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HALMOS COLLEGE OF NATURAL SCIENCES AND OCEANOGRAPHY

Bacterial Indicators of Fecal Pollution: Exploring Relationships between Fecal Coliform and *Enterococcus* Groups in Central and South Florida Surface Waters

By

Shelby G. Craig

Submitted to the Faculty of
Halmos College of Natural Sciences and Oceanography
in partial fulfillment of the requirements for
the degree of Master of Science with a specialty in:

Marine Environmental Science

Nova Southeastern University

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Thesis of
Shelby G. Craig

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Masters of Science:
Marine Environmental Science

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ABSTRACT

Ambient and recreational surface waters worldwide experience fecal pollution due to a variety of anthropogenic sources. Fecal waste has been proven, for over a century, to harbor pathogenic microorganisms which subsequently cause a variety of disease and illness in human hosts. The benefits of utilizing fecal indicator bacteria (FIB) as a simple, inexpensive means to detect fitful human pathogens within a variety of water matrices are vast. However, no universal agreement exists in regard to which indicator is best suited for detection of fecal contamination and pathogens in environmental waters, and no single standard for bacterial indicators has been federally mandated.

This study sought to explore the potential benefits of a multiple-indicator approach to water quality analysis of fresh and brackish surface waters. The distribution and fluctuation of two frequently used, EPA approved groups of FIB – fecal coliform and *Enterococcus* – were explored, and relationships between the two FIB groups were examined in fresh and brackish surface waters of Central and South Florida. Samples were collected over a period of 12 consecutive months, spanning April 2015 through March 2016, and analyzed using membrane filtration procedures outlined in *Standard Methods* 9222D and EPA method 1600. Raw and log transformed colony forming unit (CFU) data, per 100 mL, was analyzed annually and seasonally through linear regression, Spearman correlation, and exploratory data analysis techniques performed in R-Studio.

The results of this study showed a moderate to strong relationship between fecal coliform and *Enterococcus* under both fresh and brackish conditions. The presence of a positive, linear relationship between fecal coliform and *Enterococcus* in both fresh and brackish water was apparent in both seasonal and annual regression analysis; upward and downward fluctuation(s) in one variable was shown to predict similar fluctuation(s) in the other year-round. However, while fecal coliform and *Enterococcus* showed moderate to strong correlations, causation was not implied. Low R^2 values showed that the FIB groups were *not* dependent upon one another in any case, either annually or seasonally. The results of this study challenge previously accepted views of fecal coliform and *Enterococcus* effectiveness as ideal fresh and brackish water FIB, their suitability as sole indicators of fecal pollution, and their ideal usage as indicators for waters of varying salinities; results support those previously seen in studies such as Hanes and Fragala 1967, which emphasize the need for a multiple indicator approach to water quality analysis of ambient and recreational waters experiencing brackish conditions.

Key Words: water quality, Enterococcus, fecal coliform, fecal indicator, pathogens, fecal pollution, bacterial indicator

TABLE OF CONTENTS

Abstract ii

List of Figures vi

List of Tables vii

List of Appendices viii

Introduction 1

Materials and Methods 10

Data Acquisition 11

1. Surface Water Sampling Procedure 11
2. Conductivity Procedure 11

Analytical Methods 12

1. Membrane Filtration Procedure 12
   a. Fecal Coliform Analysis 12
   b. Enterococcus Analysis 14
2. Quality Control/Quality Assurance Procedures 14
   a. Sample Collection and Transport 14
   b. Media and Reagents 14
   c. Supportive Equipment 15

3. Data Analysis 16

Results 17

Fecal Coliform Analysis 17

a. Freshwater 18
b. Brackish Water 18
c. Annual Fecal Coliform Trends 18

Enterococcus Analysis 19

a. Freshwater 19
b. Brackish water 19
c. Annual Enterococcus Trends 20

Fecal Coliform vs. Enterococcus 21

a. Freshwater Trends 21
b. Annual Freshwater Trends 23
c. Seasonal Freshwater Trends 24
d. Brackish Water Trends 26
e. Annual Brackish Water Trends 28
f. Seasonal Brackish Water Trends 29

Discussion 32

Conclusion 36
Current and Future Research 39
Works Cited 51

LIST OF FIGURES

Figure 1. Membrane filtration apparatus. 13

Figure 2. Fresh and brackish water fecal coliform counts. 18

Figure 3. Fresh and brackish water fecal coliform counts expressed as monthly averages over a period of 12 consecutive months. 19

Figure 4. Fresh and brackish water *Enterococcus* counts 20

Figure 5. Fresh and brackish water *Enterococcus* counts expressed as monthly averages over a period of 12 consecutive months. 21

Figure 6. Histogram analysis of freshwater fecal coliform and *Enterococcus* 22

Figure 7. Freshwater fecal coliform and *Enterococcus* counts; violin and box plots........22

Figure 8. Freshwater fecal coliform and *Enterococcus*; linear regression........................23

Figure 9. Freshwater fecal coliform and *Enterococcus* counts expressed as monthly averages over a period of 12 consecutive months. 24

Figure 10. Seasonal freshwater fecal coliform counts; violin and box plot.......................25

Figure 11. Seasonal freshwater *Enterococcus* counts; violin and box plot 25

Figure 12. Freshwater fecal coliform and *Enterococcus*; seasonal linear regression. 26

Figure 13. Histogram analysis of brackish water fecal coliform and *Enterococcus*. 27

Figure 14. Brackish water fecal coliform and *Enterococcus*; violin and box plots. 27

Figure 15. Brackish water fecal coliform and *Enterococcus*; linear regression. 28

Figure 16. Brackish water fecal coliform and *Enterococcus* counts expressed as monthly averages over a period of 12 consecutive months. 29

Figure 17. Seasonal brackish water fecal coliform counts; violin and box plot. 30

Figure 18. Seasonal brackish water *Enterococcus* counts; violin and box plot. 30

Figure 19. Brackish water fecal coliform and *Enterococcus* counts; seasonal linear regression 31

LIST OF TABLES

Table 1. Freshwater fecal coliform and *Enterococcus* counts per month..........................36

Table 2. Brackish water fecal coliform and *Enterococcus* counts per month 41

LIST OF APPENDICES

Appendix A. Freshwater CFU/100 mL data (raw) 42

Appendix B. Brackish water CFU/100 mL data (raw) 45

INTRODUCTION

In the United States, 24% of surface water bodies are listed as impaired due to elevated levels of enteric bacteria. These water bodies are too polluted, or otherwise degraded, to meet water quality criteria standards set by U.S. tribes, states, and/or territories. In the 2010 *National Water Quality Assessment*, the U.S. Environmental Protection Agency (U.S. EPA) listed pathogens as the leading cause of impairment for U.S. rivers and streams. Additionally, pathogens were listed as the second-ranked cause of impairment for U.S. wetlands and the third-ranked cause of impairment for U.S. bays and estuaries (U.S. EPA 2012b). By definition, a pathogenic microorganism is any microorganism capable of injuring its host – plant or animal. Pathogenic microorganisms may be bloodborne, foodborne, or waterborne, and include illness and disease causing bacteria, viruses, fungi, and protozoa. They are associated with a wide variety of diseases such as typhoid fever, cholera, sepsis, meningitis, hepatitis, tuberculosis, tetanus, leprosy, urinary tract infections, influenza, gastrointestinal illness, malaria, ringworm, and skin infections such as impetigo (Meals et al. 2013; Griffin et al. 2001). Waterborne pathogens associated with human fecal waste and pollution may use humans as a host organism and pose a serious public health risk, causing diarrhea, dehydration, and potentially fatal systemic infections (Meals et al. 2013). Waterborne disease outbreaks have been scientifically documented as far back as 1854, when the public health risk of pathogenic microorganisms harbored in human sewage first came to light amidst growing concern surrounding the spread of cholera (NRC 2004). Despite countless epidemiological studies and modern advances in the fields of sanitation and water quality, the World Health Organization (WHO) estimates 250 million cases of bathing-related gastroenteritis and upper respiratory disease continue to occur each year, even within the U.S. The majority of these outbreaks are caused by viruses or bacteria linked to fecal contamination, which cause disease through the fecal-oral route; organisms are ingested by a host and subsequently shed in fecal material (WHO 2009).

Fecal waste enters aquatic environments through sewage, agricultural runoff, urban/storm water runoff, direct input via defecation, boat disturbance of bottom sediments, inefficient septic systems or water treatment plants, and contaminated groundwater, soils, sands, and plant debris (Boehm et al. 2011; U.S. EPA 2006). Human

exposure to waterborne pathogens may occur during swimming and other recreational activity via ingestion, dermal contact through the skin or mucous membranes of the mouth, eyes, and nose, inhalation of mists or water particles within the air, and consumption of shellfish obtained from contaminated water bodies. Waterborne pathogens of primary concern include species of the *Campylobacter, Salmonella*, and *Shigella* families, as well as *Escherichia coli 0157:H7.* Additionally, *Vibrio cholerae, Helicobacter pylori,* and species of the *Clostridium*, *Legionella*, *Yersinia*, and *Mycobacterium* families are of secondary concern in terms of waterborne public health risk (Meals et al. 2013; NCBI 2004). Public health concern lies in the ability of waterborne pathogens to colonize the human bowl and intestinal tract, causing diarrheal illness of varying severity depending on group specific pathogenicity; while *Shigella* and *Campylobacter* species are mainly linked to simple diarrheal illness, bacterium such as *E. coli 0157: H7* and *S. typhimurium* are linked to hemorrhagic colitis and typhoid fever, which may be life threatening (Meals et al. 2013;

Public protection from waterborne pathogens and subsequent illness and disease is heavily rooted in rapid, accurate detection of pathogenic groups within the environment. However, direct testing for specific bacterial pathogens related to common waterborne illnesses is time consuming, costly, and impractical due to the erratic nature and low levels of pathogens within environmental waters (Cabral 2010). Indicator bacteria provide a practical, simple, inexpensive means to monitoring fecal pollution, pathogen concentrations, and ensuing human health risk(s) within environmental waters, and have been an integral part of the United States' public health system for over 100 years (Meals et al. 2013). Today, fecal indicator bacteria (FIB) are used worldwide as a means to closely monitor water quality and, indirectly, the risk of water-related illness which may result from contact with contaminated recreational, surface, and drinking waters (Boehm et al. 2011; Mara et al. 2003; National Research Council 2004).

FIB are native microflora colonizing the intestinal tract of humans and other warm-blooded animals. While some strains of FIB may be pathogenic, e.g. *E. coli 0157: H7*, FIB are generally not pathogenic themselves. However, their presence has been shown to coincide with that of harmful bacterial pathogens (Meals et al. 2013). Due to their enteric nature and abundance within humans and other warm-blooded animals, high

levels of FIB within fresh and marine waters is a strong indication of fecal pollution and ascertains the likelihood that human pathogens are also present within the matrix (Buckalew et al. 2006; Byappanahalli et al. 2012; Noble et al. 2003). Ideal assessors of fecal contamination traditionally possess a set of desired characteristics, outlined by the U.S. EPA. An indicator organism should be present whenever enteric pathogens are present, and in larger numbers; should have a longer survival time than the most durable enteric pathogens; should be present in intestinal systems of warm-blooded animals; their density should relate directly to a degree of pollution or contamination; they should not grow in water matrices; and should be able to be isolated from all types of water using a simple laboratory test method. Over the years, progressive guidelines outlined and revised by the U.S. EPA have led to the selection of four ideal assessors of fecal contamination in regards to surface and drinking waters – total coliforms, fecal coliforms, *E.* coli, and *Enterococcus* (U.S. EPA 2006). At present, microbiological standards of recreational water quality are based on coliform, *E. coli*, and enterococci concentrations (U.S. EPA 2006; Mara et al. 2003). While coliform and enterococcal groups are both natural parts of the human intestinal microflora, each group provides a unique insight into the microbiological quality of water.

Coliform bacteria are native microflora of the warm-blooded animal intestinal tract and may account for up to 50 percent of biological material found in fecal waste. The coliform group belongs to the family *Enterobacteriaceae* and includes *E. coli* as well as *Enterobacter*, *Klebsiella*, and *Citrobacter* species. Coliforms are rod-shaped aerobic or facultative anaerobic bacteria which are gram-negative and non-spore-forming. They are distinguished by their ability to produce acid and gas as byproducts of lactose fermentation, after a 48 hour incubation period at 35.0°C (APHA 1999; U.S. EPA 2006). Coliform bacteria have been used by public health agencies as FIB since the 1920s, traditionally as a primary indicator of potability for drinking water (NRC 2004). In 1914 the U.S. Public Health Service (USPHS) set the earliest formal drinking water standards, requiring the total absence of the coliform organism from drinking water. This standard was soon put into use across the United States (U.S. Treasury Department 1914). Although coliforms may be of fecal origin, their ubiquitous nature in plant materials and soils, as well as their ability to propagate in extraenteric environments, makes the

presence of total coliforms an unreliable indicator of fecal contamination in ambient waters (Cohen and Shuval 1973; Mark 1977). As a result, coliform methods for the detection of fecal contamination and waterborne pathogens in recreational and ambient waters have evolved towards the use of the fecal coliform group. Despite its limitations in ambient waters, the total coliform group continues to be at the forefront of modern potable water testing.

The fecal coliform group, a subset of the total coliform group, includes thermotolerant coliform bacteria distinguished by an ability to ferment lactose at elevated temperature(s) – 44.5°C. Members of the fecal coliform group include *Klebsiella* species and, most notably, *E. coli.* Several studies have shown strong correlations between the fecal coliform group and pathogenic bacteria, making fecal coliform a useful indicator of water treatment effectiveness and fecal contamination in aquatic matrices, such as drinking and recreational waters (Polo et al. 1999; Wilkes et al. 2009). Fecal coliform bacteria, while proven to be an effective indicator of fecal contamination, have several limitations to environmental biotic and abiotic factors. Due to their enteric nature and resultant low oxygen tolerance, the fecal coliform group has demonstrated short survival rates outside of a host environment (Savichtcheva and Okabi 2006). In addition, fecal coliform bacteria, most notably *E. coli* species, have shown high sensitivity to saline environments; specifically, large increases in death rates with seawater concentration (Anderson et al. 1979; Ayres et al. 1977; Hanes and Fragala 1967; Švec et al. 2009). Low levels of fecal coliform correlation to pathogens, and low sensitivity of fecal coliform detection methods have also been reported (Horman et al. 2004; Winfield and Groisman 2003). Finally, fecal coliform bacteria have been shown to multiply after release into the water column, and some fecal coliform species, e.g. *Klebsiella pneumoniae,* have been proven to originate from non-fecal sources (Desmarais et al. 2002; Scott et al. 2002; Simpson et al. 2002; Solo-Gabriele et al. 2000).

Fecal coliform was proposed for use in recreational water quality criteria in 1968 by the National Technical Advisory Committee (NTAC) of the U.S. Federal Water Pollution Control Administration, and officially adopted as a recreational water quality indicator in 1976 by the U.S. EPA (U.S. EPA 1976). In 1986, the U.S. EPA recommended *E. coli* as the sole indicator for monitoring freshwaters due to further

research into fecal coliform limitations in regard to saline environments (Švec et al. 2009; U.S. EPA 1986). In a 2016 revision to Florida Administrative Code 63-302.530, the Florida Department of Environmental Protection (DEP) introduced water quality criterion for *E. coli* in predominately fresh Class III and Class III-Limited surface waters. The state of Florida recognizes Class III surface waters as those used for fish consumption, recreation, and propagation and maintenance of a healthy, well-balanced population of fish and wildlife. Under the 2016 63-302.530 amendment, *E. coli* most probable number (MPN) or membrane filtration (MF) counts shall neither exceed a monthly geometric mean – based on a minimum of 10 samples taken over a 30-day period – of 35 CFUs, nor exceed 130 CFUs in 10% or more of samples during any 30-day period (DEP FAC 2016). While *E. coli* is currently recommended as the best choice for freshwater surface water monitoring programs, many regions across the state of Florida continue to utilize the DEP 2010 Surface Water Quality Standards outlined in F.A.C. 62-302.530. Under the 2010 DEP surface water standards, fecal coliform MPN or MF counts shall neither exceed a monthly average – expressed as a geometric mean– of 200 CFUs, nor exceed 400 CFUs in 10% of samples, nor exceed 800 CFUs within a single sample in predominately fresh Class III and Class III-Limited surface waters. Despite aforementioned studies into the sensitivity of fecal coliforms to saline environments, these criteria also stand for fecal coliform in predominately marine Class III and Class III-Limited surface waters (DEP FAC 2010). Today, fecal coliform use has spread to include assessment of environmental waters used for shellfish collection and consumption. They have been approved as a FIB by the U.S. Food and Drug Administration's National Shellfish Sanitation Program (NSSP) (U.S. EPA 2006; NCBI 2004; WHO 2009).

Enterococcus became a unique genus in 1984 after being previously classified within the fecal streptococci group of the genus *Streptococcus.* While the use of fecal streptococci as an indicator of recent fecal contamination is no longer considered a reliable means for monitoring water quality, the previously grouped fecal streptococci *S. faecalis, S. faecium, S. avium,* and *S. gallinarum* are now considered to be of the *Enterococcus* genus. To date, there are 36 known *Enterococcus* species, classified into five groups – *E. faecalis, E. faecium, E. avium, E. gallinarum,* and *E. cecorum*

(Byappanahalli et al. 2012; Meals et al. 2013). Enterococci are native enteric microflora of the family *Enterococcaceae,* which are found in high concentrations within the human colon. Enterococci have been found to reach numbers as high as 10^8 CFUs per gram wet weight of feces, although they represent an insignificant proportion of the total human intestinal microflora, less than 1% (Boehm et al. 2003; Tendolkar et al. 2003). Enterococci are cocci – spherical or ovoid – cells arranged in pairs or chains. They are gram-positive, non-spore-forming, catalase-negative, facultative anaerobes capable of cellular respiration in both oxygen-rich and oxygen-poor environments. Chemoorganotrophs, enterococci obtain energy needed for cellular function through the break down of chemical bonds in organic compounds such as sugars, proteins, and fats (Byappanahalli et al. 2012; Švec et al. 2009). They have an optimal growth temperature of 35°C. *Enterococcus* have gained a reputation for being naturally rugged organisms, able to survive at temperatures as high as 60°C, in broths containing high concentrations of salts – 6.5% NaCl – and in broths with a pH of 9.6. Additional attributes such as growth over a temperature range of 10 to 45°C, a tolerance of pH 4.5 to 10, and survival within 40% bile salts make *Enterococcus* well suited for extraenteric survival. Enterococci have been found to be widely distributed within a variety of heterothermic environments including tropical and temperate soils, fresh and marine water sediments and beach sands, aquatic and terrestrial vegetation – e.g. algae, submerged vegetation, and wrack – and ambient waters such as rivers, streams, and creeks (reference).

The *Enterococcus* family is commensal, providing aide during digestion and other metabolic pathways within the gut of humans and other warm-blooded animals. While enterococci from the gastrointestinal tract of healthy humans are generally non-virulent, they are traditionally classified as opportunistic pathogens capable of causing a variety of foodborne, waterborne, and nosocomial infections. Enterococcal infections include gastrointestinal illness, endocarditis, and bacteremia, as well as urinary tract, neonatal, central nervous system, and abdominal/pelvic infections. Although each *Enterococcus* group includes human pathological species, *E. faecalis* and *E. faecium* are the most commonly implicated in regard to nosocomial infection (Boehm et al. 2011; Byappanahalli et al. 2012; NCBI 2004; Tendolkar et al. 2003) Additionally, *E. faecalis* species are commonly found in surface and drinking waters while species of *E. faecium*

and *E. gallinarum* have been found in aquatic and terrestrial vegetation, as well as fresh and marine water sediments and soils (Byappanahalli et al. 2012). Due to their ubiquity in nature, positive associations have been made between enterococci concentrations and swimmer related gastrointestinal illness in both fresh and marine waters across the globe. In addition, enterococci have been linked to pathogens of the *Campylobacter* and *Salmonella* genera in surface water studies conducted by Viau et al. in 2011; Walters, Thebo, & Boehm in 2011 (Kay et al. 1994; Wade et al. 2006; Wiedenmann et al. 2006).

Although oftentimes outnumbered within the gut by other enteric species such as *E. coli* and *Bacteroidales,* the ubiquity of enterococci in human feces and the ability of the genus to survive, even thrive, under extraenteric conditions makes them a subject of extensive study as a FIB well suited for environmental waters (Boehm et al. 2011; Byappanahalli et al. 2012). However, the *Enterococcus* group has demonstrated several limitations and sensitivities to environmental biotic and abiotic factors. A loss of *Enterococcus* culturability due to sunlight inactivation has been shown in several studies by Davies-Colley et al. 1994; Fujioka et al. 1981; Noble et al. 2004. Despite the increased ability of *Enterococcus* to survive in high salt concentrations, enterococci have also shown sensitivity to saline environments. An inverse relationship between enterococci survival, detection, and salinity has been demonstrated in studies by Carr et al. 2010; Dorsey et al. 2010; Viau et al. 2011. Additionally, a 2005 study by Anderson et al. showed a two-fold increase in *Enterococcus* decay rates in marine environments versus freshwater environments. Finally, *Enterococcus* are prone to nutrient starvation when transitioned from a nutrient rich gastrointestinal system to oligotrophic waters, and predation by protozoa in both marine and freshwater environments (Boehm et al. 2005; Davies et al. 1995; Gonzalez et al. 1990; Iriberri et al. 1994; Menon et al. 2003; Sinclair et al. 1984).

In 1986, the US EPA first proposed *Enterococcus* for use as the sole indicator for monitoring oceanic waters (US EPA 1986). *Enterococcus* was officially adopted by the U.S. EPA for use in marine waters in 2016. Enterococci criteria was implemented by the DEP for predominately Class III and Class III-Limited surface waters in the 2016 revision of F.A.C. 62-302.530. Under the 2016 amendment to 62-302.530, *Enterococcus* most MPN or MF counts shall neither exceed a monthly geometric mean – based on a

minimum of 10 samples taken over a 30-day period – of 35 CFUs, nor exceed 130 CFUs in 10% or more of samples during any 30-day period (DEP FAC 2016). Today, *Enterococcus* is the only fecal indicator group recommended by the U.S. EPA for brackish and marine waters (Byappanahalli et al. 2012).

Multiple studies comparing fecal coliform and *Enterococcus* have shown strong correlations between the two bacterial indicator groups within environmental waters. In 1997, Medema et al. discovered a strong relationship between fecal coliform and *Enterococcus* in freshwater sites heavily influenced by sewage and agricultural runoff. Several years later, in 2009, Wilkes et al. also found a significant correlation between fecal coliform bacteria and *Enterococcus* in Canadian river surface waters (Medema et al. 1997; Wilkes et al. 2009). However, the aquatic environment is an unnatural place for enteric bacteria, and survival rates of FIB within aquatic matrices depends largely on organismal fitness, abundance in feces, and hydrological processes used to transport the organisms within the environment. As a result, correlations between fecal coliform and *Enterococcus* groups have been shown to vary between aquatic environments due to group-specific limitations to environmental biotic and abiotic factors. Sunlight and U.V. exposure, salinity, temperature, turbidity, suspended solids, predation, and type(s) of wastewater input have been shown to decrease or inactivate FIB (Anderson et al. 1979; Hanes and Fragala 1967; Noble et al. 2003; Noble et al. 2004; Rozen and Belkin 2001; Švec et al. 2009). In addition, FIB concentrations have been found to be significantly related to additional parameters such as time of sampling, sampling season, and location of collection (Brenniman et al. 1981; Bezirtzoglou et al. 1994; Hirn et al. 1980; Maipa et al. 2001). Seasonal variations between indicators during wet and dry periods have been seen in studies by An et al. 2002; Gannon and Busse 1989. FIB groups have been shown to vary by up to three orders of magnitude within 24-hour periods of dry weather (Dorsey et al. 2010).

Despite worldwide use of FIB for assessing recreational water quality, a universal agreement does not exist in regards to which indicator organism, or combination of organisms, is most useful. Although the U.S. EPA has outlined threshold levels and limitations for specific indicators, no single standard for bacterial indicators has been federally mandated. This is, in part, due to group-specific limitations set by

aforementioned environmental biotic and abiotic factors, and the associated challenges placed on each group of FIB. Under the Clean Water Act (CWA), each state is required to implement and uphold water quality standards which protect and maintain the chemical, physical, and biological integrity of the nation's surface waters. According to the CWA, this level of water quality "provides for the protection and propagation of fish, shellfish, and wildlife, and provides for recreation in and on the water." While threshold levels and limitations for bacterial indicators in ambient waters have been outlined by the U.S. EPA, primary authority for maintenance of water quality, implementation of water quality management programs, and the safety of recreational fresh and marine waters is given to state and local governments; states may set their own bacteriological limits for coliform and enterococci, or even use alternative indicators (NRDC 1998). As a result, variations in fecal indicator usage and levels of protection exist in water quality programs across states, countries, and regions. In a 2003 status report on bacterial water quality standards for recreational waters, the U.S. EPA reported that 6 states, 3 tribes, and 2 territories use *Enterococci* as a standard for freshwaters, while 9 states and 4 territories use *Enterococci* as a standard for marine waters; 18 states, 12 tribes, and 2 territories adopted *E. coli* as the freshwater standard (U.S. EPA 2003). Today, states such as California and Texas have set limitations above or below U.S. EPA recommendations; areas such as HI have supplemented beach water quality monitoring programs with *Clostridium perfringens*, an alternative indicator; areas such as NY and RI monitor fresh and brackish water quality through the use of both total coliform and fecal coliform groups; areas such as AL and GA monitor water quality through the use of a single indicator – fecal coliform – for both brackish and marine waters; areas such as ME and MD have implemented *E. coli* and *Enterococcus* to fresh and brackish water quality monitoring programs ,with the addition or exclusion of fecal coliform; and areas such as CA and Puerto Rico continue to monitor water quality parameters through the use of all three common FIB groups – total coliform, fecal coliform, and enterococci (Griffin et al. 2001; Noble et al. 2003; Shibata et al. 2004; U.S. EPA 2003).

The selection and subsequent use of FIB has crucial implications to the water quality assessment and management of ambient waters, as the concentration and response of fecal indicators within the environment directly affects the number of surface water

sites which pass or fail established water quality standards (Noble et al. 2003). In a 2004 study of two Florida beaches, Shibata et al. discovered discrepancies between water quality ratings – pass or fail – based on fecal coliform and those based on enterococci. It was discovered that water quality ratings for a particular beach not only depended upon the selection of sampling site, but the microbial indicator used during the assessment. In the Shibata study, enterococci consistently provided lower ratings for beach sites than other bacterial indicators based on U.S. EPA, Florida Department of Health (FDOH), and FDEP recreational water quality standards (Shibata et al 2004). Similar results were seen in earlier studies conducted by Jin et al. in 2004; Noble et al. in 2003; Crowther et al. in 2001. The results of these studies prove that choice of indicator microbe(s) for monitoring surface waters may lead to the passing or failure of a sampling site. As seen in the Shibata et al. study, different ratings can be obtained for the same body of water depending upon the indicator microbe(s) chosen (Shibata et al. 2004).

This study explored distribution(s), fluctuation(s), and associations among two U.S. EPA recommended and approved groups of FIB – fecal coliform and *Enterococcus* – in fresh and brackish surface waters of Central and South Florida. Samples were collected over a period of 12 consecutive months, spanning April 2015 to March 2016. Annual and seasonal fresh and brackish water data, reported as CFU/100 mL, was examined and analyzed in order to observe associations and potential correlations among FIB to enhance our knowledge of the potential benefit associated with a multiple indicator approach to brackish surface water quality analysis.

MATERIALS AND METHODS

Data Acquisition

Samples were collected from a variety of surface waters, both brackish and fresh, across Broward, Glades, Miami-Dade, and Palm Beach counties of Central and South Florida. Sampling took place weekly, over a period of 12 consecutive months, beginning in April of 2015 and ending in March of 2016. Freshwater samples were obtained from several regions bordering the central and southern portions of the Everglades. These freshwater areas experienced daily, minor saltwater influence via drainage canals. However, all samples obtained within the 12-month sampling period were within established freshwater limits of < 0.5 PSU. Brackish water samples were obtained from residentially influenced, southeastern coastal surface water bodies. Sample collection sites were lined with tidal-influenced drainage canals which experiencing saltwater impacts. Regardless of daily variations in salinity, all samples obtained within the 12-month sampling period were within established brackish water limits of 0.5 – 3.5 PSU.

1. Surface Water Sampling Procedure

Samples were collected following the Florida Department of Environmental Protection (DEP) FS 2100 *Surface Water Sampling* standard operating procedure. Surface water samples were collected using a direct grab technique. Samples were aseptically collected by trained field personnel into 120 mL, sterile, disposable bacteria bottles containing sodium thiosulfate for the neutralization of chlorine. Containers were submerged, upright, within the first two feet below the surface. Water was allowed to flow into the container, and sample containers were filled to a pre-labeled and verified 100 mL impression. Care was taken not to overfill containers. When filled, samples were quickly returned to the surface and secured with a tightly fitting screw top lid. Samples were placed into zip lock bags and preserved on ice for transfer to the laboratory (FL DEP FS 2100 2014).

2. Conductivity Procedure

Conductivity measurements were gathered in the field following the DEP FT 1200: *Field Measurement of Specific Conductance (Conductivity)* standard operating procedure. Values were measured directly and recorded as specific conductivity measurements (μS/cm) using a multi-probe, YSI Pro-Series conductivity meter.

Equipment was examined for air bubbles and calibrated prior to use. All conductivity measurements were taken within 15 minutes of sample collection and automatically corrected to a temperature of 25.0°C (FL DEP FT 1200 2014).

Analytical Methods

Samples were received, processed, and analyzed through the use of a private, NELAC certified laboratory in Fort Lauderdale, Florida. Samples were received as 100 mL to 120 mL aliquots within 120 mL, sterile, disposable bacteria bottles containing sodium thiosulfate for the neutralization of chlorine. Samples were received on ice, at a temperature of 4.0°C, and processed within 8 hours of the indicated collection time. Samples were analyzed for fecal coliform and enterococcus simultaneously, following the United States Environmental Protection Agency (US EPA) Method 1600: *Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-β-D-Glucoside Agar* and *Standard Methods for the Examination of Water and Wastewater 20th Edition* method SM 9222D: *Fecal Coliform Membrane Filter Procedure* (APHA 1999).

1. Membrane Filtration Procedure

Upon receipt, samples were checked individually to confirm appropriate storage temperature and absence of chlorine. A vacuum filtration system consisting of a six-spot manifold and 500 mL two-part filtration units, made up of a connected funnel and filter, was used to process samples via *Standard Methods* and EPA methods of membrane filtration (Figure 1). Prior to filtration, filter units were placed on the manifold apparatus and sterilized by running 500 mL of boiling water through each individual unit; filter units were autoclaved weekly per laboratory protocol. Using aseptic technique and flame sterilized forceps, a 0.45µm, grid-lined membrane filter was transferred onto each filtration unit. Samples were shaken 25 times to assure resuspension and uniform distribution of bacteria within the sample matrix and aliquoted into individual filter units. A vacuum was used to draw samples through filter units and subsequent filter papers. Filter units were rinsed with approximately 50 mL of phosphate buffered water to assure thorough transfer of sample(s) onto respective membrane filters. Membrane filters were

then aseptically removed from each filtration unit and transferred to specific growth media for incubation, per the appropriate method.

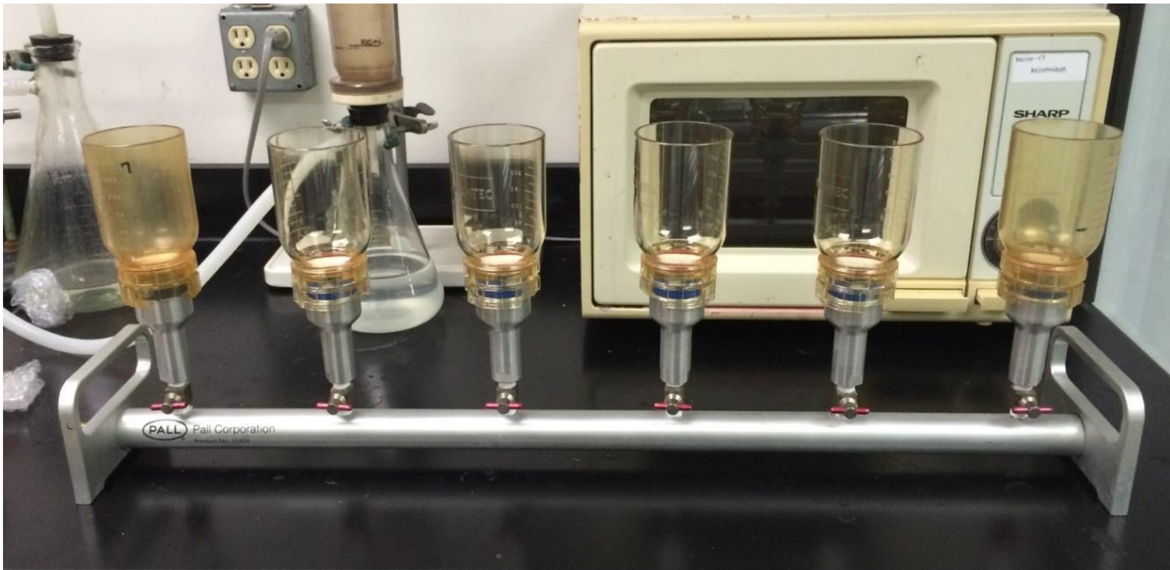

Figure 1. Membrane filtration apparatus.

a. Fecal Coliform Analysis: Samples were processed within 8 hours of collection, as outlined in *Standard Methods for the Examination of Water and Wastewater 20th Edition* method SM 9222D: *Fecal Coliform Membrane Filter Procedure* within 8 hours of collection (APHA 1999). Dilutions of 1 to 50 mL were used to obtain colony counts within the ideal range of 20 – 60 CFUs per plate. Multiple dilutions were run, per sample, to achieve this range. Following filtration, membrane filters were aseptically transferred to m-FC broth, a selective culture medium for the enumeration of fecal coliform bacteria. M-FC medium is specific to, and conforms with, *Standard Methods* SM 9222D. Individual plates were sealed with electrical tape to provide waterproofing. Plates were placed upside down in submersible containers, which were transferred to a water bath for incubation. Samples were incubated at 44.5 ± 0.2°C for 24 ± 2 hours. After incubation, colonies which were blue in color were considered fecal coliform colonies and counted as such. Colonies exhibiting all shades of blue, regardless of size, were considered fecal coliform colonies. Colonies which were pale yellow or white in color were considered to be non-fecal coliform bacteria and excluded from the final CFU count. Plates within the

ideal range of 20 – 60 CFUs were predominately used to obtain enterococcus counts representing 100 mL of sample(s). If an ideal count was not available for any dilution, a final CFU/100 mL count was determined using CFU counts obtained from the plate which represented the least diluted form of the sample matrix. Plates exhibiting counts greater than 200 CFU/plate were considered too numerous to count (TNTC) and excluded from the study, as an accurate count could not be obtained.

b. Enterococcus Analysis: Samples were processed via EPA Method 1600: *Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-β-D-Glucoside Agar* (US EPA 2002). Dilutions of 10 and 50 mL were used to obtain colony counts within the ideal range of 20 – 60 CFUs per plate. Multiple dilutions were run, per sample, to obtain this range. Following filtration, membrane filters were aseptically transferred to m-EI agar, a selective culture medium used for the chromogenic detection and enumeration of enterococcus bacterial groups. M-EI media is specific to, and conforms with, EPA 1600. Plates were placed upside down inside an incubator maintained at 41.0 ± 0.5°C for 24 ± 2 hours. After incubation, colonies which exhibited a blue "halo" surrounding a clear center were considered enterococcus colonies. Colonies exhibiting halos of any shade of blue, regardless of size, were considered enterococcus colonies and added to the final CFU count. Colonies which were clear or white were not considered enterococcus colonies and were excluded from the final CFU count. Plates within the ideal range of 20 – 60 CFUs were predominately used to obtain enterococcus counts representing 100 mL of sample(s). If an ideal count was not available for any dilution, a final CFU/100 mL count was averaged using CFU counts obtained from the plate which represented the least diluted form of the sample matrix. Plates exhibiting counts greater than 200 CFU/plate were considered TNTC and excluded from the study, as an accurate count could not be obtained.

2. Quality Control/Quality Assurance Procedures

a. Sample Collection and Transport: Samples were aseptically collected by trained field personnel into 120 mL, sterile, disposable bacteria bottles containing sodium thiosulfate for the neutralization of chlorine. Prior to use, newly received lots of bacteria bottles were tested for sterility, verified to hold 100 mL of liquid using a Class A graduated cylinder, and confirmed to neutralize 15g/L of chlorine. Samples were transported to the laboratory

on ice, in coolers, and maintained at a temperature of 4.0°C. Samples received more than 8 hours past collection time, or not received on ice, were discarded and resampled. Additionally, samples which were received in inappropriate or leaking containers were resampled. When provided, field blanks and equipment blanks were processed as samples, per SM 9222D and EPA 1600, to ensure the absence of contamination during collection and transport of samples.

b. Media and Reagents: New lots of media(s) and phosphate buffered water, made in house, were checked for sterility and proper performance prior to use. Medias were made per manufacturer instructions. Dehydrated medias were discarded 6 months after the open date. New lots of medias made in house were checked for proper performance and sterility, prior to use, via blank samples, positive control organisms, and negative control organisms per manufacturer instructions and *Standard Methods for the Examination of Water and Wastewater 20th Edition* method SM 9050: *Preparation of Culture Media* (APHA 1999). Autoclaved m-EI media was refrigerated for 3 months before disposal, and placed through quality control procedures monthly to ensure proper maintenance and performance. M-FC broth was disposed of and remade weekly.

c. Supportive equipment: Membrane filter papers and petri dishes used to process samples were checked for sterility upon receipt, prior to use, using non-selective Standard Plate Count Agar. Reusable glass pipettes were checked for appropriate volume upon receipt, cleaned and autoclaved before each use, and stored under sterile conditions. A clean, sterile pipette was used for each individual sample. Filter units were checked for appropriate volume upon receipt as well as quarterly, using a class A graduated cylinder. Filter units were autoclaved weekly and sterilized with boiling water prior to each use, per laboratory protocol.

Blank samples, consisting of 100 mL aliquots of phosphate buffered water, were used throughout the membrane filtration process per the method requirements of SM 9222D and EPA 1600 and *Standard Methods for the Examination of Water and Wastewater 20th Edition* method SM 9020: *Quality Assurance/Quality Control* (APHA 1999). Blank samples were run before beginning a filtration series, defined as 20 samples, as well as at the end of each filtration series to ensure proper aseptic technique and proper sterilization of filtration equipment. Additionally, blank samples were run

after every 10th sample, due to the absence of U.V. sterilization within the laboratory, to ensure proper rinsing technique and eliminate the possibility of cross over between filtrations.

3. Data Analysis

Fecal coliform and *Enterococcus* data collected during the 12-month sampling period was examined and analyzed, in both raw form and as log transformed data, using R-Studio software. Data was analyzed both within and between groups, for both fresh and brackish water conditions, using a traditional exploratory approach. In addition, data was analyzed collectively, both annually and seasonally, using monthly averages, linear regression, Spearman correlation, and line plot analysis. Basic, routine coding technique was used to carry out all statistical tests and graphics within R-Studio.

Exploratory data analysis was used to gain qualitative and quantitative insight into data trends and relationships, both within and between fecal coliform and *Enterococcus* groups. Calculation of range, mean, and median for each data set was used to provide a snapshot of the data as a whole. In addition, graphical analyses were used to gather insight into data distribution, and provided a simplistic means to visually observe relationships between data sets. Paired box plot and violin plots were used to visually examine overall structure, spread of data, outliers, and density distribution of both fecal coliform and *Enterococcus* data under both fresh and brackish water conditions.

Normality and skew was examined through the use of histograms paired with normal distribution curves and density curves, QQ-plots, Shapiro-Wilk, and Pearson kurtosis analysis both within and between data sets. Normality of both data sets and data residuals was determined before broadening the scope of statistical analyses. Normality was determined through the calculation of p-values, compared to a chosen significance value of $\alpha = 0.05$, and the acceptance or rejection of the null hypothesis that the data followed a normal distribution. Additionally, a Pearson kurtosis coefficient, or level of skewness, was calculated to confirm the presence of skew and its subsequent severity. Data residuals were examined for normality, both within and between groups, in order to confirm the presence of normality and thus determine if subsequent regression analysis was an accurate description of relationships between fecal coliform and *Enterococcus* groups regardless of significance level.

Annual analysis of relationships between fecal coliform and *Enterococcus* was performed using monthly averages as well as linear regression, Spearman correlation, and line plot analyses. Scatterplots were used to plot data points, and a trendline was added to visually inspect relationships during linear regression analysis. R^2 values and p-values were calculated and used to show significance in the relationship(s) between groups through the use of a chosen significance value, $\alpha = 0.05$. Monthly averages were calculated for both fecal coliform and *Enterococcus* CFU counts, under both fresh and brackish water conditions, and visualized graphically through stacked line plots. Due to indeterminate dependent variable(s) in regard to fecal coliform and enterococcal interactions and abnormal distributions within data sets, Spearman correlation coefficients were calculated to observe potential correlations between FIB groups.

Seasonal analysis of fecal coliform and *Enterococcus* relationships was performed, both within and between FIB groups, through the establishment and use of a wet and dry season. Based on historical rainfall data, the Florida dry season was defined as the months of November through April, while the Florida wet season was defined as the months of May through October. Fecal coliform and *Enterococcus* data was analyzed using coupled violin and box plots to observe overall structure, spread of data, and density distribution, as well as outliers and fluctuation(s) in data which may be dependent upon seasonal parameters. Finally, Spearman correlation coefficients were calculated to observe seasonally influenced correlations, if any, between groups.

RESULTS

Fecal Coliform (FC) Analysis

a. Freshwater: FC data ranged from a minimum count of 4 CFU/100 mL to a maximum count of 12,000 CFU/100 mL. A mean of 523 CFU/100 mL and median of 109 CFU/100 mL was calculated for all freshwater FC raw data collected over the 12-month sampling period (Appendix A). A coupled violin and box plot, displaying both FC $\log_{10}$ CFU data range and density distribution under freshwater conditions is shown in Figure 2. Shapiro-Wilk analysis of FC raw data revealed a p-value of 0.428.

b. Brackish Water: A mean of 274 CFU/100 mL and median of 106 CFU/100 mL was calculated for all brackish water FC raw data collected over the 12-month sampling period (Appendix B). A coupled violin and box plot, displaying both FC $\log_{10}$ CFU data range and density distribution under brackish water conditions is shown in Figure 2. Shapiro-Wilk analysis of FC raw data revealed a p-value of 0.05.

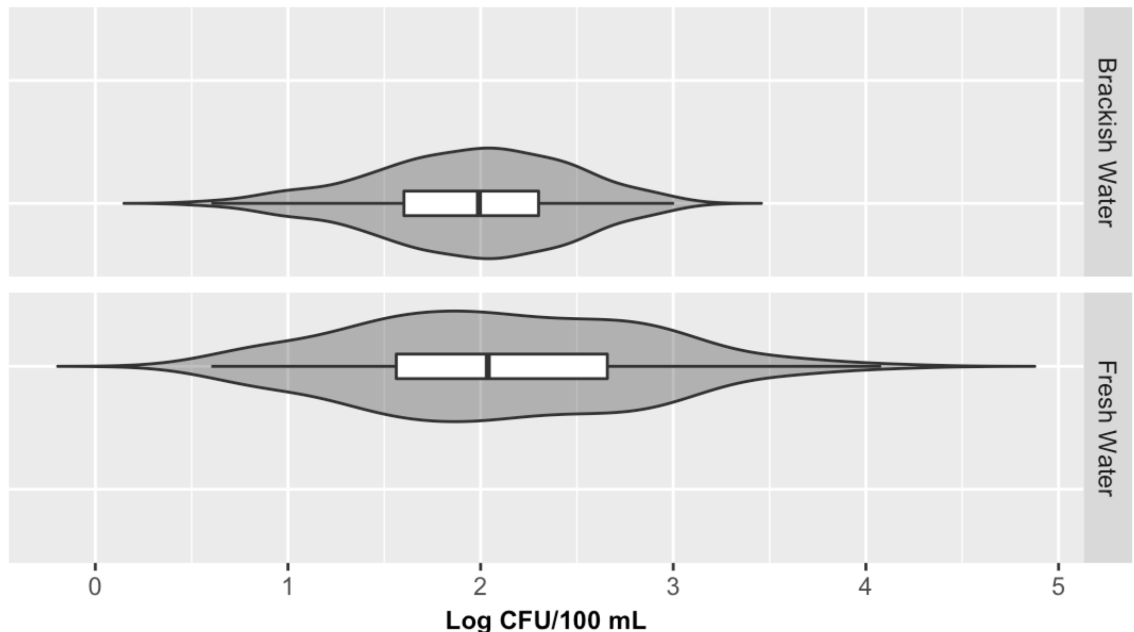

Figure 2. Freshwater and brackish water fecal coliform counts (CFU/100 mL), expressed in $\log_{10}$ formation.

c. Annual Fecal Coliform Trends: Monthly FC CFU/100 mL averages were calculated for both fresh and brackish water over the 12-month sampling period (Tables 1 and 2*)*.

Monthly averages for fresh and brackish water were graphed concurrently, in the order in which sampling took place, beginning with April 2015 and ending with March 2016 (Figure 3).

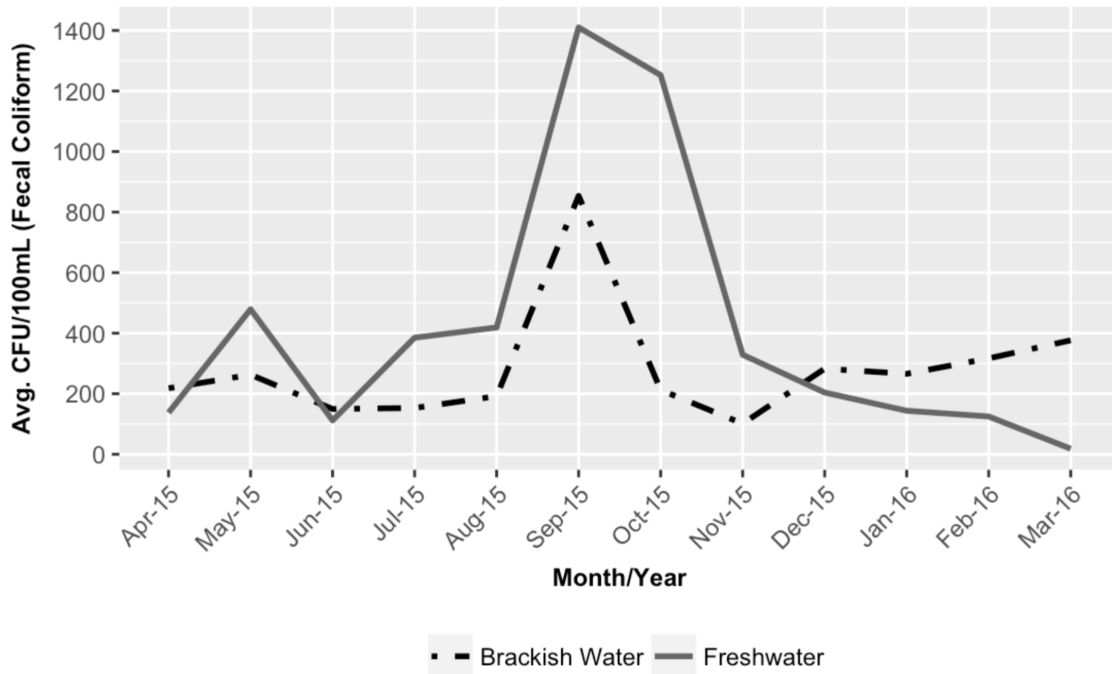

Figure 3. Freshwater and brackish water fecal coliform counts (CFU/100 mL) expressed as monthly averages over a period of 12 consecutive months.

Enterococcus (ENT) Analysis

a. Freshwater*:* The range of freshwater ENT data is described by a minimum count of 2 CFU/100 mL and maximum count of 1350 CFU/100 mL. A mean of 156 CFU/100 mL and median of 68 CFU/100 mL was calculated for all freshwater ENT raw data collected over the 12-month sampling period (Appendix A). A coupled violin and box plot, displaying both ENT $\log_{10}$ CFU data range and density distribution under freshwater conditions is shown in Figure 4. Shapiro-Wilk analysis of raw data revealed a p-value of 0.266.

b. Brackish water: A mean of 158 CFU/100 mL and median of 98 CFU/100 mL was calculated for all brackish water ENT raw data collected over the 12-month sampling period (Appendix B*).* A coupled violin and box plot, displaying both ENT $\log_{10}$ CFU data

range and density distribution under brackish water conditions is shown in Figure 4. Shapiro-Wilk analysis of raw data revealed a p-value of 0.07.

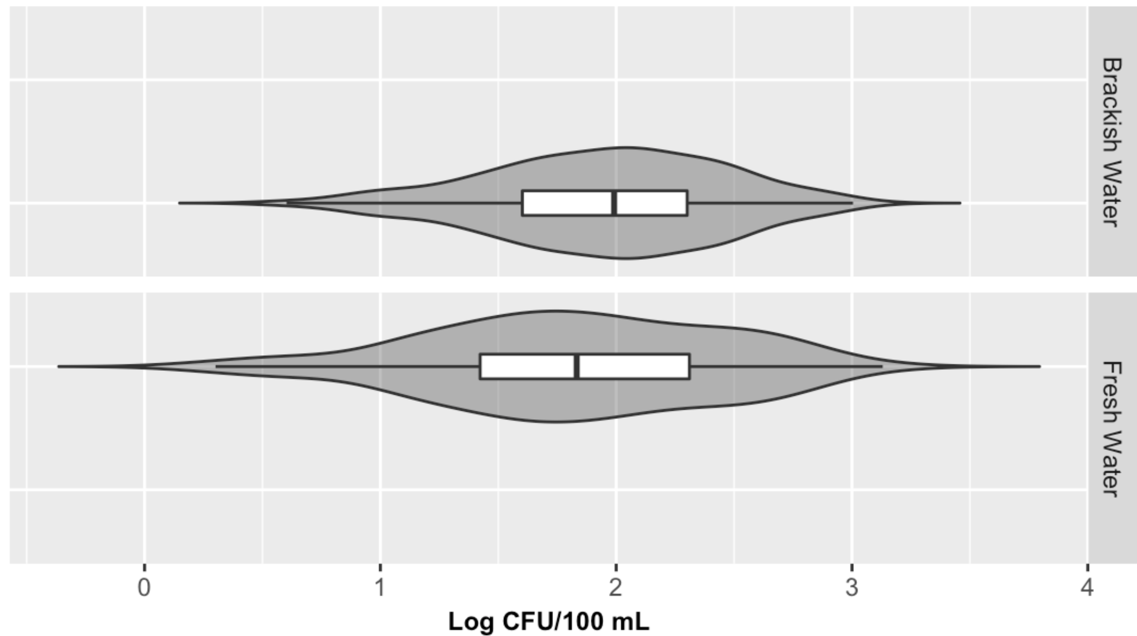

Figure 4. Freshwater and brackish water *Enterococcus* counts (CFU/100 mL), expressed in $\log_{10}$ formation.

c. Annual Enterococcus Trends*:* Monthly ENT CFU/100 mL averages were calculated for both fresh and brackish water over the 12-month sampling period (Tables 1 and 2). Calculated monthly averages for both fresh and brackish water were graphed concurrently, in the order in which sampling took place, beginning with April 2015 and ending with March 2016 (Figure 5).

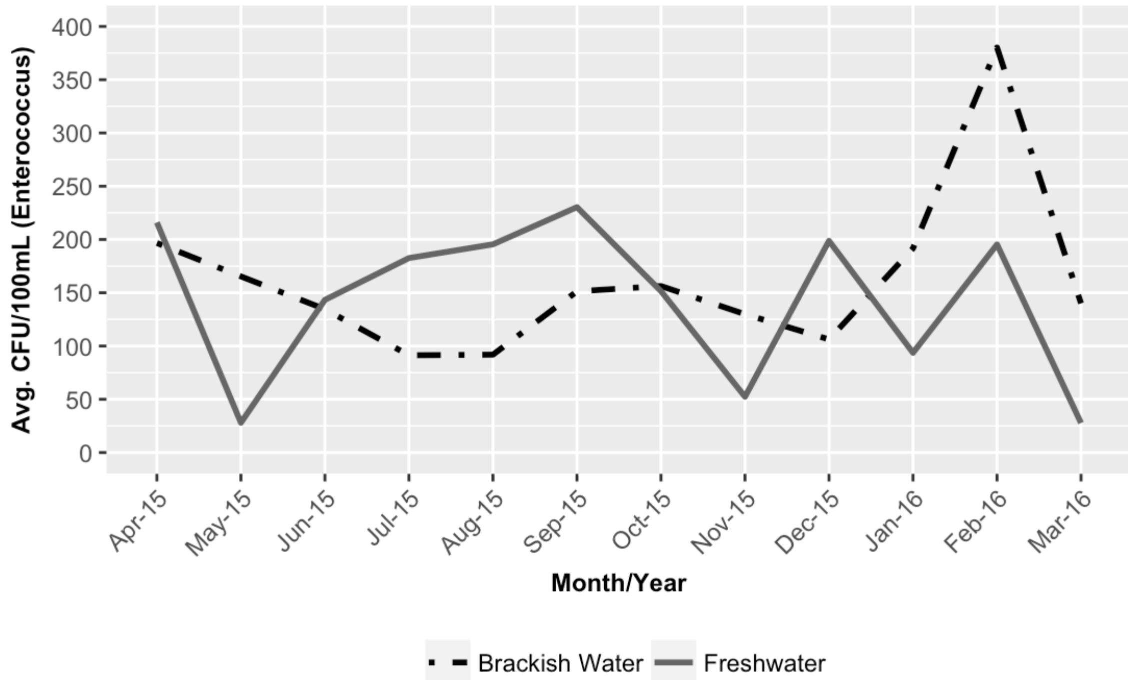

Figure 5. Freshwater and brackish water *Enterococcus* counts (CFU/100 mL) expressed as monthly averages over a period of 12 consecutive months.

Fecal Coliform vs. Enterococcus

a. Freshwater Trends*:* Histogram analysis of FC and ENT $\log_{10}$ CFU data under freshwater conditions, overlaid with both density distribution and normal curves, is shown in Figure 6. Pearson kurtosis values of 2.49 and 2.58 were calculated for FC and ENT $\log_{10}$ CFU data. A coupled violin and box plot, displaying freshwater FC and ENT $\log_{10}$ CFU data range(s), median values, and density distribution(s) is shown in Figure 7.

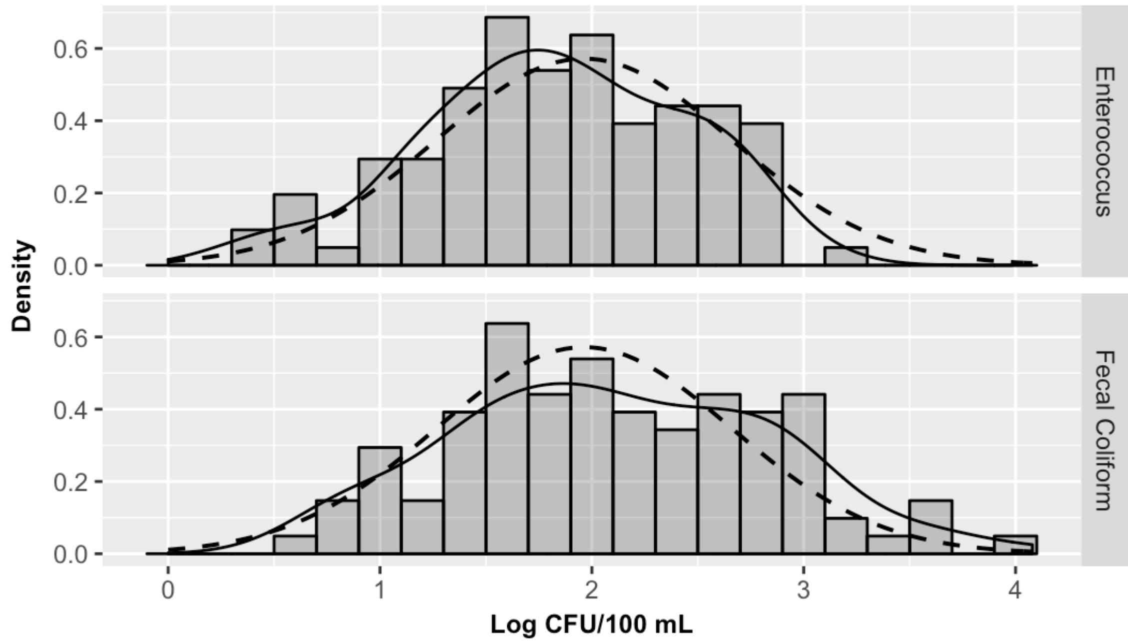

Figure 6. Histogram analysis of freshwater fecal coliform and *Enterococcus* counts (CFU/100 mL), expressed in log_{10} formation. *Note: An associated density distribution curve is expressed as a solid line; a normal distribution curve is expressed as a dashed line.*

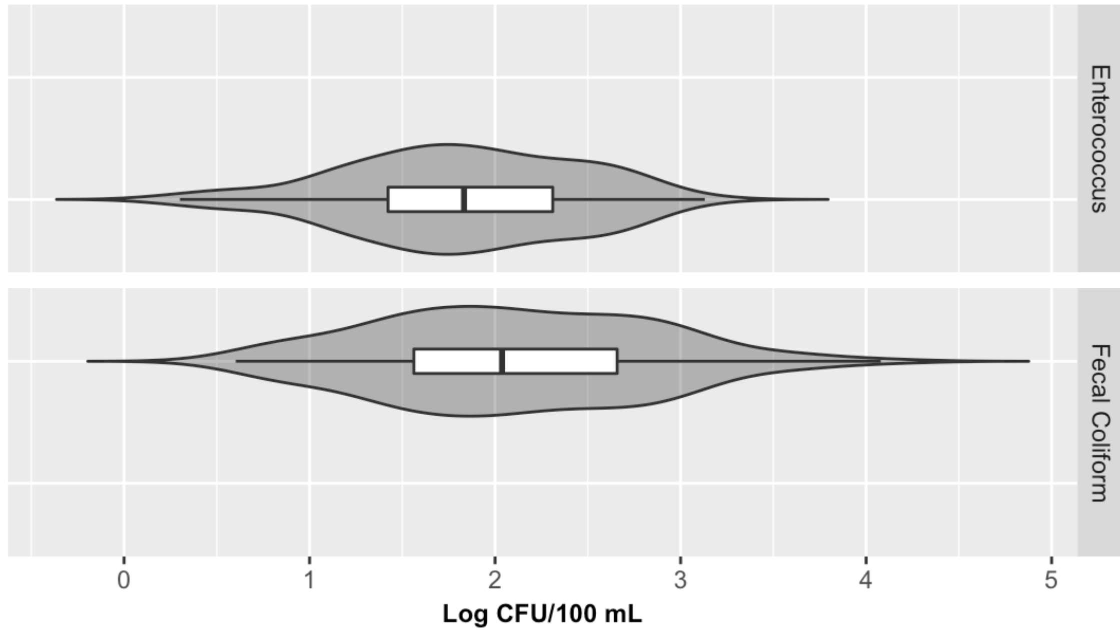

Figure 7. Violin plot and associated box plot of freshwater fecal coliform and *Enterococcus* counts (CFU/100 mL), expressed in log_{10} formation.

Linear regression analysis of all $\log_{10}$ transformed FC and ENT CFU counts obtained, per sample point, during the 12-month sampling period is shown in Figure 8. A regression line was added; an adjusted R^2 value of 0.25 and corresponding p-value of 1.1 x 10^{-7} were obtained. Graphical analysis of residual normality, using residual values vs. fitted values, revealed normal residuals. Confirmation of residual normality was obtained through the use of a normal QQ-plot. Spearman correlation analysis revealed a Spearman coefficient of r = 0.48.

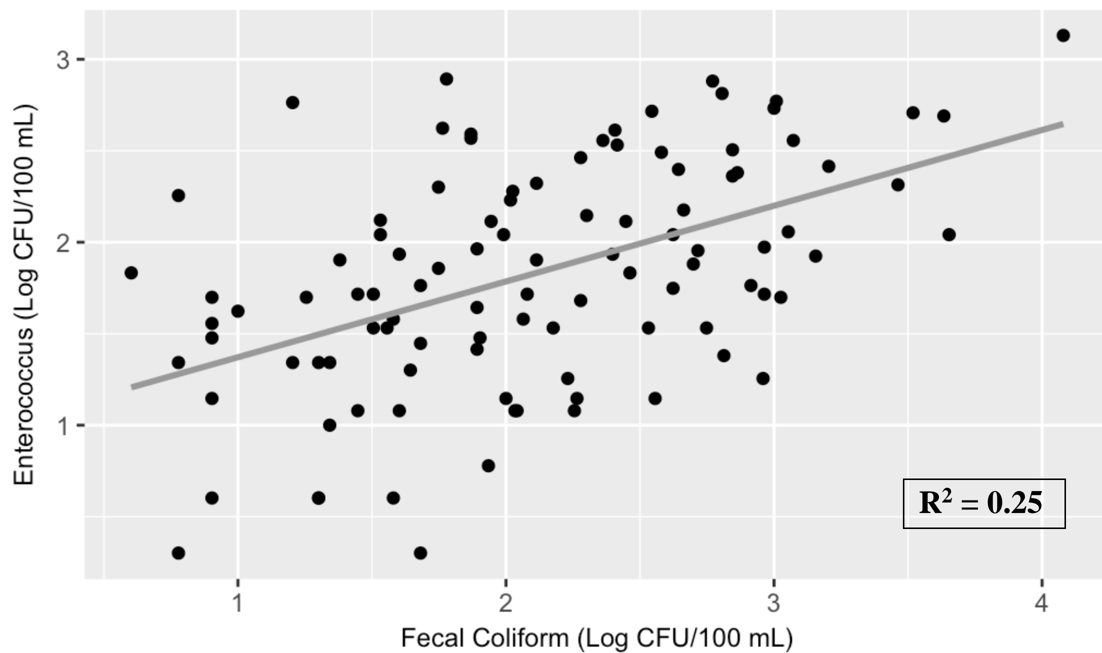

Figure 8. Linear regression analysis of fecal coliform and *Enterococcus* counts (CFU/100 mL) in freshwater, expressed in $\log_{10}$ formation.

b. Annual Freshwater Trends*:* Calculated monthly averages for FC and ENT raw CFU counts (Table 2*)* were graphed concurrently, in the order in which sampling took place, beginning with April 2015 and ending with March 2016 (Figure 9).

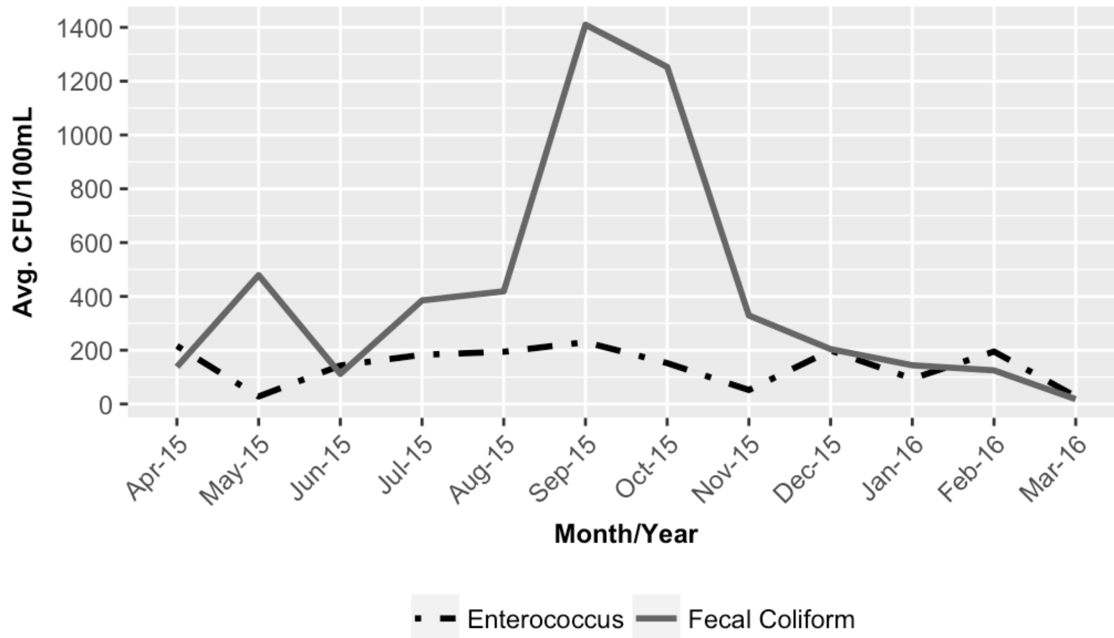

Figure 9. Freshwater fecal coliform and *Enterococcus* counts (CFU/100 mL) expressed as monthly averages over a period of 12 consecutive months.

c. Seasonal Freshwater Trends*:* Coupled violin and box plots expressing freshwater $\log_{10}$ FC and ENT counts (CFU/100 mL), per season, are shown in Figures 10 and Figure 11. Seasonal linear regression analyses of all freshwater $\log_{10}$ transformed FC and ENT CFU counts, per sample point, during the 12-month sampling period are shown in Figure 12. Based on historical rainfall data, the Florida dry season was defined as the months of November through April, while the Florida wet season was defined as the months of May through October. Adjusted R^2 values of 0.27 for the Florida dry season and 0.37 for the Florida wet season were calculated via R-Studio.

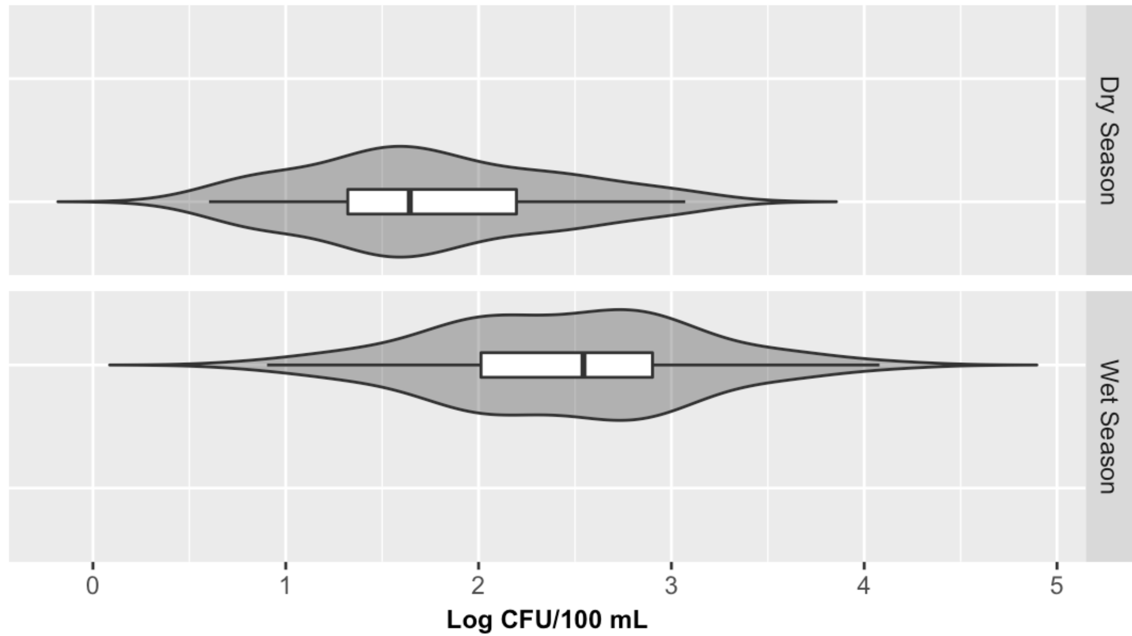

Figure 10. Violin and associated box plots for seasonal freshwater fecal coliform counts (CFU/100 mL), expressed in $\log_{10}$ formation. *Note: The Florida dry season was defined as the months of November to April, while the Florida wet season was defined as the months of May to October.*

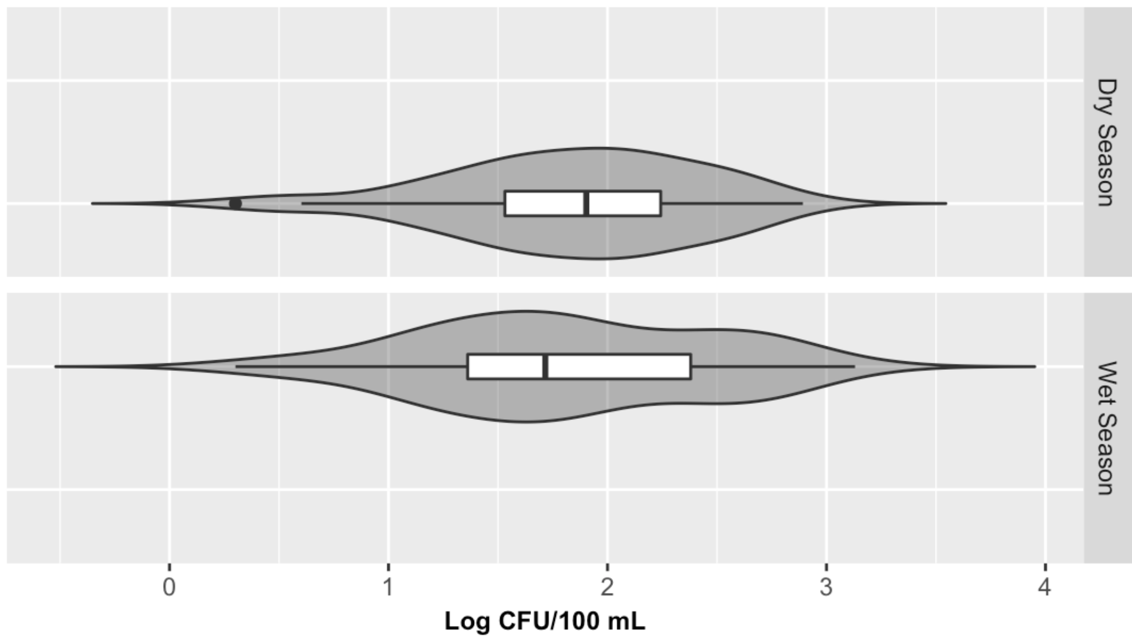

Figure 11. Violin and associated box plots for season freshwater *Enterococcus* counts (CFU/100 mL), expressed in $\log_{10}$ formation. *Note: The Florida dry season was defined as the months of November to April, while the Florida wet season was defined as the months of May to October.*

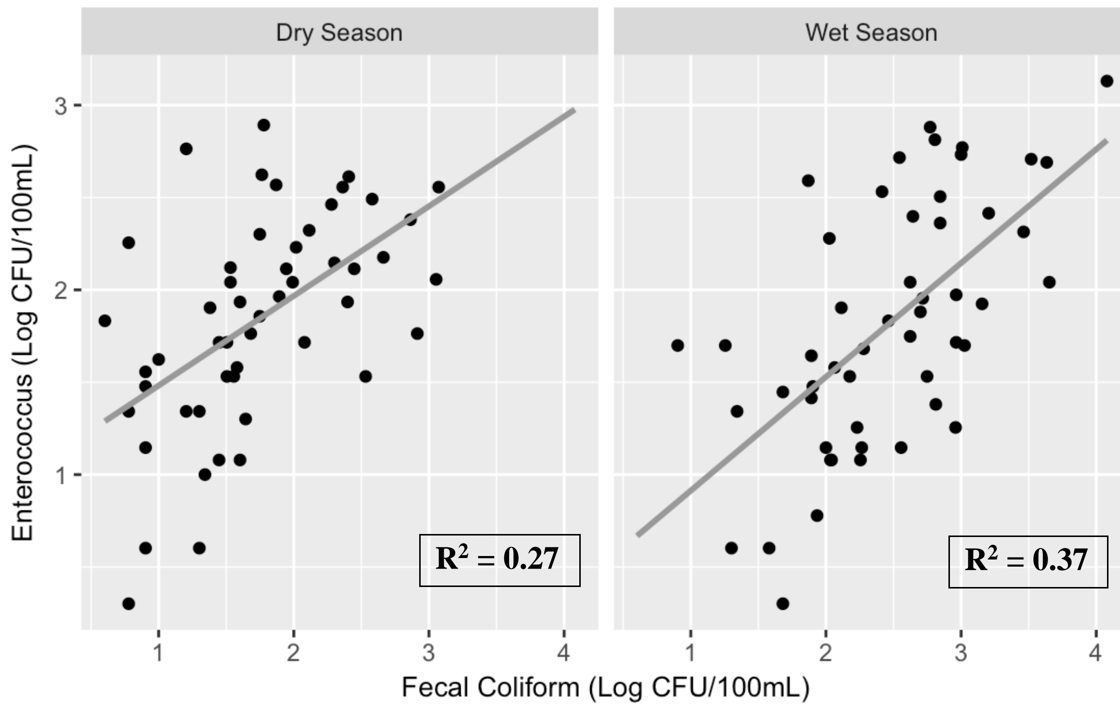

Figure 12. Seasonal regression analysis of fecal coliform and *Enterococcus* counts (CFU/100 mL) in freshwater, expressed in log_{10} formation.

d. Brackish Water Trends*:* Histogram analysis of FC and ENT log_{10} CFU data in brackish water is shown in Figure 13, overlaid with density distribution and normal curves. Pearson kurtosis values of 2.54 and 2.70 were calculated for FC and EC log_{10} CFU data. A coupled violin and box plot, displaying freshwater FC and ENT log_{10} CFU data ranges, median values, and density distribution is shown in Figure 14.

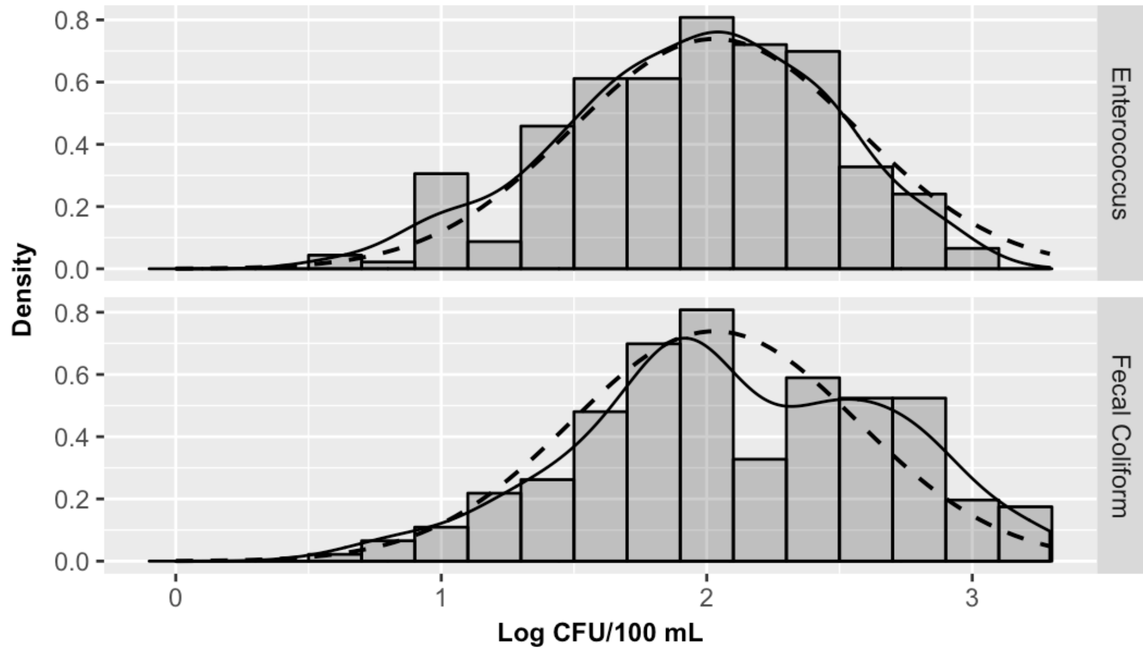

Figure 13. Histogram analysis of brackish water fecal coliform and *Enterococcus* counts (CFU/100 mL), expressed in log_{10} formation. An associated density distribution curve is expressed as a solid line; a normal distribution curve is expressed as a dashed line.

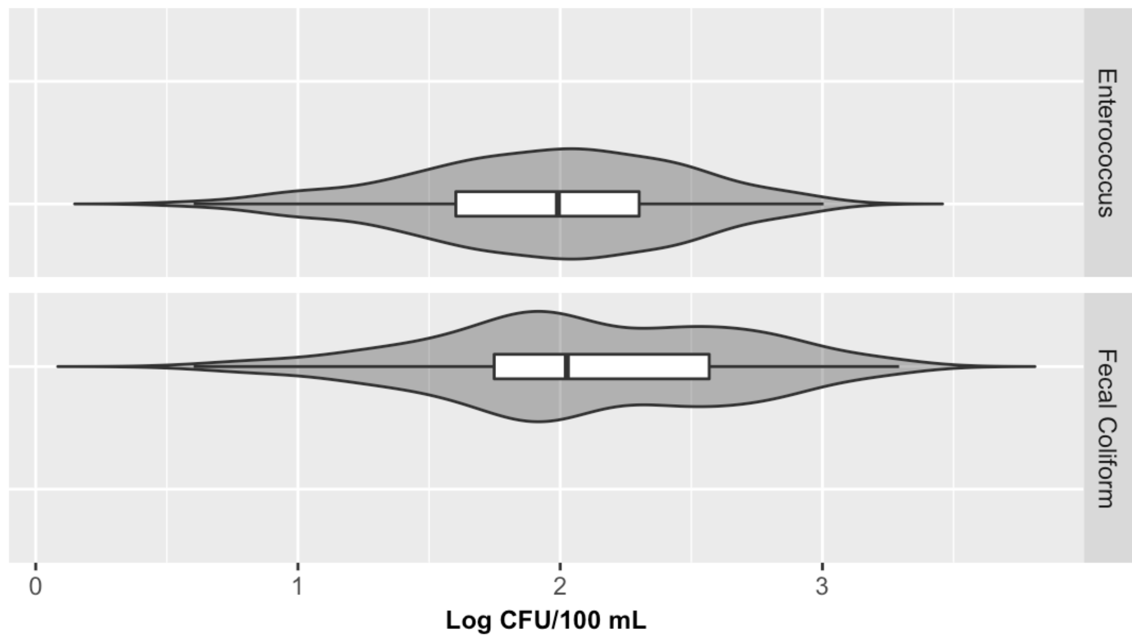

Figure 14. Violin and associated box plots of brackish water fecal coliform and *Enterococcus* counts (CFU/100 mL), expressed in log_{10} formation.

Linear regression analysis of all $\log_{10}$ transformed FC and EC CFU counts obtained, per sample point, during the 12-month sampling period is shown in Figure 15. An adjusted R^2 value of 0.34 and corresponding p-value of $< 2.2 \times 10^{-16}$ were obtained. Graphical analysis of residual normality using residual values vs. fitted values showed normal residuals. Confirmation of residual normality was obtained through the use of a normal QQ-plot. Finally, Spearman correlation analysis revealed a Spearman coefficient of r = 0.57.

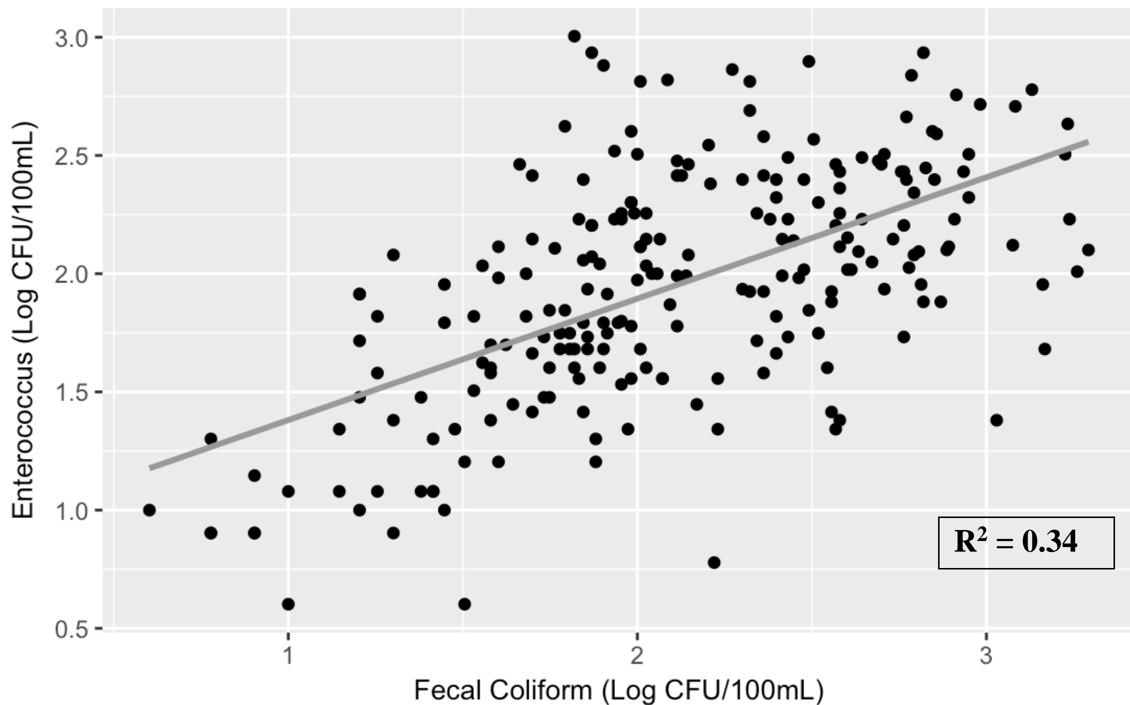

Figure 15. Linear regression analysis of fecal coliform and *Enterococcus* counts CFU/100 mL) in brackish water, expressed in $\log_{10}$ formation.

e. Annual Brackish Water Trends*:* Calculated monthly averages for FC and EC CFU raw counts (Table 2) were graphed concurrently, in the order sampling took place, beginning with April 2015 and ending with March 2016 (Figure 16).

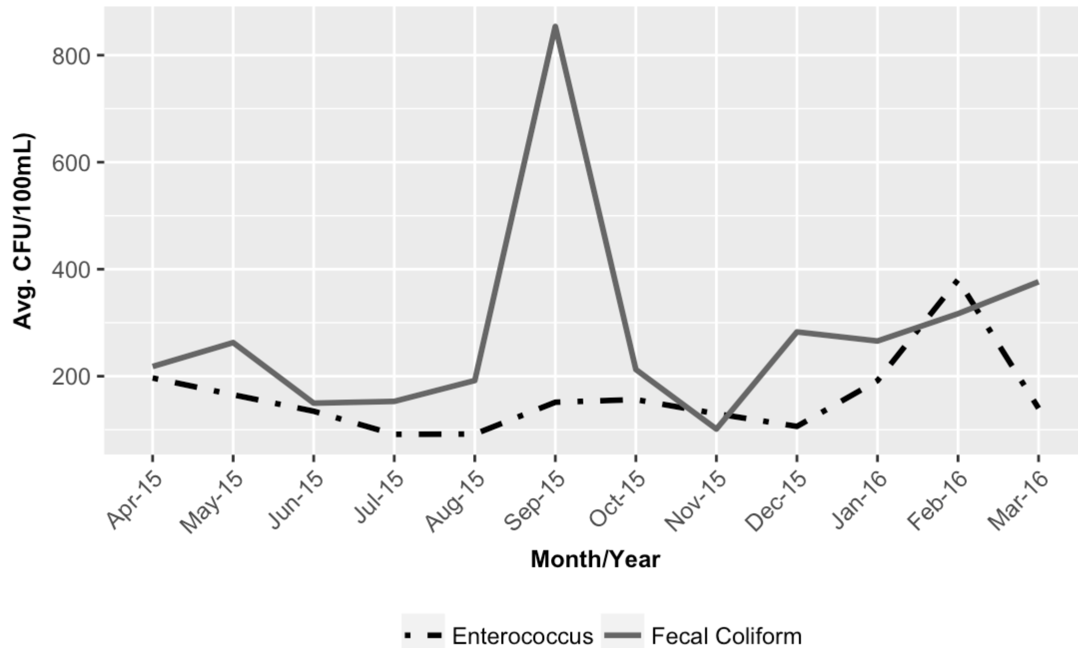

Figure 16. Brackish water fecal coliform and *Enterococcus* counts (CFU/100 mL) expressed as monthly averages over a period of 12 consecutive months.

f. Seasonal Brackish Water Trends: Coupled violin and box plots expressing freshwater log_{10} FC and ENT counts (CFU/100 mL), per season, are shown in Figures 17 and 18. Seasonal linear regression analyses of all brackish water log_{10} transformed FC and ENT CFU counts, per sample point, during the 12-month sampling period are shown in Figure 19. Adjusted R^2 values of 0.29 for the Florida dry season and 0.36 for the Florida wet season were calculated via R-Studio.

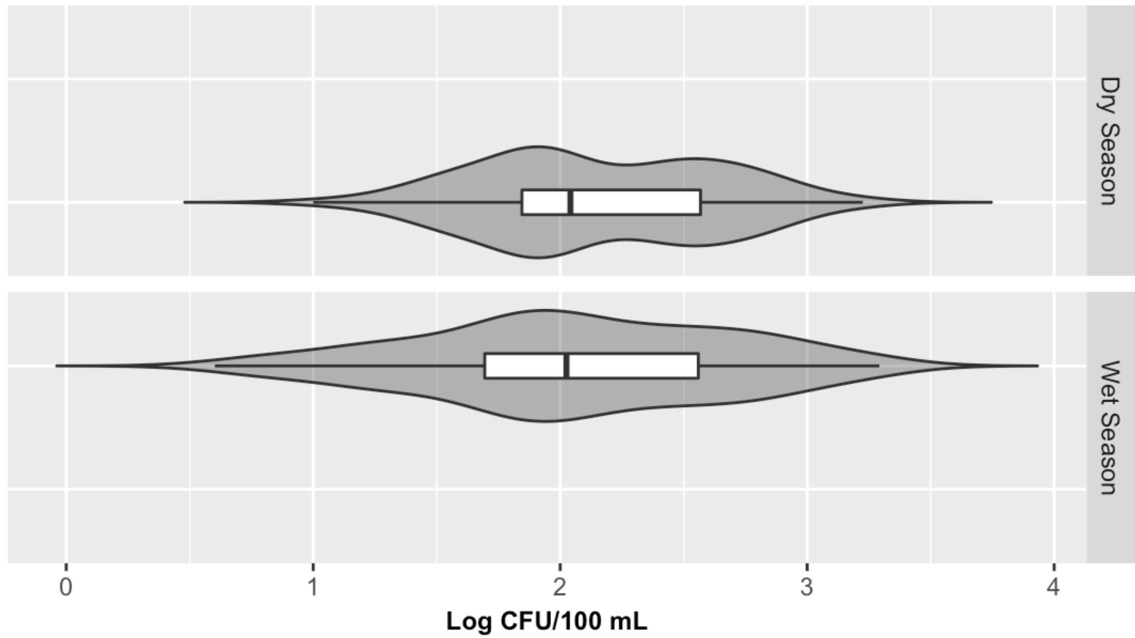

Figure 17. Violin and associated box plots for seasonal brackish water fecal coliform counts (CFU/100 mL), expressed in log_{10} formation.

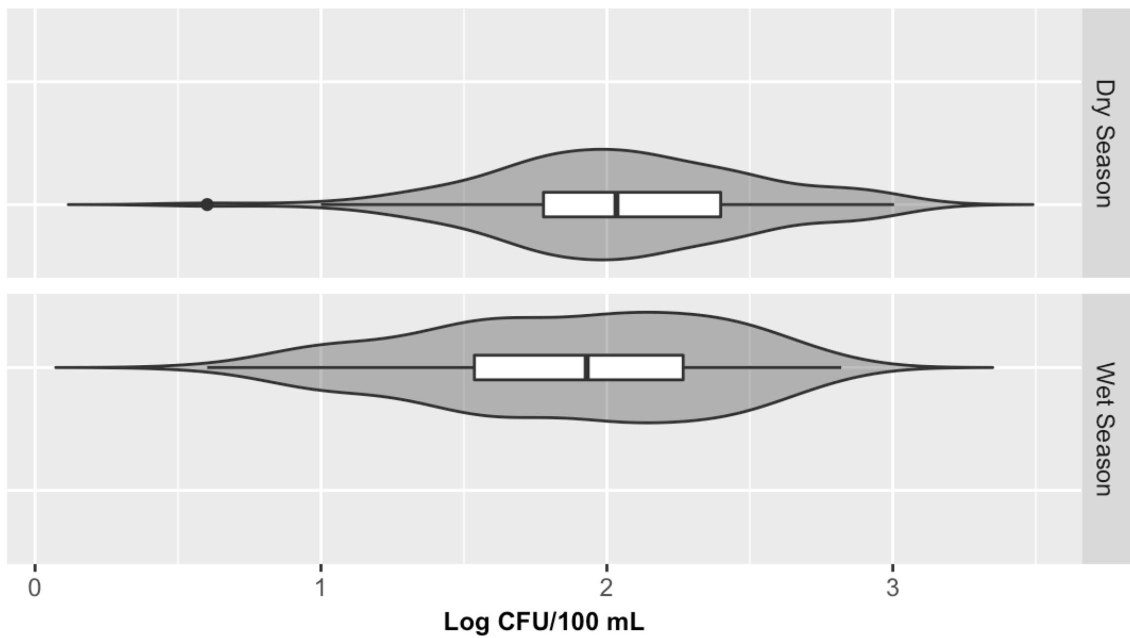

Figure 18. Violin and associated box plots for seasonal brackish water *Enterococcus* counts (CFU/100 mL), expressed in log_{10} formation.

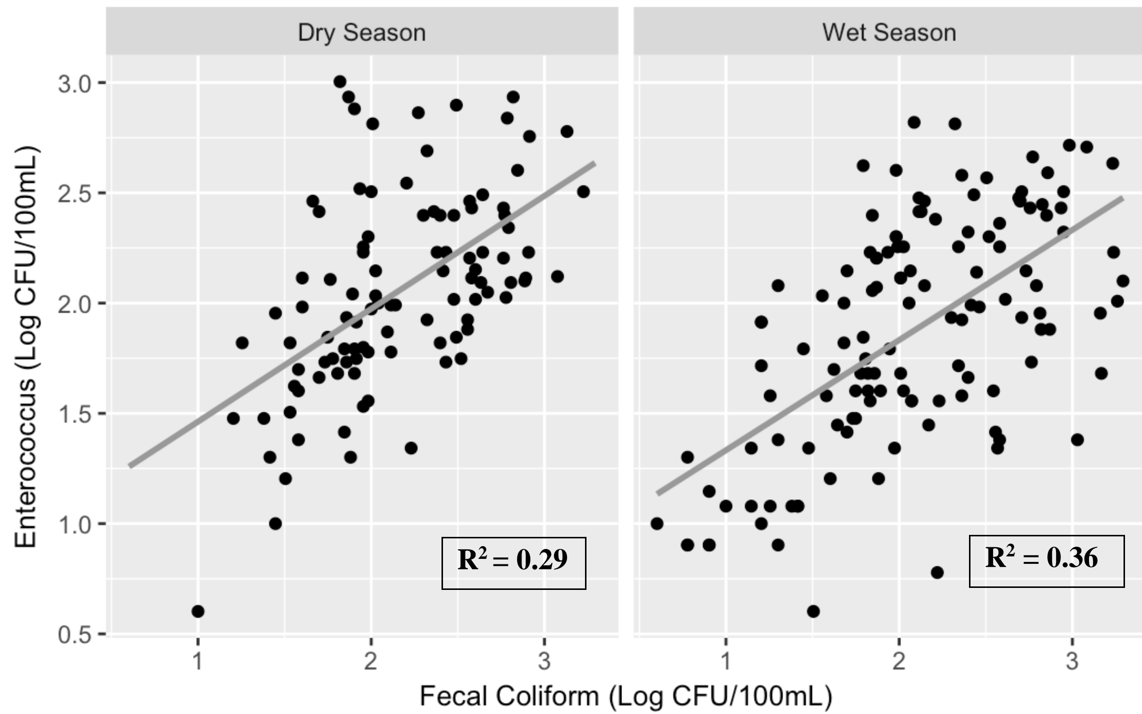

Figure 19. Seasonal regression analysis of fecal coliform and *Enterococcus* counts (CFU/100 mL) in brackish water, expressed in log_{10} formation.

DISCUSSION

Exploratory data analysis: Initial inspection of raw data distributions through boxplot and histogram analysis revealed that raw FC and ENT data for both fresh and brackish water was severely asymmetric; positively skewed. The use of QQ-plots and a Shapiro-Wilk test confirmed that raw FC and ENT CFU data did not adhere to a normal distribution under any conditions; FC: p=0.428 (fresh), 0.05 (brackish); ENT: p=0.266 (fresh), 0.07 (brackish). Additionally, due to the significant positive skew present within each data set, and the corresponding potential for mean values to be pulled towards extreme values, median CFU/100 mL counts were considered to be the best representation of central tendency within each data set.

Due to severe skew and subsequent lack of normality present in raw FC and ENT data, further data analysis was performed using FC and ENT CFU data in $\log_{10}$ formation. To verify improvement in normality based on transformation of data, a Pearson kurtosis value, or measure of skewness, was calculated for FC and ENT $\log_{10}$ data. Kurtosis values of 2.49 and 2.58 for FC and ENT freshwater data, as well as 2.54 and 2.70 for FC and ENT brackish water data indicate significantly skewed, or abnormally distributed, data. Positive skew within each data set was also presented visually via histogram. Skew, although present within $\log_{10}$ data, was shown to be significantly improved from that of raw data when compared to a normal distribution curve (Figure 6 and Figure 13).

Fecal Coliform (FC): Exploratory data analysis of overall, raw FC CFU data revealed FC variation over considerable range(s) in both fresh and brackish water; a tighter range was seen under brackish conditions (Appendix A; Appendix B). During the 12-month sampling period, FC CFU counts ranged from 4 to 12,000 CFU/100 mL in freshwater (n = 102) and 4 to 1,960 CFU/100 mL in brackish water (n = 229). Mean FC values of 523 and 274 CFU/100 mL were calculated for fresh and brackish water, respectively. Additionally, median values of 109 and 106 CFU/100 mL were calculated for fresh and brackish water, respectively. FC range(s) and distribution(s) under fresh and brackish water conditions are shown graphically through coupled violin and box plots (Figure 2). Freshwater FC $\log_{10}$ data followed a slight bimodal distribution over a significantly larger range of CFU values than data obtained under brackish conditions,

which followed a clear, unimodal distribution over a significantly smaller range. Histogram analysis, coupled with both density curves and normal distribution curves, revealed a slight negative skew in both $\log_{10}$ FC fresh and brackish water data (Figure 6; Figure 13); bimodal trends previously seen in Figure 2 were also present in histogram analysis of FC under both fresh and brackish conditions.

Monthly FC CFU/100 mL averages were calculated for both fresh and brackish water data collected over the 12-month sampling period (Table 1; Table 2*).* Monthly averages for fresh and brackish water were graphed concurrently, in the order in which sampling took place, beginning with April 2015 and ending with March 2016 (Figure 3). FC followed strikingly similar patterns within both matrices; high counts favored the months of June to September, well within the defined wet season. Freshwater FC appeared to increase earlier and persist longer in the year at high concentrations. FC [CFU] peaked during September under both fresh and brackish water conditions, before demonstrating a rapid decrease towards November. Once into the dry season, FC were shown to steadily decrease under freshwater conditions. An opposite trend was shown for FC under brackish water conditions; CFU counts were shown to steadily increase. While peak [CFU] occurred during the same month, under both conditions, annual lows were seen in March for freshwater and November for brackish water.

Enterococcus (ENT): ENT CFU data was found to vary over considerable range(s) in both fresh and brackish water; CFU values were consistently lower in both matrices than those seen for FC (Appendix A; Appendix B). During the 12-month sampling period, ENT CFU counts ranged from 2 to 1,350 CFU/100 mL in freshwater (n = 102) and 4 to 1,010 CFU/100 mL in brackish water (n = 229). A mean value of 158 CFU/100 mL was calculated for ENT values in both matrices. Median values of 168 and 98 CFU/100 mL were calculated for fresh and brackish water, respectively. ENT range(s) and distribution(s) under fresh and brackish water conditions are shown graphically through coupled violin and box plots (Figure 4). ENT $\log_{10}$ data, under both fresh and brackish conditions, followed clear, unimodal distributions over significantly smaller ranges than those seen in FC data. Histogram analysis, coupled with both density curves and normal distribution curves, show a slight negative skew in freshwater and an almost normal distribution in brackish water (Figure 6; Figure 13); the brackish ENT unimodal

trend previously seen in Figure 4 remained visible. Under freshwater conditions, however, the presence of a slight bimodal distribution in ENT data closely resembled that seen in FC freshwater data.

Monthly ENT CFU/100 mL averages were calculated for both fresh and brackish water data collected over the 12-month sampling period (Table 1; Table 2). Monthly averages for fresh and brackish water were graphed concurrently, in the order in which sampling took place, beginning with April 2015 and ending with March 2016 (Figure 5). Like FC, ENT [CFU] showed a steady increase during the wet season, under freshwater conditions, between May and September; a rapid decrease, much like that seen in FC data, was demonstrated towards November. Once into the dry season, freshwater ENT [CFU] become sporadic, experiencing a series of peaks and troughs between November and February, before rapidly decreasing into May. ENT too, like FC, experienced an opposite reaction based on matrix; under brackish water conditions, ENT peak during the dry season, reaching a maximum [CFU] in February.

Annual trends: Linear regression analysis of FC and ENT counts obtained during the 12-month sampling period revealed linear, monotonic relationships between variables in both fresh and brackish water data; as one variable increased, the other was shown to consistently increase (Figure 8; Figure 15). Relationships between FC and ENT were revealed to be stronger in brackish water, adjusted $R^2 = 0.34$, than freshwater, $R^2 = 0.25$. *Note: Due to a large discrepancy in sample size between fresh and brackish water data – $n = 102$ for freshwater and $n = 229$ for brackish water – and the corresponding effect on linear regression, all R^2 values obtained were adjusted.* Graphical analysis of residual normality using residual values vs. fitted values revealed normal residuals in both fresh and brackish water data. Confirmation of residual normality was obtained through the use of a normal QQ-plot. The presence of residual normality ensured the trustworthiness of the aforementioned linear regression analyses, despite the low R^2 values obtained.

Linear associations among FC and ENT were further supported through Spearman correlation analysis. Spearman correlation coefficients were interpreted as follows: 0.00 – 0.19 signified a very weak relationship; 0.20 – 0.39 a weak relationship; 0.40 – 0.59 a moderate relationship; 0.60 – 0.79 a strong relationship; 0.80 – 1.0 a very strong relationship. Spearman correlation coefficients of 0.48 and 0.57 were calculated for fresh

and brackish water respectively, confirming the presence of moderate to strong associations between FC and ENT in both matrices. Overall, results of Spearman correlation analyses concur with those seen for linear regression analysis; FC and ENT appear more strongly associated in brackish water.

Seasonal Trends: Marked seasonal differences between fecal coliform and *Enterococcus* [CFU] were shown. Overall, fecal coliform [CFU] revealed consistent patterns under fresh and brackish wet season conditions but an opposite trend under dry, brackish water conditions; a steady increase during the dry season in brackish water and a steady *decrease* during the dry season in freshwater. *Enterococcus* seasonal trends were shown to be more sporadic. During the wet season, a decrease was seen under brackish water conditions and an increase under freshwater conditions. A series of peaks and troughs was seen under dry conditions in both fresh and brackish waters.

Exploration of seasonal FC and ENT associations revealed a moderate correlation between FC and ENT in freshwater during the dry season ($r = 0.58$), while the highest correlation was seen in freshwater during the wet season; Spearman coefficient 0.62. Slight seasonal variations were seen in brackish water between wet and dry seasons; Spearman coefficients 0.58 and 0.56, both moderate. Similar adjusted R^2 values were found for both wet and dry seasons within both matrices, although consistently higher during the defined wet season; R^2 values of 0.37 and 0.36 were calculated for freshwater and brackish water wet season data, while R^2 values of 0.27 and 0.29 were calculated for dry season data (Figures 12 and 19).

CONCLUSION

The results of this study confirm, with confidence, a relationship between fecal coliform and *Enterococcus* indicator groups in both fresh and brackish surface waters. Significant associations between fecal coliform and *Enterococcus* were discovered both annually and seasonally; associations were found to be stronger under brackish conditions. Spearman correlation analysis of fecal coliform and *Enterococcus* demonstrated moderate to strong correlations in both fresh and brackish surface water matrices; fluctuation(s) in one variable predicted a similar fluctuation in the other, with average strength. Annual and seasonal regression analysis results may also be leaned upon with confidence, due to the verification of residual normality in both fresh and brackish water $\log_{10}$ CFU data.

The presence of a positive, linear relationship between fecal coliform and *Enterococcus* in both fresh and brackish water is apparent throughout the year. This implies that upward and downward fluctuations within one variable are also seen within the other year-round, further confirming the results seen using Spearman correlation. While seasonal variations *between* indicators are present – individual, sometimes opposite, patterns and fluctuations in fecal coliform and *Enterococcus* during wet and dry seasons– seasonal regression analysis and Spearman correlation coefficients indicate that fecal coliform and *Enterococcus* vary in a similar manner in *both* fresh and brackish water throughout the year; the FIB groups fluctuate together. This suggests that both groups of FIB are affected in a similar manner by an outside, unknown variable or variables within *both* matrices; positively and negatively. However, while fecal coliform and *Enterococcus* are proven to show moderate to strong correlation under fresh and brackish water conditions, this does not imply causation. Low R^2 values reveal that these bacterial groups are not dependent on one another in any case, either annually or seasonally.

While fecal coliform and *Enterococcus* are both proven useful FIB for the evaluation of surface water, their ability to solely and accurately describe fecal pollution within an aquatic environment is questionable. While the results of this study show *Enterococcus* to be a more reliable, conservative indicator than fecal coliform under *both* fresh and brackish conditions, following clear, unimodal distributions over significantly

smaller ranges, the fecal coliform group was shown to be more sporadic, exhibiting increased sensitivity to fluctuating abiotic and biotic parameters within the environment. In addition to significant seasonal trends among indicators, seasonal variation between indicators, and group-specific sensitivity to biotic and abiotic environmental parameters, suggest that a sole indicator is not sufficient to accurately describe annual trends of fecal pollution. Clear, linear associations coupled with moderate to strong correlations among variables, both seasonally and annually, suggest that fecal coliform and *Enterococcus* CFU data may, instead, be complimentary in regards to analysis of fecal pollution under *both* fresh and brackish conditions.

Fecal coliform CFU counts were shown to follow a similar trend within *both* fresh and brackish water matrices. In addition, it is interesting to note that fecal coliform showed annual peaks at a higher [CFU] than *Enterococcus,* under the same conditions. As a result, this study suggests that fecal coliform, despite previously demonstrated limitations in saline environments and sensitivity to high salt concentrations, may be a valuable addition to *brackish* water quality criteria due to annual and seasonal correlations to *Enterococcus*, the ideal marine indicator, under both fresh and brackish water conditions. Today, in turn, *Enterococcus* has reached status as the ideal FIB under marine conditions, due to its proven ability to thrive in saline environments. Consequently, *Enterococcus* is not commonly used as a sole indicator of freshwater fecal pollution. Interestingly, the results of this study show that freshwater *Enterococcus* [CFU] adhere to a strikingly similar range as fecal coliform under the same conditions. In addition, a clear, linear association among variables is seen under freshwater conditions. Due its conservative nature, less variation in counts throughout the year, and moderate to strong correlations with fecal coliform, the ideal freshwater indicator, this study suggests that *Enterococcus* may be just as valuable an indicator as fecal coliform in freshwater.

The results of this study have challenged previously accepted views of fecal coliform and *Enterococcus* effectiveness as ideal fresh and brackish water FIB, their suitability as sole indicators of fecal pollution, and their ideal usage as indicators for waters of varying salinities. The future of waterborne pathogen detection may lie in techniques which stray from traditional, culture-based methods and bacterial indicators. In the meantime, this study suggests that fecal coliform and *Enterococcus* have the

potential to be used interchangeably within fresh waters. However, due to group-specific fluctuations and sensitivities to a variety of biotic and abiotic factors and moderate to strong correlations between indicators, which appear complimentary, the safe bet to a brackish water quality approach appears to lie in the combined use of both FIB groups. Further exploration of associations between enteric FIB groups under a variety of environmental conditions will enhance our knowledge of the potential benefit associated with a multiple-indicator approach to bacterial water quality analyses of fresh and brackish waters.

CURRENT AND FUTURE RESEARCH

Many proposed alternative indicators are being researched and/or in use across the globe. Alternative indicators include, but are not limited to, the *Bacteroidales* family, which has shown high correlation to *Enterococcus* and *E. coli* concentrations; *Clostridium* perfringens, a hardy spore-forming organism which has proven useful in matrices experiencing heavy pollution, and may prove useful when determining pollution source(s), as concentrations vary between animal species (Hurst et al. 2002; Roll and Fujioka 1997; Sorensen et al. 1989); and viruses, mainly bacteriophage specific to humans and correlated with sewage, are being further researched for use in the detection of specific species within the *Bacteroidales* family, as well as viral pathogens. F-specific RNA bacteriophage, which have been proven useful for the detection of viral pathogens, due to their similar size and shape to enteric viruses, inability to replicate in the water column, and high correlation to sewage contamination, are of key interest (Havelaar and Pot-Hogeboom 1988).

Current research surrounding the detection and monitoring of fecal pollution and associated bacterial pathogens is heavily rooted in q-PCR techniques, which are capable of providing results more rapidly than culture-based methods. Rapid detection q-PCR techniques prove most useful in situations where rapid results are critical to avoid dangerous public health risk(s), e.g. beach monitoring programs and potential beach closures. Rapid detection methods for *E. coli* via q-PCR are developed and in use today (Lavender and Kinzelman 2009). In addition, development of U.S. EPA 1611 is currently underway, the aim of which is to provide a means for rapid detection of *Enterococcus* via q-PCR with reduced effects of environmental interference associated with problematic water samples (U.S. EPA 2012b).

In addition, research into zoonotic diseases, which may be transferred from animals to humans, and their role in public health risk is also being conducted. Several studies have linked harmful recreational water exposure to outbreaks caused by potentially zoonotic diseases (Roy et al. 2004; U.S. EPA 2009a; Valderrama et al. 2009). Source tracking of bacterial groups such as *Bacteroidales* and *Bifidobacterium* is of key interest, as it has been demonstrated that the source of contamination is essential to assessing and understanding human health risk. Organisms of primary concern and

subject to current research, in regard to zoonotic disease, include *Salmonella*, *Giardia*, *Cryptosporidium*, and *E. coli 0157: H7* (Bonjoch et al. 2004; Matsuki et al. 2004; Nebra et al. 2003).

Table 1. Freshwater fecal coliform and *Enterococcus* counts per month, expressed as CFU/100 mL.

Month	Indicator Counts (CFU/100 mL)	
	Fecal Coliform	*Enterococcus*
2015		
April	138	216
May	479	28
June	112	143
July	385	183
August	419	195
September	1410	230
October	1253	152
November	329	52
2016		
December	204	199
January	144	94
February	125	195
March	18	28

Table 2. Brackish water fecal coliform and *Enterococcus* counts per month, expressed as CFU/100 mL.

Month	Indicator Counts (CFU/100 mL)	
	Fecal Coliform	*Enterococcus*
2015		
April	218	197
May	263	165
June	150	135
July	153	91
August	192	92
September	854	151
October	212	156
November	101	130
December	283	106
2016		
January	266	192
February	317	380
March	377	140

APPENDIX A

Freshwater Raw Data

Sample	Fecal Coliform (CFU/100 mL)	Fecal Coliform (Log_{10})	*Enterococcus* (CFU/100 mL)	*Enterococcus* (Log_{10})
1	20	1.301029996	22	1.342422681
2	255	2.40654018	410	2.612783857
3	920	2.963787827	52	1.716003344
4	38	1.579783597	4	0.602059991
5	74	1.86923172	390	2.591064607
6	184	2.264817823	14	1.146128036
7	78	1.892094603	26	1.414973348
8	420	2.62324929	110	2.041392685
9	350	2.544068044	520	2.716003344
10	106	2.025305865	190	2.278753601
11	108	2.033423755	12	1.079181246
12	22	1.342422681	22	1.342422681
13	260	2.414973348	340	2.531478917
14	1430	3.155336037	84	1.924279286
15	520	2.716003344	90	1.954242509
16	170	2.230448921	18	1.255272505
17	190	2.278753601	48	1.681241237
18	500	2.698970004	76	1.880813592
19	78	1.892094603	44	1.643452676
20	290	2.462397998	68	1.832508913
21	420	2.62324929	56	1.748188027
22	130	2.113943352	80	1.903089987
23	1020	3.008600172	590	2.770852012
24	700	2.84509804	320	2.505149978
25	590	2.770852012	760	2.880813592
26	650	2.812913357	24	1.380211242
27	180	2.255272505	12	1.079181246
28	920	2.963787827	94	1.973127854
29	20	1.301029996	4	0.602059991
30	440	2.643452676	250	2.397940009
31	1600	3.204119983	260	2.414973348
32	4300	3.633468456	490	2.69019608
33	2900	3.462397998	206	2.31386722
34	3300	3.51851394	510	2.707570176
35	560	2.748188027	34	1.531478917
36	640	2.806179974	650	2.812913357
37	700	2.84509804	230	2.361727836
38	12000	4.079181246	1350	3.130333768
39	4500	3.653212514	110	2.041392685
40	1000	3	540	2.73239376

41	150	2.17609126	34	1.53147892
42	100	2	14	1.146128036
43	360	2.556302501	14	1.146128036
44	48	1.681241237	2	0.301029996
45	86	1.934498451	6	0.77815125
46	910	2.959041392	18	1.255272505
47	110	2.041392685	12	1.079181246
48	1060	3.025305865	50	1.698970004
49	48	1.681241237	28	1.447158031
50	116	2.064457989	38	1.579783597
51	80	1.903089987	30	1.477121255
52	18	1.255272505	50	1.698970004
53	8	0.903089987	50	1.698970004
54	10	1	42	1.62324929
55	1130	3.053078443	114	2.056904851
56	44	1.643452676	20	1.301029996
57	120	2.079181246	52	1.716003344
58	340	2.531478917	34	1.531478917
59	60	1.77815125	780	2.892094603
60	8	0.903089987	4	0.602059991
61	820	2.913813852	58	1.763427994
62	190	2.278753601	290	2.462397998
63	104	2.017033339	170	2.230448921
64	88	1.944482672	130	2.113943352
65	230	2.361727836	360	2.556302501
66	460	2.662757832	150	2.176091259
67	22	1.342422681	10	1
68	8	0.903089987	36	1.556302501
69	34	1.531478917	132	2.120573931
70	28	1.447158031	52	1.716003344
71	74	1.86923172	370	2.568201724
72	730	2.86332286	240	2.380211242
73	8	0.903089987	14	1.146128036
74	36	1.556302501	34	1.531478917
75	40	1.602059991	12	1.079181246
76	24	1.380211242	80	1.903089987
77	78	1.892094603	92	1.963787827
78	16	1.204119983	22	1.342422681
79	56	1.748188027	200	2.301029996
80	1180	3.071882007	360	2.556302501
81	40	1.602059991	86	1.934498451
82	48	1.681241237	58	1.763427994
83	56	1.748188027	72	1.857332496
84	16	1.204119983	580	2.763427994

85	6	0.77815125	180	2.255272505
86	34	1.531478917	110	2.041392685
87	98	1.991226076	110	2.041392685
88	380	2.579783597	310	2.491361694
89	130	2.113943352	210	2.322219295
90	200	2.301029996	140	2.146128036
91	8	0.903089987	30	1.477121255
92	280	2.447158031	130	2.113943352
93	250	2.397940009	86	1.934498451
94	58	1.763427994	420	2.62324929
95	38	1.579783597	38	1.579783597
96	6	0.77815125	22	1.342422681
97	4	0.602059991	68	1.832508913
98	6	0.77815125	2	0.301029996
99	28	1.447158031	12	1.079181246
100	20	1.301029996	4	0.602059991
101	32	1.505149978	34	1.531478917
102	32	1.505149978	52	1.716003344

APPENDIX B

Brackish Water Raw Data

Sample	Fecal Coliform (CFU/100 mL)	Fecal Coliform (Log_{10})	*Enterococcus* (CFU/100 mL)	*Enterococcus* (Log_{10})
1	90	1.954242509	63	1.799340549
2	78	1.892094603	110	2.041392685
3	90	1.954242509	180	2.255272505
4	10	1	4	0.602059991
5	36	1.556302501	42	1.62324929
6	64	1.806179974	48	1.681241237
7	56	1.748188027	70	1.84509804
8	310	2.491361694	790	2.897627091
9	96	1.982271233	200	2.301029996
10	24	1.380211242	30	1.477121255
11	100	2	320	2.505149978
12	160	2.204119983	350	2.544068044
13	580	2.763427994	160	2.204119983
14	76	1.880813592	20	1.301029996
15	370	2.568201724	160	2.204119983
16	1350	3.130333768	600	2.77815125
17	1730	3.238046103	170	2.230448921
18	36	1.556302501	108	2.033423755
19	1070	3.029383778	24	1.380211242
20	48	1.681241237	66	1.819543936
21	250	2.397940009	210	2.322219295
22	590	2.770852012	460	2.662757832
23	220	2.342422681	180	2.255272505
24	570	2.755874856	270	2.431363764
25	490	2.69019608	300	2.477121255
26	106	2.025305865	180	2.255272505
27	102	2.008600172	130	2.113943352
28	96	1.982271233	200	2.301029996
29	74	1.86923172	160	2.204119983
30	18	1.255272505	12	1.079181246
31	48	1.681241237	100	2
32	86	1.934498451	170	2.230448921
33	102	2.008600172	130	2.113943352
34	96	1.982271233	200	2.301029996

APPENDIX B (Continued)

35	74	1.86923172	160	2.204119983
36	16	1.204119983	52	1.716003344
37	116	2.064457989	140	2.146128036
38	74	1.86923172	118	2.071882007
39	66	1.819543936	48	1.681241237
40	230	2.361727836	380	2.579783597
41	130	2.113943352	300	2.477121255
42	68	1.832508913	170	2.230448921
43	130	2.113943352	260	2.414973348
44	20	1.301029996	24	1.380211242
45	200	2.301029996	86	1.934498451
46	140	2.146128036	120	2.079181246
47	38	1.579783597	38	1.579783597
48	114	2.056904851	100	2
49	162	2.209515015	240	2.380211242
50	4	0.602059991	10	1
51	68	1.832508913	36	1.556302501
52	148	2.170261715	28	1.447158031
53	16	1.204119983	82	1.913813852
54	28	1.447158031	62	1.792391689
55	70	1.84509804	114	2.056904851
56	14	1.146128036	12	1.079181246
57	96	1.982271233	400	2.602059991
58	70	1.84509804	250	2.397940009
59	360	2.556302501	26	1.414973348
60	330	2.51851394	200	2.301029996
61	510	2.707570176	320	2.505149978
62	10	1	12	1.079181246
63	64	1.806179974	56	1.748188027
64	280	2.447158031	138	2.139879086
65	670	2.826074803	280	2.447158031
66	18	1.255272505	38	1.579783597
67	380	2.579783597	230	2.361727836
68	270	2.431363764	310	2.491361694
69	24	1.380211242	12	1.079181246
70	66	1.819543936	40	1.602059991
71	380	2.579783597	180	2.255272505
72	8	0.903089987	14	1.146128036
73	14	1.146128036	22	1.342422681

74	50	1.698970004	140	2.146128036
75	98	1.991226076	180	2.255272505
76	30	1.477121255	22	1.342422681
77	16	1.204119983	10	1
78	54	1.73239376	30	1.477121255
79	62	1.792391689	70	1.84509804
80	6	0.77815125	20	1.301029996
81	32	1.505149978	4	0.602059991
82	20	1.301029996	120	2.079181246
83	20	1.301029996	8	0.903089987
84	290	2.462397998	96	1.982271233
85	410	2.612783857	104	2.017033339
86	380	2.579783597	24	1.380211242
87	170	2.230448921	36	1.556302501
88	720	2.857332496	390	2.591064607
89	106	2.025305865	40	1.602059991
90	740	2.86923172	76	1.880813592
91	230	2.361727836	84	1.924279286
92	6	0.77815125	8	0.903089987
93	8	0.903089987	8	0.903089987
94	26	1.414973348	12	1.079181246
95	166	2.220108088	6	0.77815125
96	56	1.748188027	40	1.602059991
97	60	1.77815125	48	1.681241237
98	140	2.146128036	290	2.462397998
99	134	2.127104798	260	2.414973348
100	320	2.505149978	370	2.568201724
101	860	2.934498451	270	2.431363764
102	370	2.568201724	22	1.342422681
103	6	0.77815125	8	0.903089987
104	8	0.903089987	8	0.903089987
105	26	1.414973348	12	1.079181246
106	118	2.071882007	36	1.556302501
107	220	2.342422681	52	1.716003344
108	890	2.949390007	320	2.505149978
109	118	2.071882007	36	1.556302501
110	350	2.544068044	40	1.602059991
111	620	2.792391689	120	2.079181246
112	890	2.949390007	210	2.322219295

113	650	2.812913357	90	1.954242509
114	540	2.73239376	140	2.146128036
115	960	2.982271233	520	2.716003344
116	1450	3.161368002	90	1.954242509
117	1710	3.23299611	430	2.633468456
118	710	2.851258349	250	2.397940009
119	1820	3.260071388	102	2.008600172
120	230	2.361727836	38	1.579783597
121	660	2.819543936	76	1.880813592
122	1960	3.292256071	126	2.100370545
123	1470	3.167317335	48	1.681241237
124	50	1.698970004	26	1.414973348
125	94	1.973127854	22	1.342422681
126	56	1.748188027	30	1.477121255
127	40	1.602059991	16	1.204119983
128	76	1.880813592	16	1.204119983
129	72	1.857332496	48	1.681241237
130	102	2.008600172	48	1.681241237
131	88	1.944482672	62	1.792391689
132	78	1.892094603	40	1.602059991
133	62	1.792391689	420	2.62324929
134	500	2.698970004	290	2.462397998
135	580	2.763427994	54	1.73239376
136	250	2.397940009	46	1.662757832
137	16	1.204119983	82	1.913813852
138	210	2.322219295	650	2.812913357
139	122	2.086359831	660	2.819543936
140	42	1.62324929	50	1.698970004
141	510	2.707570176	86	1.934498451
142	1210	3.08278537	510	2.707570176
143	44	1.643452676	28	1.447158031
144	260	2.414973348	98	1.991226076
145	90	1.954242509	34	1.531478917
146	138	2.139879086	98	1.991226076
147	106	2.025305865	108	2.033423755
148	240	2.380211242	170	2.230448921
149	18	1.255272505	66	1.819543936
150	34	1.531478917	32	1.505149978
151	50	1.698970004	260	2.414973348

APPENDIX B (Continued)

152	32	1.505149978	16	1.204119983
153	40	1.602059991	96	1.982271233
154	187	2.271841607	730	2.86332286
155	100	2	94	1.973127854
156	60	1.77815125	56	1.748188027
157	80	1.903089987	48	1.681241237
158	34	1.531478917	66	1.819543936
159	110	2.041392685	100	2
160	300	2.477121255	104	2.017033339
161	40	1.602059991	130	2.113943352
162	130	2.113943352	60	1.77815125
163	16	1.204119983	30	1.477121255
164	50	1.698970004	46	1.662757832
165	38	1.579783597	50	1.698970004
166	470	2.672097858	112	2.049218023
167	380	2.579783597	130	2.113943352
168	72	1.857332496	86	1.934498451
169	780	2.892094603	130	2.113943352
170	70	1.84509804	26	1.414973348
171	400	2.602059991	104	2.017033339
172	310	2.491361694	70	1.84509804
173	360	2.556302501	76	1.880813592
174	580	2.763427994	270	2.431363764
175	330	2.51851394	56	1.748188027
176	380	2.579783597	270	2.431363764
177	250	2.397940009	66	1.819543936
178	250	2.397940009	250	2.397940009
179	640	2.806179974	124	2.093421685
180	28	1.447158031	10	1
181	26	1.414973348	20	1.301029996
182	620	2.792391689	220	2.342422681
183	86	1.934498451	330	2.51851394
184	210	2.322219295	490	2.69019608
185	124	2.093421685	74	1.86923172
186	770	2.886490725	126	2.100370545
187	400	2.602059991	142	2.152288344
188	270	2.431363764	54	1.73239376
189	360	2.556302501	84	1.924279286
190	54	1.73239376	54	1.73239376

APPENDIX B (Continued)

191	58	1.763427994	128	2.10720997
192	106	2.025305865	140	2.146128036
193	96	1.982271233	60	1.77815125
194	600	2.77815125	106	2.025305865
195	82	1.913813852	82	1.913813852
196	660	2.819543936	860	2.934498451
197	230	2.361727836	260	2.414973348
198	38	1.579783597	40	1.602059991
199	200	2.301029996	250	2.397940009
200	440	2.643452676	170	2.230448921
201	96	1.982271233	36	1.556302501
202	102	2.008600172	650	2.812913357
203	700	2.84509804	400	2.602059991
204	610	2.785329835	690	2.838849091
205	80	1.903089987	760	2.880813592
206	66	1.819543936	1010	3.004321374
207	74	1.86923172	860	2.934498451
208	46	1.662757832	290	2.462397998
209	370	2.568201724	290	2.462397998
210	82	1.913813852	56	1.748188027
211	820	2.913813852	570	2.755874856
212	80	1.903089987	62	1.792391689
213	260	2.414973348	140	2.146128036
214	130	2.113943352	98	1.991226076
215	590	2.770852012	250	2.397940009
216	90	1.954242509	170	2.230448921
217	1190	3.075546961	132	2.120573931
218	70	1.84509804	62	1.792391689
219	28	1.447158031	90	1.954242509
220	170	2.230448921	22	1.342422681
221	38	1.579783597	24	1.380211242
222	210	2.322219295	84	1.924279286
223	440	2.643452676	310	2.491361694
224	72	1.857332496	54	1.73239376
225	1680	3.225309282	320	2.505149978
226	810	2.908485019	170	2.230448921
227	430	2.633468456	124	2.093421685
228	300	2.477121255	250	2.397940009
229	270	2.431363764	170	2.230448921

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