing 13 samples, produced only a thermotolerant *Campylobacter* amplicon, while 40 of these isolates (21.6%), representing ten samples, produced both a thermotolerant *Campylobacter* amplicon and an LpxA amplicon, indicating the presence of *C. jejuni*. One sample (J4) contained two different types of isolates; one isolate produced just a thermotolerant *Campylobacter* amplicon, while three others produced both a thermotolerant *Campylobacter* amplicon and an LpxA amplicon. Additionally, an isolate from sample J7 produced only an LpxA amplicon, suggesting the presence of a unique *C. jejuni* isolate. Ninety-seven isolates, representing 15 samples, did not produce any amplicons when subjected to the multiplex PCR. None of the isolates produced both a thermotolerant *Campylobacter* amplicon and a CeuE amplicon, which indicates the absence of *C. coli*.

Additionally, Table 3.2 describes multiplex PCR results obtained from analysis of each of the Newport Beach seagull fecal samples, including numbers of samples and isolates analyzed, numbers of PCR positive samples and isolates, and outcomes of the multiplex PCR analysis. As described in Chapter II, ten seagull fecal samples were homogenized in Dulbecco's PBS and 15 ml of fecal homogenate were subjected to *Campylobacter*-specific enrichment and subculture analyses. Two separate Newport Beach seagull fecal samples produced colonies presumed to be *Campylobacter*. A total of seven isolates were archived from these samples and all seven produced both a thermotolerant *Campylobacter* amplicon and an LpxA amplicon, indicating the presence of *C. jejuni*.

Talbert Marsh Water Samples

Table 3.2 describes multiplex PCR results obtained from analysis of Talbert Marsh water samples, including numbers of samples and isolates analyzed, numbers of PCR positive samples and isolates, and outcomes of the multiplex PCR analysis. As described in Chapter II, 24 one-liter volumes were subjected to *Campylobacter*-specific enrichment and subculture analyses. Fifteen separate isolates were archived from the seven Talbert Marsh water samples that were considered presumptive positive for thermotolerant *Campylobacter* by biochemical testing and Gram staining. None of these isolates produced any amplicons when subjected to the multiplex PCR.

Sand Samples

Table 3.2 describes multiplex PCR results obtained from analysis of Talbert Marsh sand samples, including numbers of samples and isolates analyzed, numbers of PCR positive samples and isolates, and outcomes of the multiplex PCR analysis. As described in Chapter II, ten sand samples were homogenized in Dulbecco's PBS and 15 ml of sand homogenate were subjected to *Campylobacter*-specific enrichment and subculture analyses. Three separate sand samples produced colonies presumed to be *Campylobacter*. A total of three isolates were archived from these samples and none of these isolates produced amplicons when subjected to the multiplex PCR.

Sensitivity of Multiplex PCR

The multiplex PCR assay performed on cells stored in TSB with 25% glycerol detected down to an equivalent of 20 CFU of *C. jejuni*, two CFU of *C. coli*, and two CFU of *C. lari*, when tested individually. These numbers are likely high estimates of the sensitivity of the

assay, as *Campylobacter* spp. contain three copies of the 23S rDNA per chromosome (Eyers, Chapelle et al. 1993). The sensitivity of the assay as performed on these isolates is better than that that of the published method, which reported simultaneous detection of 57 cells of *C. jejuni* and 69 cells of *C. coli*. This is likely explained by the fact that the published sensitivity was determined for the simultaneous detection of *C. jejuni* and *C. coli*, not for each individually. *C. upsaliensis* and UPTC strains stored in TSB with 25% glycerol were also able to be detected by the method, although the sensitivities for these strains were not determined.

C. lari PCR

Table 3.3 describes *C. lari* PCR results obtained from analysis of each of the positive multiplex PCR isolates, including numbers of samples and isolates analyzed, numbers of PCR positive samples and isolates, and outcomes of the PCR. The multiplex-positive isolates producing only a thermotolerant *Campylobacter* amplicon all originated from Talbert Marsh seagull fecal samples. When subjected to the *C. lari* PCR, 44/61 isolates (72.1%), representing 14 separate seagull fecal samples, produced both a thermotolerant *Campylobacter* amplicon and a Lari amplicon. The remaining 17 isolates (27.9%), representing 4 separate fecal specimens and which produced only a thermotolerant *Campylobacter* amplicon when subjected to multiplex PCR, again produced only a thermotolerant *Campylobacter* amplicon when subjected to the *C. lari* PCR. In total, 44/109 (40.3%) of isolates confirmed as positive campylobacters by molecular methods were identified as *C. lari*. No Talbert Marsh water samples, sand samples, or Newport Beach seagull fecal samples were determined to be *C. lari*.

Fermer PCR and Sequencing

Each of the isolates subjected to Fermer PCR and sequencing originated from Talbert Marsh seagull fecal samples; no Talbert Marsh water samples, Talbert Marsh sand samples, nor Newport Beach seagull fecal samples were able to be studied in this manner, as none produced only a thermotolerant *Campylobacter* band when subjected to the multiplex PCR. Of the subset of multiplex isolates producing only a thermotolerant *Campylobacter* amplicon when subjected to both multiplex PCR and *C. lari* PCR, 10/10 (100%) produced a thermotolerant *Campylobacter* amplicon when subjected to the Fermer PCR. The PCR amplified reaction mixtures were then purified and sequenced. Sequences were edited, entered into the BLAST database, and top hit sequences (see Table 3.4) were downloaded for comparison. Phylogenetic tree analysis, as shown in Figure 3.4, reveals that the isolates separate into five distinct clusters.

One isolate from bird feces sample P2 clustered most closely with sequence data from two *C. jejuni* sequences published in the NCBI database. *C. jejuni* TGH9011 is sequence data from *C. jejuni* ATCC 43431 and was described in a study that analyzed the organization of the rrnA operon (Kim, Gutell et al. 1995). *C. jejuni* 23S is sequence data from an non-described source in a study that amplified and sequenced variable regions in rRNA genes with conserved primers (Van Camp, Chapelle et al. 1993). Because the P2 isolate differed the most from the other seagull feces isolates analyzed, it was used as the root sequence with which to compare the remaining nine isolates.

Three Talbert Marsh seagull feces isolates, from three separate samples, clustered closely with two different DNA sequences from campylobacters isolated from river water (Linton,

Hurtado et al. 1999). Isolates T6 and O8 were highly similar in sequence to a portion of a *C. lari* 23S rRNA published sequence, while isolate J4 was highly similar to a sequence identified as *Campylobacter* spp. 1888387. Both of the published strains were described in a study that analyzed *Campylobacter coli* strains with enlarged flagellin genes isolated from river water.

Isolate G4 showed high sequence similarity to a urease-positive thermophilic *Campylobacter* (UPTC) isolate (study unpublished). This group of campylobacters have been predominantly found in bathing waters of urban coastal beaches impacted by birds (Obiri-Danso, Paul et al. 2001).

Lastly, a group of five Talbert Marsh seagull feces isolates (two from sample Q7 and three from sample Q8), clustered closely with each other and closely to the two published *C. lari* sequences. Pairwise analysis confirmed what can be visually estimated from Figure 3.4: nucleotide sequences for *C. lari* 23S and Q8(1) are 4.4% different and nucleotide sequences for *C. lari* and Q8(1) are 6.8% different.

Linton PCR

The purpose of the Linton PCR was to assess the performance of the other PCR methods used and to determine if isolates were indeed not *Campylobacter* spp. Of the three isolates previously identified as *C. jejuni*, *C. lari*, or thermotolerant *Campylobacter* by the multiplex and/or *C. lari* PCR methods, all three produced a *Campylobacter* genus amplicon when subjected to the Linton PCR. None of the other seven other isolates, which were negative by all other PCR assays and included both Talbert Marsh seagull fecal samples and Talbert Marsh water samples, produced a *Campylobacter* genus amplicon. These results further

support the reliability of the multiplex and *C. lari* methods when applied to cell suspensions stored in TBS with 25% glycerol.

Discussion

Samples originally isolated from Talbert Marsh and Newport Beach seagull feces, Talbert Marsh water, and sand from Talbert Marsh were enriched and subcultured for isolation of presumptive culture-positive *Campylobacter* spp. Colonies presumed to be *Campylobacter* were suspended in TSB with 25% glycerol and stored at -80 C. Suspensions were regrown for subsequent biochemical testing, when possible. In an attempt to confirm stored cell suspensions as thermotolerant *Campylobacter* spp., isolates were subjected to a number of molecular-based genotypic methods for identification, differentiation, and for further isolate characterization. Overall, one-third of samples from seagull feces, water, and sand that produced colonies following *Campylobacter*-specific enrichment were able to be confirmed as positive campylobacters using a series of polymerase chain reaction methods developed for *Campylobacter* identification and speciation. Each of these positive isolates originated from seagull fecal samples.

In total, about 36% of the presumptive culture-positive *Campylobacter* isolates from Talbert Marsh seagull feces were confirmed as *Campylobacter* using molecular methods. Almost half of all isolates (representing 23 Talbert Marsh seagull fecal samples) that originally produced colonies following *Campylobacter*-specific enrichment, but were unable to be regrown for biochemical testing and Gram-staining, were confirmed as positive *Campylobacter* spp. using molecular methods. However, a smaller fraction of all isolates (7.0%, representing five Talbert Marsh seagull fecal specimens) that originally produced

colonies following *Campylobacter*-specific enrichment and were determined to be presumptive positive *Campylobacter* spp. following resuscitation, biochemical tests, and Gram-staining, were also confirmed as positive *Campylobacter* spp. by molecular methods. A majority of colony isolates from these enrichments were unable to be confirmed as *Campylobacter* spp. by molecular-based methods. No *C. coli* were detected in stored isolates, however both *C. jejuni* and *C. lari* were detected in seagull feces. These two species have been previously reported in seagulls (Benjamin, Leaper et al. 1983; Moore, Gilpin et al. 2002).

Although the percentage of PCR-confirmed positives was low, thermotolerant *Campylobacter* spp. were identified in a portion of stored isolates, indicating that the conventional microbiological methods were able to correctly detect *Campylobacter* in some environmental samples. However, these results support the idea that conventional microbiological methods used for the isolation of thermophilic *Campylobacter* spp. may also enrich other bacteria present in samples and therefore lead to false positive results when used alone. It is also likely that the Preston's enrichment broth did enrich *Campylobacter* spp. present in samples, but the subculture step onto agar plates enhanced the growth of other enriched bacteria and/or suppressed the growth of campylobacters present. Re-culturing and molecular-based identification of these contaminants are necessary to support this hypothesis. Eliminating the storage aspect of this study and performing the multiplex directly on cell washes of enriched cultures would probably have resulted in a greater likelihood of *Campylobacter* detection and provided greater confidence in determining the accurate identity of *Campylobacter* in seagull feces and water samples impacted by these feces.

Furthermore, results of this study are consistent with the well-known phenomenon of limited *Campylobacter* cell viability and recovery following freezing and thawing (Humphrey and Cruickshank 1985; Humphrey 1986; Chan, Le Tran et al. 2001). Formation of intracellular ice, cell dehydration, and oxidative stress may all be responsible for this lack of culturability. Another aspect in limited cell recovery is the possibility that *Campylobacter* cells enter a viable but non-culturable (VBNC) state due to nutrient deprivation in storage media, drastic temperature changes, and variation in osmotic conditions both intracellularly and extracellularly upon freezing and thawing. This has been documented in *Campylobacter* at various temperatures (Jones, Sutcliffe et al. 1991; Medema, Schets et al. 1992), although it has not been proven to occur at sub-zero temperatures in *Campylobacter* cells. The ability to re-culture *Campylobacter* cells from frozen suspensions for colony PCR or PCR on cell enrichments would have provided DNA in larger quantities and with potentially less damage, therefore likely increasing the number of PCR positive results.

There are a few possible reasons why the molecular method used, specifically the multiplex PCR, was unable to detect *Campylobacter* potentially present in the stored isolates. Initially, it was believed that inhibitors present in the storage media decreased the efficiency of the multiplex method. Large molecules such as peptones and glycerol molecules may have prevented primers from effectively reaching their targets on template DNA. This possibility was reduced in the unmodified, published multiplex method by employing a cell-washing step, but this step was eliminated in the current work due to low remaining volumes of stored isolates. The isolates in this work were diluted 1:10 in a neutral buffer solution prior to PCR, which likely reduced the amount of inhibitors present. Generally, frozen isolates are first regrown and colony PCR or PCR on enrichment culture is performed for

molecular characterization. In the case of the isolates in this study, bacterial isolates were unable to be resuscitated for this purpose. Limited data is available in the published literature that describes the effects of glycerol (i.e. present in storage media) on the performance of PCR methods.

It may be suggested that too few cells were present in the amount of diluted sample subjected to the PCR assays. However, previous quantitative microscopy analysis on these isolates suggested that average cell concentrations in the storage media were in the $10^5 – 10^6$ cells/µl range (see Chapter II). According to the method's published sensitivity and the sensitivity determined for isolates in the present study, 1:10 dilution of these stored isolates should have provided more than enough cells for positive detection. It is also likely that DNA present in stored isolates was damaged due to repeated freeze-thaws and/or exposure to oxygen radicals and was therefore less responsive to PCR amplification. Nevertheless, a number of isolates that underwent the same freeze-thaw conditions responded positively to the multiplex PCR.

Overall, the most likely possibility for the low percentage of confirmed *Campylobacter* positives is that the presumptive culture-positive isolates simply were not thermotolerant *Campylobacter*. This possibility is supported by the results of the Linton PCR, which is a robust method designed to detect all members of the thermotolerant *Campylobacter* group, including variants such as UPTC strains, that the other PCR methods used are unable to detect. The fact that this PCR method did not detect any members of the *Campylobacter* genus in seven suspensions previously non-responsive to molecular methods, but did detect *Campylobacter* species in three previously confirmed cell suspensions and in positive controls, further suggests that species of thermotolerant *Campylobacter* were not present in

the former stored suspensions. Other microbes, such as *Candida pseudotropicalis* and *Pseudomonas aeruginosa*, have been shown to grow in *Campylobacter* enrichment broths, such as Preston's, despite their selectivity (Martin, Mattick et al. 2002). To determine the identity of these potentially non-*Campylobacter* bacteria, further molecular testing, such as 16S rRNA amplification by generic primers followed by sequencing, is required.

The purpose of the Fermer PCR and subsequent sequencing was to further characterize a subset of the isolates that were non-responsive to the species-specific primers in the multiplex and *C. lari* PCR assays (i.e. isolates that only produced "thermotolerant *Campylobacter* " amplicons in each of the PCR assays). Sequencing and phylogenetic tree analyses indicate that the majority of these isolates (8/10 or 80%) were closely related to published *C. lari* sequences. Seven of these isolates were most closely related to DNA sequence from *C. lari* isolated from river water. The eighth isolate was most closely related to sequence from *C. lari* of an unpublished source. In addition, one of these isolates was found to be closely related to two published *C. jejuni* sequences, both of which were laboratory strains and one of which was isolated from human feces. Each of these ten sequenced isolates was identified as species that the PCR methods employed in this study were designed to detect. Therefore it can be suggested that the primers in both PCR assays were not broadly effective enough to amplify all strains in the collection of stored isolates.

Although seven Talbert Marsh water samples were originally considered presumptive positive by culture methods, biochemical tests, and Gram-staining, none of the stored isolates were confirmed as positive by molecular methods, including the multiplex PCR and the Linton PCR. It cannot be conclusively established whether the original bacterial growth from *Campylobacter*-specific enrichment of water samples was not *Campylobacter*, or it

truly was *Campylobacter* and isolates were unable to be confirmed. Possible reasons for lack of PCR detection could be DNA damage of positive samples, or an actual low percent positivity and apparently low concentration or absence of *Campylobacter* in samples. Not only were samples taken during a short period of time (three months), but only a limited number of samples were taken (n = 24) and volumes were small (1 L) in comparison to the volume of the marsh.

It is not surprising that no *Campylobacter* were confirmed from water samples from Talbert Marsh. If seagull feces were the only source of *Campylobacter* into the marsh, the low concentrations found in the feces (see Chapter II) may not have had sufficient impact on surrounding waters to yield *Campylobacter* isolates, especially when waters were sampled only a few times and in small volumes. Based on the median *Campylobacter* concentration of 4.9 MPN/g of Talbert Marsh seagull feces (see Chapter II), approximately 0.2 grams of seagull feces per one liter of water would be required to produce one colony-forming unit of *Campylobacter*. It is estimated that an average of about 7000 grams of seagull feces are deposited daily on the sandflats of Talbert Marsh (see Chapter II) and the average volume of water in Talbert Marsh is 120,000 L (Grant 2006). Therefore, a concentration of 0.06 grams of feces per liter of Talbert Marsh water can be very roughly estimated if all of the feces per day entered the water and it was static over a 24-hour period. Because this is not the case due to tidal exchange with the ocean, an even greater extent of dilution is expected. So, even if Talbert Marsh waters were exposed to high levels of fecal matter or high *Campylobacter* concentrations, the fact that the waters are not static and are diluted by incoming and outgoing tides would further reduce the chance of finding *Campylobacter* in 1-liter water samples. Based on the highest *Campylobacter* concentration found (~525 MPN/g of Talbert

Marsh seagull feces), approximately 0.002 grams of seagull feces per one liter of water would be required to produce one colony forming unit of *Campylobacter*. Hence, there is a possibility of finding *Campylobacter* in the water of Talbert Marsh, but it is slight.

Additionally, if detectable levels of *Campylobacter* had been initially present in these seagull feces and exposed waters, cells may have been damaged or quickly died off due to a number of environmental factors, including salinity, temperature, or UV exposure, making them undetectable even with attempted enrichment culture. Again, because these estuarine marsh waters are further diluted as they travel to the outlet of the marsh and mix with the surf zone waters at Huntington Beach, the chances of finding *Campylobacter* in small sample volumes are further reduced.

Due to all of the above factors contributing to the presence of only very low concentrations of *Campylobacter* in Talbert Marsh water samples, and the fact that a previous human feeding study showed approximately 400-500 *Campylobacter* cells are required for human infection (U.S. Food & Drug Administration Center for Food Safety & Applied Nutrition 2003), it is unlikely that *Campylobacter* from seagull feces in Talbert Marsh pose a significant health risk to swimmers in the ocean waters at Huntington Beach. However, more recently, the dose-response relation for *Campylobacter* has been reconsidered, as studies have found significantly fewer cells (as low as 15.2 CFU/L) may be responsible for human infection. Data from a human feeding study and outbreak data from two similar incidents found "increased infectivity at low doses and a steeper increase with dose than the one previously reported (Teunis, Van den Brandhof et al. 2005)." These findings indicate that the low concentrations of *Campylobacter* in Talbert Marsh water samples may be a greater

potential human health threat than previously believed, and this topic deserves further attention.

Although these data suggest only a low presence of thermotolerant *Campylobacter*, more effective recovery and detection methods may have resulted in higher numbers of positive samples. Additional work needs to incorporate larger numbers of water samples, larger water sample volumes, and samples collected throughout each season of the year, especially during periods when bird populations in Talbert Marsh are greatest. Furthermore, molecular methods could be improved upon by attempting different DNA extraction methods and different volumes of template submitted to the PCR assays. Sequencing of additional PCR-positive *Campylobacter* isolates from seagulls would provide more information on their genetic relatedness to isolates from other sources such as seagulls from different geographic locations, humans, and other animals. Due to the small amount of cell suspension remaining for each of the isolates in this study, further attempts such as these were limited.

Substantial research gaps in this field of study make comparisons to similar environments difficult and even impossible. A number of studies have assessed the presence of *Campylobacter* in seagull feces (Rosef 1981; Kapperud and Rosef 1983; Glunder and Petermann 1989; Quessy and Messier 1992; Palmgren, Sellin et al. 1997; Sixl, Karpiskova et al. 1997; Broman, Palmgren et al. 2002; Moore, Gilpin et al. 2002; Wahlstrom, Tysen et al. 2003). Carriage rates in these studies vary widely, with percent positive samples ranging from 13.7% (Moore, Gilpin et al. 2002) to 63.0% (Sixl, Karpiskova et al. 1997). In relation to recreational water, one study (Obiri-Danso, Paul et al. 2001) looked at environmental effects on *Campylobacter* survival in surface and found that *Campylobacter lari* and urease-positive thermophilic campylobacters (UPTC) from birds were the predominant

campylobacters in bathing waters of an urban coastal beach. While the above studies did find evidence of *Campylobacter* of seagull origin, none have specifically focused on assessing the microbial impact of seagulls on marine coastal water quality. Furthermore, differences in methodologies, especially in the use of culture-based versus molecular-based detection methods, hinder such comparisons between studies.

In conclusion, molecular methods provided a basis for definitive identification of culture-positive thermotolerant *Campylobacter* spp. in stored isolates from seagull feces of Talbert Marsh. An important outcome of this work is that the multiplex PCR method is somewhat effective on characterization of culture-positive *Campylobacter* isolates that have been stored at -80 C temperatures. The results of this study limit the reliability of the percent-positivity and concentrations of presumptive culture-positive *Campylobacter* in samples as reported in Chapter II and therefore draw into question the sole use of culture-based methods for the analysis of environmental samples for the presence of *Campylobacter* spp. However, molecular-based methods employed in this study also have drawbacks. Primers in the PCR methods used were not robust enough to detect all strains of species present, as ten sequenced isolates were identified as species that the PCR methods were designed, but unable, to detect. Although thermotolerant *Campylobacter* spp. were positively identified in stored Talbert Marsh seagull feces cell suspensions, lack of positive molecular data for *Campylobacter* in Talbert Marsh water samples is a drawback to understanding the impact that seagull feces from the sandflats of Talbert Marsh may have on surrounding estuarine waters. We initially set out to determine if there was a relationship between *Campylobacter* in seagull feces and surrounding estuarine waters using molecular methods, but were unable to document or prove such a relationship. Subsequent resuscitation attempts for most of the stored

presumptive culture-positive *Campylobacter* isolates were unsuccessful, eliminating the possibility of other types of analyses to compare these isolates. Further work should focus on more sampling, larger sample volumes, the elimination of the storage element, and the optimization of the molecular-based methods to provide the most accurate representation of the impact seagull feces have on Talbert Marsh estuarine waters.

Therm 1M: AAA TTG GTT AAT ATT CCA ATA CCA ACA TTA G

Sequences producing significant alignments:	Score (Bits)	E Value
gi|57165696|gb|CP000025.1| Campylobacter jejuni RM1221, complete	61.9	5e-08
gi|6968128|emb|AL139076.2|CJ11168X3 Campylobacter jejuni subs...	61.9	5e-08
gi|6967817|emb|AL139075.2|CJ11168X2 Campylobacter jejuni subs...	61.9	5e-08
gi|6967505|emb|AL139074.2|CJ11168X1 Campylobacter jejuni subs...	61.9	5e-08
gi|62867370|dbj|AB211985.1| Campylobacter lari pnp gene for p...	61.9	5e-08
gi|467630|emb|X66616.1|CJ23SR C.jejuni 23S rRNA	61.9	5e-08
gi|50957138|gb|AY596223.1| Helicobacter pullorum strain NCTC ...	61.9	5e-08
gi|50957137|gb|AY596222.1| Helicobacter pullorum strain NCTC ...	61.9	5e-08
gi|50957136|gb|AY596221.1| Helicobacter canadensis strain ATC...	61.9	5e-08
gi|50957135|gb|AY596220.1| Helicobacter canadensis strain ATC...	61.9	5e-08
gi|440400|emb|Z29326.1|CJ16SRNA C.jejuni TGH9011(ATCC43431) gene	61.9	5e-08
gi|1888387|emb|Y11766.1|CS23SRNA Campylobacter sp. 23S rRNA gene	61.9	5e-08
gi|1888384|emb|Y11765.1|CH23SRNA C.hyoilei 23S rRNA gene	61.9	5e-08

Therm 2M: GGT TTA CGG TAC GGG CAA CAT TAG

Sequences producing significant alignments:	Score (Bits)	E Value
gi|57165696|gb|CP000025.1| Campylobacter jejuni RM1221, complete	48.1	3e-04
gi|6968128|emb|AL139076.2|CJ11168X3 Campylobacter jejuni subs...	48.1	3e-04
gi|6967817|emb|AL139075.2|CJ11168X2 Campylobacter jejuni subs...	48.1	3e-04
gi|6967505|emb|AL139074.2|CJ11168X1 Campylobacter jejuni subs...	48.1	3e-04
gi|62867370|dbj|AB211985.1| Campylobacter lari pnp gene for p...	48.1	3e-04
gi|467630|emb|X66616.1|CJ23SR C.jejuni 23S rRNA	48.1	3e-04
gi|56607125|gb|AY686612.1| Helicobacter winghamensis strain A...	48.1	3e-04
gi|440400|emb|Z29326.1|CJ16SRNA C.jejuni TGH9011(ATCC43431) gene	48.1	3e-04
gi|1888387|emb|Y11766.1|CS23SRNA Campylobacter sp. 23S rRNA gene	48.1	3e-04
gi|1888384|emb|Y11765.1|CH23SRNA C.hyoilei 23S rRNA gene	48.1	3e-04
gi|1888385|emb|Y11764.1|CL23SRNA C.lari 23S rRNA gene	48.1	3e-04
gi|434940|emb|X67774.1|CU8854 C.upsaliensis (LMG 8854) 23S rRNA	48.1	3e-04
gi|1359538|emb|X67770.1|CC7535 C.coli (LMG 7535) 23S rRNA gene	48.1	3e-04
gi|434936|emb|X67766.1|CJD23S C.jejuni ssp.doylei 23S rRNA gene	48.1	3e-04
gi|434929|emb|X67765.1|CJJ23S C.jejuni ssp.jejuni (LMG 6629) 23S	48.1	3e-04
gi|434923|emb|X67764.1|CC6440 C.coli (LMG 6440) 23S rRNA gene	48.1	3e-04
gi|1359594|emb|X67769.1|CL23S C.lari 23S rRNA gene	48.1	3e-04
gi|434939|emb|X67763.1|CU23S C.upsaliensis (LMG 7533) 23S rRNA g	48.1	3e-04
gi|498572|gb|U09611.1|CCU09611 Campylobacter coli 23S ribosomal	48.1	3e-04

Figure 3.1: BLAST results for multiplex primers

Ceu F: CAT GCC CTA AGA CTT AAC GAT AAA GTT

Sequences producing significant alignments:	Score (Bits)	E Value
gi|1107527|emb|X88849.1|CCCEUBCDE C.coli ceuB, ceuC, ceuD, ceuE,	54.0	8e-06
gi|15872176|emb|AJ327758.1|HSA327758 Homo sapiens genomic seq...	38.2	0.45
gi|7267495|emb|AL161512.2|ATCHRIV24 Arabidopsis thaliana DNA chr	34.2	7.0
gi|7321058|emb|AL161813.1|ATT32A17 Arabidopsis thaliana DNA c...	34.2	7.0
gi|4220637|dbj|AB023038.1| Arabidopsis thaliana genomic DNA, chr	34.2	7.0
gi|26798864|emb|AJ530604.1|ATH530604 Arabidopsis thaliana T-D...	34.2	7.0

Ceu R: GAT TCT AAG CCA TTG CCA CTT GCT AG

Sequences producing significant alignments:	Score (Bits)	E Value
gi|1107527|emb|X88849.1|CCCEUBCDE C.coli ceuB, ceuC, ceuD, ceuE,	52.0	3e-05
gi|33147474|gb|AC121767.3| Mus musculus BAC clone RP23-339E1 ...	36.2	1.6
gi|62912455|ref|NM_002860.3| Homo sapiens aldehyde dehydrogen...	34.2	6.1
gi|62912456|ref|NM_001017423.1| Homo sapiens aldehyde dehydro...	34.2	6.1
gi|79559610|ref|NM_127754.3| Arabidopsis thaliana protein bin...	34.2	6.1
gi|76779855|gb|BC106054.1| Homo sapiens aldehyde dehydrogenas...	34.2	6.1
gi|62632712|ref|NM_001015039.1| Mus musculus zinc finger, FYV...	34.2	6.1
gi|2791577|emb|AL021171.1|CEH04D03 Caenorhabditis elegans Fosmid	34.2	6.1
gi|13750889|emb|AL390917.12| Human DNA sequence from clone RP...	34.2	6.1
gi|16214623|emb|AL356632.12| Human DNA sequence from clone RP...	34.2	6.1
gi|1304313|emb|X94453.1|HSP5CS H.sapiens mRNA for pyrroline 5-ca	34.2	6.1
gi|42269349|gb|AC145807.2| Xenopus tropicalis clone CH216-9P15,	34.2	6.1

Figure 3.1: BLAST results for multiplex primers (cont.)

LpxA F: CCG AGC TTA AAG CTA TGA TAG TGG AT

Sequences producing significant alignments:		Score (Bits)	E Value
gi|57165696|gb|CP000025.1|	Campylobacter jejuni RM1221, complete	52.0	3e-05
gi|6967505|emb|AL139074.2|CJ11168X1	Campylobacter jejuni subs...	52.0	3e-05
gi|76626170|ref|XM_584360.2|	PREDICTED: Bos taurus similar to CG	36.2	1.6
gi|60605965|gb|AY812484.1|	Schistosoma japonicum SJCHGC05593 pro	36.2	1.6
gi|9453885|dbj|AB045975.1|	Chrysophrys major COLV/XIA1 mRNA f...	36.2	1.6
gi|70999603|ref|XM_749426.1|	Aspergillus fumigatus Af293 tran...	34.2	6.1
gi|51011232|gb|AC034265.9|	Mus musculus chromosome 1, clone RP23	34.2	6.1
gi|74203580|dbj|AK136582.1|	Mus musculus adult male cecum cDN...	34.2	6.1
gi|15778824|gb|AC084391.1|	Mus musculus BAC clone RP23-294I17 fr	34.2	6.1

LpxA R: TCT ACT ACA ACA TCG TCA CCA AGT TGT

Sequences producing significant alignments:		Score (Bits)	E Value
gi|57165696|gb|CP000025.1|	Campylobacter jejuni RM1221, complete	50.1	1e-04
gi|51449813|gb|AY598976.1|	Campylobacter jejuni strain FZ917T...	50.1	1e-04
gi|51449809|gb|AY598974.1|	Campylobacter jejuni strain S2b LpxA	50.1	1e-04
gi|51449807|gb|AY598973.1|	Campylobacter jejuni strain M129 LpxA	50.1	1e-04
gi|47524453|gb|AY531521.1|	Campylobacter jejuni strain RM1221...	50.1	1e-04
gi|47524451|gb|AY531520.1|	Campylobacter jejuni strain F38011...	50.1	1e-04
gi|47524435|gb|AY531512.1|	Campylobacter jejuni strain RM3672...	50.1	1e-04
gi|47524433|gb|AY531511.1|	Campylobacter jejuni strain RM3673...	50.1	1e-04
gi|51449823|gb|AY598981.1|	Campylobacter jejuni strain ZJ638R...	44.1	0.007
gi|51449821|gb|AY598980.1|	Campylobacter jejuni strain KLC427...	44.1	0.007
gi|51449819|gb|AY598979.1|	Campylobacter jejuni strain 081 LpxA	44.1	0.007
gi|51449817|gb|AY598978.1|	Campylobacter jejuni strain YG936T...	44.1	0.007
gi|51449815|gb|AY598977.1|	Campylobacter jejuni strain YG108S...	44.1	0.007
gi|51449811|gb|AY598975.1|	Campylobacter jejuni strain 81116 Lpx	44.1	0.007
gi|47524457|gb|AY531523.1|	Campylobacter jejuni strain ANRO49...	44.1	0.007
gi|47524455|gb|AY531522.1|	Campylobacter jejuni strain KLC285...	44.1	0.007
gi|47524449|gb|AY531519.1|	Campylobacter jejuni strain RM3664...	44.1	0.007
gi|47524447|gb|AY531518.1|	Campylobacter jejuni strain RM3665...	44.1	0.007

Figure 3.1: BLAST results for multiplex primers (cont.)

Therm 1: TAT TCC AAT ACC AAC ATT AGT

Sequences producing significant alignments:	Score (Bits)	E Value
gi|57165696|gb|CP000025.1| Campylobacter jejuni RM1221, complete	42.1	0.011
gi|6968128|emb|AL139076.2|CJ11168X3 Campylobacter jejuni subs...	42.1	0.011
gi|6967817|emb|AL139075.2|CJ11168X2 Campylobacter jejuni subs...	42.1	0.011
gi|6967505|emb|AL139074.2|CJ11168X1 Campylobacter jejuni subs...	42.1	0.011
gi|62867370|dbj|AB211985.1| Campylobacter lari pnp gene for p...	42.1	0.011
gi|467630|emb|X66616.1|CJ23SR C.jejuni 23S rRNA	42.1	0.011
gi|50957138|gb|AY596223.1| Helicobacter pullorum strain NCTC ...	42.1	0.011
gi|50957137|gb|AY596222.1| Helicobacter pullorum strain NCTC ...	42.1	0.011
gi|50957136|gb|AY596221.1| Helicobacter canadensis strain ATC...	42.1	0.011
gi|50957135|gb|AY596220.1| Helicobacter canadensis strain ATC...	42.1	0.011
gi|440400|emb|Z29326.1|CJ16SRNA C.jejuni TGH9011(ATCC43431) gene	42.1	0.011
gi|1888387|emb|Y11766.1|CS23SRNA Campylobacter sp. 23S rRNA gene	42.1	0.011
gi|1888384|emb|Y11765.1|CH23SRNA C.hyoilei 23S rRNA gene	42.1	0.011
gi|1888385|emb|Y11764.1|CL23SRNA C.lari 23S rRNA gene	42.1	0.011
gi|434940|emb|X67774.1|CU8854 C.upsaliensis (LMG 8854) 23S rRNA	42.1	0.011
gi|1359538|emb|X67770.1|CC7535 C.coli (LMG 7535) 23S rRNA gene	42.1	0.011
gi|434930|emb|X67767.1|CJJ6444 C.jejuni ssp.jejuni (LMG 6444) 23	42.1	0.011
gi|434936|emb|X67766.1|CJD23S C.jejuni ssp.doylei 23S rRNA gene	42.1	0.011

Therm 2: CGG TAC GGG CAA CAT TAG

Sequences producing significant alignments:	Score (Bits)	E Value
gi|57165696|gb|CP000025.1| Campylobacter jejuni RM1221, complete	36.2	0.44
gi|6968128|emb|AL139076.2|CJ11168X3 Campylobacter jejuni subs...	36.2	0.44
gi|6967817|emb|AL139075.2|CJ11168X2 Campylobacter jejuni subs...	36.2	0.44
gi|6967505|emb|AL139074.2|CJ11168X1 Campylobacter jejuni subs...	36.2	0.44
gi|62867370|dbj|AB211985.1| Campylobacter lari pnp gene for p...	36.2	0.44
gi|467630|emb|X66616.1|CJ23SR C.jejuni 23S rRNA	36.2	0.44
gi|56607125|gb|AY686612.1| Helicobacter winghamensis strain A...	36.2	0.44
gi|56607123|gb|AY686610.1| Helicobacter sp. 'Flexispira taxon...	36.2	0.44
gi|50957176|gb|AY596261.1| Helicobacter cinaedi strain MIT 01...	36.2	0.44
gi|50957175|gb|AY596260.1| Helicobacter cinaedi strain CCUG 3...	36.2	0.44
gi|50957173|gb|AY596258.1| Helicobacter sp. MIT 01-5529A 23S ...	36.2	0.44
gi|50957172|gb|AY596257.1| Helicobacter sp. 'Flexispira taxon...	36.2	0.44
gi|50957171|gb|AY596256.1| Helicobacter sp. 'Flexispira taxon...	36.2	0.44
gi|50957170|gb|AY596255.1| Helicobacter bilis strain ATCC 516...	36.2	0.44
gi|50957169|gb|AY596254.1| Helicobacter cinaedi strain CCUG 1...	36.2	0.44
gi|50957168|gb|AY596253.1| Helicobacter sp. 'Schauer DBS59' 2...	36.2	0.44
gi|50957167|gb|AY596252.1| Helicobacter sp. 'Flexispira taxon...	36.2	0.44
gi|50957166|gb|AY596251.1| Helicobacter trogontum strain ATCC...	36.2	0.44
gi|50957165|gb|AY596250.1| Helicobacter trogontum strain ATCC...	36.2	0.44
gi|50957164|gb|AY596249.1| Helicobacter trogontum strain ATCC...	36.2	0.44

Figure 3.2: BLAST results for *C. lari* primers

Lari: ACG GCA TCA GCA ATT CTC

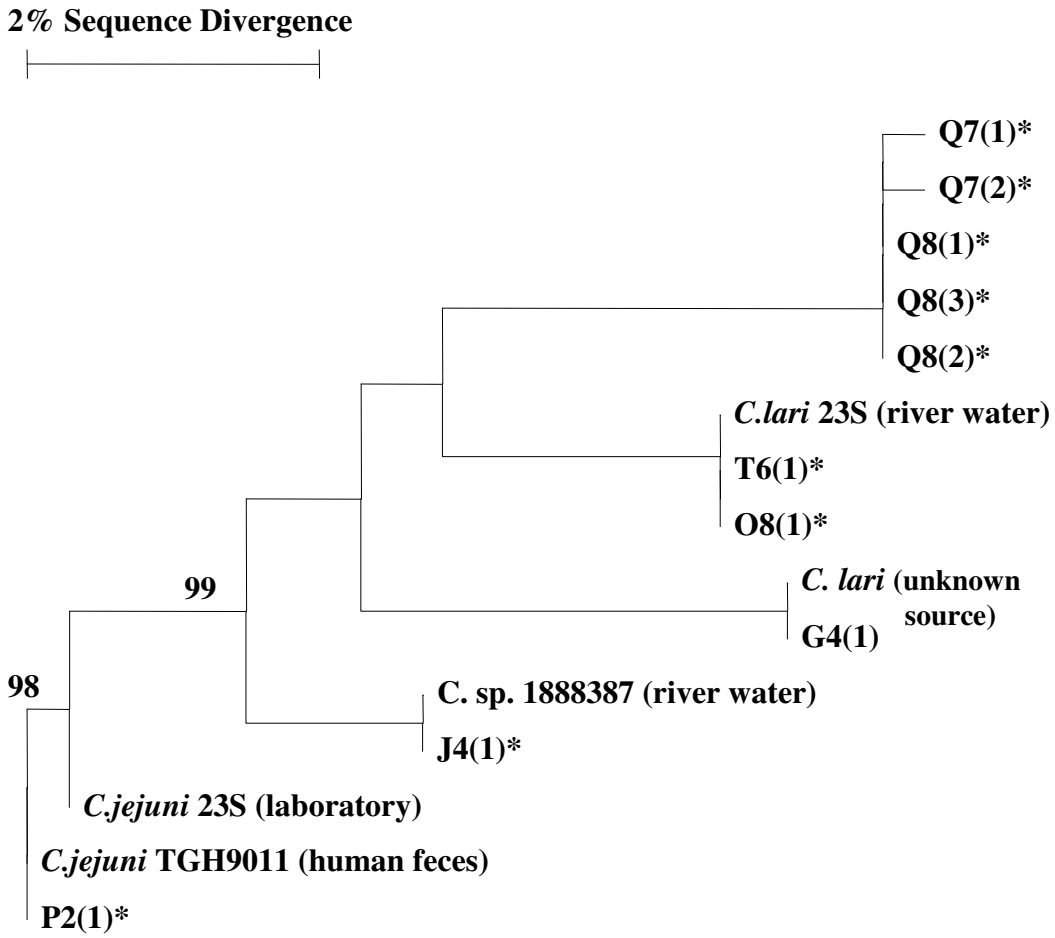
Sequences producing significant alignments:	Score (Bits)	E Value
gi|62867370|dbj|AB211985.1| Campylobacter lari pnp gene for p...	36.2	0.44
gi|1888385|emb|Y11764.1|CL23SRNA C.lari 23S rRNA gene	36.2	0.44
gi|1359594|emb|X67769.1|CL23S C.lari 23S rRNA gene	36.2	0.44
gi|56121942|gb|AC152985.1| Mus musculus 6 BAC RP23-118B18 (Ro...	32.2	6.9
gi|71852596|gb|CP000017.1| Streptococcus pyogenes MGAS5005, comp	32.2	6.9
gi|2780183|emb|Z99496.1|HS509L4 Human DNA sequence from clone...	32.2	6.9
gi|10443372|emb|AL137179.14| Human DNA sequence from clone RP...	32.2	6.9
gi|50902420|gb|CP000003.1| Streptococcus pyogenes MGAS10394, com	32.2	6.9
gi|62700976|emb|CR854907.6| Zebrafish DNA sequence from clone...	32.2	6.9
gi|13622883|gb|AE006611.1| Streptococcus pyogenes M1 GAS, sec...	32.2	6.9
gi|71801762|gb|CP000056.1| Streptococcus pyogenes MGAS6180, comp	32.2	6.9
gi|34740409|gb|AC116123.7| Mus musculus chromosome 6, clone RP23	32.2	6.9
gi|21905337|gb|AE014167.1| Streptococcus pyogenes MGAS315, se...	32.2	6.9

Figure 3.2: BLAST results for *C. lari* primers (cont.)

Therm 4: CTT CGC TAA TGC TAA CCC

Sequences producing significant alignments:	Score (Bits)	E Value
gi|57165696|gb|CP000025.1| Campylobacter jejuni RM1221, complete	36.2	0.44
gi|6968128|emb|AL139076.2|CJ11168X3 Campylobacter jejuni subs...	36.2	0.44
gi|6967817|emb|AL139075.2|CJ11168X2 Campylobacter jejuni subs...	36.2	0.44
gi|6967505|emb|AL139074.2|CJ11168X1 Campylobacter jejuni subs...	36.2	0.44
gi|62867370|dbj|AB211985.1| Campylobacter lari pnp gene for p...	36.2	0.44
gi|467630|emb|X66616.1|CJ23SR C.jejuni 23S rRNA	36.2	0.44
gi|30908776|gb|AY277527.1| Campylobacter coli isolate 00027 2...	36.2	0.44
gi|30908775|gb|AY277526.1| Campylobacter coli isolate 00247 2...	36.2	0.44
gi|30908774|gb|AY277525.1| Campylobacter coli isolate 00254 2...	36.2	0.44
gi|30908773|gb|AY277524.1| Campylobacter coli isolate 00258 2...	36.2	0.44
gi|30908772|gb|AY277523.1| Campylobacter coli isolate 00260 2...	36.2	0.44
gi|30908771|gb|AY277522.1| Campylobacter coli isolate 00300 2...	36.2	0.44
gi|30908770|gb|AY277521.1| Campylobacter jejuni isolate 00346...	36.2	0.44
gi|30908769|gb|AY277520.1| Campylobacter jejuni isolate 00358...	36.2	0.44
gi|30908768|gb|AY277519.1| Campylobacter jejuni isolate 01197...	36.2	0.44
gi|30908767|gb|AY277518.1| Campylobacter coli isolate 00261 2...	36.2	0.44
gi|30908766|gb|AY277517.1| Campylobacter coli isolate 00262 2...	36.2	0.44
gi|30908765|gb|AY277516.1| Campylobacter coli isolate 98134 2...	36.2	0.44

Figure 3.3: BLAST results for Therm 4 primer



*Seagull feces isolates isolated from this study

Figure 3.4: Phylogenetic tree of a subset of thermotolerant *Campylobacter* nucleotide sequences. A majority of isolates sequenced were most similar to *C. lari* strains in the region amplified.

Sample Type	Total Samples Analyzed	Volume Homogenate Analyzed (ml)	# Samples presumptive culture-positive *Campylobacter* (%)	# Isolates Archived
TM seagull feces	148	15	84 (56.8)	380
TM sand samples	10	15	3 (30.0)	3
TM water samples	24	1000	7 (29.2)	15
NB seagull feces	10	15	2 (20.0)	7

TM: Talbert Marsh
NB: Newport Beach

Table 3.1: Presumptive culture-positive *Campylobacter* in samples analyzed

Sample Type	# Samp. presumptive culture + *Camp.* (%)	BC +	# Isolates	Total # PCR + Samp. (%)	Total # PCR + Isolates (%)	Total # *C. jej.* + Isolates (%)	Total # Therm-only + Isolates (%)
TM seagull	40*	Y	184	5 (12.5)	15 (8.2)	1 (0.5)	14 (7.6)
	38	N/A	184	23 (60.5)	87 (47.3)	40 (21.7)	47 (25.5)
TM sand	3	Y	3	0 (0.0)	0 (0.0	0 (0.0)	0 (0.0)
TM water	7	N/A	15	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
NB seagull	2	N/A	7	2 (100.0)	7 (100.0)	7 (100.0)	0 (0.0)

TM: Talbert Marsh
NB: Newport Beach
BC: Biochemical tests and Gram-staining
*does not include 6 samples (11 isolates) that were unavailable during molecular analyses

Table 3.2: Multiplex PCR results

Sample Type	# Samp. presumptive culture + *Camp.* (%)*	BC +	# Isolates	Total # *C. lari* PCR + Samp. (%)	Total # *C. lari* PCR + Isolates (%)	Total # Therm-only PCR + Samp. (%)	Total # Therm-only PCR + Isolates (%)
TM seagull	40	Y	184	4 (10.0)	13 (7.1)	1 (2.5)	1 (0.5)
	38	N/A	184	10 (26.3)	31 (16.8)	3 (7.9)	16 (8.7)

TM: Talbert Marsh
BC: Biochemical tests and Gram-staining

Table 3.3*: C. lari* PCR results

Name	Source	NCBI Accession #	Reference
C. jejuni TGH9011	human	Z29326	Kim et al 1995
C. jejuni 23S	laboratory	X66616	Van Camp et al 1993
C. sp. 1888387	river water	Y11764	Linton et al 1999
C. lari	unknown	AB211985	unpublished
C. lari 23S rRNA	river water	Y11764	Linton et al 1999

Table 3.4: Published sequences used for comparison of *Campylobacter* isolated from Talbert Marsh seagull feces

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CHAPTER IV

Molecular Analysis of F+ Coliphages Isolated from Seagull Feces and Water of an Estuarine Environment

Introduction

Previously, F+ coliphages were isolated from seagull feces and estuarine water samples using conventional microbiological techniques and isolates were stored in TSB with 25% glycerol (v/v) at -80 C for at least one year. Molecular analyses for detection and genotyping of F-RNA and F-DNA coliphages were performed on stored isolates, or enrichments of these isolates, to further classify and compare them. These results allow a better understanding of the types of F+ coliphages present in seagull feces and waters impacted by these feces for microbial source tracking purposes.

Background

Indicators of Fecal Contamination

Around the beginning of the 20th century, it became apparent that monitoring for specific bacterial pathogens in drinking water was not pragmatic because there are a large number of pathogens, they are often present in small numbers, and methods for their detection are expensive, time-consuming, and often ineffective. At this time, microbiologists agreed that monitoring for fecal pollution, specifically by monitoring for coliform bacteria, including *Escherichia coli*, as indicators of fecal pollution, was a more practical approach. Methods for detecting these enteric bacteria involved culturing for the fermentation of lactose in

differential and selective growth media and they were easy, inexpensive, and rapid. In 1986, the U.S. EPA published recommended water quality criteria for recreational waters, which proposed the use of enterococci in marine water and enterococci and/or *Escherichia coli* in fresh water as indicator organisms. Monitoring for fecal indicator bacteria is still an effective tool in management activities for the prevention of waterborne bacterial disease worldwide (Leclerc, Edberg et al. 2000).

More recently, studies began to focus on the transmission of enteric viruses in drinking water and other environmental routes, and it was found that the fate of coliforms possibly differs biologically from that of animal viruses (Leclerc, Edberg et al. 2000). In 1948, Guelin was the first to recommend the use of bacteriophages, or viruses that infect bacteria, as models for enteric viruses in the environment. As with bacterial pathogens, there are a great number of viral pathogens that are usually present in low numbers and whose detection methods are expensive, difficult, and time-consuming. Not only are bacteriophages present in the gastro-intestinal tracts of humans and warm-blooded animals, but their composition, structure, and morphology share many fundamental properties with enteric viruses (Grabow 2001). The role of bacteriophages as indicators of human enteric viruses has been greatly debated over the past several decades.

Bacteriophages

Bacteriophages, or phages, were first discovered independently by Twort and d'Herelle in 1915 and 1918, respectively (Grabow 2001). Because they possess the ability to kill bacteria, they initially were studied in hopes that they could treat and prevent bacterial

disease. Bacteriophages infect and replicate in bacteria cells in several stages (IAWPRC Study Group on Health Related Water Microbiology 1991):

Adsorption of the phage particles to host cell

Entry of phage nucleic acid into the host cell

Synthesis of phage macromolecules and subunits by the host cell

Assembly of mature phage particles

Lysis of the host cell and release of phages

Attachment sites on bacteriophages adsorb to receptor sites on host bacteria. A somatic phage is one that attaches directly to receptor sites on the cell wall of the host bacterium, while a F+ phage is one that attaches to receptor sites on the bacterial F-pilus (described below). Host-specificity ranges, with some bacteriophages only infecting a very restricted group of bacterial strains and others infecting a much broader host range (IAWPRC Study Group on Health Related Water Microbiology 1991).

The formal classification of bacteriophages is generally based on the nature of nucleic acid, i.e. single- or double-stranded, and the morphology of the capsid, or protein coat that surrounds the nucleic acid. Figure 4.1, adapted from the bacteriophage review paper written by the IAWPRC Study Groups on Health Related Water Microbiology (IAWPRC Study Group on Health Related Water Microbiology 1991), describes the six major families of bacteriophages.

A number of methods for the recovery of phages from fresh-, sea-, and estuarine waters have been outlined by Grabow (Grabow 2001) and include adsorption-elution methods (using either negatively- or positively-charge filter media), ultrafiltration, flocculation, hydro-extraction, secondary concentration methods, direct plaque assays on large volumes,

qualitative presence-absence enrichment tests, and rapid detection methods. Molecular techniques, such as PCR and nucleic acid hybridization assays, for the detection of phages have also recently been developed.

Bacteriophages as Indicators of Fecal Pollution

The three types of bacteriophages commonly used as indicators of enteric viruses in water quality assessment are somatic coliphages, F+ coliphages (specifically F-RNA coliphages), and phages that infect *Bacteriodes fragilis* (IAWPRC Study Group on Health Related Water Microbiology 1991). The term coliphage refers to a subset of bacteriophages that infect coliforms, including *Escherichia coli* and possibly other Enterobacteriaceae, which are found in the intestinal tracts of all warm-blooded animals. The presence of coliphages indicates the likely presence of coliforms, and therefore the likely presence of fecal contamination.

Somatic Coliphages

Somatic coliphages are heterogeneous group of phages belonging to the *Myoviridae*, *Styloviridae*, *Podoviridae*, and *Microviridae* families. Again, these phages infect host bacteria through receptors located on the cell wall. They are abundant in human and animal excrement (Havelaar, Furuse et al. 1986; Grabow 2001), sewage sludge (Lasobras, Dellunde et al. 1999), wastewater (Grabow 2001), and freshwater environments impacted with fecal pollution (Araujo, Puig et al. 1997; Lucena, Mendez et al. 2003; Muniesa and Jofre 2004; Skraber, Gassilloud et al. 2004). They have not only been found to be less sensitive to environmental factors and able to survive longer than bacterial indicators (Lucena, Mendez et al. 2003; Skraber, Gassilloud et al. 2004), but have also been found to persist longer than

enteroviruses in wastewaters, surface waters, and sand columns (reviewed in (Leclerc, Edberg et al. 2000)). While some studies describe the usefulness of somatic coliphages as indicators of enteric viruses in water environments (Grabow 2001; Skraber, Gassilloud et al. 2004; Skraber, Gassilloud et al. 2004), others have been unable to find a significant association between the concentration of somatic coliphages and the presence of either infectious enteroviruses or non-enterovirus viral pathogens (Hot, Legeay et al. 2003).

Muniesa et al. (Muniesa, Lucena et al. 1999) describe the measurement of somatic coliphages as easier, cheaper, and faster than that of the other bacteriophage groups. However several shortcomings with the use of somatic coliphages as indicators have been described, notably the heterogeneity of the group and their ability to be non-exclusively detected by a range of host bacteria (Ackermann and Nguyen 1983). Common host bacteria used for their isolation include *E. coli* strains B, C, CN, CN13, C-3000, F-amp, and K-12 derivatives (Leclerc, Edberg et al. 2000). Another longstanding drawback associated with somatic coliphages is the possibility that they may replicate in *E. coli* and other naturally occurring enterobacteria in water. However, recent research indicates "that the phage and bacterium densities and the bacterial physiological conditions needed for phage replication are rarely expected to be found in the natural water environments (Muniesa and Jofre 2004)."

Phages of Bacteroides fragilis

Bacteroides fragilis is an obligate anaerobic bacterium present in the human colon in higher concentrations than coliform bacteria (Grabow 2001). Bacteriophages infecting *Bacteroides fragilis* are somatic phages that generally fall within the *Siphoviridae* family (Booth, Van Tassell et al. 1979). *Bacteroides fragilis* phages, especially *B. fragilis* strain

HSP40, have been detected extensively in human feces and sewage, demonstrate a high degree of host-strain specificity, lack activity against other species of *Bacteroides*, and have not been recovered in non-polluted areas nor those exclusively occupied by wild animals (reviewed in (Leclerc, Edberg et al. 2000)). Furthermore, *B. fragilis* phages have been shown to be resistant to unfavorable environmental conditions and therefore more persistent than somatic coliphages (reviewed in (Leclerc, Edberg et al. 2000); (Armon, Araujo et al. 1997; Duran, Muniesa et al. 2002)). These observations, coupled with the inability of these phages to multiply in natural water environments, suggest that they may be reliable indicators of enterovirus contamination (reviewed in (Leclerc, Edberg et al. 2000; Grabow 2001)).

Plaque assays for *B. fragilis* phages are more complicated, expensive, labor-intensive, and time-consuming than those for other phages. Not only is a complex growth medium required, but cells must be incubated under strictly anaerobic conditions (Grabow 2001). Additionally, although more persistent, *B. fragilis* phages are detected in lower concentrations than other phages in fresh water (Araujo, Puig et al. 1997; Armon, Araujo et al. 1997; Duran, Muniesa et al. 2002; Lucena, Mendez et al. 2003), and it is therefore more difficult to evaluate the precise ratio of the phages with enteric viruses (Leclerc, Edberg et al. 2000). In order to find more prevalent *Bacteroides* phages in human and animal guts, Puig et al. (Puig, Queralt et al. 1999) identified additional host strains of *Bacteroides*. Despite the ability to also detect phages in animal feces, one strain, RYC2056, detected greater numbers of *Bacteroides* phage in waters with known human impact. A recent PCR procedure for the detection of *B. fragilis* HSP40 phages has also been shown to be more sensitive than the plaque assays and results in higher counts of phages in the environmental water samples analyzed (Puig, Pina et al. 2000).

F+ Coliphages

F+ coliphages are composed of two families, Family *Leviviridae* (F-RNA coliphages) and Family *Inoviridae* (F-DNA coliphages), which consist of single-stranded genetic material and do not contain tails (Leclerc, Edberg et al. 2000). Both types infect host bacteria via the F+ pilus, a bacterial appendage used in the transfer of genetic material between two bacterial cells. The pilus is encoded by the classical F-plasmid of *E. coli* K-12 and related plasmids of the IncF-incompatibility group (IAWPRC Study Group on Health Related Water Microbiology 1991). F-RNA coliphages infect through the sides of the pilus, whereas F-DNA coliphages infect through the tip of the pilus (Duckworth 1987). A variety of bacterial hosts have been described for isolation of F+ coliphages. Generally, growth in the presence of RNase is used to differentiate F-DNA from F-RNA coliphages, however a recent molecular method (Vinje, Oudejans et al. 2004) (described below) has been developed to both detect and differentiate between F+ coliphages.

Several characteristics of F+ coliphages make them promising indicators of viral contamination. They are nonpathogenic to humans, are not likely to replicate in environments other than the gastrointestinal tracts of warm-blooded animals, and are more similar to enteric viruses in terms of physical characteristics, environmental persistence, and resistance to treatment processes than are indicator bacteria. F+ coliphages are consistently present in both raw and treated sewage, often in concentrations ranging from 10^3 to 10^7 PFU/L (reviewed in (Grabow 2001; Cole, Long et al. 2003)). Furthermore, they have also been found in surface waters with potential sewage or fecal contamination, but not in waters considered unimpacted by fecal contamination. In a number of studies, the presence of F+ coliphages has been significantly correlated to the presence of mammalian enteric viruses in

polluted surface waters (reviewed in (Long, El-Khoury et al. 2005)). Although high numbers of F+ coliphages are not generally isolated from the feces of domestic animals such as cows, pigs, and horses (Dhillon, Dhillon et al. 1976; Osawa, Furuse et al. 1981), several studies have found that many avian species, including seagulls, may be constant sources of these microbes (Calci, Burkhardt et al. 1998; Muniesa, Jofre et al. 1999; Cole, Long et al. 2003).

The F-DNA family is composed of large, filamentous phages that are nonenveloped, flexible, rod-shaped, and contain circular single-stranded DNA. F-DNA phages do not resemble human enteric viruses morphologically and little information is known about their ecology (Leclerc, Edberg et al. 2000). Recent work has shown that F-DNA phages are concurrently detected with F-RNA coliphages in a variety of wastewaters, including human, swine, and bovine and are detected from surface waters in higher numbers than F-RNA coliphages during the summer and fall months (Cole, Long et al. 2003). Futhermore, F-DNA phages are reported to be more resistant to sunlight exposure than F-RNA coliphages. Although limited, these observations suggest that F-DNA phages may be useful as year-round indicators (Vinje, Oudejans et al. 2004), however the sanitary significance of F-DNA coliphages has yet to be fully assessed (Sinton, Finlay et al. 1999).

The F-RNA family is composed of positive-sense, linear, single-stranded, tail-less phages with small, hexagonal capsomeres (components of the protein capsid). The family contains two genera: *Levivirus* and *Allolevivirus* and each of these contains two distinct subgroups based upon serological cross-reactivity, molecular weight and density of the virion, sedimentation velocity of the viral particle, and replicase activity (reviewed in (Bollback and Huelsenbeck 2001)). The genus *Levivirus* contains groups I and II phages, while the genus *Allolevivirus* contains groups III and IV phages.

F-RNA coliphages have been more fully studied for their use as viral indicators of fecal pollution than F-DNA phages for a number of reasons. Of the bacteriophages, F-RNA coliphages are the most similar to many human enteric viruses in physical structure, composition, and morphology. Additionally, the four serogroups are selectively excreted by humans or animals. In most cases, groups II and III F-RNA coliphages are found in environments affected by human waste and groups I and IV in environments affected by animal waste. Serological classification of F-RNA coliphages has previously been used to classify the four general F-RNA coliphage groups, however, recent work is shifting to more specific and conclusive nucleic acid techniques to provide some insight into the origin of fecal contamination (Hsu, Shieh et al. 1995; Vinje, Oudejans et al. 2004). Although the F-RNA classifications are not absolute, many studies continue to focus on the use of F-RNA coliphages for the distinction between fecal pollution of human and animal origin (reviewed in (Grabow 2001)) .

Molecular Detection of F-RNA Coliphages

Hybridization with Oligonucleotide Probes

Serotyping F-RNA coliphages has traditionally been used to distinguish between human and animal fecal contamination. However, this method poses problems as it is laborious, antisera for the F-RNA coliphages are not readily available, isolates are sometimes difficult to serotype, and some isolates may be neutralized by two heterologous antisera (reviewed in (Hsu, Shieh et al. 1995)). Genotyping by nucleic acid hybridization methods may offer a number of benefits over traditional serotyping methods, in that they are often reliable, rapid, simple, and inexpensive.

In 1995, Hsu et al. (Hsu, Shieh et al. 1995) developed a nucleic acid hybridization genotyping method to group F-RNA coliphages with nonradioactive oligonucleotide probes and compared this method with serotyping. Briefly, F-RNA coliphages were first detected in environmental samples using a double agar layer plaque assay. Individual plaques were isolated and suspended in PBS with glycerol. These suspensions were tested with RNase A to distinguish between RNA and DNA phages and were serotyped by a spot test. Suspensions were again spotted onto lawns of host cells and methods for phage transfer from zones of lysis to candidate membrane filters and fixation of genomic nucleic acid on the membranes were optimized. Oligoprobes designed to detect groups I, II, III, IV, I plus II, and III plus IV phages and end labeled with digoxigenin were applied in DNA-RNA hybridizations and hybrids were observed by colorimetric, immunoenzymatic detection.

There was no significant difference between overall percentages of classification by serotyping and hybridization of over 200 isolates from water, wastes, and shellfish tested in this study. Hybridization demonstrated several clear advantages over serotyping, including the ability to readily recognize mixed populations of coliphages and to resolve problematic serological differences between group intermediates. This study further confirms that F-RNA groups II and III suggest primarily human fecal contamination sources, while groups I and IV suggest primarily animal sources. Grouping allows the determination of sources of contamination and may strengthen the associations of F-RNA coliphages with human enteric viruses. Overall the genotyping method is effective, technically feasible, and strengthens the argument that F-RNA coliphages are promising indicators for monitoring and distinguishing fecal contamination sources in water and food (Hsu, Shieh et al. 1995).

RT-PCR and Reverse Line Blot Hybridization

More recently, Vinje et al. (Vinje, Oudejans et al. 2004) described a method that uses reverse-transcription PCR (RT-PCR) and a reverse line blot hybridization (RLB) assay to molecularly detect and genotype F+ coliphages. A similar RLB method was previously described for the simultaneous detection and genotyping of noroviruses in an assay in which virus-specific amplicons hybridize to one of multiple probes covalently linked to a nylon membrane. The coliphage method uses membrane-bound genotype-specific probes that react with biotinylated RT-PCR products.

The genomes of both leviviruses and alloleviruses contain four genes: a maturase gene, a coat gene, a lysis gene, and a replicase gene. Initially, a generic F-RNA duplex RT-PCR assay with one forward primer and two genus-specific reverse primers was developed based on the replicase gene of published nucleotide sequences and F-RNA sequences generated in the study. A generic PCR assay was also developed for F-DNA coliphages based on gene IV, which encodes the outer membrane pore pIV through which F-DNA phages exits from their host. This region exhibited the largest nucleotide sequences variation among prototype strains analyzed.

Four probes for F-DNA (representing three clusters and one consensus sequences) and six probes for F-RNA (MS2, GA, Qβ, M11, FI, and SP), based on PCR and RT-PCR products, respectively, were designed for use in the RLB hybridization method. Briefly, to prepare for RLB hybridization a nylon membrane was activated and then placed in a miniblotter where the oligonucleotide probes were then covalently bound to the activated membrane. For hybridization, the membrane was placed in the miniblotter with slots perpendicular to the lines of the probes. Diluted and heat-separated RT-PCR product generated with biotin-

labeled reverse primers were then loaded into the miniblotter slots. After a one-hour hybridization and incubation with a labeling compound, bound RT-PCR product was visualized using chemiluminescence.

All 216 F-DNA PCR-positive samples and 98% of 135 RT-PCR-positives F-RNA samples were confirmed by RLB in this study. Furthermore, a potential novel genotype (JS) within the *Levivirus* genus was identified and these data are the first on the ecology of F-DNA phages isolated from different sources. Both of these aspects require further molecular studies for confirmation. Although the RT-PCR step requires access to a thermocycler, the one-step RT-PCR kits now available simplify this portion of the assay. This method allows the detection and genotyping of phages that are unable to be re-propagated after isolation and provides a rapid, reproducible, and easy alternative to serological classification methods. It is also inexpensive, as membranes can be stripped of products and reused. Overall, the method serves as an ideal candidate to become a standardized method for genotyping of F+ coliphages as a tool for microbial source tracking (Vinje, Oudejans et al. 2004).

The use of F+ coliphages, specifically subgroups of F-RNA coliphages, has recently become an important tool in microbial source tracking. A number of characteristics of these coliphages make them beneficial indicators of viral contamination, including their presence in surface waters with potential sewage or fecal contamination and their absence in waters not impacted by fecal contamination. Furthermore, several studies have shown that seagulls may be constant sources of these microbes. Potential control measures based on manipulating water systems and their release of fecally-contaminated water must first be based on reliable identification and quantification of fecal contamination sources. This study

served to assess the genotypes of F+ coliphages found in both the feces of seagulls in Talbert Marsh and in the surrounding estuarine waters.

Objectives

To use molecular-based genotypic methods to confirm the presence of, to further type, and to compare F+ coliphages in presumptive positive plaque suspensions, or enrichments of these suspensions, isolated from Talbert Marsh seagull feces and estuarine water samples and stored in TSB with 25% glycerol (v/v) at -80 C.

Approaches

In an attempt to confirm stored plaque suspensions as F+ coliphages, isolates were subjected to a duplex RT-PCR method developed for the detection of F-RNA coliphages and for the differentiation between *Allolevivirus* and *Levivirus*. For positive isolates, this was followed by a reverse line blot hybridization assay to further classify F-RNA coliphages into Groups I, II, III, or IV. For negative F-RNA isolates, a generic F-DNA PCR method was performed to detect the presence of F-DNA coliphages. Finally, a subset of F-RNA coliphage isolates were sequenced and analyzed for microbial source tracking purposes.

Methods

Virus strains and host cells

F-DNA positive control strain f1 and F-RNA positive control strains MS2 (serogroup I), GA (serogroup II), Qβ (serogroup III), M11 (serogroup III), FI (serogroup IV), and SP (serogroup IV) were provided by Dr. Jan Vinje (UNC-Chapel Hill). As seen in Table 4.2, a

total of 275 previously-picked isolated plaques (or secondary enrichments of original isolated plaques[1]) were analyzed in this study: 96 were isolated from 31 separate seagull feces collected in Talbert Marsh; four were isolated from four Talbert Marsh sand samples; two were isolated from one Newport Beach seagull fecal sample; 44 were isolated from ten Talbert Marsh estuarine water samples using the Two-Step Enrichment Procedure (EPA 1601); and 112 were isolated from 11 Talbert Marsh estuarine water samples using the Single Agar Layer (SAL) Procedure (EPA 1602). Thirty-seven additional isolates from six Talbert Marsh estuarine water samples were enrichment samples of non-plaquing chloroform extractions performed on original enrichments (EPA 1601) of estuarine water samples[2]. Isolates and chloroform extracted enrichments were stored in TSB/glycerol (80:20 v/v) at -80^{o}C. *E. coli* Famp was used as the host strain and was grown in Tryptic Soy Broth (TSB) with streptomycin-sulphate and ampicillin (0.0015% w/v each). The isolation methods used were semi-quantitative and gave a crude estimate of concentrations of phages in both waterfowl feces and water samples.

F-RNA coliphage RT-PCR

A modified version of the duplex RT-PCR assay described by Vinje et al (Vinje, Oudejans et al. 2004) was used for F-RNA coliphage detection. The assay targets the replicase gene of F-RNA coliphages using genus-specific primer pairs. Detection was achieved using one forward primer (MJV82) and two biotin-labeled genus (*Levivirus* and *Allolevivirus*)-specific

[1] Some of the original plaque isolates were low in volume after various attempts to re-isolate, so they were re-enriched to become the new stored isolate pool

[2] These isolates are chloroform extractions of enrichments that did not plaque, likely because of *E. coli* host growth problems; after they did not form plaques the chloroform extracts were re-enriched and stored

reverse primers (JV41 and JV81, respectively; see Table 4.1). Strains from each genus produced different-sized RT-PCR products allowing a genus level of identification.

Picked plaques and chloroform extracted enrichments were diluted 1:50 in sterile distilled water and viral RNA was released by incubation at 99°C for five minutes. Dilutions were then iced for two minutes, followed by centrifugation for 10 seconds. The RT-PCR was performed on 2.5 µl aliquots of this material in a 25 µl reaction using OneStep RT-PCR kit (QIAGEN, Valencia, CA) consisting of: OneStep RT-PCR Enzyme Mix (1 µl); 1X OneStep RT-PCR Buffer with 12.5 mM $MgCl_2$; and 10 mM deoxynucleotide triphosphates. RNasin RNA Inhibitor (0.4 Units/µl) was added to increase sensitivity. Primers used were 0.8 µM MJV82 (IDT), 0.8 µM JV41 (IDT), and 0.6 µM JV81 (IDT).

Thermal cycling conditions for the MJ Research Gradient Thermal Cycler were: an RT step at 45°C for 30 minutes, followed by heat activation of the *Taq* polymerase at 95°C for 15 minutes. PCR consisted of 40 cycles of denaturation at 94°C for 30 s, 45°C annealing for 30 s, 72°C extension for 30 s, with a final 10 min extension step at 72°C. For each RT-PCR run, positive (2.5 µl each of diluted *Levivirus* and *Allolevivirus* control strains) and negative (2.5 µl of molecular biology grade water) controls were included. RT-PCR products (Leviviruses = 266 bp; Alloleviviruses = 229 bp) were visualized by agarose gel electrophoresis (2%) stained with ethidium bromide.

Reverse Line Blot Hybridization Assay

The RLB hybridization method used was described by Vinje et al (Vinje, Oudejans et al. 2004) to genotype all six subgroups of F-RNA coliphages. All isolates producing F-RNA RT-PCR products were subjected to RLB. A nylon membrane was activated for 10 min with

16% (w/v) 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma, St. Louis, Mo.) at room temperature, then placed in a miniblotter. Two-fold dilutions of oligonucleotide probes (see Table 4.1) were covalently bound to the carboxyl groups of the activated membrane and the remaining activated esters were hydrolyzed by incubation in 0.1 M NaOH. Following several washing steps, the membrane was then placed in the miniblotter with the slots perpendicular to the lines of the probes. RT-PCR biotin-labeled products were diluted in 2X SSPE/0.1% SDS, heat-denatured, loaded into the slots of the miniblotter, and hybridized at 42^{o}C for 1 hour. After several washing steps the membrane was incubated in streptavidin peroxidase at 42^{o}C for 45 min. Again, the membrane was washed and bound PCR product was detected by chemiluminsecence using ECL detection liquid and visualized after 30 min of exposure to X-ray film.

Enrichment of Isolates (and Chloroform Extract Enrichments)

Isolates (and chloroform extract enrichments) that did not produce RT-PCR products or produced RT-PCR products but did not produce a visible RLB product were enriched as described in EPA Method 1601 to compensate for possible die-off while frozen. Enrichments were prepared in sterile 10 ml glass fermentation tubes and included: 500 µl TSB, 6.25 $MgCl_2$, 5 µl streptomycin sulfate/ampicillin, 2.5 µl log-phage *E. coli* Famp, and 10 µl of the thawed and vortexed isolate or chloroform extract enrichment. Tubes were capped loosely and incubated at 37^{o}C on a shaking platform overnight. The following day, enrichments were transferred using separate sterile 1 ml pipets into sterile 1.5 ml microcentrifuge tubes. Tubes were micro-centrifuged (Eppendorf centrifuge 5415C) for 30 minutes at 16,000 x g. Supernatants were carefully transferred using separate sterile 1 ml

pipets into 0.65 ml microcentifuge tubes. Tubes were stored at 4°C until molecular analysis. Enrichments were diluted 1:50 in sterile distilled water and viral RNA was released by incubation at 99°C for 5 minutes. Dilutions were then iced for two minutes, followed by centrifugation for 10 seconds. The RT-PCR was performed on 2.5 µl aliquots of this material in a 25 µl reaction as described above. Enrichments producing F-RNA products were subjected to RLB as also described above.

Generic F-DNA PCR

Coliphage enrichments that did not produce products using the F-RNA RT-PCR assay described above were subjected to an F-DNA PCR assay, also described by Vinje et al (Vinje, Oudejans et al. 2004), which targets the gene encoding the outer membrane pore (secretin) pIV through which F-DNA phages exit their hosts. For all sample types, a subset of enrichments was assayed individually and the remaining enrichments were pooled into groups of five, with 5 µl of each isolate. Pooled isolates were diluted 1:50 in sterile distilled water and viral DNA was released by incubation at 99°C for 5 min. For both pooled and non-pooled isolates, the PCR was performed on 2.5 µl of this material in a 25 µl reaction volume using 2X HotStarTaq mastermix (HotStarTaq DNA polymerase, PCR Buffer with 3mM $MgCl_2$, and 400 uM each dNTP) (QIAGEN, Valencia, CA) and 0.6 µM of each SL2 and SL3 primers (see Table 4.1).

Thermal cycling conditions for the MJ Research Gradient Thermal Cycler were: heat activation of the *Taq* polymerase at 95°C for 15 minutes, followed by 40 cycles of denaturation at 94°C for 1 min, 50°C annealing for 1 min, 72°C extension for 1 min, with a final 10 min extension step at 72°C. For each PCR run, positive (2.5 µl each of diluted

Inovirus control strain f1) and negative (2.5 µl of molecular biology grade water) controls were included. PCR products (Inoviruses = 256 bp) were visualized by agarose gel electrophoresis stained with ethidium bromide.

Spot Plating

To compare the sensitivity of the RT-PCR method to a traditional plaque assay method, subsets of enriched isolates that were RT-PCR positive and RT-PCR negative were spot-plated in 10 µl aliquots. Spot plates consisted of TSB, bacteriological grade agar, log phase *E. coli* Famp host, and streptomycin sulfate/ampicillin. After incubation at 37^{o}C overnight, a clearing in the agar lawn of bacteria indicated the presence of coliphage infection.

Sequencing for Identification and Comparison

The F-RNA products of the seagull feces isolates that did not produce RLB bands were purified using a QIA-quick PCR purification kit (Qiagen Inc.) according to the manufacturer's instructions and sequenced at Lineberger Sequence facility (University of North Carolina, Chapel Hill) with the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and using the MJV82 forward primer.

Each sequence was edited using BioEdit software and compared with previously described sequences. Multiple sequence alignments of the remaining seven sequences and available sequences of known prototype strains were generated using Clustal W and imported into TreeCon (V 1.3b). Phylogenetic trees were drawn with Jukes and Cantor correction for evolutionary rate. A clustering method was used and the confidence values of the internal nodes were calculated by performing 100 bootstrap analyses.

Sequence Comparison Between Sample Types

Ten seagull feces isolates and nine estuarine water sample isolates all genotyped as MS2-like by the RT-PCR and RLB methods were sequenced using the forward primer MJV82 (as described above) for sequence similarity analyses. Multiple sequence alignments of the nucleotide sequences and available nucleotide sequences of known prototype strains were generated using Clustal W and imported into TreeCon (V 1.3b). Phylogenetic trees were drawn with Jukes and Cantor correction for evolutionary rate. A neighbor-joining method was used (with the MS2 prototype sequence serving as the root) and the confidence values of the internal nodes were calculated by performing 100 bootstrap analyses.

As differences in nucleotide sequence do not necessarily indicate differences in amino acid sequence, each of the 19 sequences was further analyzed. To compare these isolates, the correct reading frame of each nucleotide sequence was determined using the known amino acid sequence of the MS2 positive control. Nucleotide sequences were transformed into their amino acid sequences and aligned using Vector NTI® software (V 10.1). Phylogenetic trees were drawn with Jukes and Cantor correction for evolutionary rate. A neighbor-joining method was used (with the MS2 prototype sequence serving as the root).

Results

F+ Coliphages Isolated from Seagull Feces

Original Isolates

As seen in Tables 4.3 and 4.4, a total of 96 individual plaques were originally isolated from 31 separate Talbert Marsh seagull fecal samples. 25/96 (26.0%) picked plaque suspensions representing 10 separate fecal samples, produced F-RNA product when

subjected to RT-PCR. Of these, 14/25 (56.0%) produced RLB bands: 3/14 (21.4%) were MS2 and were isolated from 3 separate bird feces samples; 6/14 were GA-like (GII) (42.9%) and were isolated from 4 separate bird feces samples; and 5/14 (35.7%) were Qβ-like (G III) and were isolated from 2 separate bird feces samples. None of the four isolates from Talbert Marsh sand samples nor the two isolates from Newport Beach seagull feces samples produced F-RNA product when subjected to RT-PCR.

Enriched Seagull Feces Isolates

RT-PCR & RLB

11/25 (44.0%) of the picked plaque suspensions described above produced an F-RNA product but did not produce an RLB band. These 11 isolates plus the remaining 71 picked plaque suspensions from Talbert Marsh seagull feces samples that did not produce an F-RNA product were enriched. Also seen in Tables 4.3 and 4.4, 37/82 (45.1%) of these enrichments, representing 14 separate fecal samples, produced F-RNA product when subjected to RT-PCR. The 11 enrichments whose plaque suspensions originally produced F-RNA products, but did not produce an RLB band, did so again. Nine of these isolates then produced RLB bands. The other 26 enrichments, representing 11 fecal samples, 6 of which were previously not identified as positive fecal samples, produced F-RNA products following enrichment. Of the 37 total enrichments that produced product, 29/37 (78.4%) produced RLB bands: 8/29 (25.6%) were MS2-like (G I) and were isolated from 6 separate fecal samples and 21/29 (72.4%) were GA-like (G II) and were isolated from 7 separate fecal samples. No RT-PCR product was produced following enrichment of the four isolates from four Talbert Marsh sand samples or of the two isolates from one Newport Beach seagull feces sample.

F-DNA PCR

Coliphage enrichments that did not produce products using the F-RNA RT-PCR assay were subjected to an F-DNA PCR assay. Ten enrichments were assayed individually and enrichments of the remaining 42 isolates were pooled into groups of five, using 5 ul of each isolate. As seen in Table 4.3, neither individual nor pooled isolates produced an F-DNA product.

Spot Plating

All F-RNA RT-PCR positive isolates that were spot plated produced plaques except one. This isolate had a very faint RT-PCR reaction band and did not produce an RLB band. Nine of the ten F-RNA RT-PCR negative isolates that were spot plated did not produce plaques.

Sequencing

F-RNA RT-PCR products of the eight isolates that did not produce RLB bands were purified and sequenced using the MJV82 forward primer. One isolate (T33) did not produce useable sequence data upon several sequencing attempts.

Results Summary of Seagull Feces Isolates

In total, 51/96 (53.1%) bird feces isolates, including both originals and enrichments of originals, from 21 separate seagull fecal samples, produced RT-PCR bands, confirming the presence of F-RNA coliphages. No F-DNA coliphages were detected in any of the original isolates or enrichments of original isolates. As indicated in Table 4.4, 43/96 (44.8%) F-RNA isolates were able to be typed by the RLB method. Overall, isolates from fifteen different

seagull fecal samples were able to be typed by the RLB method. Two of these samples had mixed F-RNA coliphage populations; samples Q9 and U7 each had both MS2-like (G I) and GA-like (G II) F-RNA coliphages.

Seven of the isolates that were unable to be typed by RLB were sequenced and analyzed (see Figures 4.2 and 4.3). Four of these isolates are categorized as belonging to the *Levivirus* family by sequence analysis. Within the family they were most similar to the GA prototype strain. However, pairwise analyses indicate that sequences of these four strains are an average of 16.1% different than the GA prototype strain in the region sequenced. These sequences cluster most closely with each other, with an average of 95.0% similarity in the region sequenced. They also cluster closely with H5-1, one of the gull isolates that was positively identified as GA-like by RLB and was previously sequenced for the database.

The three remaining isolates, designated P22, P24, and P27, are categorized as belonging to the *Allolevivirus* family by sequence analysis. Within the family, these sequences are most similar to the Fi prototype strain. However, pairwise analyses indicate that sequences of these three strains are an average of 22.8% different than the Fi prototype strain in the region sequenced. Two of the isolates (P24 and P27) have identical sequences in the region amplified. A pairwise alignment shows that the third isolate is 83.0% similar to the other two strains in the region sequenced. Isolate P22 clusters closely with a previously sequenced strain (G3&4_7) that was also unable to be genotyped using the RLB method.

F+ Coliphages Isolated from Estuarine Waters--EPA Method 1601

Original Isolates

Forty-four discrete plaques were originally isolated from ten separate water samples. As seen in Tables 4.3 and 4.4, 5/44 (11.4%) of picked plaque suspensions representing 2 separate water samples, produced F-RNA product when subjected to F-RNA RT-PCR. Of these, 4/5 (80.0%) produced RLB bands: 4/4 (100%) were MS2-like (G I).

Enriched Water Isolates

RT-PCR & RLB

One of original picked plaque suspensions produced an F-RNA product but did not produce an RLB band. This isolate plus the remaining 39 picked plaque suspensions (or their secondary enrichments[1]) that did not produce an F-RNA product were enriched. Additionally, 37 aliquots of non-plaquing chloroform extracts[3] originally obtained from enrichments (EPA 1601) of six estuarine water samples were again enriched. Of these 77 total enrichments, six isolates (7.8%) representing four separate water samples, produced F-RNA product when subjected to F-RNA RT-PCR. The one enrichment whose plaque suspension originally produced F-RNA products did so again. The remaining five enrichments, three separate water samples in total, each of which were previously unidentified as a positive water sample, produced F-RNA products following enrichment. One of these enrichments was a chloroform-extracted enrichment. Of the total six

[3] These isolates are chloroform extractions of enrichments that did not plaque, likely because of *E. coli* host growth problems; after they did not form plaques the chloroform extractions were re-enriched and stored; three of these six samples also contained enrichments that did originally produce plaques on *E. coli* host agar

enrichments that produced product, 4/6 (66.7%) were MS2-like (G I) while the remaining two isolates did not produce an RLB band.

Spot Plating

All F-RNA RT-PCR positive isolates produced plaques when spotted on agar containing host cells. Eight of the ten F-RNA RT-PCR negative isolates that were spot plated did not produce plaques.

F-DNA PCR

Coliphage enrichments that did not produce products using the F-RNA RT-PCR assay were subjected to an F-DNA PCR assay. Ten enrichments were assayed individually and enrichments of the remaining 62 water sample isolates were pooled into groups of five, using 5 ul of each isolate. As seen in Table 4.3, neither individual nor pooled isolates produced an F-DNA product.

Sequencing

F-RNA RT-PCR products of the two isolates that did not produce RLB bands were purified and sequenced using the MJV82 forward primer. One isolate (X300-2) did not produce useable sequence data upon several sequencing attempts. Isolate I300-2 was sequenced and determined to be MS2-like (G I) upon comparison with available sequences of known prototype strains using Clustal W. In the region amplified, this isolate had identical sequence as the group I (MS2-like) prototype strain (data not shown). Because the RLB probes were designed using the prototype strains, this isolate should have produced an

RLB band. Lack of RLB data for this isolate suggests a technical error. Further comparison of this isolate and other MS2-like isolates from both estuarine water and seagull feces is presented below under "Sequence Comparison Between Sample Types" and in Figure 4.4.

F+ Coliphages Isolated from Estuarine Waters--EPA Method 1602

Original Isolates

One-hundred twelve discrete plaques were originally isolated from 11 separate water samples. As seen in Table 4.3, none of the isolates produced F-RNA product when subjected to F-RNA RT-PCR.

Enriched Water Isolates

RT-PCR & RLB

Ninety-nine of the original 112 plaque isolates were enriched. As seen in Table 4.3, none of the enrichments produced F-RNA product when subjected to F-RNA RT-PCR.

Spot Plating

None of F-RNA RT-PCR negative isolates that were spot plated produced plaques. There were no F-RNA RT-PCR positive isolates to assay.

F-DNA PCR

Coliphage enrichments that did not produce products using the F-RNA RT-PCR assay were subjected to an F-DNA PCR assay. Eight enrichments were assayed individually and enrichments of the remaining 92 water sample isolates were pooled into groups of five, using

5 ul of each isolate. As seen in Table 4.3, neither individual isolates nor pooled isolates produced an F-DNA product.

Results Summary of Estuarine Water Isolates

In total, for both detection methods used, 10/193 (5.2%) estuarine water sample isolates, from five water samples, including both originals and enrichments of originals, produced RT-PCR bands, confirming the presence of F-RNA coliphages. Positive samples were only found using Method 1601. As indicated in Table 4.4, 8/193 (4.1%) isolates were typed as MS-like (G I) F-RNA coliphages by the RLB method. No F-DNA coliphages were detected in any of the original isolates or enrichments of original isolates.

Sequence Comparison Between Sample Types

As seen in Figure 4.4, 17 of the 19 seagull feces and water strains typed as MS2-like (G I) fell within 90% nucleotide sequence similarity when sequenced and analyzed. Two isolates (U72 and U81), both seagull feces isolates, were slightly less similar to the other isolates (~88% similarity), but both were very similar to each other. Six water isolates collected at various times were 100% similar to the prototype MS2 strain in the nucleotide sequence analyzed. In general, each cluster within the phylogenetic tree is comprised of isolates that originate from the same sample type (i.e. seagull feces or water). However, isolates originating from the same sample of water or feces collected at a specific time (i.e. U1-1 and U1-4; F3001 and F3003) often clustered separately.

To further analyze the differences between isolates, nucleotide sequences were converted to amino acid sequences and aligned. As seen in Figure 4.5, amino acid sequence differences

were much less pronounced (~1.9% sequence difference). Sixteen of the isolates and the MS2 prototype strain had identical amino acid sequence in the region studied (data not shown). This indicates that although nucleotide differences did exist, the differences overall did not change the amino acids produced, resulting in production of identical proteins in the region studied. Two seagull feces isolates (U7-2 and U8-1) and one water isolate (F300-3) each produced one differing amino acid (isoleucine versus valine) in the same location. As seen in Figure 4.4, these three isolates were the least similar to the other isolates studied and the MS-2 prototype strain.

Discussion

A subset of F+ coliphages originally isolated from seagull feces and estuarine water samples were genotyped using RT-PCR and RLB analyses. In some cases, picked plaques stored in TSB/glycerol contained enough coliphage template to produce a positive F-RNA RT-PCR product. In other cases, low numbers of coliphages in picked plaques that were stored at -80 C required enrichment before F-RNA RT-PCR analyses were identified as positive. In order to determine whether enrichment had an effect on the nucleic acid sequence of coliphages, two original isolates and their enrichments were sequenced and compared. In the region that was amplified, sequences were identical between original and enriched phages. This indicates that the enrichment process did not alter nucleic acid sequence.

F-RNA coliphages were detected by RT-PCR in one-half of the presumptive positive seagull feces isolates, while very few F-RNA coliphages (approximately 5% of total isolates analyzed) were detected by RT-PCR in presumptive positive water samples. A majority of

the isolates positive for F-RNA by RT-PCR (84.3% of gull isolates and 80.0% of water isolates) were typeable by the RLB analysis. Although Cole et al. (Cole, Long et al. 2003) reported a majority of F-RNA coliphages from seagull feces as being group IV, most bird feces phages in this study were typed as GA-like (G II), which is the group generally used to indicate the presence of human fecal contamination. It is important to note that seagulls in the current study had frequent access to a nearby sewage treatment plant (McGee 2005). Previous studies have shown that seagulls consume food at or near these types of plants (Snoeyenbos, Morin et al. 1967; Fenlon 1981; Butterfield, Coulson et al. 1983; Reed, Meece et al. 2003) and GA-like (G II) coliphages have been detected in large numbers in human sewage (reviewed in (Grabow 2001)). Analysis of F-RNA coliphages isolated from the Orange County Sewage District sewage and sewage sludge where these seagulls are known to feed is necessary to establish this link. However, such analysis was not done in this study.

F-RNA coliphages from water samples genotyped as MS2-like (G I); similar reports have been documented in the available literature (Griffin, Stokes et al. 2000; Brion, Meschke et al. 2002; Cole, Long et al. 2003). The absence of GA-like (G II) coliphages, which were the majority found in seagull feces, suggests that gull feces may not have an impact on surrounding waters. However, a number studies have found that the majority of F-RNA coliphages isolated from environmental waters were MS2-like (G I) (Griffin, Stokes et al. 2000; Brion, Meschke et al. 2002; Cole, Long et al. 2003). Furthermore, Long et al (Long, El-Khoury et al. 2005) found that MS2-like (G I) coliphages survive longer in lake water at both 4 and 20 C than do the other subgroups. Therefore, other subgroups of F-RNA coliphages (i.e. GA-like coliphages) may have originally been present in water, but did not persist as well as the MS2-like coliphages. It is likely that the coliphages isolated from water

samples had more exposure to adverse environmental conditions prior to isolation than did the coliphages isolated from seagull feces. DNA damage due to these unfavorable conditions, which was likely increased by freeze-thawing events after isolation, may have led to these low detection levels.

In environmental waters, F-DNA coliphages have been reported to occur concurrently with F-RNA coliphages, and often in greater numbers than F-RNA coliphages during certain months of the year. However, in these studies, waters were impacted by human and swine wastewaters (Cole, Long et al. 2003). Although the Talbert Marsh seagulls likely feed at the adjacent sewage treatment plant, no F-DNA phages were detected in either seagull feces in this study. The lack of F-DNA coliphages in seagull feces is somewhat supported by a study that reports very low numbers of F-DNA coliphages from seagull feces (4% of F+ coliphages evaluated) (Cole, Long et al. 2003). The fact that no F-DNA coliphages were found in seagull feces is therefore consistent with the absence of F-DNA coliphages in estuarine waters impacted by these feces. The presence of F-DNA coliphages in Talbert Marsh would likely suggest sources of fecal contamination other than seagulls. As described for F-RNA coliphages above, freeze-thaw events may have damaged the DNA in the annealing regions for PCR primers, thereby preventing amplification. Again it is possible, although unlikely, that F-DNA coliphages had unique nucleic acid sequences in the regions targeted by primers and were therefore undetected by the PCR assay.

To compare the sensitivity of the RT-PCR method to a traditional plaque assay method, subsets of enriched isolates that were RT-PCR positive and RT-PCR negative were spot-plated on agar containing *E. coli* host cells. Of all RT-PCR-positive isolates, only one isolate (T33) was RT-PCR positive for F-RNA coliphage but unable to produce a plaque on a host

agar plate. After three separate sequencing attempts, no useable sequence data for the T33 isolate were produced. The implication is that significant portions of the RNA were damaged, which likely caused the loss of infectivity. Indeed, the use of molecular assays such as RT-PCR requires only that the correct nucleic acid template be present, not that an organism be infectious or even viable. For this subset of isolates, RT-PCR was a more reliable indicator of the presence of F-RNA coliphages than was plaque formation. Overall, these results demonstrate an advantage that molecular-based methods have over traditional microbiological methods, especially when applied to isolate samples stored frozen such that degradation of the coliphage or its nucleic acid could occur.

On the other hand, three RT-PCR-negative enrichments produced plaques on agar-host cell lawns. One possibility is that these were F-RNA coliphages that had unique nucleic acid sequences in the regions targeted by the primers and were therefore undetected by the RT-PCR assay. However, because the RT-PCR primers were designed and tested on a wide range of environmental isolates (Vinje, Oudejans et al. 2004), this is unlikely. The results from these three isolates seem to diminish the reliability of the RT-PCR method over the traditional plaque assay. Further analyses, such as sensitivity to RNase, serotyping, and nucleotide sequencing, on the coliphages present in these three enrichments are necessary before definitive conclusions about their identity can be made.

In general, methodological problems, including plaque-picking inaccuracies or the presence of air bubbles mistaken for plaques, may have resulted in the lack of F-RNA coliphage during the original isolation from both seagull feces and water samples. Furthermore, F-RNA coliphage nucleic acid may have been present in the stored suspensions at one time, but suffered damage in the RT-PCR annealing regions (likely during freeze-

thaws), thereby preventing RT-PCR amplification. Lastly, another possibility is that F-RNA coliphages isolated in this study had unique nucleic acid sequences in the regions targeted by the primers and were therefore undetected by the RT-PCR assay. However, this is unlikely because the RT-PCR primers were designed and previously tested on a wide range of environmental isolates (Vinje, Oudejans et al. 2004).

It is important to note that several biases based on methodologies may have been introduced during the original isolation of and subsequent microbiological work on coliphages, therefore affecting the outcome of molecular analyses. One possibility is that the enrichment process originally used on a subset of water samples (EPA method 1601) preferentially enriched one type or subgroup of coliphage over others. Selection of only a subset of plaques from host agar plates may have also introduced a form of bias. It is possible that both F-DNA and more than one group of F-RNA coliphages were present on plates, but were not all picked for further molecular analyses, therefore underestimating the number and types present.

Based on sequence analyses performed in this study, isolates in which F-RNA coliphages were detected by RT-PCR but were not typed by RLB clustered separately from prototype strains and other previously genotyped coliphages. These isolates did cluster closely to each other, suggesting that they were not random oddities, but likely represent a subset of phages undescribed in the available database. These findings are somewhat analogous to those recently reported by Stewart et al. (Stewart, Vinje et al. 2006) who detected unique Qβ-like (G III) isolates of F+ coliphages from water and fecal waste samples that were not detectable by available G III nucleic acid probes. Although the RT-PCR primers were general enough to detect and separate these coliphages by family, RLB probes may need to be redesigned to

incorporate sequence differences making possible more specific differentiation between subgroups.

It is difficult to definitively say whether the similarities among the MS2-like nucleotide sequences of F-RNA coliphages isolated in this study are compelling enough to link those coliphages from Talbert Marsh estuarine waters samples to those from seagull feces. The isolates analyzed in this study are overall relatively similar in both nucleotide and amino acid sequence and therefore may have come from the same population. However, the replicase gene that was used for identification represents one portion of the F-RNA coliphage genome. Similarities within subgroups in this one gene do not necessarily reflect similarities over the genome as a whole. While this gene is useful for some level of differentiation, other regions may have more sequence differences that can further differentiate subgroups. No other studies to date have looked at other regions in the F-RNA genome for differences between the different subgroups and this represents an area that requires further investigation.

For microbial source tracking purposes, we seek genetically identical isolates to link a microbe found in the environment with one from a known source of contamination. However, this is often difficult to accomplish due to a number of factors, largely because of the biological properties of the microbes. F-RNA coliphages are positive-sense RNA viruses, meaning upon entry into the host cell, viruses take over host cellular machinery. The genomic RNA, which is identical with the mRNA, is copied into a minus strand, which then acts as a template for efficient synthesis of more plus strands, or mRNA. Despite their efficiency however, RNA viruses have an extremely high rate of mutation due to the lack of DNA polymerases that find and repair sequence mistakes. As Drake et al. (Drake and Holland 1999) explain, “because a single cycle of infection produces a mutant frequency

twice that of the rate per genome replication, few progeny viruses escape mutation." In this case, it is highly possible that the viral nucleotide sequence infecting a host cell will not be exactly the same sequence as the progeny viruses that are released. In many and most cases, the mutations that occur in RNA viruses are deleterious (Drake and Holland 1999). Therefore it is likely that silent mutations in RNA viruses, or as those that do not alter the amino acid sequence, as seen in most of the sequenced isolates in this study, are generated variants of a limited number of strains and are able to persist because natural selection has no effect on their ability to persist (van Belkum, Struelens et al. 2001).

Furthermore, genetic recombination is also common in RNA viruses. In the case that more than one F-RNA coliphage infects a cell, genetic recombination between viral particles may lead to the formation of quasi-species. These viruses are closely related, but non-identical mutants of parental strains, which may then persist and/or continue to mutate upon subsequent infection cycles. Based on this occurrence and the high replication and mutation rates found in RNA viruses, it is understandable that microbial source tracking methods based on very similar or identical nucleotide sequences of F-RNA coliphages is problematic.

While microbiological methods detected F-RNA coliphages in both seagull feces and water samples, molecular methods were unable to confirm all of these isolates. Further work and a larger dataset of isolates, especially from water samples, are required to fully compare the types of F+ coliphages present in seagull feces with those in Talbert Marsh estuarine waters. Furthermore, all plaques on host cell agar should be picked and analyzed to more completely classify the range of F+ coliphage types present. Finally, molecular analyses should ideally be performed upon fresh coliphage enrichments, since picked plaques may not

provide enough template, and over time, freezer storage may result in RNA damage and loss of detectability.

Overall, reliable identification and quantification of fecal contamination sources is necessary for manipulating water systems and their release of fecally-contaminated water. This study assessed the genotypes of F+ coliphages found in both the feces of seagulls in Talbert Marsh and in the surrounding estuarine waters impacted by seagull feces. The data provide an initial assessment of seagull fecal contamination and a foundation to be built upon. Further research is required in order to make a more definitive determination of the impact of seagull feces on Talbert Marsh waters.

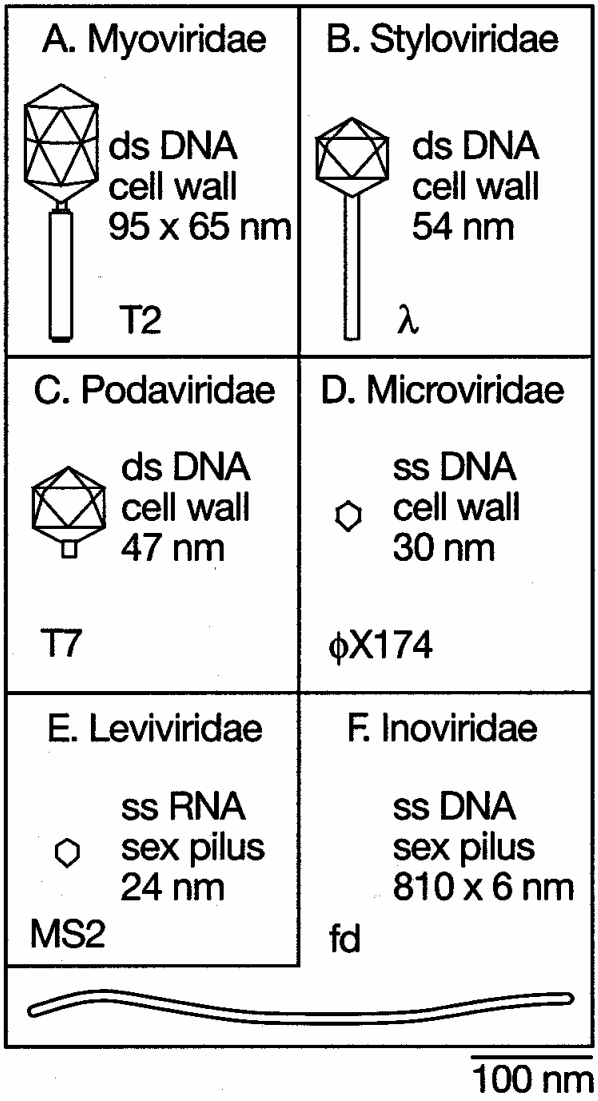

Figure 4.1: Major families of bacteriophages. Each box shows: morphological group, family name, morphology and name of type species, type of nucleic acid, situation of receptor in host-cell, dimensions of capsid (head in case of tailed bacteriophages) of type species (IAWPRC Study Group on Health Related Water Microbiology 1991)

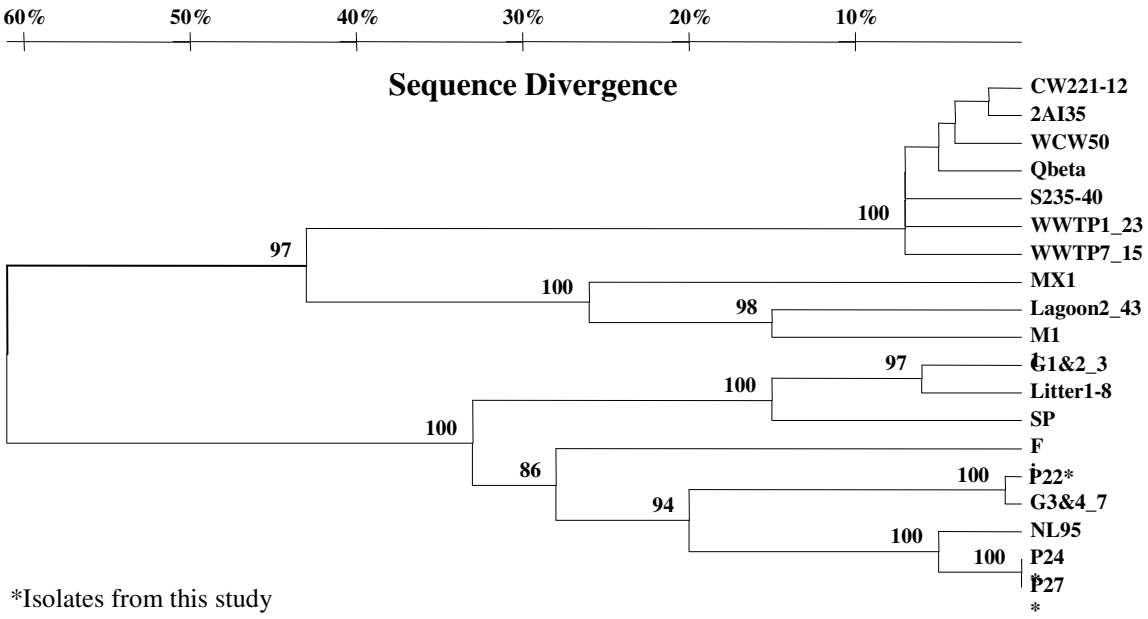

Figure 4.2: ***Allolevivirus*** **nucleotide sequence analysis. The four strains analyzed were most similar to the GA-like (G II) prototype strain, however, pairwise analyses indicate that these sequences were an average of 16.1% different than the GA-like prototype strain in the region sequenced.**

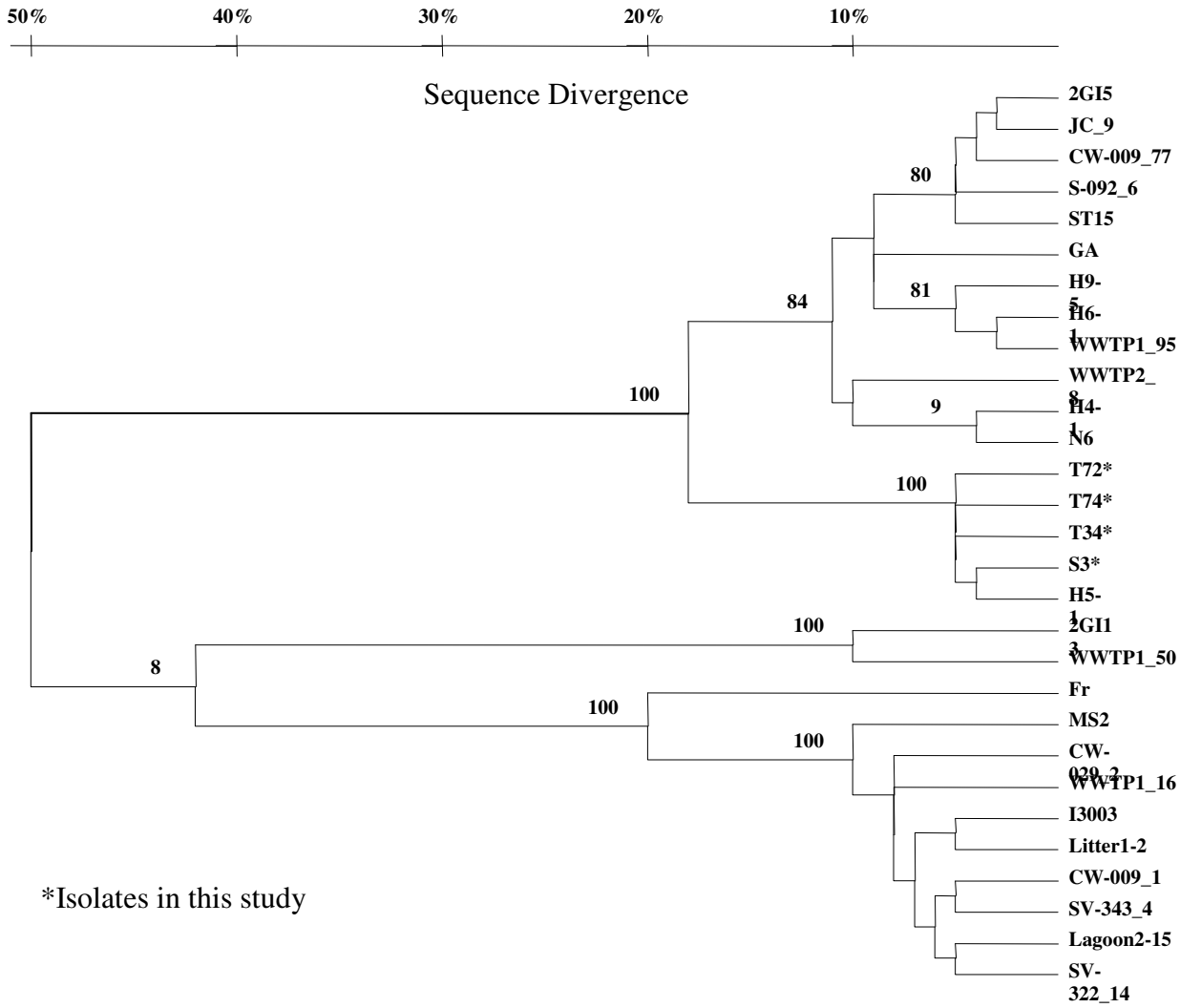

Figure 4.3: ***Levivirus*** **nucleotide sequence analysis. The three strains analyzed are most similar to the Fi-like (G IV) prototype strain, , however, pairwise analyses indicate that these sequences were an average of 22.8% different than the Fi-like prototype strain in the region sequenced.**

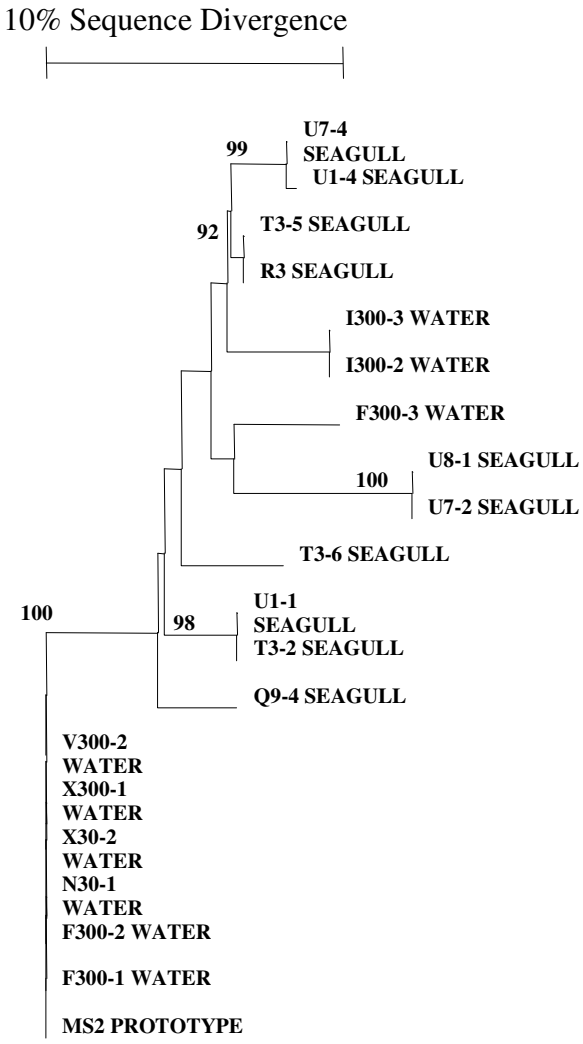

Figure 4.4: Comparison of nucleotide sequences of MS2-like (G I) coliphage isolates from Talbert Marsh seagull feces and water samples. A majority (89%) of the strains were within 90% nucleotide sequence similarity. Two isolates (U72 and U81) were slightly less similar to the other isolates (~88% similarity), but both were very similar to each other. Six water isolates collected at various times were 100% similar to the MS2-like prototype strain in the nucleotide sequence analyzed.

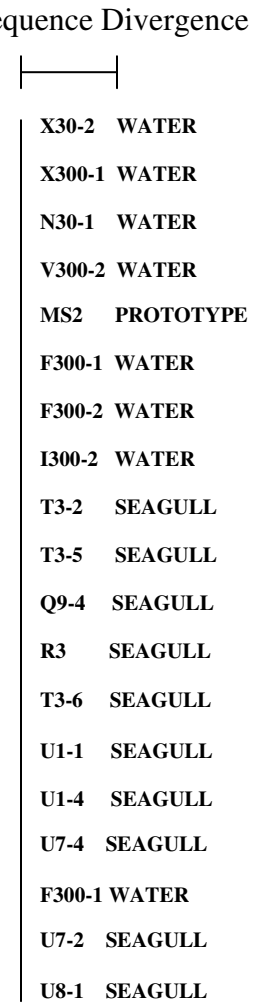

Figure 4.5: Comparison of amino acid sequences of (MS2-like (G I) coliphage isolates from Talbert Marsh seagull feces and water samples. Amino acid sequence differences were much less pronounced than nucleotide sequence differences. Sixteen of the isolates and the MS2-like prototype strain had identical amino acid sequence in the region studied.

Oligonucleotide Identity[a]	Sequence (5' - 3')[b]							Orientation	Target[c]
Primer									
JV41	CCC	ATD	GAR	GAW	ATY	TTC	TC	-	3
JV81	CCA	RAA	DAT	CAT	GGA	CTC		-	2
MJV82	GTA	TAG	AYC	TKA	AYG	AYC	A	+	2 and 3
SL2	GTA	ACT	TGG	TAT	TCA	AAG	CA	+	1
SL3	AAG	GAG	CTT	AAT	TAT	CAT	CA	-	1
Probe									
MS2	GAG	ACG	ATA	CGA	TGG	GAA	C	+	2
GA	AAT	CAG	GAR	TTA	GCC	CTA		+	2
Qβ	CAC	GAA	GGC	TCC	GTT	AMT	AAT	+	3
M11	GGC	AGC	CGT	GAY	GAT	AA		+	3
SP	GAT	GGG	TCT	YTG	CTA	AAT	CAT	+	3
FI	GCA	GCC	AGC	GAC	TCT	AT		+	3

[a] Probes are 5' hexylamine labeled
[b] IUPAC codes to indicate degenerate positions
[c] 1, inovirus; 2, levivirus; 3, allolevivirus

Table 4.1: Oligonucleotides for detection and genotyping of F+ coliphages used in this study (Vinje, Oudejans et al. 2004)

Sample Type	Total Samples Analyzed	Volume Homogenate Analyzed (ml)	# Plaque-forming Samples (%)	# Isolates Archived
TM seagull feces	109	1	31 (28.4)	96
TM sand samples	10	1	4 (40.0)	4
NB seagull feces	10	1	1 (10.0)	2
TM water samples				
1601*	18	1000	10 (55.6)	44
1602	20	100	10 (20.0)	112

TM: Talbert Marsh
NB: Newport Beach

Table 4.2: Presumptive positive F+ coliphages in samples analyzed

Sample Type	# Samp. presumptive MSP + (%)	# Isolates presumptive MSP + (%)	Total # F-RNA RT-PCR + Samp. (%)	Total # F-RNA RT-PCR + Isolates (%)	Total # F-DNA PCR + Samp. (%)	Total F-DNA PCR + Isolates (%)
TM seagull	31	96	21 (67.7)	51 (53.1)	0 (0.0)	0 (0.0)
TM sand	4	4	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
NB seagull	2	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TM water						
1601	13	81	5 (38.5)	10 (12.3)	0 (0.0)	0 (0.0)
1602	11	112	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

TM: Talbert Marsh
NB: Newport Beach

Table 4.3: F-RNA RT-PCR and F-DNA PCR results for samples analyzed

Sample Type	Total # F-RNA RT-PCR + Samp. (%)	Total # F-RNA RT-PCR + Isolates (%)	Total # MS2-like Isolates (%)	Total GA-like Isolates (%)	Total Qβ-like Isolates (%)
TM seagull	21 (67.7)	51 (53.1)	11 (21.6)	27 (52.9)	5 (9.8)
TM water (1601)	5 (38.5)	10 (12.3)	8 (80.0)	0 (0.0)	0 (0.0)

TM: Talbert Marsh

Table 4.4: RLB results for F-RNA RT-PCR-positive isolates

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Chapter V

Final Discussion

A growing public health concern in our coastal environments is the impact of fecal contamination from both human and animal sources. Often the presence of fecal contamination in these environments carries the threat of exposure to waterborne pathogens capable of causing infectious disease in the millions of humans who come into contact with coastal waters. Multiple adverse health outcomes have been linked to exposure to marine recreational water of poor microbiologic quality. As highly urbanized coastal areas rapidly grow, multiple sources of both point and non-point fecal contamination develop and significant environmental degradation often occurs. Despite regulations and management systems to address these impacts, there is inadequate and uncertain information on them in many coastal environmental settings.

Talbert Marsh, located in Orange County, CA, is a tidally-influenced constructed wetland that receives large inputs of urban runoff and serves as a main outlet from Talbert Watershed to the Pacific Ocean at Huntington Beach. During flood tides, water flows from the Pacific Ocean through Talbert Marsh and inland along the channel network; during ebb tides, water flows out of the channel network through Talbert Marsh and into the Pacific Ocean. Talbert Marsh was built to not only provide control of urban runoff but, among a number of other purposes, to create a wildlife habitat. Hundreds of wild birds, specifically seagulls and terns, take daily refuge on the sandflats of the marsh for nesting and feeding purposes.

Wild birds have long been considered important non-point sources of fecal contamination to surface waters, excreting both fecal indicator bacteria and enteric pathogens. Two common bacterial human pathogens commonly harbored by birds are *Salmonella* and *Campylobacter*, both of which cause acute gastroenteritis in humans. Indeed, *Campylobacter* is the leading cause of bacterial gastroenteritis in the United States. In rare cases, both pathogens can lead to serious sequelae, including arthritic symptoms, recurrent colitis in the case of *Salmonella* infection and Guillain-Barré syndrome in the case of *Campylobacter* infection. Additionally, fecal indicator microbes, such as fecal coliforms, *Escherichia coli*, enterococci, and F+ coliphages, are commonly found in the feces of warm-blooded animals. These microbes have similar structure and persistence in the environment as many human pathogenic enteric bacteria and viruses, respectively, and are often used as indicators of fecal contamination.

A number of beach closures at Huntington State and City Beaches due to elevated fecal indicator bacteria levels in the summer months of 1999 prompted numerous investigations by local agencies to determine the source of contamination. Studies conducted at Huntington State and City Beaches made a connection between the elevated levels of fecal indicator bacteria in the surf zone of the beaches and the presence of these bacteria in bird feces, marsh sediments, and on marine vegetation in Talbert Marsh. These findings prompted this current research to look more closely at not only the potential sources of microbial contamination within Talbert Marsh, but at the types of bacterial pathogens that also could be present in the marsh.

The focus of this study was on the role of seagull fecal contamination as a potentially important contributor to the non-point sources of fecal contamination and in particular

Campylobacter species and *Salmonella* species as bacterial pathogens potentially present in this system. Additionally, the presence of fecal indicator microbes, such as fecal coliforms, *Escherichia coli*, enterococci, and F+ coliphages, was assessed. Finally, several genotypic methods were employed in this study to identify sources of fecal contamination as human or animal in origin. Source-specific information is becoming an important tool in decision making to manage fecal contamination of water as the analytical methods improve and become further validated.

The specific research aims of the study were to use conventional phenotypic and newer genotypic methods to identify and quantify *Salmonella*, *Campylobacter*, and F+ coliphages in the feces of seagulls present in Talbert Marsh. We also aimed to use conventional phenotypic and newer genotypic methods to identify and quantify *Salmonella*, *Campylobacter*, F+ coliphages, and fecal indicator bacteria, including fecal coliforms, *E. coli*, and enterococci, in estuarine waters that surround the sandflats in Talbert Marsh. Finally, we used microbial source tracking methods to characterize the presence and impacts of fecal contamination from seagulls in Talbert Marsh and its potential transport and presence into the nearby estuarine waters

The following sections represent the major research questions presented in this study and an overall summary and interpretation of findings.

1. **Do the feces of seagulls in Talbert Marsh contain the pathogens *Salmonella* and *Campylobacter* and/or specific groups of F+ coliphages, which serve as indicators of fecal contamination?**

Previous studies conducted in Talbert Marsh found an average of 228 birds present during the day and the largest congregation consisting of 1180 birds. Although no seagull-specific

census has been conducted in Talbert Marsh, studies estimate that gulls and elegant turns represent about 80% of the birds present. Based on the average deposition rate of seagulls in the marsh of one feces per bird every three hours, it can be estimated that about 7 kg of seagull feces are deposited in Talbert Marsh on a daily basis, and up to 36 kg on a worst-case basis. The sandflats were chosen as a sampling point for seagull feces, as they provide a wide, open area in the marsh for seagulls to congregate and feed. It can be suggested that this region is likely where the majority of seagull feces are deposited in Talbert Marsh.

For detection and enumeration of *Salmonella* and *Campylobacter*, seagull fecal samples were first submitted to a series of enrichment culture steps and subcultured onto appropriate selective agars for isolation of distinct colonies and subsequent biochemical testing to phenotypically identify the bacterial complexes or species present. To detect and enumerate F+ coliphages in the feces of seagulls present in Talbert Marsh, a known volume of freshly-deposited waterfowl feces was submitted to a Double Agar Layer plaque assay in which *E. coli* Famp host cell infection by F+ coliphages present in the feces was visualized and quantified. Using these conventional microbiological methods, we were able to determine that *Salmonella*, *Campylobacter*, and F+ coliphages were present in the feces of seagulls that congregate on the sandflats within Talbert Marsh.

The seagull *Salmonella* carriage rate of 8.0% and the arithmetic average concentration of positive *Salmonella* seagull fecal samples samples of 82 MPN/g both fall within the ranges found in the literature. Additionally, the carriage rate of *Campylobacter* in seagulls of 37% found in this study also falls within the range of carriage rates found in the literature. However, the arithmetic average MPN/g of positive *Campylobacter* seagull samples was 48, well below the concentrations found in broiler chickens. To the author's knowledge, this is

the first report on concentrations of *Campylobacter* found in seagull feces. Finally, the carriage rate of F+ coliphages in seagull feces in this study was found to be 28%, which is lower than the rates previously reported in the literature. The arithmetic average of 1.4×10^3 PFU/g for positive F+ coliphage seagull samples was within the range of one reported study, but substantially higher than another.

Although low numbers of *Salmonella* were isolated from seagull feces, these preliminary data represent important findings. PFGE analysis found that there was a wide variation in the strain types of *Salmonella* found between and among seagull feces samples present in Talbert Marsh. Furthermore, similarities were noted between PFGE and MIC results among isolates, with unique PFGE strains often exhibiting intermediately-resistant or resistant responses to the antimicrobials against they were tested.

In general, low numbers of seagull feces positive for microbes prevented rigorous statistical analysis of seasonal patterns both in presence and concentrations. However, for *Campylobacter*, Autumn and Winter months had considerably lower observed values than expected values, while Spring and Summer months had considerably higher observed values than expected values. Co-occurrence of the microbes found in seagull feces was generally low and the presence of one microbe could not predict the presence of another microbe. Some results do indicate a slight degree of positive correlation, however, data may be skewed by the low numbers of positive samples and the dominance of one or a few outlying values. Overall results from statistical analyses based on both of these factors were generally inconclusive.

Because carriage rates and concentrations of the microbes in individual seagull feces were relatively low, coupled with anticipated short carriage and excretion periods (average of four

days), it seems unlikely that gull feces could have a consistently large microbial impact on the microbiological quality of environmental waters. However, large numbers of gulls often congregate near waters and the collective contribution of gulls, especially ones who have previously fed at sewage or dump sites such as OCDS, may potentially have a great impact on water quality, at least periodically. In this study, average estimated daily loads into Talbert Marsh for *Salmonella*, *Campylobacter*, and F+ coliphages were determined to be 5.7 x 10^5, 8.4 x 10^4, and 1.1 x 10^5, respectively. Using highest bird counts and microbe concentrations recorded in Talbert Marsh, these numbers become elevated by three to four $\log_{10}$.

Furthermore, the carriage rates described in this study may be underestimated for several reasons. Single fecal samples, and not entire cloacal washings, were collected only from a subset of seagulls, and may not reflect representative sampling from the entire population present in Talbert Marsh. Additionally, because samples were collected post-defecation and analyzed 24 hours post-collection, temperature fluctuations, exposure to UV light, and exposure to low pH due to uric acid in the fecal samples and other environmental stressors could have likely decreased numbers of culturable microbes present.

Following isolation of presumptive positive *Salmonella*, *Campylobacter*, and F+ coliphages, from seagull feces, each of the isolates were preserved in appropriate storage media and stored at -80 C. Stored presumptive culture-positive *Campylobacter* cell suspensions were subjected to a number of molecular-based genotypic methods for identification, differentiation, and for further isolate characterization. In total, about 36% of the presumptive culture-positive *Campylobacter* isolates from Talbert Marsh seagull feces were confirmed as *Campylobacter* using molecular methods. However, a majority of colony

isolates from these enrichments were unable to be confirmed as *Campylobacter* spp. by these methods. The most likely possibility for this low percentage of positive PCR confirmation is that the original presumptive culture-positive isolates were not thermotolerant *Campylobacter*, but other bacterial genera resulting from the enrichment process. To determine the identity of these potentially non-*Campylobacter* bacteria, further molecular testing is required. The possibility of isolation of non-*Campylobacter* bacteria was also supported by the negative results obtained from the application of a robust *Campylobacter* genus PCR to the isolates deemed negative by the other PCR assays. No *C. coli* were detected in stored isolates, however both *C. jejuni* and *C. lari* were detected in seagull feces, as has been previously reported in seagulls. Of the 375 *Campylobacter* presumptive culture-positive isolates tested, 13% were found to be *C. jejuni* and 12% were found to be *C. lari*.

Lack of *Campylobacter* resuscitation in this study is consistent with the well-known phenomenon of limited *Campylobacter* cell viability and recovery following freezing and thawing. Genotypic methods were also possibly hampered by several factors, including the presence of inhibitors in the storage media and substantial DNA damage due to freeze-thaw events, limiting the reliability of the PCR assays. Lack of enough cells or DNA for PCR analysis was discredited, as microscopic techniques employed on stored cell suspensions indicated that cell concentrations were in the 10^5 to 10^6 cells/µl range, and at least a portion of the cells were determined to be viable. Furthermore, PCR species-specific primers were not broadly effective enough to identify species in isolates that were later confirmed as closely related to *C. lari* or *C. jejuni* by nucleotide sequence analysis.

In conclusion, data from studies focused on the detection and identification of *Campylobacter* in seagull feces samples resulted in three important findings. First, these

results are the first to report on concentrations of *Campylobacter* found in seagull feces. Secondly, results support the idea that conventional microbiological methods used for the isolation of thermophilic *Campylobacter* spp. may also enrich other bacteria present in samples and therefore lead to false positive results when used alone. Thirdly, the modified multiplex PCR method used was somewhat effective on characterization of culture-positive *Campylobacter* isolates that have been stored at -80 C temperatures.

Stored presumptive positive F+ coliphage plaque suspensions (or enrichments of these suspensions) isolated from Talbert Marsh seagull feces were also subjected to a number of molecular-based genotypic methods for identification, differentiation, and for further characterization. A recently described method that uses reverse-transcription PCR (RT-PCR) and a reverse line blot hybridization (RLB) assay was used to molecularly detect and genotype F+ coliphages. F-RNA coliphages were detected by RT-PCR in one-half of the presumptive positive seagull feces isolates and a majority of the isolates (84%) positive for F-RNA were further typeable by RLB. Although other studies report a majority of F-RNA coliphages in seagull feces as being from subgroup four (G IV), most seagull feces phages in this study were typed as GA-like (G II), the group generally used to indicate the presence of human fecal contamination. Seagulls in this study had frequent access to a nearby sewage treatment plant and previous studies have shown that seagulls consume food at or near these types of plants and could have therefore picked up these coliphages during feeding. Analysis of F-RNA coliphages isolated from the Orange County Sewage District sewage and sewage sludge where these seagulls are known to feed is necessary to conclusively establish this link. No F-DNA coliphages were isolated from seagull feces, which somewhat supports previous research that found very low occurrence of these coliphages in seagulls, despite that fact that

F-DNA coliphages are also generally found in human sewage and wastewaters, such as the ones that these seagulls are known to feed upon.

In general, methodological problems and biases introduced during the isolation from seagull feces may have resulted in the low numbers of F-RNA coliphages originally isolated. Furthermore, F-RNA coliphage nucleic acid present in the stored suspensions may have suffered damage in the RT-PCR annealing regions (likely during freeze-thaws), thereby preventing RT-PCR amplification. Lastly, another possibility is that F-RNA coliphages isolated in this study had unique nucleic acid sequences in the regions targeted by the primers and were therefore undetected by the RT-PCR assay. A number of F-RNA coliphages that were detected by RT-PCR but were unable to be typed by RLB were sequenced for analysis. These isolates clustered closely together, but separately from previous-characterized isolates that were used to design the RLB probes. These findings are somewhat analogous to recent reports of the detection of unique Qβ-like (G III) isolates of F-RNA coliphages from water and fecal waste samples that were not detectable by available Qβ-like (G III) nucleic acid probes. Although the RT-PCR primers were general enough to detect and separate these coliphages by family in the present study, RLB probes may need to be redesigned to incorporate sequence differences making possible more specific differentiation between subgroups.

In conclusion, data from studies focused on the detection and identification of F+ coliphages in seagull feces samples resulted in two important findings. First, these studies found a majority of GA-like (G II) F-RNA coliphages in seagull feces, which contradicts previous reports on the human specificity of this F-RNA coliphage group. These results suggest the possibility that seagulls acquire these GA-like (G II) F-RNA coliphages by

feeding on human fecal waste sources at the adjacent sewage treatment plant prior to defecation in Talbert Marsh. Seagulls may therefore serve as a potential vector of pathogens also present in human sewage and sewage wastewater into Talbert Marsh. Further analysis of F-RNA coliphages isolated from the sewage and sewage sludge where these seagulls are known to feed is necessary to conclusively establish this link. Secondly, these results suggest that, while the available RLB method does successfully genotype F-RNA coliphages isolated from this environmental source, the probes involved are not robust enough to detect all potential F-RNA coliphages present in seagull feces. Further probe sequence modifications are required for more specific characterization of F-RNA coliphages.

2. **Do the waters surrounding the sandflats at which seagulls and other waterfowl congregate contain the pathogens *Salmonella* and *Campylobacter* and/or the same specific groups of F+ coliphages which serve as indicators of fecal contamination?**

Water samples collected upmarsh were intended to assess the microbial presence in the waters prior to contact with the sandflats or the impact of incoming tides and currents that could carry contaminants picked up from the sandflats; likewise, waters collected adjacent to and downmarsh of the sandflats were intended to assess microbial presence in the waters following contact with the sandflats and associated with outgoing tides and currents. For detection and enumeration of *Salmonella* and *Campylobacter*, known volumes of water samples were first submitted to a series of enrichment culture steps and subcultured onto appropriate selective agars for isolation of distinct colonies and subsequent biochemical testing to phenotypically identify the bacterial complexes or species present. Because coliphage concentration in the estuarine waters was unknown, two different US EPA

methods were employed to identify and quantify F+ coliphages in known volumes of estuarine water, one of which included an enrichment step. Both methods use *E. coli* Famp host cell for detection of infection by F+ coliphages. Additionally, water samples were analyzed for fecal indicator bacteria, including fecal coliforms, *Escherichia coli*, and enterococci, using Colilert and Enterolert defined-substrate quantal assays, respectively.

Although no *Salmonella* were isolated from Talbert Marsh water samples, *Campylobacter* spp., F+ coliphages, and fecal indicator bacteria, including fecal coliforms, *E. coli*, and enterococci, were detected. Approximately 29% of water samples analyzed in the study were positive for *Campylobacter* with MPN/L ranging from 1.4 to 11. Although these concentrations fall below the most recently estimated concentration required for infection, *Campylobacter* in Talbert Marsh water samples may be a greater potential human health threat than previously believed, and this topic deserves further attention. Eighty percent of water samples were positive for F+ coliphages, with PFU/L ranging from 10 – 630. Unexpectedly, the detection method that does not include an enrichment step detected F+ coliphages in a greater number of samples, and detected higher concentrations of F+ coliphages. Finally, all Talbert Marsh water samples analyzed were positive for enterococci and all but one of the samples analyzed were positive for fecal coliforms. Concentrations for fecal coliforms ranged from 0.0 – 398 MPN/100 ml, with a median concentration of 25 MPN/100 ml. All fecal coliform concentrations fell below California's current fecal coliform standard for contact recreation in ocean and bay waters of 400 MPN/100ml in a single sample. However, almost 57% of the samples analyzed exceeded California's current enterococci standard for contact recreation in ocean and bay waters of 104/100ml in a single sample. The range of concentrations of enterococci was 39.1 – 1300 MPN/100 ml, with a

median concentration of 108 MPN/100 ml. Finally, less than one-half of the samples analyzed were positive for *E. coli* and concentrations of these were relatively low, ranging from 0 – 27 MPN/100 ml. California's current standards for contact recreation in ocean and bay water do not include *E. coli*.

Seasonal trends for prevalence of microbes in Talbert Marsh water samples were not able to be confirmed statistically, as the total numbers of samples analyzed were too low for valid and meaningful analysis. However, prevalence of *Campylobacter* and F+ coliphages did appear to vary seasonally. In contrast to the seasonal differences described for *Campylobacter* in seagull feces, a higher percent-positivity of samples was noted in Autumn than in Summer in water samples. However, for F+ coliphages, a higher percent-positivity of samples was noted in Summer months, as also described for F+ coliphages in seagull feces. For *Campylobacter*, fecal coliforms, *E. coli*, and enterococci, analyses indicated that variations between concentration medians for each season were not significantly different at the 5% level, however, for F+ coliphages, a significant difference (at the 5% level) in concentrations occurred between the two seasons, with a higher median concentration in the Summer months than the Autumn months.

Co-occurrence analyses were performed for each pair of microbes isolated from water. As seen in similar analyses for microbes in seagull feces, some results do indicate a slight degree of correlation, but data may be skewed by the low numbers of positive samples and the dominance of one or a few outlying values. Overall, results from these statistical analyses were generally inconclusive.

Water sample location was also assessed in this study, although statistical analyses were unable to be performed, as numbers of samples was too low for valid and meaningful

analysis. However, results suggest that the number of positive samples for *Campylobacter*, F+ coliphages, and *E. coli* did vary by location. A higher number of observed than expected positive *Campylobacter* samples were found adjacent to the sandflats (although positive samples were also found upmarsh of the sandflats), while a higher number of observed than expected positive *E. coli* samples were found upmarsh of the sandflats. Neither *Campylobacter* nor *E. coli* were detected in samples taken from downmarsh of the sandflats. Finally, a higher number of observed than expected positive F+ coliphage samples were found downmarsh of the sandflats, although positive samples were also found both upmarsh and adjacent to the sandflats. It is important to note that factors including low sample volume, dilution, tidal stage, and environmental conditions all may have had an impact on prevalence and concentrations of microbes detected in water samples.

The low volume of water collected from this area of the marsh, in comparison to the average total volume of water in Talbert Marsh (approximately 120,000 L), may simply not have been a large enough volume for the detection of microbes, especially due to the daily flushing of Talbert Marsh waters. Furthermore, water samples were only collected during two seasons of the year and were therefore unrepresentative of the microbe levels and concentrations in the marsh on a yearly basis. Additionally, it is likely that all microbes originally present in these water samples did not survive environmental conditions, such as higher salinity and longer exposure to UV light, during travel within the marsh. Overall, it is important to note that factors including low sample volume, dilution, tidal stage, and environmental conditions all may have had an impact on prevalence and concentrations of microbes detected in water samples.

Following isolation of presumptive positive *Campylobacter* and F+ coliphages, isolates of each were preserved in appropriate storage media and stored at -80 C. Unfortunately, although seven Talbert Marsh water samples were originally considered presumptive positive by culture methods, biochemical tests, and Gram-staining, none of the stored isolates could be resuscitated by culture methods nor could they be confirmed as positive by genotypic methods. It cannot be conclusively established whether the original bacterial growth from *Campylobacter*-specific enrichment of water samples was not *Campylobacter*, or it truly was *Campylobacter* and isolates were unable to be confirmed. As described above, lack of *Campylobacter* resuscitation is a well-known phenomenon following freezing and thawing and a number of other factors may be responsible for the inability of the PCR assays to detect *Campylobacter* in stored cell suspensions. It is also possible that PCR species-specific primers were not broadly effective enough to identify species in these stored isolates. This idea is diminished by the fact that the robust *Campylobacter* genus PCR did not detect *Campylobacter* in these stored water isolates. Initial isolation of non-*Campylobacter* bacteria is the most likely explanation for these results.

As described above, a recently described method that uses reverse-transcription PCR (RT-PCR) and a reverse line blot hybridization (RLB) assay was used to molecularly detect and genotype F+ coliphages. Very few F-RNA coliphages (approximately 5% of total isolates analyzed) were detected by RT-PCR in presumptive positive water samples, however 80% of RT-PCR positive isolates were able to be typed by RLB analysis. F-RNA coliphages from water samples genotyped as MS2-like (G I) and similar reports have been documented in the available literature. Certain subgroups of F-RNA coliphages (i.e. G II) have been found to survive less well than others in the environment (i.e. G I) and this fact may be the reason that

only this subgroup was detected in water. No F-DNA coliphages were detected in Talbert Marsh water samples.

Methodological problems, F+ coliphage *E. coli* host contamination issues, and biases introduced during the isolation from water samples may have resulted in the low numbers of F-RNA coliphages originally isolated and may account for the differences in concentrations found between methods. Furthermore, F-RNA coliphage nucleic acid present in the stored suspensions may have suffered damage in the RT-PCR annealing regions (likely during freeze-thaws), or had unique nucleic acid sequences in the regions targeted by the RT-PCR primers.

Overall, low numbers of *Campylobacter*, *Salmonella*, and F+ coliphages were isolated from Talbert Marsh water samples, and when possible, even fewer were able to be further characterized by genotypic methods. Bacterial indicators including total coliforms, fecal coliforms, and enterococci were present in most, if not all, water samples analyzed, suggesting that although they may represent the presence of fecal contamination, they may not necessarily represent the presence of human pathogens such as *Campylobacter* and *Salmonella* originating from seagull fecal contamination. Future work should incorporate larger numbers of water samples, larger water sample volumes, and samples collected throughout each season of the year.

3. **Is it likely that the seagull population in Talbert Marsh contributes pathogens and fecal indicators into the estuarine waters of lower Talbert Marsh and ultimately flow into the Huntington Beach surfzone? Can microbial source tracking methods, including strain-typing and genetic analysis, be used to better understand the impact waterfowl have on the Talbert Marsh environment?**

Human bacterial pathogens *Campylobacter* and *Salmonella* and indicators of organisms, specifically GA-like (G II) F-RNA coliphages indicative of human fecal contamination, were found in the feces of seagulls that feed, preen, and defecate on the sandflats surrounding Talbert Marsh. However, no *Salmonella*, no GA-like (G II) F-RNA coliphages, and very low numbers of *Campylobacter* (which could not be confirmed genotypically), were detected in the estuarine waters surrounding the sandflats. Based upon these facts alone, it is unlikely that seagull feces deposited on Talbert Marsh sandflats are appreciably responsible for contamination of the surrounding estuarine waters. Overall, it is unlikely that pathogens from seagull feces in Talbert Marsh pose a significant health risk to swimmers in the ocean waters at Huntington Beach.

However, there are a number of limitations presented within this study that prevent a complete and definitive answer to this question. First, and perhaps most importantly, too few water samples were analyzed in the study. While nearly 150 seagull fecal samples were collected over the course of one year, only 24 water samples were collected within a three-month period, barely spanning two seasons. Furthermore, low sample volumes (1 L) were analyzed. While a few samples of this volume did produce low concentrations of presumptive culture-positive *Campylobacter*, the total volume analyzed was hardly representative of a nearly 120,000 L estuarine system. Furthermore, this system is not stagnant, but is constantly influenced by incoming and outgoing tides. Therefore, dilution plays a much bigger role in the distribution of introduced microbes than it would in a stagnant system. Overall, larger volumes and a greater number of samples needed to be collected over a longer time period, especially when bird populations are highest in the estuary.

Another limitation presented within the study is the occurrence of microbial die-off, both prior to sample collection and during the transport of the samples from California to North Carolina. Temperature fluctuations, exposure to UV light, exposure to low pH due to uric acid in the fecal samples, and other environmental stressors could have likely decreased numbers of microbes present. This aspect also limits the numbers of microbes in seagull feces that are ultimately entering marsh estuarine waters, as fecal samples may be exposed to these antagonistic conditions for several hours between tides. Furthermore, once in the estuarine waters, microbes are again subjected to a number of environmental stressors, including temperature effects, high salinity, nutrient-deprivation, and UV light exposure from sunlight. All of the above combined effects likely reduced concentrations of microbes found in both seagull feces, and especially in estuarine waters. These factors therefore limit the reliability of the results presented.

Additionally, the isolate storage aspect of the study reduced the reliability of the genotypic methods used. Repeated freezing and thawing of cell suspensions, even in the presence of cryoprotective additives such as glycerol, can have a deleterious effect on cells and the integrity of nucleic acids. This is especially pronounced in fragile cells such as *Campylobacter*. DNA and RNA are both required to be present and intact for different extraction techniques and other PCR optimization tactics to have a positive impact on the ability of nucleic acid targets to be successfully amplified. Each freeze-thaw event diminishes the integrity of nucleic acids present and therefore decreases the likelihood of PCR amplification. Furthermore, it is possible that inhibitors present in the storage media used for microbes hindered or even prevented successful amplification.

Storage at -80 C and freeze-thawing is also the likely reason that *Campylobacter* cells were unable to be regrown for further analyses, as they were likely dead or had entered a VBNC state. Lack of resuscitation prevented further analyses such as PFGE that potentially would have provided valuable insights into the relationships between isolates from seagull feces and those from water.

Despite the limitations presented above, there was some evidence supporting the idea that seagull feces deposited on the sandflats of Talbert Marsh did impact the surrounding estuarine waters. Although unable to be shown statistically, as sample numbers were too low for valid and meaningful analysis, variability was noted within *Campylobacter*, F+ coliphage, and *E. coli* occurrence based on location of water sample collection. For presumptive culture-positive *Campylobacter*, observed values both upmarsh and downmarsh of the sandflats were lower than expected values (central tendency), while observed values adjacent to the sandflats were slightly higher than expected values. These results suggest that the number of positive samples did vary between sample locations and was higher than expected at the location adjacent to the sandflats. For presumptive positive F+ coliphages, the observed value upmarsh was lower than the expected value, while the observed value downmarsh was higher than the expected value. It can be suggested that the increased number of positive samples downmarsh was due to outgoing tides moving water from upmarsh regions (i.e. the sandflats) to the outlet of the marsh. Finally, for *E. coli*, the observed value upmarsh was higher than the expected value, while the observed values both adjacent to the sandflats and downmarsh were lower than the expected values. In this case, it is possible that the increased number of positive samples upmarsh was due to incoming tides moving water from the sandflats region to the upper reaches of the marsh. A more thorough

investigation of the tidal cycles during sample collection is required to substantiate these possibilities.

As F+ coliphages were genotypically detected in both seagull feces and water samples, this dataset allows further investigation into the relatedness of isolates from seagull feces and estuarine waters. Isolates from both sources that were analyzed in this study are overall relatively similar in both nucleotide and amino acid sequence and therefore may have come from the same population. However, it is difficult to definitively say whether the similarities among nucleotide sequences of are compelling enough to link the two sources. The replicase gene that was used for identification represents one portion of the F-RNA coliphage genome, however similarities within subgroups in this one gene do not necessarily reflect similarities over the genome as a whole. Further studies in other regions of the F-RNA coliphage genome are necessary to confirm these differences between subgroups. Additionally, the extremely high rate of mutation found in RNA viruses and the potential for recombination between two or more isolates, opens up the possibility that the viral nucleotide sequence of an F-RNA coliphage infecting a host cell will not be exactly the same sequence as the progeny viruses that are released. These factors have an important impact on the use of F-RNA coliphages for microbial source tracking purposes.

Results of these studies are significant for a number of reasons. Talbert Marsh is a constructed wetland whose water depths vary both tidally and seasonally. Due to these fluctuations in water levels in the marsh, feces deposited on the sandflats of the marsh come into daily contact with rising marsh waters. Therefore, both fecal material and sands containing pathogens are likely to be submerged within the waters and ultimately distributed both upmarsh of the sandflats and downmarsh where the marsh empties into the surf zone at

Huntington Beach. However, the hydrodynamics of the marsh are controlled by a system of flood-controlled channels within a network of urban runoff management systems, therefore allowing some level of manipulation. Results of these studies may aid in the management of marsh water flow in ways that would minimize impacts on the microbial quality of adjacent ocean beach water. Furthermore, these results may be useful for comparison with similar estuarine and associated tidal marsh environments impacted by seagulls.

An important aspect of any scientific study is to identify further work required. As mentioned above, additional work needs to incorporate larger numbers of water samples, larger water sample volumes, and samples collected throughout each season of the year. Consistency of water sampling collection time is required to assess variability due to tidal stage. Further analysis of sand samples, vegetation, and silt samples from the marsh bed, where accumulation of seagull feces is likely, will provide a more thorough undertanding of the impact the seagulls have on this environment. Natural microbial die-off in the marsh environment is also an important topic to specifically address.

Molecular methods could be improved upon by attempting different DNA or RNA extraction methods and different volumes of template submitted to the PCR or RT-PCR assays. Sequencing of additional PCR- or RT-PCR-positive isolates from both seagulls and water samples would provide more information on their genetic relatedness and relatedness to isolates from other sources such as seagulls from different geographic locations, humans, sewage sludge and wastewaters, and other animals. Finally, molecular analyses should ideally be performed upon fresh microbial enrichments to eliminate the deleterious consequences of storage that may hinder further genotypic analyses by causing nucleic acid damage and ultimately the loss of detectability.

Currently, researchers at UC-Irvine are completing a manuscript assessing fecal indicator bacteria loading from Talbert Marsh to the surf zone at Huntington Beach. These analyses, combined with this study's findings, will allow speculation on the levels of microbes present in seagull feces that are required to make an impact in the surf zone and to potentially affect human health of bathers using these waters. The results and experiences of this study provide useful insights and directions for future research on the enteric microbial impacts of marsh seagull fecal contamination on urban coastal estuarine and sea waters.

APPENDIX

I. Chi-squared Test for Independence for *Campylobacter* isolated from Seagull Feces

Chi-square: 19.174
Degrees of Freedom: 3

Table size: 4 rows, 2 columns.
The P value is 0.0003.
The row and column variables are significantly associated.

Chi-Squared Test for Trend.
Note: This analysis is useful only if the categories defining the rows are arranged in a natural order (i.e. age groups, dose or time), with equal spacing between rows.

Chi-squared for trend = 13.248 (1 degree of freedom)
The P value is 0.0003.
There is a significant linear trend among the ordered categories defining the rows and the proportion of subjects in the left column.

Summary of Data

Row	Total	Percent
1	30	20.13%
2	50	33.56%
3	59	39.60%
4	10	6.71%
Total	149	100.00%

Column	Total	Percent
Campy	75	50.34%
No Campy	74	49.66%
Total	149	100.00%

II. Kruskal-Wallis Tests (Nonparametric ANOVA)

a. *Campylobacter* concentrations in seagull feces by season

The P value is < 0.0001, considered extremely significant.
Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean of Ranks
Autumn	30	1880.5	62.683
Winter	50	2882.5	57.650
Spring	58	5193.0	89.534
Summer	10	1070.0	107.00

Kruskal-Wallis Statistic KW = 26.008 (corrected for ties)

Dunn's Multiple Comparisons Test

Comparison	Mean Rank Difference	P value
Autumn vs. Winter	5.033	ns P>0.05
Autumn vs. Spring	-26.851	* P<0.05
Autumn vs. Summer	-44.317	* P<0.05
Winter vs. Spring	-31.884	*** P<0.001
Winter vs. Summer	-49.350	** P<0.01
Spring vs. Summer	-17.466	ns P>0.05

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
Autumn	30	0.000	0.000	500.20
Winter	50	0.000	0.000	46.200
Spring	58	2.950	0.000	69.200
Summer	10	57.300	0.000	524.90

b. *Salmonella* concentrations in seagull feces by season

The P value is 0.0282, considered significant.
Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean of Ranks
Autumn	30	1931.0	64.367
Winter	26	1632.0	62.769
Spring	59	3539.0	59.983
Summer	10	773.00	77.300

Kruskal-Wallis Statistic KW = 9.086 (corrected for ties)

Dunn's Multiple Comparisons Test

Comparison	Mean Rank Difference	P value
Autumn vs. Winter	1.597	ns P>0.05
Autumn vs. Spring	4.384	ns P>0.05
Autumn vs. Summer	-12.933	ns P>0.05
Winter vs. Spring	2.786	ns P>0.05
Winter vs. Summer	-14.531	ns P>0.05
Spring vs. Summer	-17.317	* P<0.05

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
Autumn	30	0.000	0.000	34.300
Winter	26	0.000	0.000	5.900
Spring	59	0.000	0.000	0.6000
Summer	10	0.000	0.000	737.00

c. F+ coliphage concentrations in seagull feces by season

The P value is 0.0006, considered extremely significant.
Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean of Ranks
Autumn	10	636.00	63.600
Winter	30	1417.0	47.233
Spring	59	3105.5	52.636
Summer	10	836.50	83.650

Kruskal-Wallis Statistic KW = 17.514 (corrected for ties)

Dunn's Multiple Comparisons Test

Comparison	Mean Rank Difference	P value
Autumn vs. Winter	16.367	ns P>0.05
Autumn vs. Spring	10.964	ns P>0.05
Autumn vs. Summer	-20.050	ns P>0.05
Winter vs. Spring	-5.402	ns P>0.05
Winter vs. Summer	-36.417	*** P<0.001
Spring vs. Summer	-31.014	** P<0.01

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
Autumn	10	0.000	0.000	216.40
Winter	30	0.000	0.000	210.20
Spring	59	0.000	0.000	54.400
Summer	10	125.85	0.000	19932

III. Mann-Whitney Test (Nonparametric unpaired)

a. *Campylobacter* concentrations in Talbert Marsh water samples by season

Do the medians of Summer and Autumn differ significantly?

The two-tailed P value is 0.1802, considered not significant.
The P value is an estimate based on a normal approximation.
The 'exact' method would not be exact, due to tied ranks.

Calculation details
Mann-Whitney U-statistic = 49.000
U' = 95.000
Sum of ranks in Summer = 173.00. Sum of ranks in Autumn = 127.00.

Summary of Data

Parameter:	Summer	Autumn
Mean:	2.558	0.2333
# of points:	12	12
Std deviation:	3.780	0.5449
Std error:	1.091	0.1573
Minimum:	0.000	0.000
Maximum:	11.400	1.400
Median:	0.000	0.000
Lower 95% CI:	0.1568	-0.1129
Upper 95% CI:	4.960	0.5796

b. F+ coliphage concentrations in Talbert Marsh water samples by season

Do the medians of Summer and Autumn differ significantly?

The two-tailed P value is 0.0487, considered significant.
The P value is an estimate based on a normal approximation.
The 'exact' method would not be exact, due to tied ranks.

Calculation details
Mann-Whitney U-statistic = 37.500
U' = 106.50
Sum of ranks in Summer = 184.50. Sum of ranks in Autumn = 115.50.

Summary of Data

Parameter:	Summer	Autumn
Mean:	113.94	3.600
# of points:	12	12
Std deviation:	185.46	5.957
Std error:	53.538	1.720
Minimum:	0.000	0.000
Maximum:	630.00	20.000
Median:	15.000	1.100
Lower 95% CI:	-3.896	-0.1851
Upper 95% CI:	231.78	7.385

c. Fecal coliform concentrations in Talbert Marsh water samples by season

Do the medians of Summer and Autumn differ significantly?

The two-tailed P value is 0.2014, considered not significant.
The P value is exact.

Calculation details
Mann-Whitney U-statistic = 32.000
U' = 67.000
Sum of ranks in Summer = 112.00. Sum of ranks in Autumn = 98.000.

Summary of Data

Parameter:	Summer	Autumn
Mean:	582.11	531.45
# of points:	9	11
Std deviation:	591.69	1123.5
Std error:	197.23	338.76
Minimum:	63.000	0.000
Maximum:	1727.0	3873.0
Median:	350.00	148.00
Lower 95% CI:	127.30	-223.31
Upper 95% CI:	1036.9	1286.2

d. *E. coli* concentrations in Talbert Marsh water samples by season

Do the medians of Summer and Autumn differ significantly?

The two-tailed P value is 0.2973, considered not significant.
The P value is an estimate based on a normal approximation.
The 'exact' method would not be exact, due to tied ranks.

Calculation details
Mann-Whitney U-statistic = 35.500
U' = 63.500
Sum of ranks in Summer = 108.50. Sum of ranks in Autumn = 101.50.

Summary of Data

Parameter:	Summer	Autumn
Mean:	54.000	12.364
# of points:	9	11
Std deviation:	87.740	25.719
Std error:	29.247	7.754
Minimum:	0.000	0.000
Maximum:	266.00	86.000
Median:	10.000	0.000
Lower 95% CI:	-13.443	-4.913
Upper 95% CI:	121.44	29.641

e. Enterococci concentrations in Talbert Marsh water samples by season

Do the medians of Summer and Autumn differ significantly?

The two-tailed P value is 0.2300, considered not significant.
The P value is an estimate based on a normal approximation.
The 'exact' method would not be exact, due to tied ranks.

Calculation details
Mann-Whitney U-statistic = 46.000
U' = 86.000
Sum of ranks in Summer = 124.00. Sum of ranks in Autumn = 152.00.

Summary of Data

Parameter:	Summer	Autumn
Mean:	2298.3	3102.4
# of points:	12	11
Std deviation:	2899.9	3731.8
Std error:	837.13	1125.2
Minimum:	391.00	496.00
Maximum:	8164.0	12997
Median:	730.00	1391.0
Lower 95% CI:	455.73	595.47
Upper 95% CI:	4140.8	5609.3