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The microbiology of the Las Vegas Wash

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THE MICROBIOLOGY OF THE

LAS VEGAS WASH

by

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ABSTRACT

The Microbiology of the Las Vegas Wash

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The Las Vegas Wash, the only drainage channel for the Las Vegas hydrographic basin, drains to Lake Mead, the major source of drinking water for the Las Vegas valley. High levels of fecally-associated bacteria are observed in the Wash, particularly during the summer months.

Several studies were undertaken to investigate the indicator organisms in the Las Vegas Wash. Attempts were made to determine the source(s) of fecal bacteria, with results pointing toward overall species being environmentally-associated as opposed to being a result of human contributions. Wash enterococci showed high resistance to low levels of vancomycin. Regrowth potential of *Escherichia coli* in Wash water generally demonstrated low growth. Coliform resuscitation from wastewater effluent was attempted from wastewater disinfected by chlorination, chloramination or UV. Data indicate statistically insignificant recovery from wastewater disinfected by chlorination or chloramination, but UV irradiation induced bacterial resuscitation in UV-disinfected wastewater effluent.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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CHAPTER 1

INTRODUCTION

As a large city in the desert, water availability and water quality in Las Vegas, Nevada and its surrounding areas have always been of concern. Metropolitan Las Vegas, which means "the meadows" in Spanish, exists only because of the artesian groundwater present in the area. Current archeological finds indicate human use as far back as 12,000 years. Artesian waters surfaced in pools near the current Las Vegas Springs preserve and then drained into the Las Vegas Wash (Las Vegas Wash Coordination Committee, n.d.). The area surrounding the Las Vegas Wash has been inhabited since 600 AD. Native Americans have long taken advantage of the Wash and it served as a watering stop along the Spanish Trail in the 18th century (Las Vegas Wash Coordination Committee, n.d.). Today, the Las Vegas Wash is both the drainage channel for the metropolitan area and the place where treated wastewater for the area is discharged. It is, therefore apparent, that the Las Vegas Wash is intimately tied to the lives of people living in this desert. Permanent settlement began as a town in 1905, and population growth in Las Vegas/southern Nevada increased significantly after World War II, with a tripling of the population size in the last 20 years (Piechota *et al.* 2002). The artesian groundwater supply is no longer available due to increasing urban pressure on the area's natural resources; however importation of Colorado River water began in 1971.

Located in southeastern Las Vegas, Nevada, the Las Vegas Wash is a 12-mile long, naturally occurring and engineered waterway. Along with its major tributaries, the Range Wash, Las Vegas Creek, Red Rock Wash, Flamingo Wash, Tropicana Wash, Duck Creek, Pittman Wash and C-1 Channel, the Las Vegas Wash drains the entire area of the 1600 square mile Las Vegas valley.

Flows in the Las Vegas Wash have been perennial since the onset of treated sewage discharges in the 1950s (Johnson 2000). Additionally, the Las Vegas Wash is the outlet for all stormwater flows, urban runoff and shallow groundwater seepage. Treated wastewater discharges are generated by the City of Las Vegas, Clark County and City of Henderson, along with raw water returns from Basic Management Incorporated. The Las Vegas area currently (2004) generates approximately 160 million gallons per day of treated wastewater from three wastewater treatment plants operated by the Clark County Water Reclamation District, the City of Las Vegas and the City of Henderson. A fertile wetland area has been created as a result of the nutrients from these waters (Las Vegas Wash Vegetation Study 2000). In the 1960s and 1970s, the wetlands aided in polishing the wastewater that flowed through them, but due to increased flows and erosion, they have been reduced in size from 2,000 to 200 acres since the early 1980s (Southern Nevada Water Authority n.d.).

The Las Vegas Wash discharges into Lake Mead, the principal drinking water supply for the area, at Las Vegas Bay. The confluence of the Wash and Lake is located approximately six river-miles upstream from the drinking water uptake site at Saddle Island. Lake Mead was created by the construction of Hoover Dam in the 1930s and is mainly filled with flows from the Colorado River. Flows from the Virgin and Muddy

Rivers, and the Las Vegas Wash, account for approximately 3% of Lake Mead's non-drought year inflows (Southern Nevada Water Authority n.d.). Eighty-eight percent of the annual water used for the Las Vegas valley is withdrawn from Lake Mead (Southern Nevada Water Authority n.d.). The combined net withdrawal, under the 1922 Colorado River contract allowed for all Southern Nevada users is 300,000 acre-feet a year. This represents 2-3% of the average non-drought year Colorado River flow that ranges from 10,000,000 to 14,000,000 acre-feet per year.

In the late 1980s, the Nevada Division of Environmental Protection (NDEP) established regulations beneficial use standards (BUS) for pH, total phosphate, chlorophyll-a, un-ionized ammonia, and total dissolved solids for the Las Vegas Wash and Lake Mead. Total maximum daily loads (TMDLs) for ammonia and phosphate were established to achieve the BUS in the Lake. In accordance with their National Pollutant Discharge Elimination System (NPDES) permits, the wastewater discharging agencies treat, monitor and report the water quality of their respective treated effluents. All three plants discharge treated effluent to the Las Vegas Wash and also monitor the receiving water quality in the Wash and the Lake. With very rare exceptions, the wastewater treatment plants comply with water quality standards imposed upon them. However, concerns have been raised about high levels of microorganisms, chemicals and suspended solids that are observed in the Las Vegas Wash and its tributaries (Montgomery Watson 2000). Nonpoint sources (surface irrigation, stormwater drainage and shallow groundwater infiltration) are thought to contribute to these observed high levels, but identification of actual sources has been difficult with standard monitoring protocols. Additionally, it is difficult to control non-point sources introduced into the Las Vegas

Wash and Lake Mead. Non-point dry weather nutrient loads are small, however wet weather "spike" loads could be significant and occasionally contribute to algal blooms and other transient water quality violations in Lake Mead.

Water quality in the Las Vegas Wash and Lake Mead is an extremely high profile environmental issue in southern Nevada. Potential health risks that could develop from poor water quality have contributed to a public view that there is a substantial relationship between quality of the water in the Las Vegas Wash and drinking water safety for the Las Vegas population. The fact is that, although important, the flows from the Las Vegas Wash contribute minimally to the overall Lake Mead volume and the substantial dilution of the Wash discharge that usually occurs in Lake Mead and aggressive treatment by the Southern Nevada Water Authority's water treatment plants are thought to minimize any health risks (Las Vegas Wash Coordination Committee 2000).

The microbiological quality of treated effluent is monitored daily by all three water reclamation plants. The Wash is monitored biweekly by the City of Henderson. Data generated from monitoring indicate that numbers of coliforms discharged from the treatment plants are nearly always below permit levels and are often non-detectable. Every summer, however, the Las Vegas Wash experiences an explosion of microbial growth as detected by high total and fecal coliform and *Escherichia coli* levels.

Historically, fecal coliforms and, more recently, *E. coli* have been used as the indicators for water quality monitoring (*Standard Methods for the Examination of Water and Wastewater* 1998). Several groups of bacteria, total and fecal coliforms, fecal streptococci and *E. coli,* are known as indicator organisms. Routine and relatively simple

culture methods for detection of these microbes have been developed and generally adopted by United States treatment plants. As both healthy humans and animals ubiquitously excrete these indicator organisms, they are likely to be present in high concentrations in wastewaters as compared to other specific pathogens. Their presence or absence is therefore thought to be a good indicator of the quality of the water in question (Moe 2002).

Las Vegas Wash water typically yields coliform most probable number (MPN) levels ranging from the hundreds in the winter to magnitudes as high as a million per 100 mL in the summer months (City of Henderson 2003). Questions have arisen as to why there are such disproportionate numbers between the treated effluent and the bulk Wash water associated with seasonal variations. There are several hypotheses as to why these differences occur.

One possible source is runoff from seasonal rainstorms, which may cause a spike in the indicator organism levels. Kistemann *et al.* (2002) found that coliform, fecal streptococci and *E. coli* levels increased considerably during extreme runoff events in drinking water reservoir tributaries in Germany, demonstrating that considerable portions of the total microbial load may be due to rainfall and extreme runoff episodes (Kistemann *et al.* 2002). *E. coli* and enterococci have been found to be ubiquitous, including along the nearshore water and beach sand of Lake Michigan during the summer of 2002 (Whitman *et al.* 2003). The coastal waters of southern California are similarly affected by urban runoff. Jiang *et al.* (2000) found that many of their sampling sites routinely exceeded water quality limits for total coliforms, fecal coliforms, and enterococci (Jiang *et al.* 2000). Other experiments designed to simulate subtropical tidal conditions

demonstrated large pulses of *E. coli* concentrations during storm conditions (Solo-Gabrielle *et al.* 1999).

Microbial contributions of runoff to the Las Vegas Wash system have been previously investigated. Piechota *et al.* (2002) found that wet weather events resulted in high overall bacterial densities in the Las Vegas Wash and its tributaries. Cold weather produced the lowest bacterial densities as determined by heterotrophic plate count. Controlled runoff experiments were conducted and extrapolated to estimate the total microbial contribution of runoff for the area, resulting in significant counts for this watershed system. Because of the extremely variable conditions of samplings, it was difficult to assess the potential contribution of fecal indicator organisms (Piechota *et al.* 2002).

Tied to microbial assessment is survivability of the specific microbes over time. Fish and Pettibone found that *E. coli* was able to survive in autoclaved water and freshwater sediment for at least 28 days (Fish and Pettibone 1994). They concluded that the sediment was protective in nature, possibly acting as a nutrient source and a reservoir for bacteria. Davies *et al.* (1995) demonstrated that *E. coli* remained fully culturable, at the same level, for 68 days in seeded marine sediments (Davies *et al.* 1995). Sediments may, in fact, contain up to 1000 times the number of fecal microbes found in the waters above them (Van Donsel and Geldreich 1971). The addition of sterile sediment to water under simulated tidal conditions in a subtropical environment demonstrated the high potential for regrowth, or the multiplication of microbes, of both enterococci and *E. coli* (Desmarais *et al.* 2002).

Bacteria may be undetected by traditional culture methods, but may be present and, under the appropriate conditions, resuscitate and become detectable (Colwell and Grimes 2000). The current investigation involves assessing the ability of the Las Vegas Wash water to serve as a growth medium by seeding autoclaved Wash water with a known concentration of *E. coli* and monitoring its growth over time.

A second possible source is based on the natural sources of fecal contamination (e.g. wildlife) using specific target bacterial types, i.e., enterococci. This investigation aims at estimating the approximate origin(s) of the fecal bacteria, and whether the sources are naturally occurring or are the result of human contributions. Accompanying this phase of research is an analysis of the antimicrobial resistance patterns of Las Vegas Wash enterococci, and thereby, the potential for human health effects.

In Washington, D.C. waterways, birds, dogs and wildlife were found to be major contributors of fecal microorganisms, while human inputs were found notably following storm events (Porter *et al.* 2003). In rural Virginia, the source of fecal streptococci was found to be mainly from cattle (Hagedorn *et al.* 1999). Harwood *et al.*(2000) reported that the subtropical waters of Florida showed direct anthropogenic impacts of indicator bacteria from septic tanks (Harwood *et al.* 2000).

A third possible source involves resuscitation of injured microorganisms from treated wastewater. In the Las Vegas valley, three wastewater plants are responsible for treatment and subsequent discharge of wastewater in southern Nevada. These are the City of Henderson Water Reclamation Facility, the City of Las Vegas Water Pollution Control Facility and the Clark County Water Reclamation District. Each of these entities produces high-quality, tertiary-treated wastewater and operates under strict National

Pollutant Discharge Elimination System (NPDES) permits as issued by the U.S. Environmental Protection Agency. The effluent is disinfected by one of two methods: chlorine (City of Las Vegas, City of Henderson) or ultraviolet (UV) treatment (Clark County Water Reclamation District). Chloramines are added to the reclaimed water used for watering golf courses and parks in the City of Henderson to further disinfect it as they tend to provide a long-term residual.

In 1982, the term "non-culturable" was coined to explain the survival strategy of dormancy that was employed by actively dividing cultures of *Vibrio cholerae* or *E. coli* after transfer to a nutrient deficient environment (Colwell and Grimes 2000). These cells would not grow under traditional culture conditions, but were proven to be metabolically active. The expression "viable but nonculturable" (VBNC) was thus invoked. Through countless studies, it has become understood that it is an unfavorable environmental condition, such as starvation, pH change, radiation exposure or temperature stress that may induce this physiological state (Colwell and Grimes 2000).

It is well documented that *E. coli* can undergo repair (i.e. resuscitation) following injury from radiation. This includes both artificial UV and sunlight. Zimmer and Slawson demonstrated the repair after exposure to a low-pressure UV source (Zimmer and Slawson 2002). Solar radiation has been reported to dramatically decrease enteric bacterial numbers in the ocean as well as inactivate *E. coli* in river water (Gauthier 2000). Entry of bacteria into the VBNC state may also be induced by exposure to nuclear radiation, as described by Pitonzo *et al.*(1999), with studies on injured heterotrophic bacteria isolated from rock of the proposed Yucca Mountain Nuclear Waste Repository (Pitonzo *et al.* 1999).

Chlorine has also been shown to initiate cellular damage in bacteria. Exposure of *E. coli* and other enteric bacteria to sublethal levels of chlorine has been shown to result in nonculturability of the microbes. Additionally, after undergoing a "recovery" process consisting of less selective growth conditions, they were able to regrow. The human host may be one such less selective environment, allowing for bacterial recovery. Injury to bacteria has been extensively documented in the food industry, oftentimes resulting in the gross underestimation of indicator organisms in the food product (McFeters and LeChevallier 2000). It follows that there would be, and is in fact, also bacterial injury occurring in water, wastewater and distribution systems. It is not, however, the practice of regulatory laboratories to employ recovery enhancement techniques in their monitoring protocols (McFeters and LeChevallier 2000). Here, too, the number of viable indicator organisms may be largely miscalculated and may result in a public health effect.

E. coli, specifically, has been reported to enter the VBNC state in the following environments: culture medium, fresh water, buffered fresh water, lake water, river water, wastewater, brackish water, seawater and sediment. The conditions associated with its entry into the VBNC state include temperature, visible light, ultraviolet light, toxic forms of oxygen, salts, osmolarity, nutrient scarcity, humic acids and biocidal agents (Gauthier 2000). It has been estimated that between 50% and 90% of viable coliforms present in potable water systems may be injured and, are therefore not being detected. This is significant, as coliform bacteria are one of the standard indicators of microbial contamination in water and wastewater systems. Current methods have largely been shown to be insensitive in the detection of stressed microorganisms, among them, VBNC. Detection of these nonculturable bacteria may allow for remediation at the source and

may possibly identify treatment problems before the water enters the distribution systems (McFeters and LeChevallier 2000).

It is possible that some microorganisms are injured in the course of disinfection in the Las Vegas valley wastewater treatment plants and are subsequently capable of resuscitation when placed in favorable environmental conditions, such as the Las Vegas Wash environment. In this investigation, the issue of indicator bacterial resuscitation following disinfection by UV, chlorination and chloramination is addressed.

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CHAPTER 2

ENTEROCOCCAL POPULATIONS IN THE LAS VEGAS WASH

This chapter has been prepared for submission to the *Journal of Water and Health* and is presented in the style of that journal.

Abstract

This study was initiated to investigate the microbiology of the Las Vegas Wash, a naturally occurring tributary to Lake Mead. The Las Vegas Wash is the primary outlet for all stormwater flow, urban runoff and shallow ground water seepage as well as the pathway by which treated wastewater travels. Wastewater discharges contain low to non-detectable levels of fecally-associated microbes. Much higher levels of fecally associated microbes are observed in the Wash, particularly during the summer months. As it flows to Lake Mead, the major source of drinking water for the Las Vegas valley, the fecally-associated microbes in the Wash are a concern. This research investigation is an attempt to estimate the approximate origins of the contamination, whether human, bird, or other sources.

The studies were conducted at two time points: a winter or cold weather sampling, and a summer or hot weather sampling. Three matrices were investigated: water, sediment and plant material. Samples were analyzed using API speciation test strips for *Streptococcus*. Results suggest that the highest overall enterococcal populations were avian-associated species, *Enterococcus avium* (63% of total species) and to a lesser

extent *Enterococcus gallinarum* (18%), with some contribution from the human associated species *Enterococcus faecium* (15%) and *Enterococcus faecalis* (3%). These data suggest that the fecal contamination is naturally occurring and is not an effect of the treated wastewater flow. A second investigation aimed at examining the antibiotic resistance patterns of the enterococci in the Las Vegas Wash. Enterococci isolated from the Las Vegas Wash showed little resistance to doxycycline, imipenem or nitrofurantoin. 11.5% showed resistance to ofloxacin, 13.4% to ampicillin and 38.7% of the isolates demonstrated resistance to low levels of vancomycin.

Introduction

Located in southeastern Las Vegas, Nevada, the Las Vegas Wash is a twelve-mile long naturally occurring and engineered waterway. Along with its major tributaries, Range Wash, Las Vegas Creek, Red Rock Wash, Flamingo Wash, Tropicana Wash, Duck Creek, Pittman Wash and C-1 Channel, the Las Vegas Wash drains the entire 1600 square mile valley and is the only drainage channel for the whole Las Vegas area. As the outlet for all stormwater flows, urban runoff and shallow groundwater seepage, the Las Vegas Wash is additionally the conduit through which treated wastewater generated by the City of Las Vegas, Clark County and City of Henderson, along with raw water returns from Basic Management Incorporated, travels. The residents of the Las Vegas area generate sufficient wastewater to account for approximately 160 million gallons per day of reclaimed water from the three wastewater treatment plants.

The Las Vegas Wash empties into Lake Mead, the principal drinking water supply for the area. The confluence of the Wash with the Lake is located approximately six miles upstream from the drinking water uptake site at Saddle Island. Eighty-eight percent of

the water used by the Las Vegas valley is from Lake Mead (Southern Nevada Water Authority n.d.).

In accordance with their National Pollutant Discharge Elimination System (NPDES) permits, the wastewater discharging agencies treat, monitor and report their respective effluents. The wastewater treatment plants comply with water quality standards imposed upon them, but concerns have been raised about the microorganisms, chemicals and sediment due to high levels of erosion that are introduced into Lake Mead through the Las Vegas Wash (Montgomery Watson 2000). Additionally, it is difficult to control the non-point sources introduced into the Las Vegas Wash and Lake Mead which, if nutrient levels became high enough, could lead to increasing eutrophication in Lake Mead.

The Las Vegas Wash and Lake Mead water quality are extremely high profile environmental issues in southern Nevada. The potential health issues that could develop from poor water quality have led to a public view that there is a substantial relationship between quality of the water in the Las Vegas Wash and drinking water safety for the Las Vegas population, when, although important, the flows from the Las Vegas Wash contribute minimally to Lake inflow (Las Vegas Wash Coordination Committee 2000).

The microbiological quality of the treated effluent is monitored daily, and the Wash is monitored biweekly. Data generated from monitoring indicate that coliforms discharged from the treatment plants are low to non-detectable. Every summer, however, the Las Vegas Wash experiences an explosion of microbial growth as detected by high coliform and *Escherichia coli* levels.

Historically, fecal coliforms and, more recently, *E. coli* have been used as the indicators for water quality monitoring (*Standard Methods for the Examination of Water*

and Wastewater 1998). Several groups of bacteria, total and fecal coliforms, fecal streptococci and *E. coli* are known as indicator organisms. Routine and relatively simple culture methods for detection of these microbes have been developed. As both healthy humans and animals ubiquitously excrete these indicator organisms, they are likely to be in high concentrations in wastewaters as compared to other specific pathogens, which are both less numerous and often more difficult to culture. Their presence or absence is therefore thought to be a good indicator of the quality of the water in question (Moe 2002).

Las Vegas Wash water typically yields coliform most probable number (MPN) levels ranging from the hundreds in the winter to magnitudes equal to a million per 100 mL in the summer months (City of Henderson 2003). Questions of why there are such disproportionate numbers between the treated effluent and the bulk Wash water associated with seasonal variations have arisen.

Fecal streptococci have been proposed as an alternate water quality indicator (*Standard Methods for the Examination of Water and Wastewater* 1998). Previously classified as fecal streptococci, the group is now known as the enterococci. These bacteria are members of the genus *Enterococcus*, inhabit the gastrointestinal tracts and female genital tracts of humans and other animals, and are ubiquitous, occurring in soil, food, water, plants, animals, birds and insects. Enterococci are oxygen tolerant anaerobes which are gram-positive, catalase-negative cocci that hydrolyze esculin in the presence of 40% bile salts (Holt 1994). The entrococci may be differentiated from other streptococcal groups by their ability to grow between 10 and 45°C, at a pH of 9.6, and in broth containing 6.5% NaCl (Holt 1994).

There have been studies suggesting that the enterococci and *E. coli* may be better indicator organisms than fecal coliforms, as their survival rate through wastewater treatment processes is higher than that of fecal coliforms (Miescier and Cabelli 1982). Enterococcal numbers correlate better with human gastrointestinal disease and human pathogens better than fecal coliforms (Cabelli 1980, Dufour 1984, Kay *et. al.* 1994). The USEPA demonstrated, through epidemiological studies, a direct correlation between *E. coli* and enterococcal densities in surface waters and increases in swimmer-associated gastroenteritis (U.S. Environmental Protection Agency 1986).

Kistemann *et al.*(2002) found that coliform, fecal streptococcal and *E. coli* levels increased considerably during extreme runoff events in drinking water reservoir tributaries in Germany, demonstrating that considerable portions of the total microbial load may be due to rainfall and extreme runoff episodes (Kistemann *et al.* 2002). During storm conditions, *E. coli* entered coastal waterways in Ft. Lauderdale, Florida in a large pulse (Solo-Gabrielle *et. al.* 2000). They also found that the primary source of *E. coli* in this waterway was from the soils along the riverbanks. Other studies have suggested large concentrations of *E. coli* during rainfall events and sewer overflows as potential *E. coli* contributors (Richman 1996). *E. coli* and enterococci have been found to occur ubiquitously along the nearshore water and beach sand of Lake Michigan during the summer of 2002 (Whitman *et al.* 2003). The coastal waters of southern California are similarly affected by urban runoff. Jiang *et al.*(2000) found that many of their sampling sites routinely exceeded water quality limits for total coliforms, fecal coliforms, and enterococci (Jiang *et al.* 2000). Other experiments designed to simulate subtropical tidal conditions demonstrated large pulses of *E. coli* during storm conditions (Solo-Gabrielle *et*

al. 1999). Shehane *et al.* (2003) demonstrated that the sources of fecal coliform bacteria from the lower St. Johns River Basin in Florida were generally of wild animal origin during dry season samplings. During periods of higher rainfall, however, the majority of isolates from several sites were of human origin, demonstrating the potential impact of climate change, combined with high septic tank usage and urban runoff on surface waters (Shehane *et al.* 2003).

The microbial contributions of runoff to the Las Vegas Wash system have been previously investigated. Piechota *et al.* (2002) found that wet weather events resulted in high overall bacterial densities in the Las Vegas Wash and its tributaries. Cold weather produced the lowest bacterial densities, as determined by heterotrophic plate count. Controlled runoff experiments were conducted and extrapolated to estimate total microbial contribution of runoff for the area, resulting in significant counts for this watershed system. Because of the extremely variable conditions of samplings, it was found to be difficult to assess the potential contribution of fecal indicator organisms (Piechota *et al.* 2002).

In this investigation, an attempt was made to analyze seasonal effects on the Las Vegas Wash system on the microbes that are found in the Wash. It was thought that runoff from seasonal rainstorms may cause a spike in the indicator organism levels.

Our experimental approach involves determining the actual source of fecal contamination using specific target bacterial types, i.e., enterococci. In Washington, D.C. waterways, birds, dogs and wildlife were found to be major contributors of fecal microorganisms, while human inputs were found notably following storm events (Porter *et al.* 2003). Seasonal trends were also seen in birds contributing substantial fecal loads

during peak summer and spring seasons (Porter *et al.* 2003). In rural Virginia, fecal streptococci were found to be mainly from cattle (Hagedorn *et al.* 1999). Harwood *et al.* (2000) reported that the subtropical waters of Florida showed direct anthropogenic impacts of indicator bacteria from septic tanks (Harwood *et al.* 2000).

This investigation aims at estimating the approximate origin(s) of the fecal bacteria, and whether the sources are naturally occurring or are the result of human contribution. Accompanying this phase of research is an analysis of the antimicrobial resistance patterns of Las Vegas Wash enterococci. Rice *et al.* (1995) observed high levels of resistance to aminoglycosides in environmental enterococcal isolates, including *E. faecalis, E. faecium* and *E. gallinarum* (Rice *et al.* 1995). In clinical settings, enterococci have been found to be resistant to most commonly used antibiotics, including vancomycin (Lukasova and Sustackova 2003). Additionally, vancomycin resistant enterococci isolated from municipal sewage, activated sludge and pharmaceutical waste were shown to engage in interspecies plasmid transfer (Guardabassi and Dalsgaard 2004). This presents an emerging health problem, also making it hard to associate antibiotic resistance with a particular source.

Materials and Methods

Sample Collection

Five sample sites were chosen in the Las Vegas Wash: (1) in the Las Vegas Wash, upstream of treated wastewater effluent (LW 10.75), (2) treated wastewater effluent (LW 8.85), (3) the Wash at the intersection of Pabco and Hollywood roads, downstream of all three wastewater treatment plants (LW 6.05), (4) the upper and (5) lower reaches of the Duck Creek tributary (Figure 1). The numbers associated with the sample sites refer to

the distance, in miles, of the sample site in relation to the entry of the Las Vegas Wash into Lake Mead. Water, sediment and plant matter were aseptically collected at each site for analysis. Additionally, raw wastewater influent was aseptically collected from the City of Henderson wastewater treatment plant.

Laboratory Analysis

Using standard membrane filtration technique (*Standard Methods for the Examination of Water and Wastewater* 1998), water samples of the appropriate dilution were filtered and placed on mE (Difco, Sparks, MD) agar plates. The plant and sediment samples were processed in the following manner: a 1:10 sample dilution in 0.1% sodium pyrophosphate dilution was both placed in a stomacher (Dynatech Laboratories, Alexandria, VA) for two minutes (plant material) or in a sterile 125 mL flask and sonicated (Aquasonic, West Chester, PA) for the same amount of time (sediment). The supernatant was used for analysis: enumeration via the spread plate method onto mE (Difco, Sparks, MD) agar plates. All plates were incubated at 41°C for 48 hours.

Isolates were randomly selected from the mE plates and transferred into a tube of Buffered Azide Glucose Glycerol Medium (BAGG) broth (Difco, Sparks, MD). These were incubated at 45°C for 48 hours. The tubes were read for growth and acid production, indicated by turbidity and color change from purple to yellow, respectively. Each positive isolate from the BAGG broth was transferred to Bile Esculin Azide Agar (Difco, Sparks, MD) plates and incubated at 35°C for 18 – 24 hours. Positive colonies from the Bile Esculin Azide Agar plates were tested for catalase. Positive colonies, indicated by bubbling, were discarded. Negative colonies were transferred to Sheep's Blood Agar Plates and the hemolysis type was noted as part of the API strep strip

(Biomerieux, Hazelwood, MO) speciation protocol. Speciation was conducted utilizing API speciation strips for *Streptococcus* and species was determined by interpretation from the API code book (Biomerieux, Hazelwood, MO). API is a phenotypic speciation method that utilizes carbon utilization profiles to determine the specific species of bacteria. In the tests for streptococci, 20 different biochemical assays are performed on a single strip, the results are scored, tabulated and speciated by reference to the API system (Biomerieux, Hazelwood, MO). All species were typed to a 95% confidence.

Antibiotic Profile Testing

Using standard membrane filtration technique *(Standard Methods for the Examination of Water and Wastewater* 1998), Wash and Creek water samples of the appropriate dilution were filtered and placed on mE agar plates. All plates were incubated at 31.5°C for 24 hours.

Isolates were randomly selected from the mE plates and transferred to Bile Esculin Agar plates and incubated at 35°C for 18 – 24 hours. Positive colonies from the Bile Esculin Agar plates were tested for catalase. Catalase positive colonies, indicated by bubbling, were discarded. Negative colonies were transferred to Brain Heart Infusion (BHI) Agar + 6.5% NaCl. Colonies unable to grow in BHI + 6.5% NaCl were discarded. Positive colonies were transferred to Tryptone Glucose Yeast (TGY) broth (Difco, Sparks, MD) and incubated overnight at 35°C. Isolates were transferred to Plate Count Agar (Difco, Sparks, MD) and resistance to ampicillin (10 μg), doxycycline (30μg), imipenem (10 μg), nitrofurantoin (100 μg), ofloxacin (5 μg) and vancomycin (5 μg) was tested using antibiotic disks.

The same isolation and antibiotic susceptibility procedures as described above were followed for City of Henderson raw wastewater influent.

Results

Enterococcal Speciation

Combined results from both summer (warm water) and winter (colder water) time points suggested that the highest overall enterococcal populations were the avian-associated species *Enterococcus avium* (63% of total species) and to a lesser extent *Enterococcus gallinarum* (18%). There was some contribution from the human associated species, *Enterococcus faecium* (15%) and *Enterococcus faecalis* (3%).

An analysis of each sample site, with all matrices included, demonstrated that again, *E. avium* is in the greatest abundance at all five sample sites (Figure 1). Duck Creek exhibited the greatest species diversity and evenness. Rawhide Channel and LW 10.75 contained higher proportions of *E. faecium* than *E. gallinarum*, while LW 8.85 contained more than double the proportion of *E. gallinarum* present as compared with *E. faecium*. LW 6.05 demonstrated the highest proportion of *E. avium* with similar amounts of *E. gallinarum* and *E. faecium*.

Figure 2 shows the enterococcal populations separated by the matrix type, that is water, plant or sediment. In all matrices, *E. avium* was present in the highest proportion. *E. gallinarum* was slightly more abundant than *E. faecium* in the plant matter, while *E. faecium* was higher in the sediment samples. The largest inconsistency between the sample matrices was in the sediment. Sediment samples contained significant levels of *E. faecalis* when compared with the water and plant matter samples.

Analysis of single sampling of influent speciation data reveals that 10% of the isolates were *E. avium* (Figure 3). The remaining 90% of the isolates were human associated species of which 24% were *E. faecalis* and 66% were *E. faecium*.

Although *E. avium* was generally present in highest proportion at all the summer and winter sampling points, its overall proportion was lower during cold weather sampling (53.27%) than warm weather sampling (77.33%). Additionally, the winter sampling demonstrated a population of *E. faecalis* (5.61%) that was not present in the summer sampling.

Antibiotic Profiles

Analyzing the data from all sites shows that 13.4% (n = 269) of all isolates were resistant to ampicillin, 1.9% (n = 265) to doxycycline, 0% (n = 267) to imipenem, 1.1% (n = 269) to nitrofurantoin, 11.5% (n = 269) to ofloxacin and 38.7% (n = 269) to vancomycin (Table 1). Separating these data by sample site; for Duck Creek tributary, 6.3% were resistant to ampicillin, 2.1% to doxycycline, 0% to imipenem, 1.1% to nitrofurantoin, 8.5% to ofloxacin and 22.1% to vancomycin; LW 10.75, 13.3% to ampicillin, 1.2% to doxycycline, 0% to imipenem, 1.2% to nitrofurantoin, 8.3% to ofloxacin and 30.1% to vancomycin; LW 6.05, 0% resistant to ampicillin, 1.1% to doxycycline, 0% to imipenem, 1.1% to nitrofurantoin, 17.6% to ofloxacin and 63.7% to vancomycin. The City of Henderson influent showed the following antibiotic resistance profile: 2% resistance to ampicillin, 6% to doxycycline, 0% to imipenem, 2% to ofloxacin, and 0% to vancomycin.

Discussion

Results showing a strong proportion of avian-associated species demonstrate that at all sample sites, the large majority of the enterococcal populations collected at the sampling points along the Las Vegas Wash and its tributaries is most likely of environmental origin (i.e. not human). When these data are compared with the enterococcal populations in the raw sewage water, the conclusion of the environmental origin of enterococcal populations is further supported as the very strong human-associated signal found in the isolated human wastewater source lends strength to the reliability of the speciation methods employed. Similar results have been found in other urban water systems (Hagedorn *et al.* 1999). Wastewater data (Figure 3) demonstrated that approximately 90% of the enterococci in the system were human associated species, *E. faecium* and *E. faecalis*.

As an open waterway in the desert with high flows, the Las Vegas Wash is subject to a large amount of erosion and sediment in its waters. It is well documented that indicator organisms have been shown to survive in riparian sediments and often enter into waterways in high concentration during runoff or storm conditions (Kistemann *et al.* 2002, Piechota *et al.* 2002, Porter *et al.* 2003, Shehane *et al.* 2003, Solo-Gabrielle *et al.* 1999). Data indicated that Las Vegas Wash sediment was better able to confer survivability to a larger number of species than could Wash water or plant matter.

We also observed a seasonal trend in the enterococcal species concentrations. The Las Vegas Wash is located near a bird viewing preserve and a wetlands demonstration park. It may be that the trend is associated with the seasonal migrations of birds in the

area. Others have found seasonal trends in indicator organisms (Porter *et al.* 2003), most particularly related to storm events.

The data generated by this investigation demonstrate that the enterococcal populations of the Las Vegas Wash show a high degree of resistance to low levels of vancomycin. This is to be contrasted with the complete lack of vancomycin resistant enterococci isolated from the raw wastewater influent (Table 1). Others have isolated VRE from sewage and chicken feces. The same study also found VRE from hospital wastewater (resistant to $\geq$20 μg of vancomycin/mL) that had the *vanA* gen and residental wastewater and chicken feces (resistant to 3 to 5 μg of vancomycin/mL) that had the *vanC* gene (Harwood *et al.* 2001). Thousands of fecal streptococci were isolated from wastewater, animal feces and surface water by the same group as part of a different study, some of which were resistant to >32 μg/mL vancomycin (Harwood *et al.* 2000).

True vancomycin resistant enterococci are defined as those resistant to vancomycin at a concentration greater than or equal to 32 μg/mL (Canadian External Quality Assessment Advisory Group for Antibiotic Resistance 1998). We are not suggesting that any of the enterococci in this study are truly vancomycin resistant, however, it is interesting to note the differences in the populations isolated from the different sources. Further, we did not conduct any molecular testing to determine if these isolates possessed any *van* genes. It is possible, that the environment in the Las Vegas Wash, particularily downstream from the wastewater effluent discharge, may be responsible for these variations, as the large volume of treated wastewater would contain a variety of wasted pharmaceuticals. It is additionally possible the the Las Vegas Wash environment could favor growth or regrowth of antibiotic resistant strains or that natural but similar

mechanisms have developed in these bacteria to allow for them to survive in the presence of low levels of vancomycin. Interspecies transfer of transposable elements between environmental and clinical isolates has been proposed (Guardabassi and Dalsgaard 2004) and is therefore of concern. This is especialy true in the case of vancomycin, which is often the last antibiotic used in treatment of *Staphylococcus aureus* infections.

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Figure 1: Map of the sampling sites.

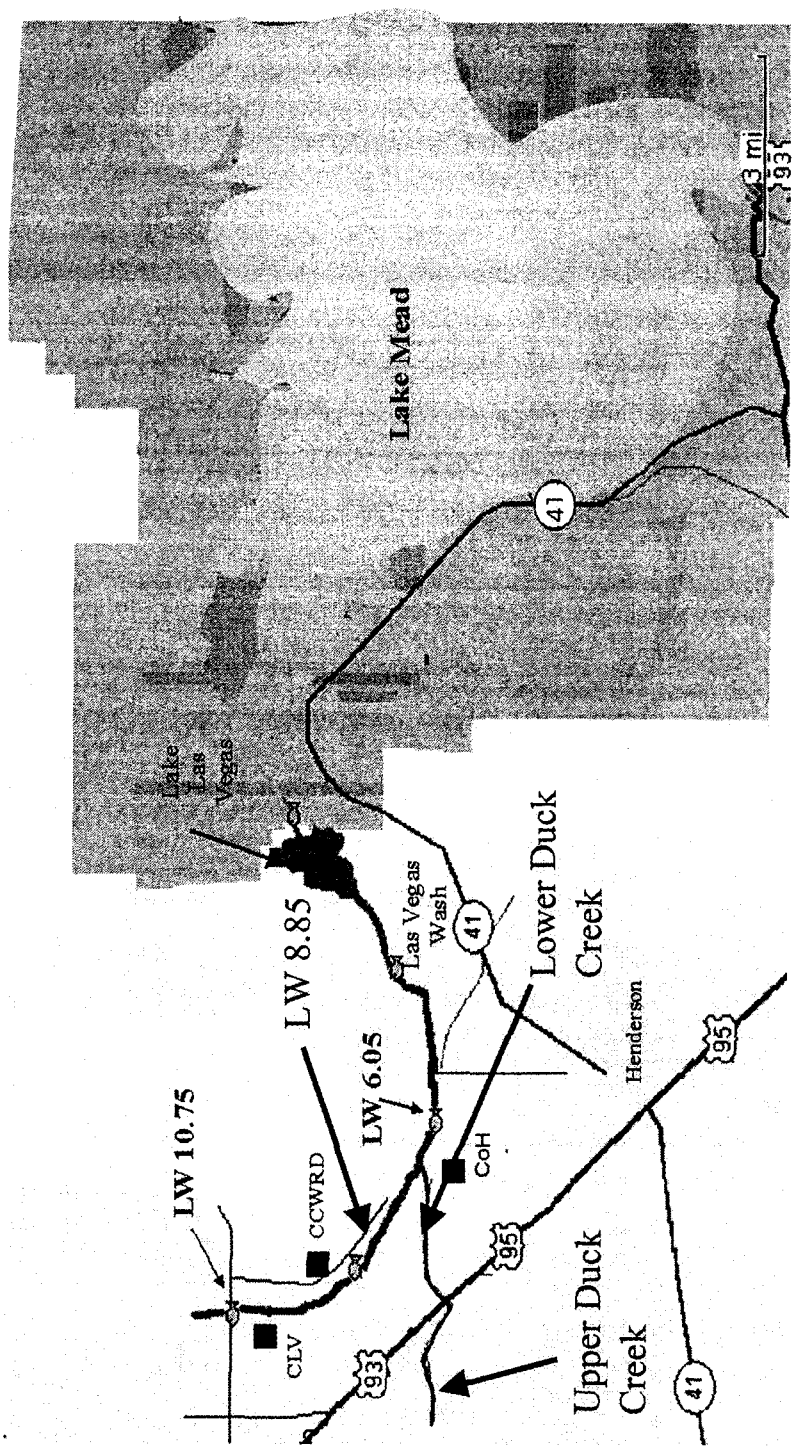

Figure 2: Speciation of enterococcal isolates from all five sample sites (Upper and Lower Duck Creek, LW 10.75, LW 8.85 and LW 6.05). Data include sum of isolates from all three matrices (water, plant and sediment) from both winter and summer time points. Figure shows the percentage of each species of the total isolates for each sample site. The isolates designated by crosshatching are environmentally-associated species. The isolates designated by stripes are human-associated species.

Figure 3: Enterococcal populations by matrix type, water, plant or sediment. The isolates designated by crosshatching are environmentally-associated species. The isolates designated by stripes are human-associated species.

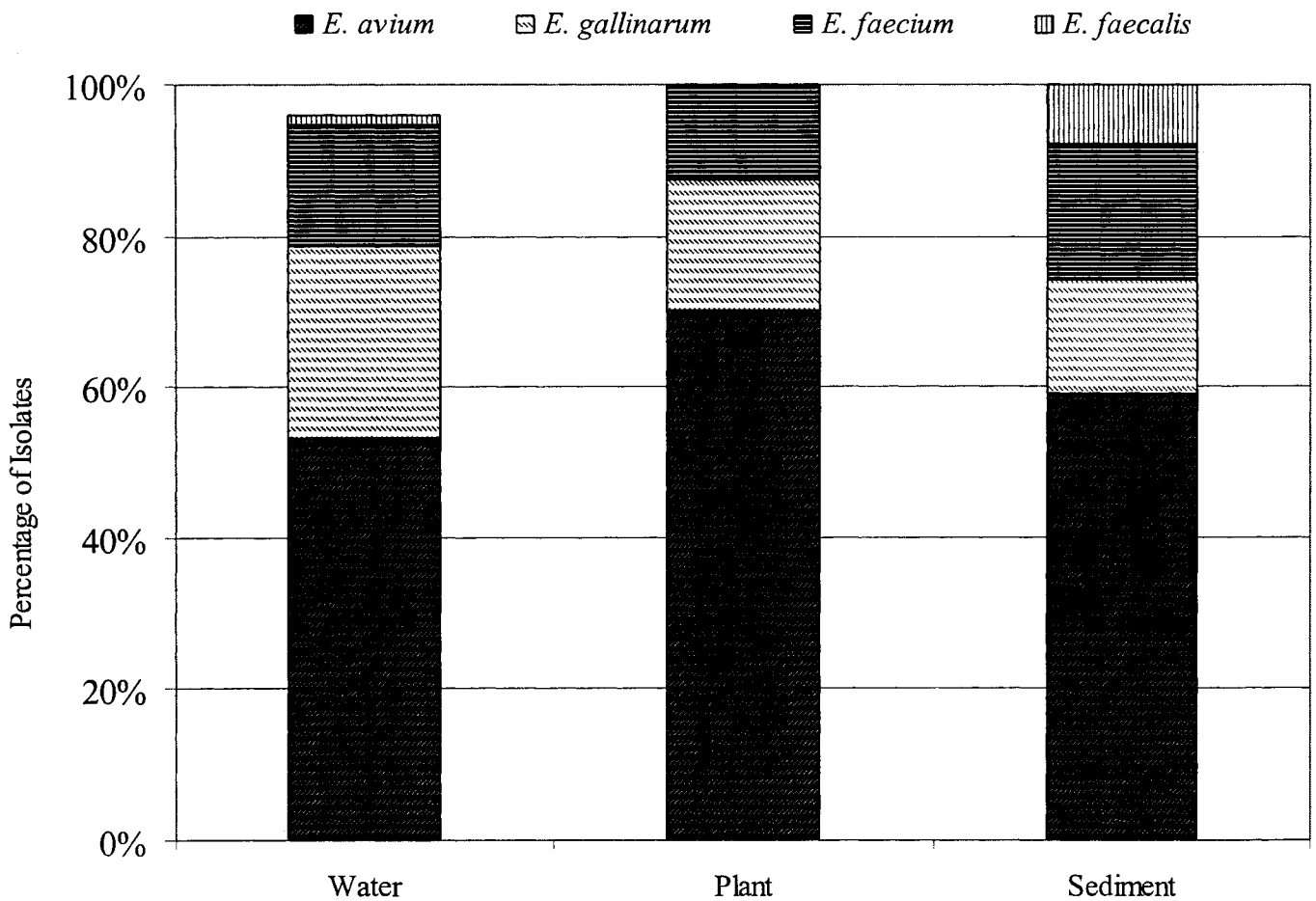

Figure 4: Enterococcal species in wastewater influent. The isolates designated by crosshatching are environmentally-associated species. The isolates designated by stripes are human-associated species.

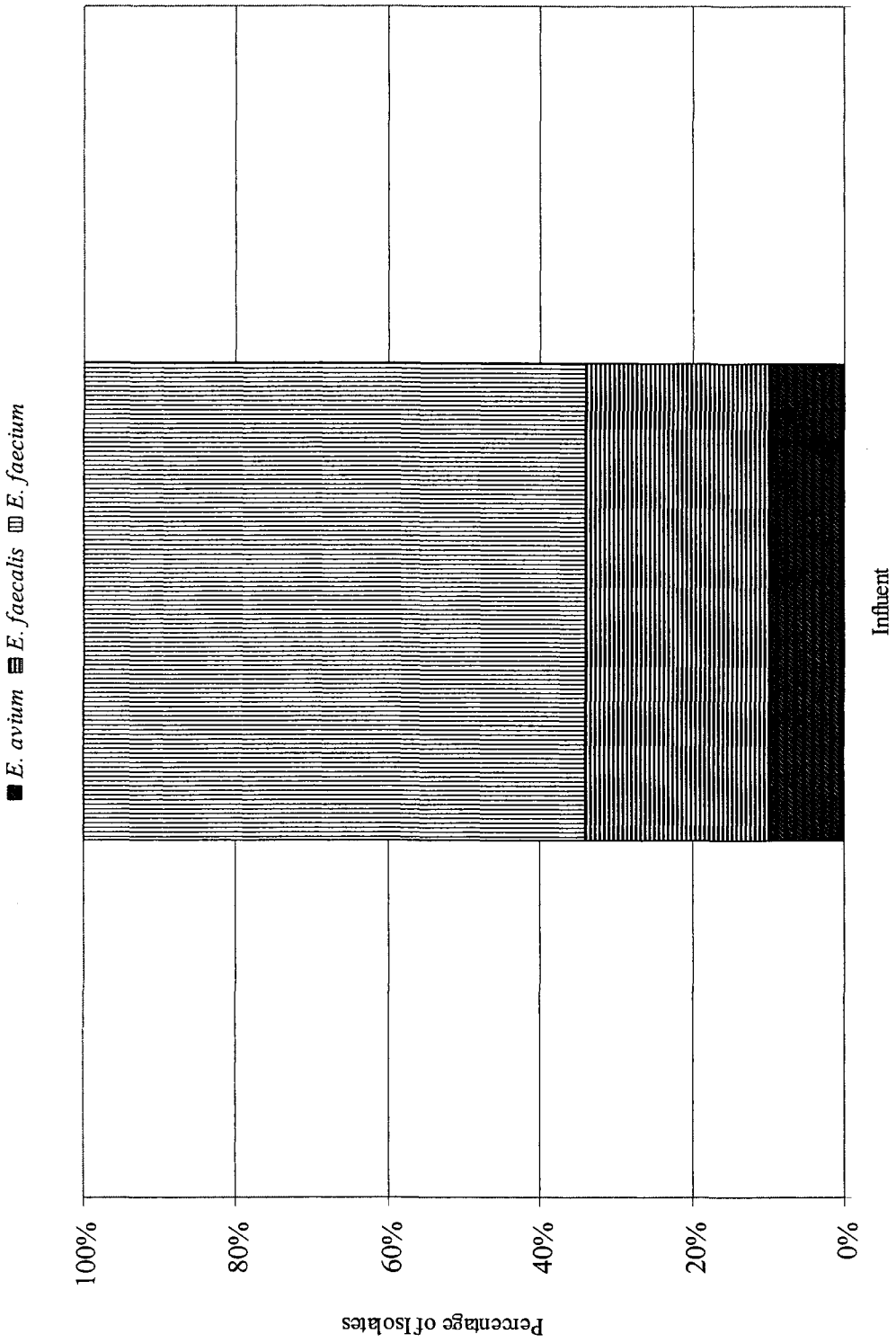

Figure 5: Enterococci in winter sediment. The isolates designated by crosshatching are environmentally-associated species. The isolates designated by stripes are human-associated species.

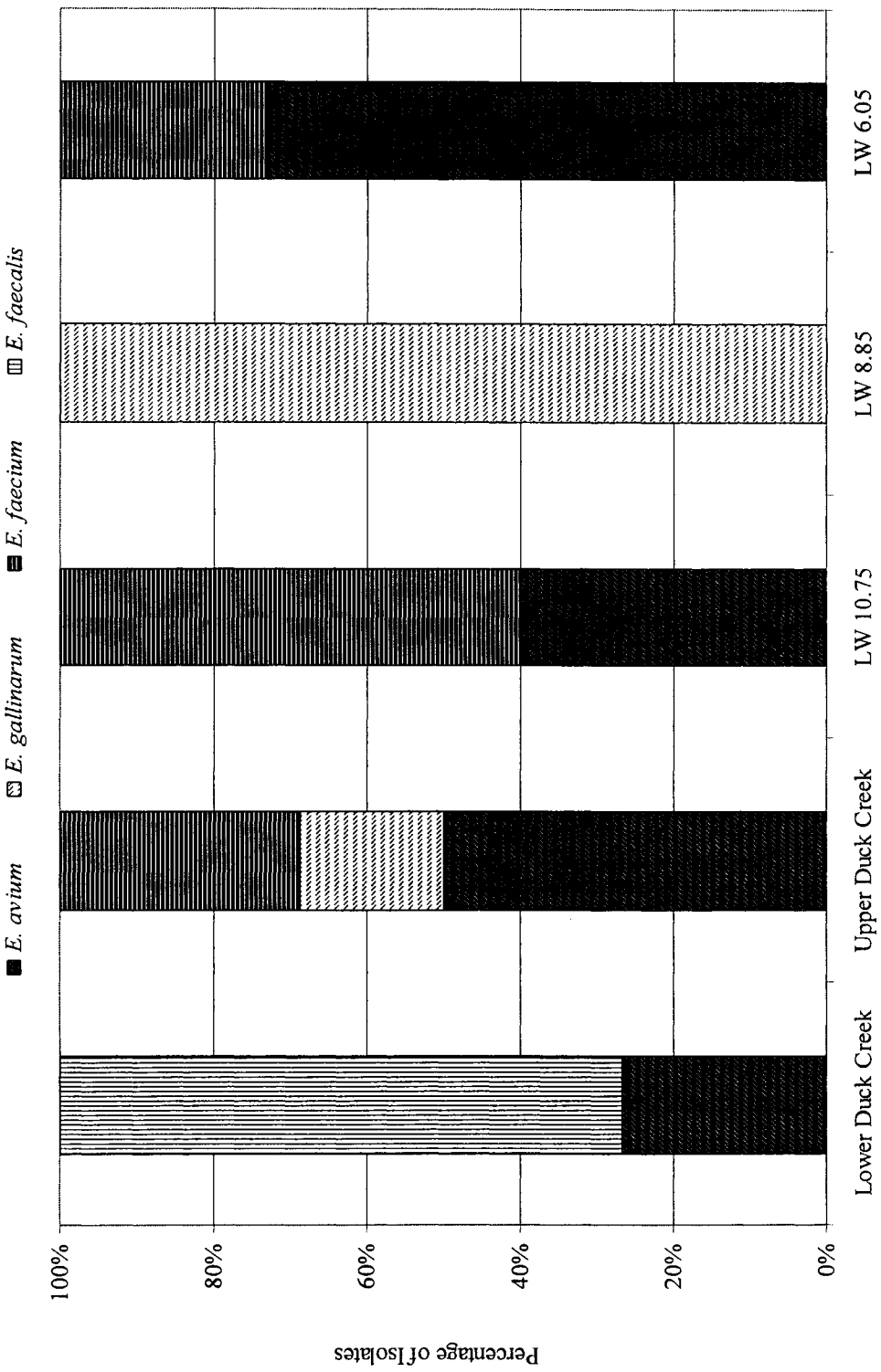

Figure 6: Enterococci in winter water. The isolates designated by crosshatching are environmentally-associated species. The isolates designated by stripes are human-associated species.

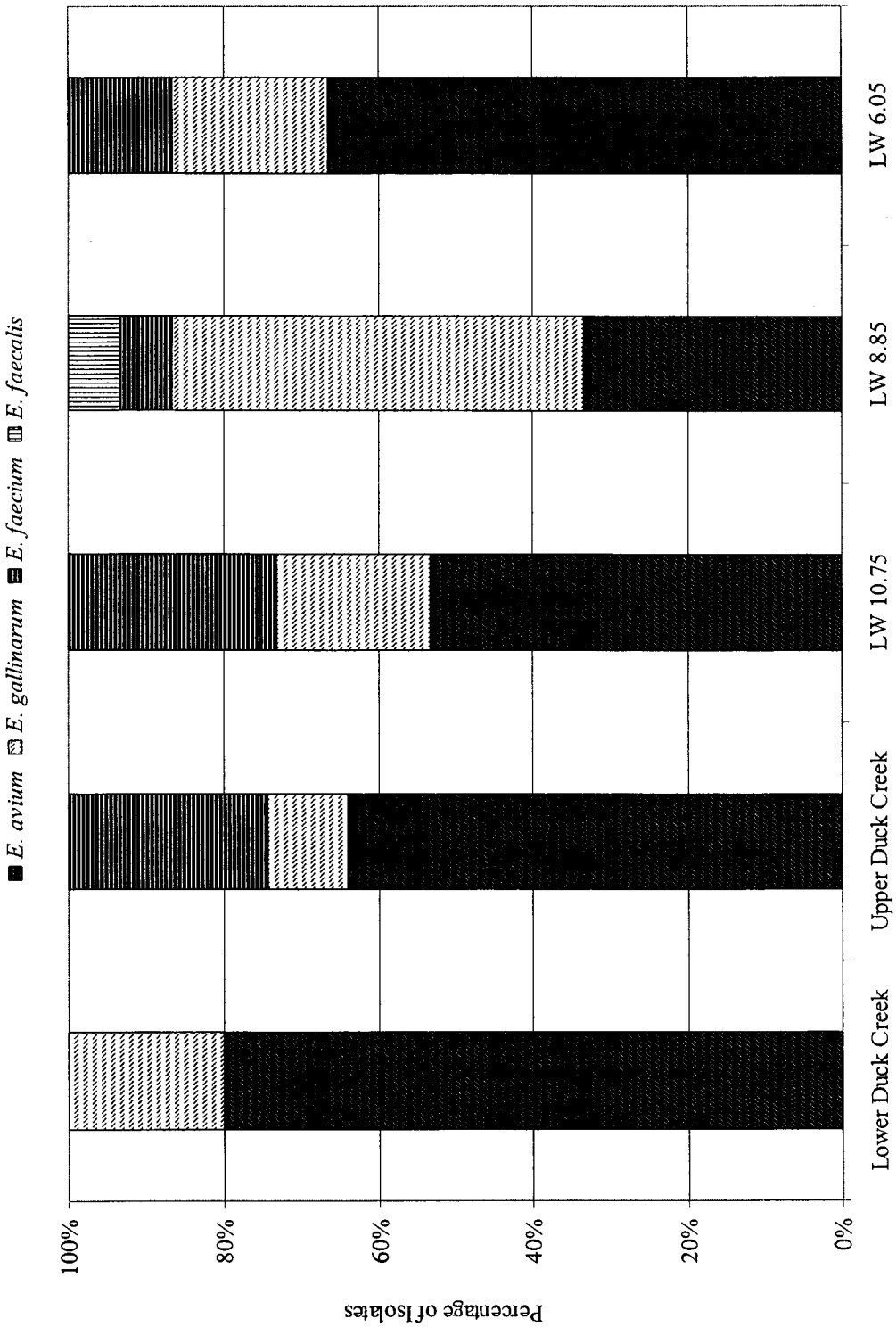

Figure 7: Enterococci in winter plants. The isolates designated by crosshatching are environmentally-associated species. The isolates designated by stripes are human-associated species.

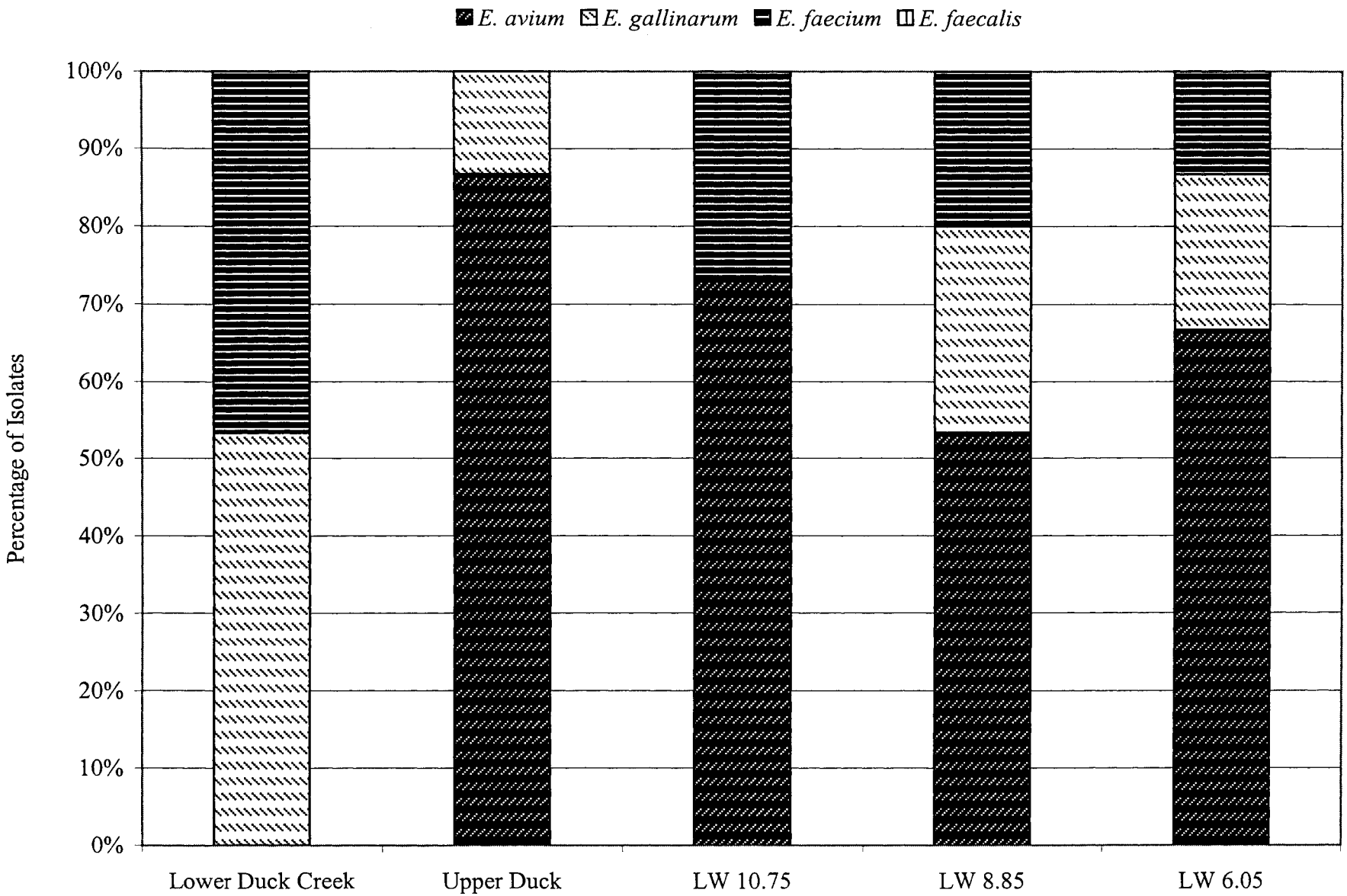

Figure 8: Enterococci in summer sediment. The isolates designated by crosshatching are environmentally-associated species. The isolates designated by stripes are human-associated species.

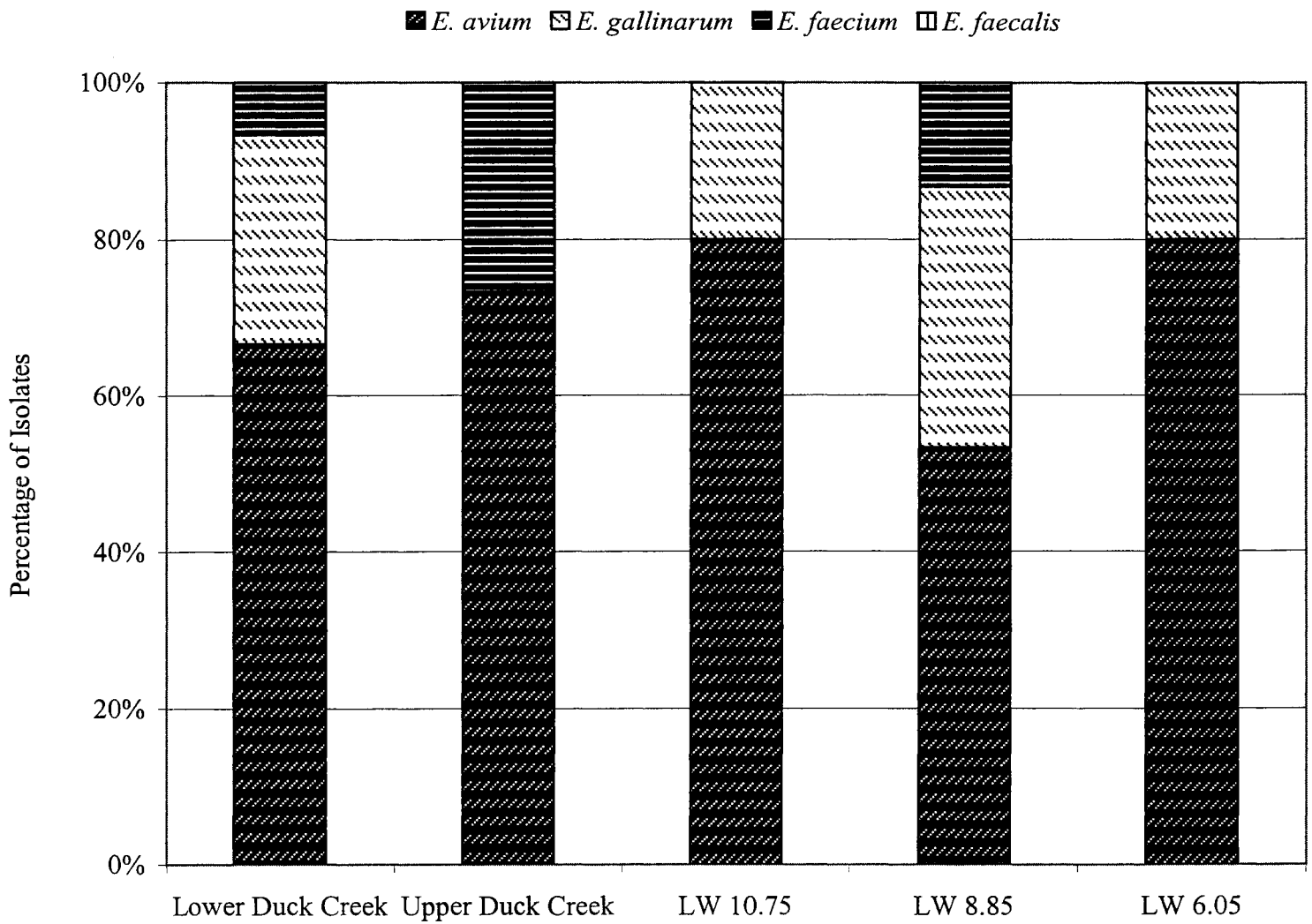

Figure 9: Enterococci in summer plants. The isolates designated by crosshatching are environmentally-associated species. The isolates designated by stripes are human-associated species.

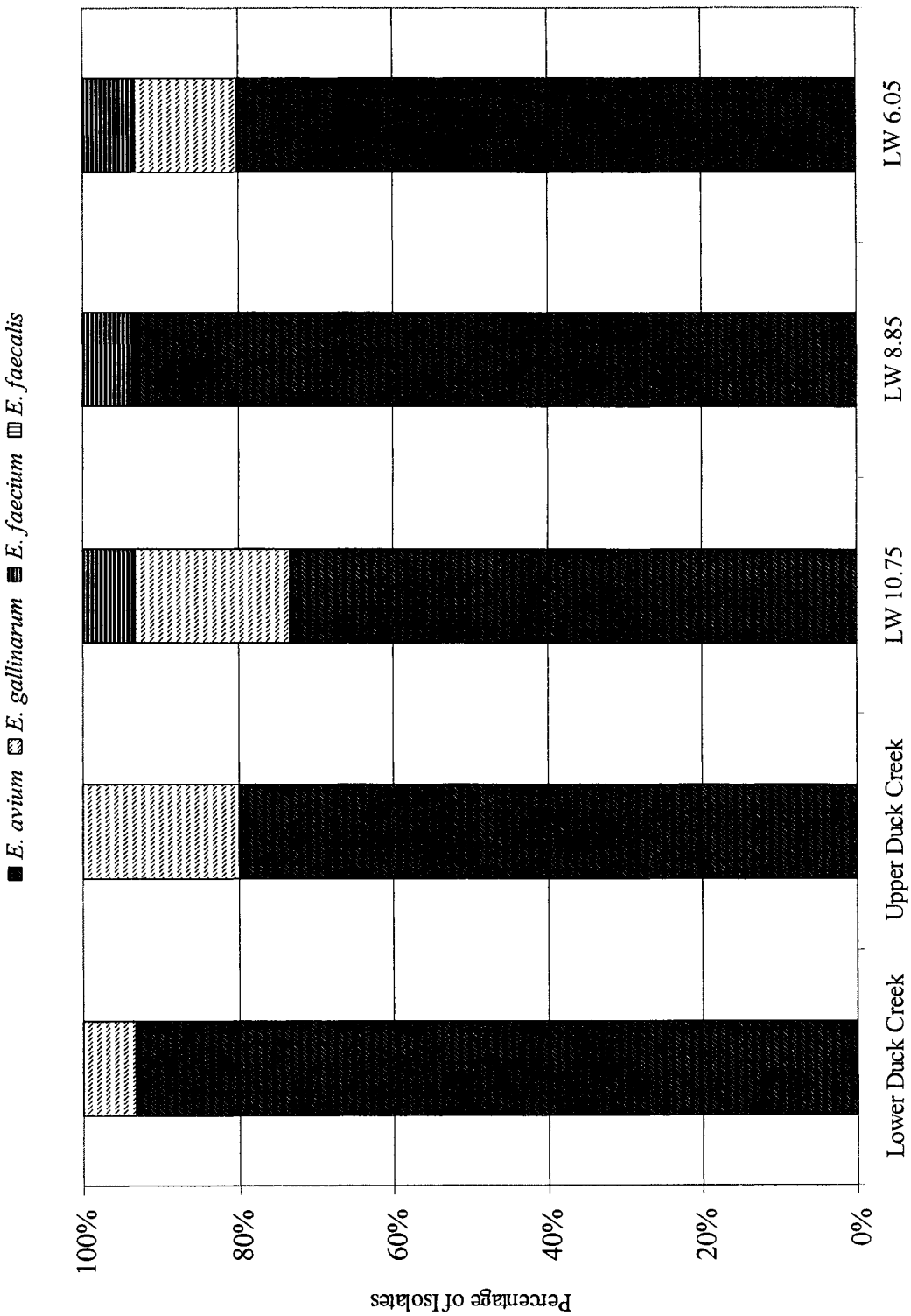

Figure 10: Enterococci by season. Figure includes species from all sample sites and matrices combined. The isolates designated by crosshatching are environmentally-associated species. The isolates designated by stripes are human-associated species.

Table 1: Antibiotic resistance of enterococci isolated from the Las Vegas Wash and from City of Henderson wastewater influent. Numbers shown are the percentage of isolates that were resistant to the antibiotics shown on the left of the table.

	Sample Site Percent of Resistant Isolates			
Antibiotic	Duck Creek	LW 10.75	LW 6.05	City of Henderson Influent
Ampicilin	6.3	13.3	0	2
Doxycycline	2.1	1.2	1.1	6
Imipenem	0	0	0	0
Nitrofurantoin	1.1	1.2	1.1	0
Ofloxacin	8.5	8.3	17.6	2
Vancomycin	22.1	30.1	63.7	0

CHAPTER 3

ESCHERICHIA COLI GROWTH IN THE LAS VEGAS WASH

This chapter has been prepared for submission to the *Journal of Water and Health* and is presented in the style of that journal.

Abstract

The ability of a natural and engineered tributary in the Las Vegas area to sustain microbial growth over time was assessed. Autoclaved water samples were seeded with a known concentration of *Escherichia coli*. Sub-samples were incubated at 25°C, and *E. coli* growth was measured by heterotrophic plate counts at 0, 1, 2, 3, 4, 5, 6 and 24 hours. At different sample times, bacterial growth varied from none or even a decrease in culturable numbers to a 10^5 increase in growth over the 24-hour period.

Introduction

As a large city in the desert, water availability and water quality in Las Vegas, Nevada and its surrounding areas has always been of significant concern. Growth in the Las Vegas area exploded in the late 20th century, culminating in a tripling of the population size in the last 20 years (Piechota *et al.* 2002). Much of the original supply of groundwater is no longer available due to increasing urban pressure on the area's natural resources.

The Las Vegas Wash is a twelve-mile long naturally occurring and engineered waterway located in southeastern Las Vegas. Along with its major tributaries, the Las

Vegas Wash drains the entire 1600 square mile valley and is the only drainage channel for the whole Las Vegas area. As the outlet for all stormwater flows, urban runoff and shallow groundwater seepage, the Las Vegas Wash is also the route through which travels approximately 160 million gallons per day of treated wastewater. Treated wastewater is generated by the City of Las Vegas, Clark County and City of Henderson, along with raw water returns from Basic Management Incorporated. About 10% of the flow in the Wash is estimated to be from nonpoint sources, including infiltrating groundwater. The Las Vegas Wash empties into Lake Mead, the principal drinking water supply for the area, at Las Vegas Bay. Flows from the Virgin and Muddy Rivers and, the Las Vegas Wash, account for approximately 3% of Lake Mead's volume. Eighty-eight percent of the water used by the Las Vegas valley is from Lake Mead (Southern Nevada Water Authority n.d.).

The wastewater treatment plants comply with water quality standards imposed upon them, but concerns have been raised about the microorganisms, chemicals and sediment due to high levels of erosion that are being introduced into Lake Mead through the Las Vegas Wash (Montgomery Watson 2000). Additionally, it is difficult to control the non-point sources introduced into the Las Vegas Wash and Lake Mead which, if nutrient levels became high enough, could lead to increased eutrophication of Lake Mead.

The Las Vegas Wash and Lake Mead water quality is an extremely high profile environmental issue in southern Nevada. The potential health issues that could develop from poor water quality have led to a public view that there is a substantial relationship between the quality of the water in the Las Vegas Wash and drinking water safety for the Las Vegas population, when, although important, the flows from the Las Vegas Wash are

currently thought to contribute minimally to water quality in the Lake (Las Vegas Wash Coordination Committee 2000).

The microbiological quality of the treated effluent is monitored daily and the Wash is monitored biweekly. Monitoring data indicate that coliform density inflows discharged from the treatment plants are usually low to non-detectable. Every summer, however, the Las Vegas Wash experiences an explosion of microbial growth as detected by high coliform and *E. coli* levels.

Historically, fecal coliforms and, more recently, *E. coli* have been used as the indicators for water quality monitoring *(Standard Methods for the Examination of Water and Wastewater* 1998). Several groups of bacteria, total and fecal coliforms, fecal streptococci and *E. coli* are known as indicator organisms. Routine and relatively simple culture methods for detection of these microbes have been developed. As both healthy humans and animals ubiquitously excrete these indicator organisms, they are likely to be present in high concentrations in wastewaters as compared to other specific pathogens. Their presence or absence is therefore thought to be a good indicator of the quality of the water in question (Moe 2002).

Las Vegas Wash water typically yields coliform most probable number (MPN) levels ranging from the hundreds in the winter up to a million per 100 mL in the summer months (City of Henderson 2003). Questions have arisen as to why there are such seasonally varying disproportionate density differences between the treated effluent and the bulk Wash water. There are several theories as to why these differences occur.

Microbial contributions of runoff to the Las Vegas Wash system have been previously investigated. Piechota *et al.* (2002) found that wet weather events resulted in

high overall bacterial densities in the Las Vegas Wash and its tributaries. Cold weather produced the lowest bacterial densities as determined by heterotrophic plate count. Controlled runoff experiments were conducted and extrapolated to estimate total microbial contribution of runoff for the area, resulting in significant counts for this watershed system. Because of the extremely variable conditions of samplings, it was found to be difficult to assess the potential contribution of fecal indicator organisms (Piechota *et al.* 2002).

Tied to microbial assessment is survivability of the specific microbes over time. It has been estimated that the half-life of *E. coli* in water is approximately one day (Winfield and Groisman 2003). Fish and Pettibone found that *E. coli* was able to survive in autoclaved water and freshwater sediment for at least 28 days (Fish and Pettibone 1994). They concluded that the sediment was protective in nature, possibly acting as a nutrient source and a reservoir for bacteria. Davies *et al.* (1995) demonstrated that *E. coli* remained fully culturable, at the same level, for 68 days in seeded marine sediments (Davies *et al.* 1995). Sediments may, in fact, contain up to 1000 times the number of fecal microbes found in the water above them (Van Donsel and Geldreich 1971). The addition of sterile sediment to water under simulated tidal conditions in a subtropical environment demonstrated the high potential for regrowth of both enterococci and *E. coli* in sand (Desmarais *et al.* 2002).

E. coli has been shown to be able to sustain growth under a variety of conditions. In experimental treatments involving the addition of acetic, citric, lactic, malic and tartaric acids, at 25°C, *E. coli* populations increased $10^2 - 10^4$ CFU/mL over eight weeks (Conner and Kotrola 1995). *E. coli* is also able to proliferate in tropical climates in the absence of

fecal contamination, with the indication that soils that are tidally influenced are able to help support such populations (Doyle *et al.* 1992, Fujioka *et al.* 1995). *E. coli* and enterococci have been shown to be able to multiply in simulated environmental settings, with findings pointing to the sediment providing favorable growth conditions either by increased nutrient concentration or decreased microbial predation (Desmarais *et al.* 2002). Urine has been shown to be an adequate growth medium for some strains of *E. coli* (Hull and Hull 1997).

It appears that the survival and subsequent growth of *E. coli* in a secondary habitat is largely dependent upon overcoming low nutrient conditions, temperature fluctuations, and shielding from radiation, among other environmental factors. This was demonstrated by the high concentration of coliforms in bromeliad water, which typically contains high concentrations of nitrates and nitrites (Winfield and Groisman 2003). In drinking water, phosphorus has been shown to be a limiting nutrient for microbial growth (Miettinen *et al.* 1997), even though organic carbon compounds are often a limiting growth factor for microbial growth (Kovarova-Kovar and Egli 1998). It is thought, however, that in aquatic systems, bacterial growth is most likely controlled by not by one but by several nutrients (Kovarova-Kovar and Egli 1998). In the Las Vegas Wash, average phosphate levels for the 2003 year at the upper site were 0.07 mg/L. Average nitrate concentrations were 3.70 mg/L and nitrite concentrations were 0.07 mg/L. At the same time, in the sampling site below wastewater discharge, average phosphate concentrations were 0.22 mg/L, nitrate concentrations were 13.86 mg/L and nitrite levels were 0.07 mg/L (City of Henderson 2003). Therefore, nitrogen concentrations are most likely not a limiting growth factor, but low levels of phosphate could be contributing to a limitation of growth.

The current investigation involves assessing the ability of the Las Vegas Wash water to serve as a growth medium by seeding autoclaved Wash water with a known concentration of *E. coli* and monitoring its growth over time. We have attempted to estimate the amount of microbial growth that Las Vegas Wash water is able to sustain over the time it travels through the Wash before entering into Lake Mead.

Materials and Methods

Sample Collection

Two sample sites were chosen along the Las Vegas Wash: upstream of treated wastewater effluent (LW 10.75) and the Wash at the intersection of Pabco Road and Hollywood Road (LW 6.05), downstream of wastewater effluent discharge points (Figure 1). The numbers associated with the sample sites refer to the distance, in river miles, upstream from the confluence with Lake Mead. Water samples were aseptically collected in 1000 mL sterile glass bottles at each site for analysis, at both summer and winter time points. Samples were transported on ice (<4°C) to the laboratory. Samples were collected on February 21, 2002, June 6, 2002, July 11, 2002 and August 5, 2004.

Laboratory Analysis

Upon return to the laboratory, water samples were removed from ice and immediately sterilized at 121°C by autoclaving. Sample volumes of 100 mL were placed in duplicate sterile flasks. Duplicate flasks were seeded with one mL of *E. coli* culture of a previously grown culture in R2B. The flasks were incubated at 25°C, with shaking in room light. *E. coli* concentrations were determined by the optical density of the culture. Triplicate plates were prepared for culturable heterotrophic plate count (HPC) on R2A using the spread plate method. HPCs were conducted after 1, 2, 3, 4, 5, 6 and 24 hours of

incubation. Various dilutions were prepared in sterile water. Plates were incubated at 25°C for 48 hours prior to counting. The experiment was repeated four times. On the June 6, 2002 and the August 5, 2004 samplings only single flasks but triplicate plates were prepared.

Results

The first growth experiment showed no significant growth or decay of bacterial densities over time (Figures 2-7). There was not a noteworthy amount of growth for either site in the winter, however at the 6 hour time point there was an actual decrease in bacterial density as compared with the 5 hour count (Figures 2 and 3). Densities did increase slightly at the end of 24 hours of incubation (Figures 2 and 3). An experiment using summer growth rates also showed little decay or growth for either LW 10.75 or LW 6.05 (Figures 5 and 6).

Figure 4 shows a repeat of this experiment using samples collected on a different date. These data indicate a 5×10^5 increase in microbial densities over the 24-hour time period for both sample sites. Growth remained relatively constant until reaching the 24-hour time point, at which there was a significant increase in bacterial density (5×10^5). This was found in both LW 10.75 and LW 6.05.

A fourth experiment was conducted using samples collected in the summer. Data indicate a 10^1 increase in microbial densities over the 24-hour time period for both LW 10.75 and LW 6.05 (Figure 7). There was a greater increase in density for LW 10.75 as compared with LW 6.05.

Discussion

Experimental data have shown a wide range of microbial growth rates. One trial demonstrated virtually no growth nor decay. A second trial showed a large increase ($5x10^5$) in bacterial density. A third time point resulted in a lesser, but measurable, amount of bacterial growth. There did not seem to be any consistent association between bacterial growth and season. Out of eight separate experiments in the Wash, four showed no real measurable growth or decay. One showed slight decay and a second showed a 10^1 increase in growth. The samples collected on July 11, 2002 demonstrated a very large amount of growth (10^5).

Sufficient repetitions were performed to have discerned an obvious pattern if there was one present since duplicate flasks and triplicate platings were prepared and the experiment was performed four times. There is also no seasonal pattern associated with nitrate, nitrite and phosphorus concentrations at either sample site in the Wash (City of Henderson 2003).

The Las Vegas Wash is a dynamic system. Over the last 30 years, the large volume of treated wastewater had allowed for the deposition of a large amount of sediment and erosion throughout the channel resulting in a destabilization of the Wash and increased sedimentation of Lake Mead (Southern Nevada Water Authority n.d.). Further sedimentation has occurred by the erection of a resort community which contains a large lake, under which the Las Vegas Wash travels through a large concrete pipe. Truckloads of sediment are regularly removed from this area as a result of this structure. Kistemann *et al.* (2002) demonstrated the substantial share of the total microbial load in water reservoirs that can result from rainfall or extreme runoff events (Kistemann *et al.* 2002).

Previous studies (Chapter 2) have shown that Las Vegas Wash sediment is able to serve as a reservoir for indicator bacterial species. Controlled runoff experiments have demonstrated potential microbial contributions to the Wash system (Piechota *et al.* 2002). Nutrient level spikes are well documented in the Las Vegas Wash system after a storm or runoff event (City of Henderson 2003).

Additionally, storm events significantly affect the Wash system. As the only drainage channel for the entire Las Vegas hydrographic basin, during a storm event urban runoff will carry high concentrations of fertilizers, pesticides, herbicides, oil, nutrients and bacteria. Desert storms make for highly unpredictable Wash flows, often resulting in extremely destructive consequences. In July, 1999, the Las Vegas area experienced a large storm event, in which 16,000 cubic feet per second (cfs) of stormwater entered into the Wash. Normal daily Wash flows are 225-250 cfs. The increased flows ended with the Wash channel widening up to 300 feet at some points (Las Vegas Wash Coordination Committee n.d.). LW 6.05 experienced significant changes in channel location as a result of this storm. Efforts are being made to stabilize the Wash by erecting erosion control structures along the channel (Southern Nevada Water Authority n.d.), however these storm events would easily contribute to a difference of water quality and composition and could therefore change the ability of the water to sustain microbial growth.

Although there was no discernable pattern in either the amount of growth in the Wash or any seasonal trend, this is not altogether inconsistent with the variable nature of the source and channel water. It is quite possible that there could be increased concentrations of pesticides or another chemical that would affect bacterial growth at one of the

sampling points. It is similarly possible that there might have been increased nutrient supplies due to particular Wash conditions that could allow for increased growth.

It is not only possible, but probable that the particular conditions of the Wash system are significantly affecting the ability of the water to sustain microbial growth over time. Each of the factors that have been described significantly affect microbial growth. It is well documented that the addition of sediment to natural waters will allow for an increase in bacterial counts (Davies *et al.* 1995, Desmarais *et al.* 2002, Doyle *et al.* 1992, Fish and Pettibone 1994, Fujioka *et al.* 1995, Van Donsel and Geldreich 1971). There is a strong possibility that similar events occur in the Las Vegas Wash system. These variable conditions may account for the difference in growth rates that was observed in the Las Vegas Wash.

The travel time in the Wash is approximately six hours from the discharge of the City of Las Vegas wastewater treatment plant to the confluence of the Wash with Lake Mead (James 2004) and from these data it appears that the fecal coliform level is not able to consistently regrow to a high degree during this time period. At this time, the only clear conclusion is that oxygenated Las Vegas Wash water is able, under favorable conditions, to sustain *E. coli* densities for up to one day at 25°C.

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Figure 1: Map of Sample Sites.

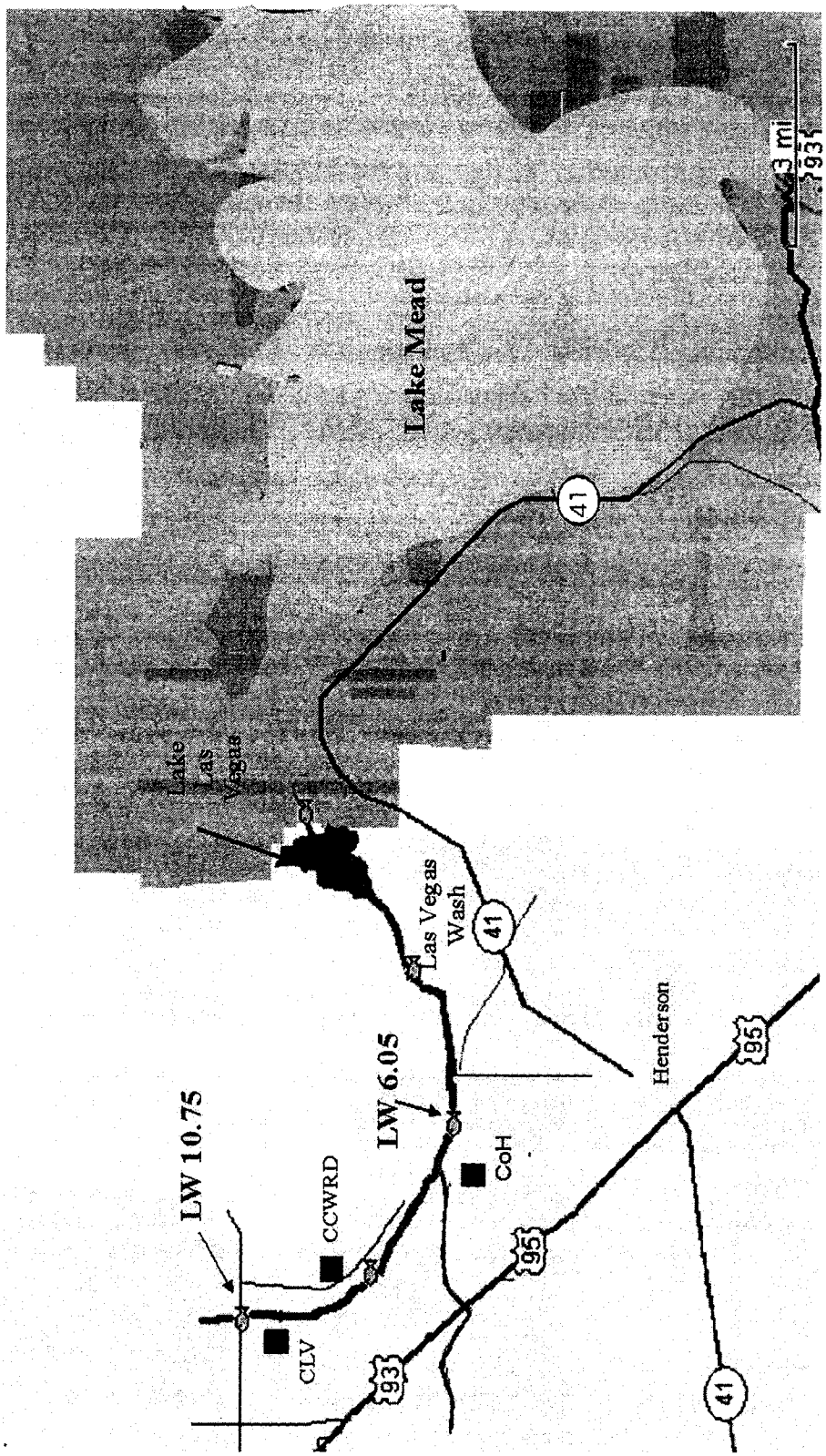


Figure 2: Growth of *E. coli* when placed in autoclaved water collected from LW 10.75. Collection Date - February 21, 2002.

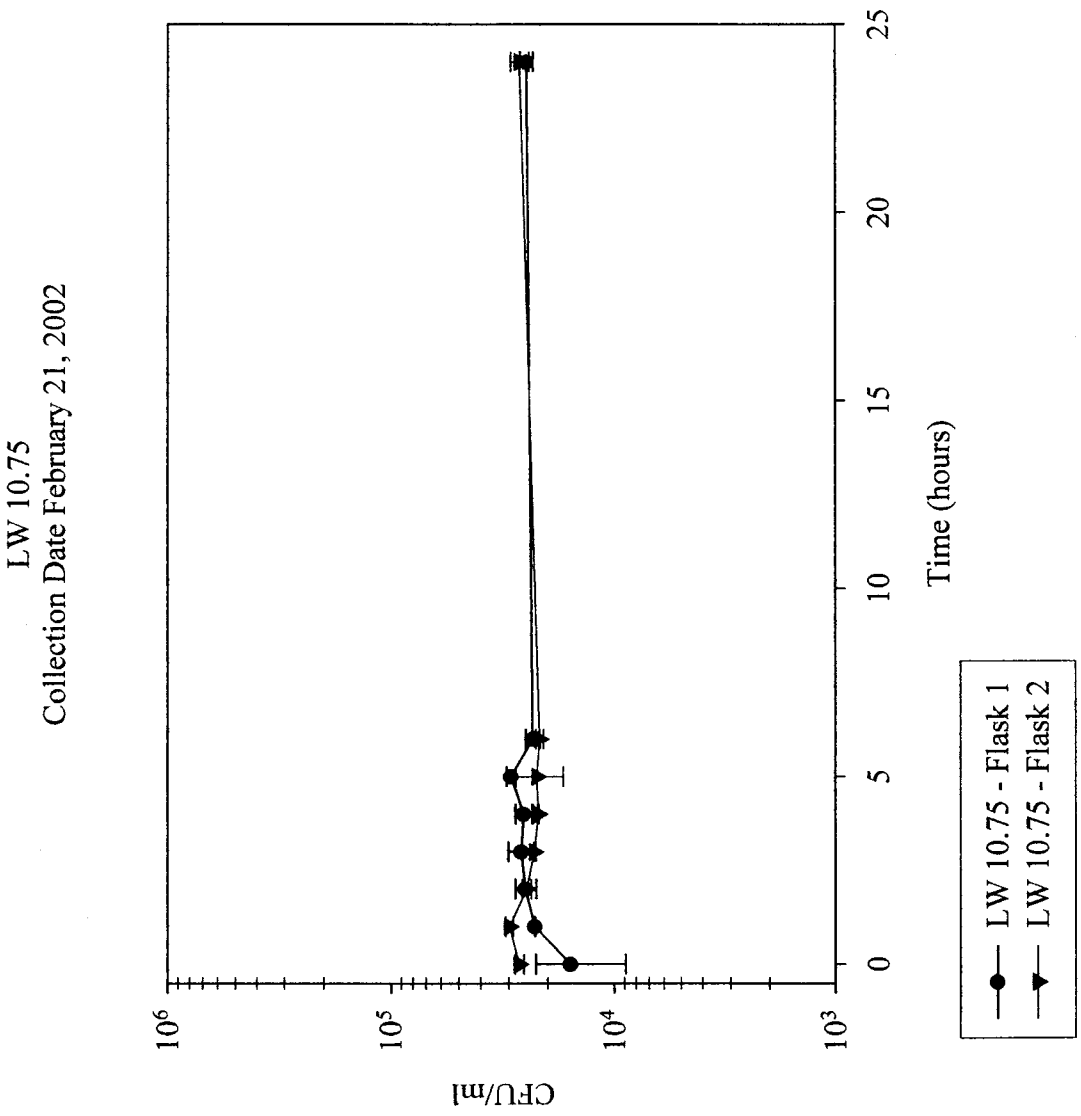


Figure 3: Growth of *E. coli* when placed in autoclaved water collected from LW 6.05. Collection Date - February 21, 2002.

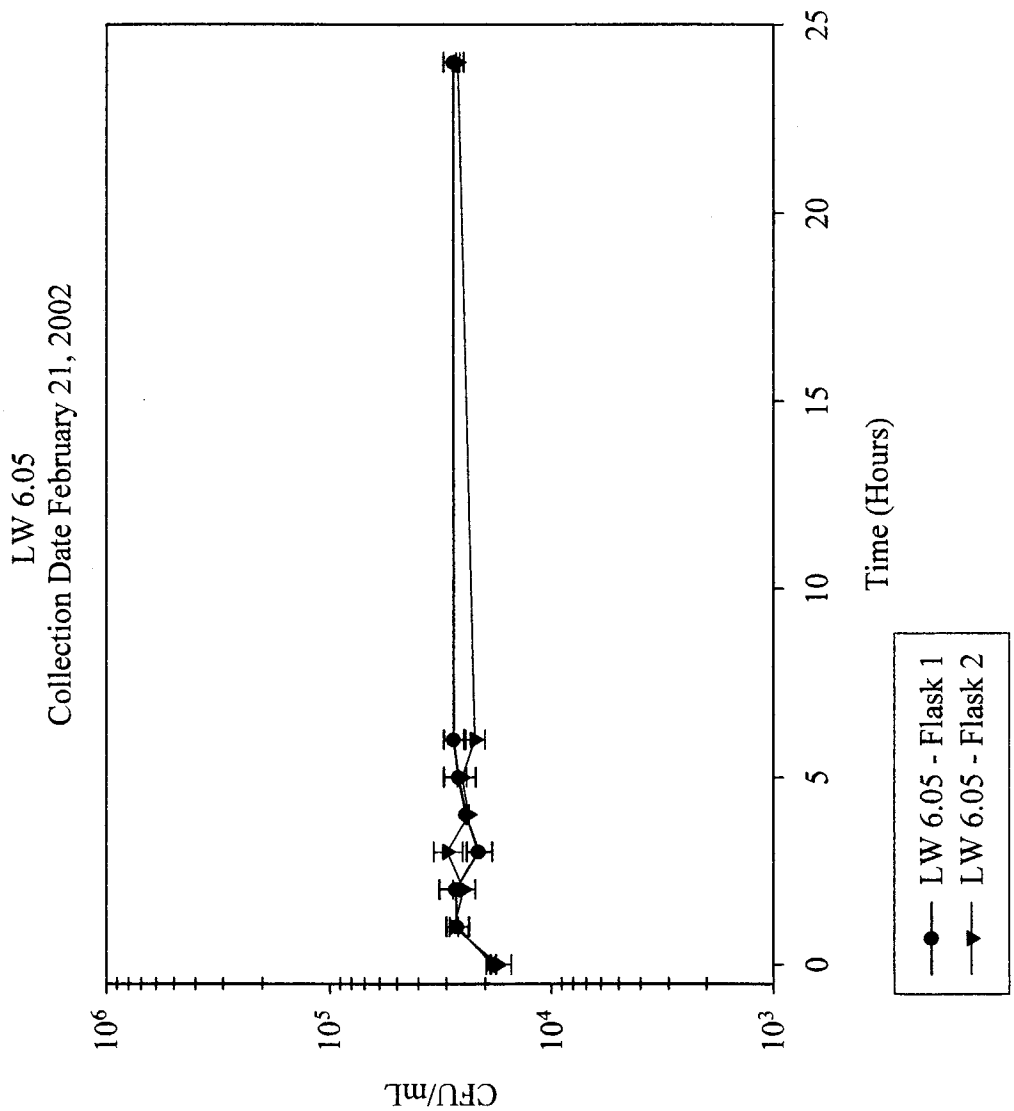


Figure 4: Growth of *E. coli* when placed in autoclaved water collected from LW 10.75 and LW 6.05. Collection Date – June 6, 2002.

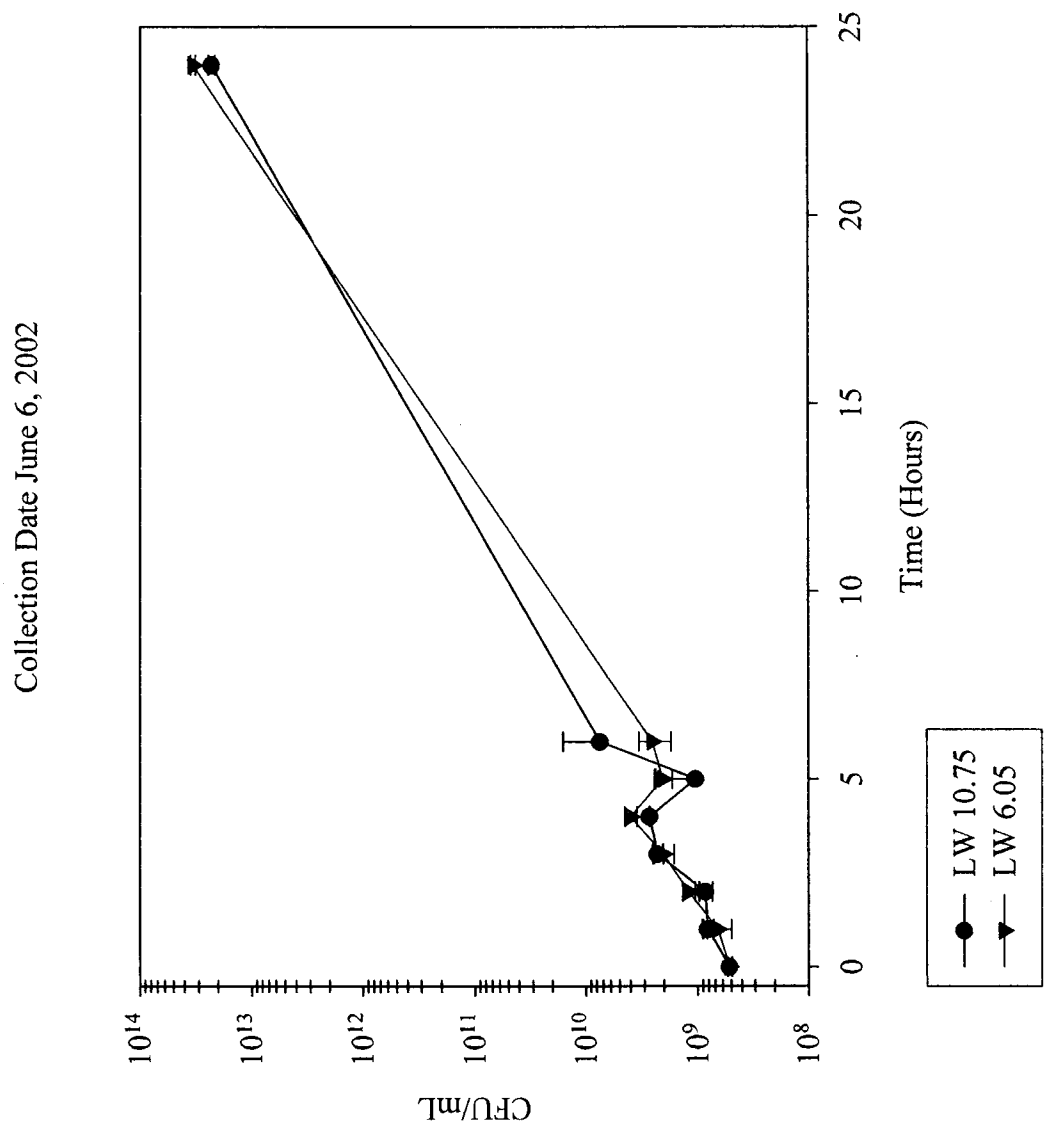


Figure 5: Growth of *E. coli* when placed in autoclaved water collected from LW 10.75. Collection Date – July 11, 2002.

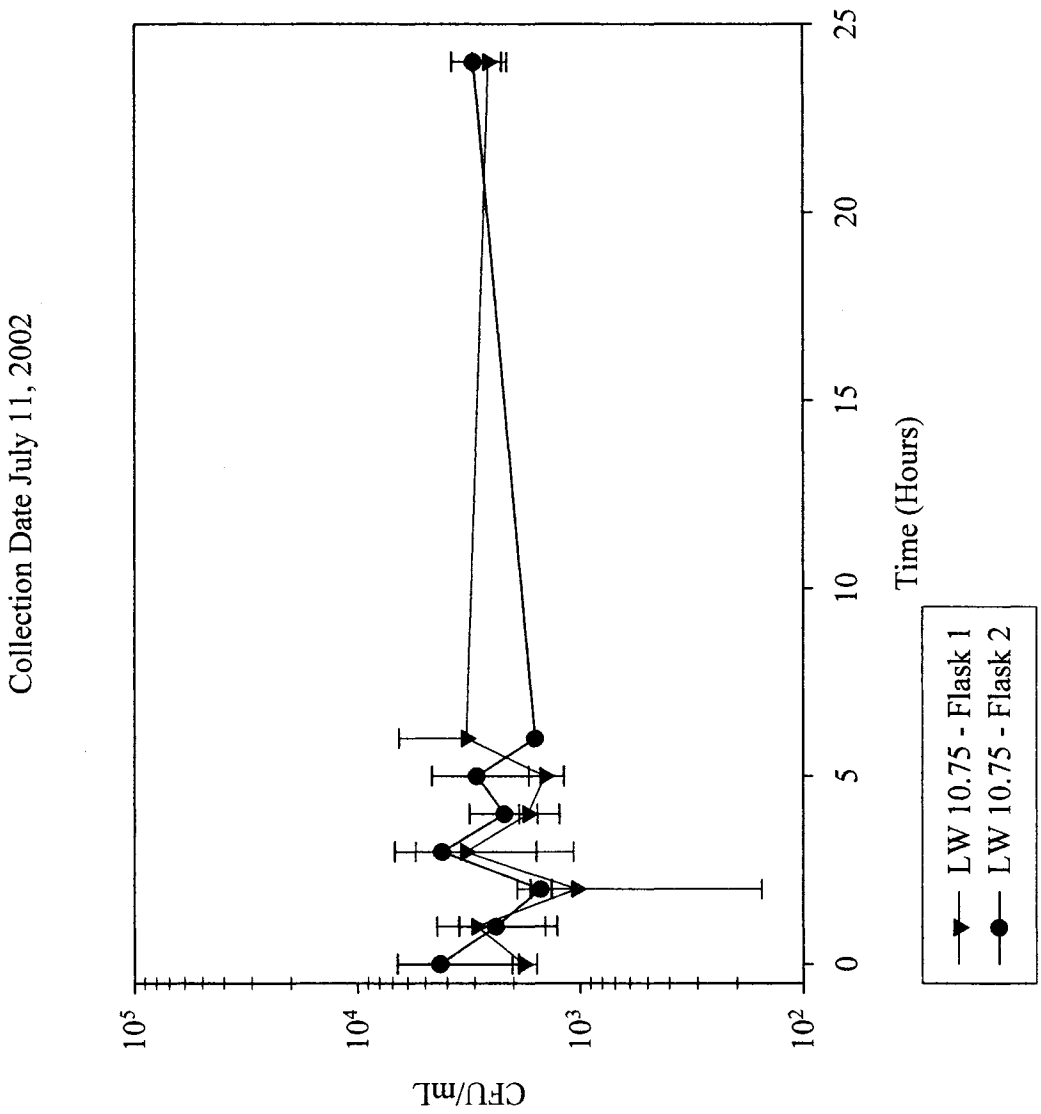


Figure 6: Growth of *E. coli* when placed in autoclaved water collected from LW 6.05. Collection Date – July 11, 2002.

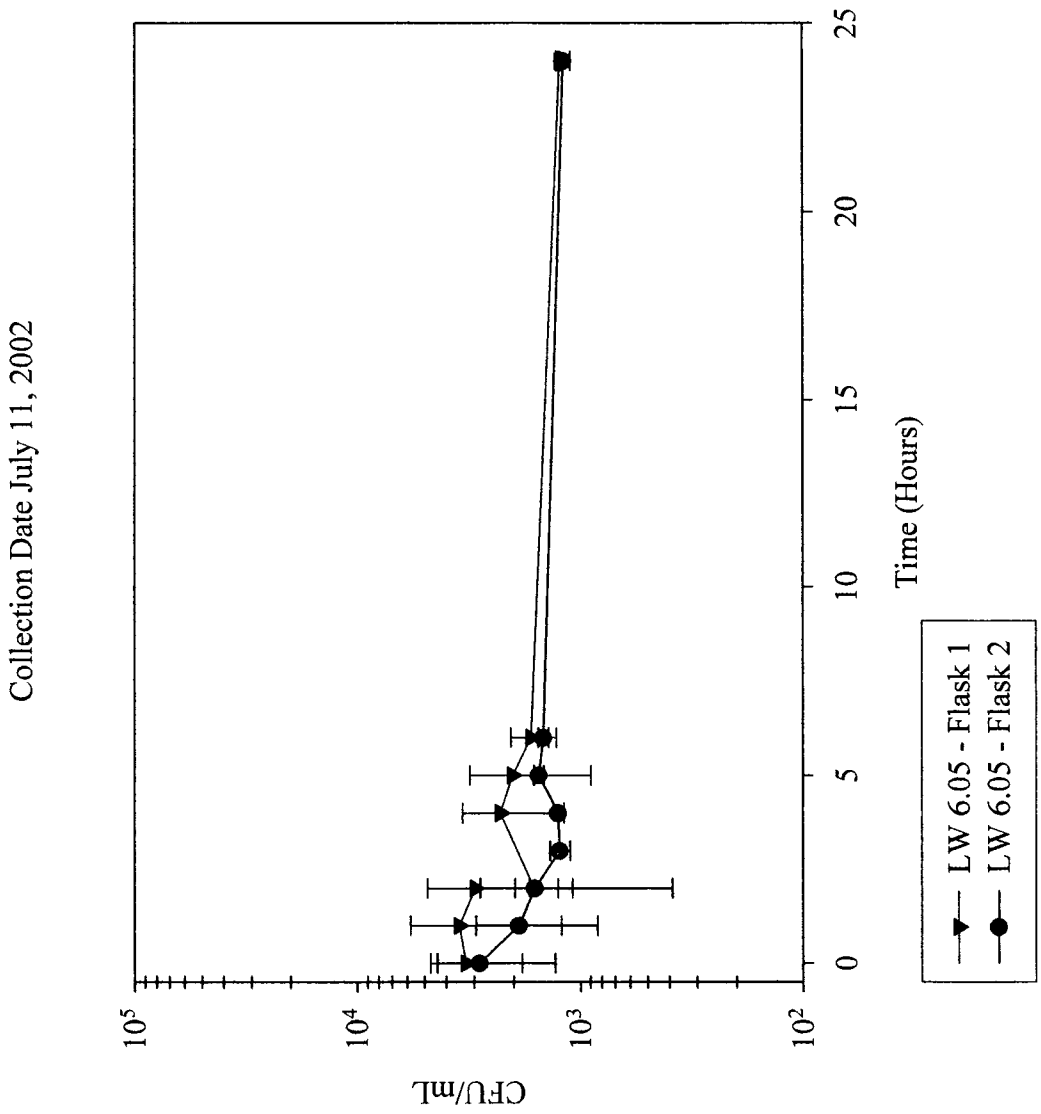
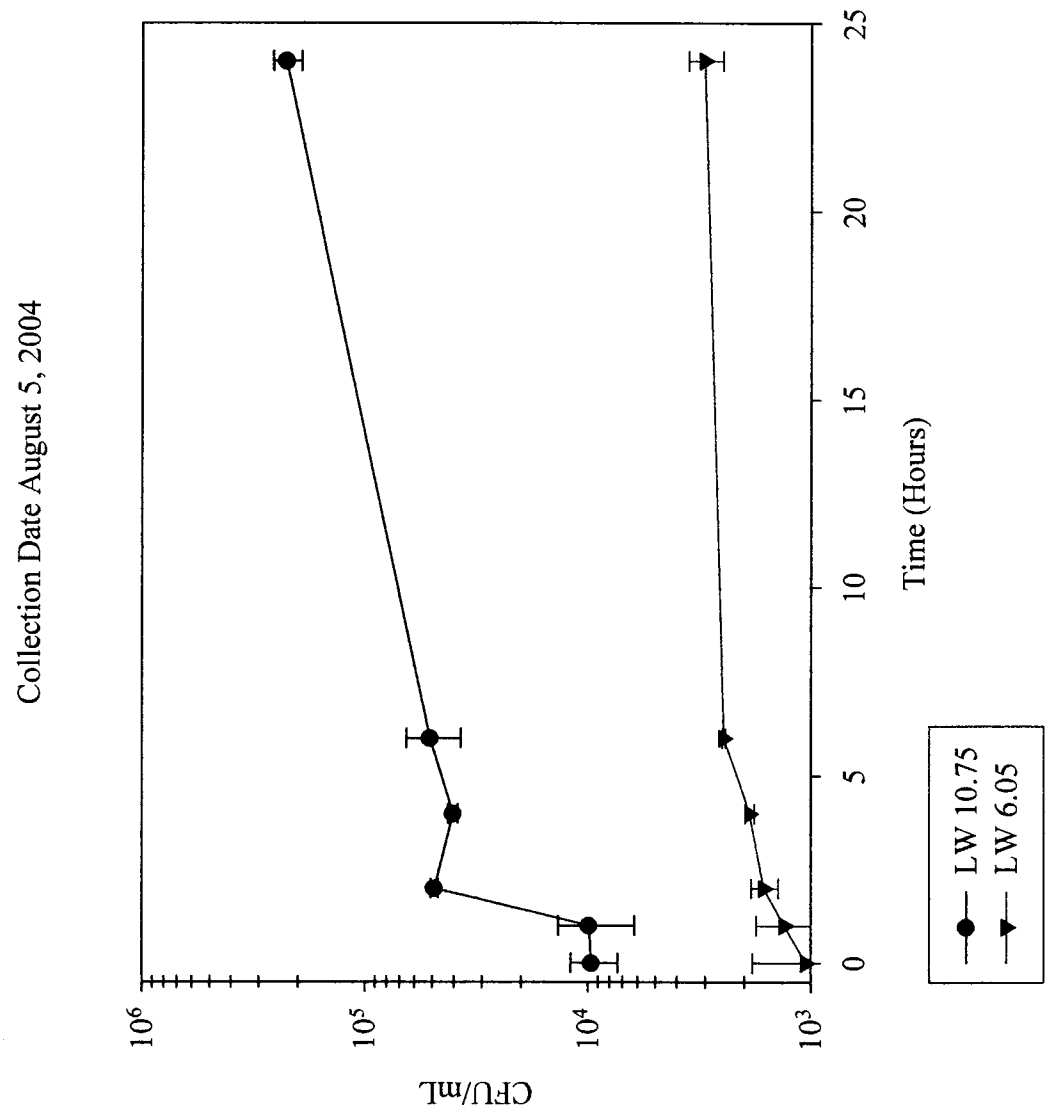


Figure 7: Growth of *E. coli* when placed in autoclaved water collected from LW 10.75 and LW 6.05. Collection Date – August 5, 2004.



CHAPTER 4

COLIFORM RECOVERY IN TERTIARY-TREATED EFFLUENT IN SOUTHERN NEVADA

This chapter has been prepared for submission to the *Journal of Water and Health* and is presented in the style of that journal.

Abstract

During wastewater treatment, indicator bacteria may become injured or stressed, rendering them unable to grow on conventional culture media. Microbes retain their viability and are capable of recovery in a favorable environment. Tertiary-treated effluent from two wastewater treatment plants was collected and analyzed for bacterial resuscitation. Standard recovery enhancement methods were used for recovery of fecal coliforms. Data indicate statistically insignificant recovery from wastewater disinfected by chlorination or chloramination. Wastewater disinfected by UV irradiation demonstrated statistically significant differences when compared with control samples. Both nutrient addition and temperature acclimation were shown to induce bacterial resuscitation from the viable but non culturable (VBNC) state in UV-disinfected wastewater effluent.

Introduction

The Las Vegas region is home to 1.3 million people and has 30 million visitors annually; it currently (2004) generates 160 million gallons of wastewater per day. Three

wastewater treatment plants are responsible for the treatment and subsequent discharge of wastewater in Southern Nevada. Each of the three entities, the City of Las Vegas Water Pollution Control Facility, the City of Henderson Water Reclamation Facility and the Clark County Water Reclamation District, produces tertiary-treated wastewater, operating under strict National Pollutant Discharge Elimination System (NPDES) permits as issued by the U.S. Environmental Protection Agency through the Nevada Division of Environmental Protection (NDEP). The effluent is disinfected by one of two methods: chlorination (City of Henderson, City of Las Vegas) or UV treatment (Clark County Water Reclamation District). Reclaimed water used for the watering of golf courses and parks in the City of Henderson is disinfected using chloramines.

The Las Vegas Wash is a naturally occurring and engineered tributary to Lake Mead, the principal drinking water source for the Las Vegas area. As the primary outlet for all treated wastewater, stormwater flows, urban runoff and groundwater seepage, the Las Vegas Wash is the only drainage channel for the entire 1600 square mile Las Vegas hydrographic basin.

The water quality of the Las Vegas Wash is of particular import to the Las Vegas population as the intake for the drinking water supply for the Las Vegas area is located five miles downstream from the outlet of the Wash into Lake Mead. Due to a multiple-year regional drought, the water level of Lake Mead has dropped severely. This decrease in the water supply and the prediction that the arid states in the West will likely "run out of water" in 2015, the reuse of water and its treatment to drinking water standards has become a major focal point. Specific attention has been given to the microbiological quality of the Wash for public safety.

Historically, fecal coliforms and, more recently, *Escherichia coli* have been used as indicators for water quality monitoring (*Standard Methods for the Examination of Water and Wastewater* 1998). Several groups of bacteria, total and fecal coliforms, fecal streptococci and *E. coli* are known as indicator organisms. Routine and relatively simple culture methods for detection of these microbes have been developed. As both healthy humans and animals ubiquitously excrete these indicator organisms, they are likely to be in high concentrations in wastewaters as compared to other specific pathogens. Their presence or absence is therefore thought to be a good indicator of the quality of the water in question (Moe 2002).

The area's three wastewater treatment plants usually discharge low to non-detectable most probable number (MPN) fecal coliform levels into the Las Vegas Wash. However levels as high as 10^6 per 100 mL have been observed during the course of Wash monitoring (City of Henderson 2003). The discharge permits dictate that effluent fecal coliform limits should not exceed "a log mean of 200 CFU [colony forming units] or MPN per 100 mL over a thirty day period, nor may more than 10% of the total samples taken exceed 400 CFU or MPN per 100 mL" (Nevada Division of Environmental Protection *Authorization to Discharge* issued to the City of Henderson 2001). Reuse water that is discharged to golf courses and parks may not exceed 2.2 CFU or MPN per 100 mL as a 30-day average with a maximum of 23 CFU or MPN per 100 mL as a daily maximum for fecal coliform levels (Nevada Division of Environmental Protection *Authorization to Discharge* issued to the City of Henderson 1999).

The source of high fecal coliform levels in the Wash itself is unknown. Previous studies conducted in the Las Vegas Wash attempted to estimate the origin of microbial

indicators through enterococcal speciation (Chapter 2). These data demonstrate that the majority of the Wash enterococci were naturally occurring and not a result of humans. The high bacterial densities that are observed in the Wash may also be due to direct deposition of fecal material from wildlife, plants, or from high levels of sedimentation that may enter into the Wash from runoff, storm events or urban pressures. There is the additional possibility that microbes deposited in the Wash may be able to use the environment to grow substantially. A third possiblity is the resuscitation of previously undetected bacteria.

There are two microbial physiology terms that are often incorrectly used interchangeably. These are bacterial regrowth and bacterial resuscitation. Regrowth, in the context of water and wastewater treatment, generally refers to the multiplication of microbes in a closed distribution system, as either planktonic cells or on surfaces of pipes, as biofilms (White 1999).

Our hypothesis focuses on bacterial resuscitation. Resuscitation involves a condition known as viable but nonculturable (VBNC) which bacteria have been found to enter. In this state, the bacterial cell will fail to grow on the media in which it is routinely cultured, but it is, in fact, still alive. This term was first used in 1982 to describe the dormant survival strategy of both *Vibrio cholerae* and *E. coli* after being subjected to nutrient stress. These cells would not grow under traditional culture conditions, but were proven to be metabolically active. Previous studies hae shown that unfavorable conditions, such as extreme temperatures, starvation, UV exposure, or exposure to metals or other substances such as chlorine may induce the VBNC state (Colwell and Grimes 2000).

E. coli, specifically, has been shown to undergo cellular repair following injury from radiation, including both UV and sunlight (Zimmer and Slawson 2002). McFeters and LeChevallier (2000) have estimated that between 50 and 90% of viable coliforms present in potable water systems may be injured, and are therefore not detected. Solar radiation has been reported to dramatically decay enteric bacteria numbers in the ocean as well as inactivate *E. coli* in river water (Gauthier 2000).

Pitonzo *et al.* (1999) described the entry of bacteria into the VBNC state due to exposure to gamma radiation in bacteria isolated from rock of the proposed Yucca Mountain Nuclear Waste Repository. Few details are known about what conditions allow VBNC to resuscitate to a culturable state, however some treatments are known to assist this process. *The Standard Methods for the Examination of Water and Wastewater* (1998) detail some of these treatments. Environmental microbiologists have repeatedly recounted microbial recovery on various types of media (Balkwill *et al.* 1989, Haldeman *et al.* 1994, Hattori 1980, Martin 1975, Olsen and Bakken 1987). Many studies suggest that VBNC organisms can be resuscitated by both physical and chemical means and may, in fact, be a significant part of natural microbial populations (Byrd *et al.* 1991, Haldeman *et al.* 1994, Kaprelyants and Kell 1993, Lopez and Vela 1981, Nilsson *et al.* 1991).

The cities of Las Vegas and Henderson utilize chlorination for disinfection of their wastewater effluent. Chlorination is well-studied as an effective means of disinfection. There are primitive methods documented as far back as 1879 when William Soper described the use of chlorinated lime to treat the feces of infected typhoid patients. The chlorine reactor was developed in 1913, fostering the use of chlorine as the integral part of wastewater treatment (White 1999). There are concerns, however, about some

potential adverse conditions associated with chlorination, chief among them is potentially hazardous by-products, possible chemical spills and negative effects on the surrounding environment.

Chlorine has also been shown to initiate bacterial cellular damage. *E. coli* and other enteric bacteria have become nonculturable after exposure to sublethal levels of chlorine. After undergoing a "recovery" process, they were able to regrow. Bacterial injury has been extensively documented in the food industry, resulting in the gross underestimation of indicator organisms (McFeters and LeChevallier 2000). It follows that this underestimation could also occur in water distribution and wastewater systems. Culturing methods associated with routine monitoring have largely been shown to be insensitive in the detection of stressed microorganisms, among them VBNC. Detection of these nonculturable bacteria may allow for remediation at the source and possibly identify treatment problems before the water enters the distribution systems (McFeters and LeChevallier 2000).

The City of Henderson uses chloramination for the disinfection of its reclaimed water for use on golf courses and parks. Chloramines were discovered in 1907 by Fritz Raschig. He noticed that sodium hypochlorite and ammonia reacted to produce a faint yellow compound he termed "chloramine" according to the following reaction: $NaOCl + NH_3 \rightarrow NH_2Cl + NaOH$. The process was used for water disinfection largely in the 1930s, after which other methods became more popular. Chloramination is the separate addition of ammonia and chlorine compounds to a water treatment system with the resulting formation of chloramines (White 1999).

The Clark County Water Reclamation District utilizes a Trojan UV system for its wastewater disinfection. This is a medium pressure system that uses lamps which produce a broad UV spectrum. Absorption of UV light disrupts the DNA/RNA sequence of the organisms, resulting in disinfection.

The Las Vegas area provides a unique opportunity to study similar and high quality tertiary-treated effluents that are disinfected by different methods, namely UV, chlorination and chloramination. In this study, standard recovery enhancement methods were employed to assess and compare the potential for resuscitation from wastewater effluent disinfected by UV, chlorine and chloramination in the Las Vegas valley.

Materials and Methods

Sample collection and preparation

Effluent samples were aseptically collected in autoclaved glass liter bottles from two wastewater treatment plants. Reuse water samples were aseptically collected in autoclaved glass liter bottles from one of the wastewater treatment plants. Samples were transported on ice and analyzed immediately upon returning to the laboratory. Measurements of chlorine residual (Hach Pocket Colorimeter Analysis System; Hach, Loveland, CO), temperature and pH were taken on all samples. Chlorinated samples were dechlorinated using sodium thiosulfate (140 mg tablets; Nasco, Fort Atkinson, WI). One tablet was used for each 100 mL of sample.

Laboratory analysis

Recovery enhancement method

A standard enrichment-temperature acclimation method was used to recover stressed fecal coliforms (*Standard Methods for the Examination of Water and Wastewater* 1998).

Samples were pre-filtered through sterile 8.0 μm filters and then through sterile 0.45 μm membrane filters. Sample volumes of 100 to 1000 mL were tested. The 0.45 μm filters were placed on bilayer mFC agar (Difco, Sparks, MD) with an overlay of tryptic soy agar (Difco, Sparks, MD). These samples were called the experimental or recovery samples. As a control, filter samples of the same volumes were placed on standard mFC agar. All samples were prepared in triplicate or more repetitions. Blanks were prepared at the beginning and the end of each test batch to ensure that no contamination was present. The experimental samples were placed in bags and into a water bath at 35°C for two, four or six hrs. and then transferred to 44.5°C until a total of 24 hrs. of incubation had been reached. The control samples were placed in bags and directly into a water bath at 44.5°C for 24 hrs. Colony forming units (CFU) per 100 mL were counted and recorded for all samples. Colonies were verified by growth in selective media (lauryl tryptose broth, brilliant green bile, EC medium and EC medium with mug (fluorescence; Difco, Sparks, MD).

Recovery of fecal coliforms using Wash water

Three liters of UV treated effluent were concentrated onto 0.45 μm membrane filters. These were placed in a flask of sterile Las Vegas Wash water and vortexed for 20 minutes to remove the bacteria from the filters. The flask was held at 35°C. Subsamples were filtered onto mFC agar in triplicate, placed in bags, and then into a water bath at 44.5°C for 24 hrs. Each sample was prepared in triplicate. Blanks were prepared at the beginning and the end of each test batch to ensure that no contamination was present. CFU per 100 mL of fecal coliforms were counted and recorded for all samples. Colonies were verified in selective media as described above.

Estimation of the effects of temperature and enrichment on recovery

UV treated effluent was collected and 100 mL samples were pre-filtered through sterile 8.0 μm filters and then through sterile 0.45 μm membrane filters in triplicate. Sample volumes of 100 mL were used. Blanks were prepared at the beginning and the end of each test batch to ensure that no contamination was present. Samples were placed into one of four groups: (1) -/-, filters were placed on bilayer mFC agar with an overlay of tryptic soy agar, incubated in a water bath at 35°C for 2, 4 or 6 hrs. and then transferred to 44.5°C for a total of 24 hrs. of incubation; (2) +/-, filters were placed on standard mFC agar and incubated in a water bath at 35°C for 2, 4 or 6 hrs. and then transferred to 44.5°C for a total of 24 hrs. of incubation; (3) -/+, filters were placed on bilayer mFC agar with an overlay of tryptic soy agar and incubated in a water bath at 44.5°C for 24 hrs.; (4) +/+, filters were placed on standard mFC agar and incubated in a water bath at 44.5°C for 24 hrs. CFU per 100 mL were counted and recorded for all samples. Colonies were verified in selective media as described above.

Sample Statistics

The mean, variance, standard deviation and student's t-test were conducted on the chlorinated samples to determine significance. The percent difference of growth compared to control was performed on chlorinated and UV-treated samples. Analysis of variance calculations were performed on UV-treated bilayer, standard mFC samples and on UV-treated samples placed in sterile Wash water. The least significant difference (LSD) and Duncan's Multiple Range tests were also conducted on these samples to determine if significant difference among samples occurred (Alder and Roessler 1960).

Results

Recovery enhancement

Recovery of fecal coliforms from UV, chlorine or chloramine-treated effluent is depicted in Table 1. The average number, in CFU/100 mL, of fecal coliforms that were recovered using the experimental method (enrichment/temperature acclimation) is compared to control (standard mFC at 44.5°C) samples. Two collection dates are shown for each experimental group.

In the case of disinfection using chlorine, the data indicate an average recovery of 235%. Although the data show an increase in viable mean numbers with resuscitation treatments, they are statistically insignificant when held to a 10% confidence level. The results for the recovery of fecal coliforms from effluent disinfected by UV irradiation are depicted in Table 1. The data indicate an average recovery of 1154%, ranging from a ten to twenty-fold increase in countable colonies.

The data depicting the recovery of fecal coliforms from effluent disinfected by chloramination are depicted in Table 1. The data show no recovery within limits of detection of fecal coliforms from the experimental group as compared with the control group.

Recovery of fecal coliforms using Wash water

The recovery of fecal coliforms using Las Vegas Wash water as a recovery medium is presented in Figure 1. Figure 1 shows the recovery of fecal coliforms over six hours using Las Vegas Wash water as a recovery medium. Even though the data show an increase in viable mean numbers with the increasing resuscitation times, none were significantly different based on the LSD test at 10% confidence level.

Estimation of the effects of temperature and enrichment on recovery

The recovery of fecal coliforms from UV-treated effluent, where temperature effects and nutrient effects were compared, is depicted in Tables 2 and 3. An analysis of variance of the samples (Table 3) that estimated the effect of temperature on recovery demonstrates a statistically significant difference to a 2.5% (p <.025) confidence level. Significance was confirmed by LSD and Duncan's Multiple Range tests (Table 4), which showed that there was a statistically significant difference between the time points to a p < 0.05 level.

An analysis of variance (Table 3) of the samples that estimated the effect of nutrients on recovery demonstrates a statistically significant difference to a 1% confidence level. Significance was confirmed by LSD and Duncan's Multiple Range tests (Table 4), which showed that there was a statistically significant difference between the time points to a p < 0.01 level.

Discussion

The data generated by this study indicate that there is the potential for fecal coliform resuscitation in some tertiary treated wastewater effluents. Chlorination and chloramination seem to be highly effective methods for the disinfection of wastewater, preventing recovery of fecal coliforms. These finding are based on experiments using large sample volumes (1000 mL), while monitoring requirements are based on 100 mL sample volumes.

Wastewater disinfection using UV light appears to allow for some recovery of fecal coliforms. Discharge permits set fecal coliform limits at 200 CFU/100 mL as a monthly average with 400 CFU/100 mL as a daily maximum. Although we observed a ten to

twenty-fold recovery of fecal coliforms from the wastewater effluent from the UV-treated effluent, none of our data exceeded the permit requirements under which these wastewater plants operate after 24 hrs. of culturing. It is possible, however, if coliform levels are approaching discharge limits in UV-treated effluent, that the effluent will contain enough VBNC organisms to violate those limits when bacteria in the sample are allowed to resuscitate in a favorable environment.

Further, using Wash water as a resuscitation medium, we were able to demonstrate that fecal coliforms are able to resuscitate in the Las Vegas Wash. This experiment demonstrates the possibility of bacterial resuscitation in a non-artificial system, one of which is in place to return treated wastewater to a potable water source. Resuscitation of fecal coliforms was less than that using the enrichment-temperature acclimation procedure, but nonetheless shows that this recovery may account for some of the fecal coliforms found in the Las Vegas Wash.

The final experiment presented here was an attempt to determine which factor, temperature or nutrient enrichment, was the most crucial in allowing for fecal coliform resuscitation. The data indicate both temperature acclimation and nutrient enrichment allow for significant amounts of bacterial resuscitation. The combination of the nutrient enrichment and low temperature acclimation did significantly allow for more fecal coliform resuscitation.

With decreasing water supplies in the western United States, water reuse and reclamation are becoming a increasingly important topics. We have demonstrated that tertiary treated effluent disinfected by UV light allows for the resuscitation of fecal

coliforms which were previously not detected. These studies show the need for a possible further evaluation of current monitoring practices in wastewater treatment.

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Table 1: Recovery of fecal coliforms from effluent disinfected by chlorine, UV and chloramines.

Chlorine			UV			Chloramines		
Collection Date	Average CFU/100 mL	% of Control	Collection Date	Average CFU/100 mL	% of Control	Collection Date	Average CFU/100 mL	% of Control
12/2/02	0.219	305%	1/17/03	9.83	97.80%	12/15/02	ND*	ND*
12/8/02	0.2	166%	1/21/03	2.1	2133%	1/19/03	ND*	ND*

*ND = Not Detected at 0.1 CFU/100 mL

Figure 1: Recovery of fecal coliforms using Las Vegas Wash water as a recovery medium. Fecal coliforms from UV-treated effluent were held at 35°C for 0, 2, 4, and 6 hrs. prior to transfer to 44.5°C for 24 hours. Error bars are (±) 1 standard deviation after 24 hours of recovery.

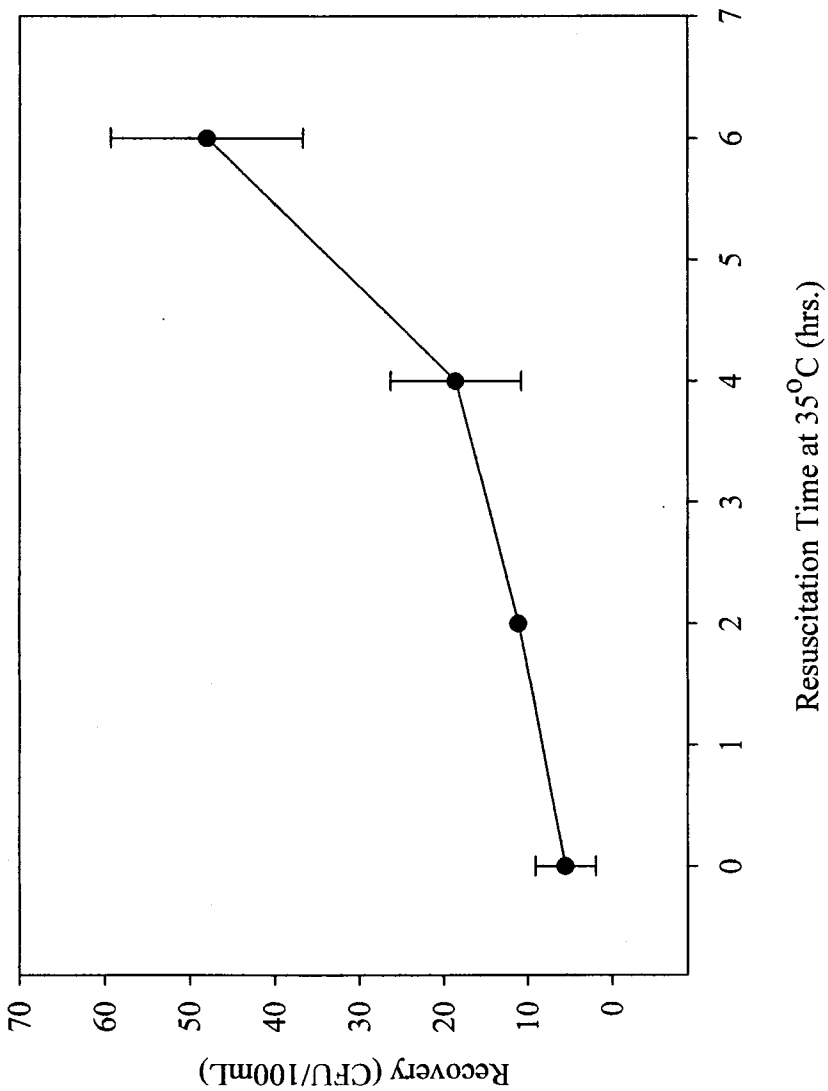

Table 2: Recovery of fecal coliforms from UV treated effluent. The +/- treatment is mFC agar incubated at 35°C for 2, 3, or 6 hrs., followed by incubation at 44.5°C for a total incubation period of 24 hrs. The -/- treatment is bilayer mFC agar with an overlay of tryptic soy agar incubated at 35°C for 2, 4, or 6 hrs., followed by incubation at 44.5°C for a total incubation period of 24 hrs. Recovery of fecal coliforms from UV treated effluent. The +/+ treatment is a control. It shows standard conditions of mFC agar incubated at 44.5°C for 24 hours. The -/+ treatment is bilayer mFC agar with an overlay of tryptic soy agar incubated at 44.5°C for 24 hrs.

Treatment	Average CFU/100 mL
+/+[a]	13
-/+[b]	24.3

	Average CFU/100 mL		
Treatment	2 hour	4 hour	6 hour
+/-[c]	18	63	78.5
-/-[d]	24.7	75.6	221.6

[a]Control – mFC at 44.5°C for 24 hrs.

[b]mFC with overlay of TSA at 44.5°C for 24 hrs.

[c]mFC at 35°C for 2, 4 or 6 hrs. prior to transfer to 44.5°C for total incubation of 24 hrs.

[d]mFC with overlay of TSA at 35°C for 2, 4 or 6 hrs. prior to transfer to 44.5°C for total incubation of 24 hrs.

Table 3: Analysis of variance (ANOVA) tables for three experiments: The first is UV-treated samples placed in a simulated Wash environment. The second is UV-treated samples placed on bilayer agar with temperature acclimation for 0, 2, 4, or 6 hours. The third is UV-treated samples placed on mFC agar with temperature acclimation for 0, 2, 4, or 6 hours.

Analysis of Variance Table -	For UV-treated samples placed in simulated Wash environment				
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Calculated	F.95, v1=3,v2=4
Total	4073.75	7		0.96	6.59
Between-means (or treatments)	2150.5	3	716.83		
Within samples (or error)	2998.5	4	749.63		

Analysis of Variance Table -	For UV-treated samples placed on bilayer agar with temperature acclimation for 0, 2, 4, or 6 hrs.				
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Calculated	F.99, v1=3,v2=8
Total	80684.25	11		178.44	7.59
Between-means (or treatments)	79496.25	3	26498.75		
Within samples (or error)	1188	8	148.50		

Analysis of Variance Table -	For UV-treated samples placed on mFC agar with temperature acclimation for 0, 2, 4 or 6 hrs.				
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Calculated	F.975, v1=3, v2=7
Total	11428.67	10		6.20	5.89
Between-means (or treatments)	8304.17	3	2768.06		
Within samples (or error)	3124.50	7	446.36		

Table 4: Duncan's multiple ranges for three experiments. The first is UV-treated samples placed on bilayer agar with temperature acclimation for 0, 2, 4, or 6 hours. The second is UV-treated samples placed on mFC agar with temperature acclimation for 0, 2, 4, or 6 hours. The third is UV-treated samples placed in a simulated Wash environment.

Analysis of Variance Table -	For UV-treated samples placed in simulated Wash environment				
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Calculated	F.95, v1=3,v2=4
Total	4073.75	7		0.96	6.59
Between-means (or treatments)	2150.5	3	716.83		
Within samples (or error)	2998.5	4	749.63		

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Within samples (or error)	1188	8	148.50		

Analysis of Variance Table -	For UV-treated samples placed on mFC agar with temperature acclimation for 0, 2, 4 or 6 hrs.				
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Calculated	F.975, v1=3, v2=7
Total	11428.67	10		6.20	5.89
Between-means (or treatments)	8304.17	3	2768.06		
Within samples (or error)	3124.50	7	446.36		

CHAPTER 5

GENERAL DISCUSSION

A significant and long-term drought in southern Nevada has made return flows in the Las Vegas Wash extremely important to southern Nevada's water resources. High levels of indicator microorganisms have been found in the Las Vegas Wash, prompting concern about its water quality, and particular attention is being paid to the possible health effects that may be associated with these levels as wastewater is returned to the source of drinking water.

Characterization of indicator organisms in the Las Vegas Wash was performed. Speciation of indicator organisms, specifically enterococci, was undertaken. As presented in Chapter 2, the majority of isolates were found to be avian or bird-associated species, with smaller inputs from human-associated species. Hagedorn *et al.* (1999) found in rural Virginia that 95% of fecally-associated microbes were from cattle, while in Washington, D.C. waterways birds, dogs and wildlife were found to be major contributors of fecal microorganisms with human inputs most notably only after storm events (Hagedorn *et al.* 1999, Porter *et al.* 2003).

The data indicate that a significant proportion of the enterococcal populations in the Las Vegas Wash are likely to be from environmental sources. Further, LW 6.05, Duck Creek and LW 10.75 fecal coliform and enterococcal population data suggest that the microbial populations are coming from non-point sources and are not a result of the large

discharges from the three wastewater treatment plants in the area. In contrast, raw wastewater influent, a mainly human source of fecal matter, contained mainly (90%) human-associated species (*E. faecium* and *E. faecalis*). Since the enterococcal populations in the Wash are mainly environmentally-associated species and the raw sewage influent species are mainly human-associated, it is likely that the fecal contamination is likely to be naturally occurring and not a result of the treated wastewater effluent that travels through this system. Although there is not a strict species-specific association of enterococci with hosts, analysis of samples comprised mainly of wastewater effluent, environmental samples, and raw wastewater effluent lead us to believe that there is validity in drawing conclusions as to the origin of contamination based upon enterococcal speciation. The data strongly suggest enterococci in the Wash are coming from non-point sources if the treated wastewater is not the main source of fecal microbial contamination.

Current research is underway that is studying the survivability of enterococcal species in Las Vegas Wash water. Enterococci were found to persist for weeks rather than days under mimicked Las Vegas Wash conditions. *E. faecium* and *E. gallinarum* survived the longest (up to 73 days). The length of survival correlates with the relative concentration of species in the Wash as the four most common enterococcal species found (Chapter 2) were also able to survive longest in simulated Wash conditions (unpublished data).

There is a possible source of the bird-associated microbes found in the Wash. A seasonal trend was observed in the Wash, in which during the winter, or cooler temperatures, there was greater species diversity compared with the summer. The Las Vegas Wash is located near a bird viewing preserve and a wetlands demonstration park.

It may be that the trend is associated with the over-wintering of birds in the area. Other researchers have found seasonal trends in indicator organisms with birds contributing substantial fecal loads during peak summer and spring seasons in Washington, D.C. waterways (Porter *et al.* 2003).

Many researchers have reported that sediments and soils along waterways are significant sources of microbes, including *Escherichia coli* (Kistemann *et al.* 2002, Solo-Gabrielle *et al.* 2000, Whitman *et al.* 2003). These soils may contribute substantial shares of the total microbial loads into waters. Marine and freshwater sediments have been shown to provide a favorable, non-starvation environment for *E. coli* for extended periods of time (68 days), even allowing for the detection of VBNC organisms (Davies *et al.* 1995). High concentrations of *E. coli* are consistently being found in foreshore sand and submerged sediment in Lake Michigan beaches, playing a significant role in lake water quality and a non-point source of *E. coli* to lake water (Whitman and Nevers 2003).

Our findings are consistent with these studies in that we saw greater species diversity in the sediments as compared with the water and plant samples. It is probable that the sediment is a suitable reservoir for indicator organisms, allowing for greater numbers of species to survive. Enumeration studies were not undertaken, but sediments would be expected to contribute relatively high bacterial densities as compared with water samples, since there are often large "spikes" in bacterial densities after a storm event when suspended solids are typically high in the water samples.

Samples downstream of treated wastewater effluent (LW 6.05) were taken following exposure to the municipal wastewater system. Although not a major source of

fecal microbes, the water is subject to human impacts, such as homes, hotels, cooking and even the particular treatments and chemicals associated with wastewater treatment.

With the advent of even more pharmaceutical use, there is the possibility that excess drugs are being excreted into our wastewater and passing through treatment systems. Particular concern has been shown with antibiotics because microbial resistance has become a serious medical threat to the general population. The fear is that natural microbes will develop resistance to antibiotics from exposure to resistant microbes from humans or animals treated with antibiotics, which would then cause them to be ineffective as treatment options.

Tests for antibiotic resistance to various antibiotics were performed on enterococci isolated from the Las Vegas Wash. The environmental isolates showed high resistance to low levels of vancomycin, while the sewage isolates showed no resistance to vancomycin at all. Although these environmental isolates may not be classified as truly vancomycin resistant, there is nonetheless concern as interspecies transfer of genetic elements between environmental and clinical isolates has been proposed (Guardabassi and Dalsgaard 2004). The differences in resistance in the environmental and sewage-related isolates are also notable and could be a result of excreted antibiotics being discharged through the wastewater effluent.

It is possible, that the environment in the Las Vegas Wash, particularily downstream from the wastewater effluent discharge, may be responsible for these variances, as the large volume of treated wastewater would contain a variety of pharmaceuticals. It is additionally possible the the Las Vegas Wash environment could favor growth or regrowth of antibiotic resistant strains or that natural but similar mechanisms have

developed in these bacteria to allow for them to survive in the presence of low levels of vancomycin.

Studies were undertaken to estimate the ability of this system to support microbial growth over time. *E. coli*, a microbe that is an indicator of fecal pollution, was used as the target organism. It is clear from this research that the Las Vegas Wash water supports a wide range of microbial growth rates. It is possible that growth rates are dependent upon the specific nutrients within the water sample. Additionally, growth rate differences may be due to a varying amount of sediment within the sample. Others have found that sediment may be protective in nature and act as a nutrient source and bacterial reservoir (Fish and Pettibone 1994). *E. coli* has been shown to remain fully culturable, at the same level, for 68 days in seeded marine sediments (Davies *et al.* 1995). It has been estimated that sediments contain up to 1000 times the number of fecal microbes found in the surrounding water (Van Donsel and Geldreich 1971). Sediment addition to water has been shown to aid in regrowth of both enterococci and *E. coli* in sand (Desmarais *et al.* 2002).

Even without fecal contamination, *E. coli* can live in subtropical climates where tidally influenced soils are able to help support such populations (Doyle *et al.* 1992, Fujioka *et al.* 1995). Increased nutrient concentrations and decreased predation have resulted in the multiplication of *E. coli* and enterococci in simulated environmental settings, with sediment providing favorable growth conditions (Desmarais *et al.* 2002). It appears that the survival and subsequent growth of *E. coli* in a secondary habitat is largely dependent upon overcoming low nutrient conditions and temperature fluctuations among other environmental factors.

Not only is sediment a factor, but the very nature of the Wash itself as a changing system would also account for the extremely wide range of growth rates seen in this study. Out of eight separate experiments in the Wash, four showed no real measurable growth or decay, one showed slight decay, one showed a 10^1 increase in growth, and two demonstrated a very large amount of growth (10^5). Modeling has provided the estimation that the transit time for Las Vegas Wash water to enter Lake Mead is approximately six hours from the City of Las Vegas wastewater treatment plant to the confluence of the Wash with the Lake (James 2004). Our studies (Chapter 3) have shown that under suitable conditions the Las Vegas Wash can allow for significant bacterial growth, however this does not seem to be occurring on a regular basis. The travel time in the Wash is approximately six hours from the discharge point from the City of Las Vegas wastewater treatment plant to the confluent of the Wash with Lake Mead, and it appears that the fecal coliform level is not able to consistently regrow to a high degree during this time period. At this time, the only clear conclusion is that oxygenated Las Vegas Wash water is able, under favorable conditions, to sustain *E. coli* densities for up to one day at 25°C.

Indicator bacteria may become injured or stressed during the course of wastewater treatment. These can no longer grow on conventional culture media but, in a favorable environment, may be capable of recovery. Known as viable but non culturable (VBNC), these microbes are the cause of considerable concern as they may become viable after undergoing microbial resuscitation even though they have not been detected by conventional testing of the same waters. It was possible that indicator bacteria became

injured during wastewater treatment then were released into the Las Vegas Wash where they were resuscitated.

A recovery enhancement method was undertaken to allow for potential resuscitation of fecal coliforms. Our data indicate that statistically significant numbers of fecal coliforms could be resuscitated from UV-treated effluent. Further experimentation demonstrated that over a time similar to transport of water down the Wash, the Las Vegas Wash was able to serve as a medium for the resuscitation of fecal coliforms.

Resuscitation could be shown when the growth temperature or the nutrient composition was changed. We found measurable increases (nearly a doubling) in growth when selective agar was supplemented with an overlay of non-selective media. When samples were placed in a less restrictive temperature (35°C instead of 44.5°C) recovery was over 200% of the initial bacterial density. The greatest recovery was found when a combination of both nutrient supplementation and temperature acclimation was used.

The data generated by these studies are important. They demonstrate that a wastewater treatment plant may produce high quality, tertiary-treated effluent by performing the appropriate laboratory monitoring, and adhering to their discharge permits, but may be releasing higher levels of indicator organisms than conventional methods can detect into a water system. It is apparent that there is a need to further evaluate current monitoring practices especially the UV treatment system employed by the largest wastewater treatment facility in southern Nevada.

When compared with wastewater effluent, density increases of 10^2 to 10^5 occur regularly in the Las Vegas Wash. The combined results of high proportion of environmentally-associated enterococci combined with modest resuscitation and

regrowth of fecal coliforms indicate that environmental sources are likely to be the largest contributors to high observed indicator densities in Wash samples collected for monitoring purposes. It is likely that these microbes are largely not a result of wastewater flows.

Although it may occur under optimal conditions, significant *E. coli* regrowth in Wash water does not appear to be a reliable source of indicator bacterial populations. It has also been demonstrated that a measurable number of indicator organisms are capable of resuscitation in the Wash water after being undetected in the wastewater effluent. These may be increased by a factor of 10 to 20. While important from a discharger's standpoint, this 10 to 20-fold increase in fecal coliform densities is likely not the cause of the large increase in bacterial counts in the Las Vegas Wash.

Rather, the most probable source for the high levels of indicator organisms found in the Las Vegas Wash is direct deposition from sediment, animal and plant reservoirs. Truckloads of sediment are removed from parts of the Las Vegas Wash on a regular basis. The millions of gallons of treated wastewater traveling through the Wash make for enormous levels of erosion through the channel. Storm events drastically impact the system. Simulated runoff events have demonstrated the potential for the deposition of indicator organisms (Piechota *et al.* 2002).

Future research directions may include the study of the specific environmental signals associated with birds and humans, both in fresh and old fecal matter. Instead of isolation from environmental sources, isolation could be made from direct sources to assess possible differences. These could then be compared and possibly correlated with prior studies.

A second research study that is partially underway involves the determination of the growth rates of enterococci in Wash water combined with simultaneous speciation of environmental isolates. Third, a VBNC experiment may be undertaken to determine if other indicator organisms, such as the fecal streptococci, also undergo resuscitation from wastewater effluent. This experiment would also be valid in determining if there are any enterococci that may be culturable in wastewater effluent and may be skewing the assumptions made (Chapter 2) on the environmental origins of the Las Vegas Wash enterococci.

Other sampling of raw sewage could also be undertaken to see if there is any difference between what is collected at the wastewater treatment plants. The possibility that irrigation could be contributing enterococci should also be investigated. To do so, one could sample and speciate enterococci from irrigated runoff and irrigated turf.

Possible management strategies could include the need to reevaluate current monitoring for the enumeration of fecal coliforms in exclusively disinfected UV-treated effluent. An alternative monitoring procedure, such as the enrichment-temperature acclimation procedure (Chapter 4) may need to be undertaken to ensure that VBNC coliforms are not being discharged into receiving waters.

Although sediment management strategies are already in place with the erection of erosion control basins and the proposal of an alternate discharge system for some of the generated wastewater, many professionals think of sediment as "dirt" and are not aware of the extreme microbial impact that these sediments are causing upon the surrounding waters. Not only do sediment management practices need to continue, education about it being such a large non-point source of indicator bacteria must accompany it.

The studies undertaken here have revealed significant information about the microbiology of the Las Vegas Wash, but it is clear that to have a full understanding further study is needed.

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