Florida Gulf Coast University

The Water School

Estero River Bacteria-Nutrient Source Identification Project
Contract EC 2019-29

Final Report to Village of Estero

March 15, 2021

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1.0 Executive summary

This project consisted of a monitoring program, laboratory analysis, and data analysis for bacteria and related water quality parameters within the Estero River by FGCU personnel under contract with the Village of Estero. The primary objectives of the study were to gather information about temporal and spatial distribution of two types of fecal bacteria associated with human origins (*Enterococcus* and *Escherichia coli*, commonly called *E. coli*) in and near the Estero River, a tidal waterbody located in Lee County, Florida; and reach any possible preliminary findings about the locations of sources of those two species of bacteria. These species and others are used for regulatory purposes to indicate the presence or absence of human fecal matter, which may pose a health threat by carrying pathogens that could reach human hosts through contact with the waterbody. These organisms collectively are referred to as fecal indicator bacteria (FIB).

The study is intended to give preliminary information about distribution and potential sources in the river, as well as to begin to characterize other watershed factors that might affect the temporal and spatial distribution of the bacteria in a way that could interfere with identifying relationships between sources and measured concentration in the environment.

The results document that sampling and analyses of this type can detect cases where waterbodies do on some occasions, and in some locations, experience high or very high concentrations of FIB. The results do not, however, pinpoint particular activities or land uses that are demonstrably sources of FIB, and did demonstrate that FIB are a problem not of a limited location, a limited time, or a specific set of conditions, but that high and varying concentrations may recur at unpredictable times and locations on the target waterbodies.

The data lead to the following findings:

- Concentration of FIB was identified to be very high at some locations in the Estero River during nearly all of the seven 4-hour-long sampling events conducted across 13 months. On all but one sampling event, one or the other of the two target FIB was present in at least one location on the river at concentration at or above the laboratory detection limit of about 2400 MPN/100mL. On no occasions were those conditions sustained throughout the 5-mile sampled reach of the Estero River.

- Concentration of FIB varies very much on relatively short time frames and spatial scales. The known extreme variation of FIB transport and survival in the natural environment, along with the extreme variation in the kinds of human activities and presence of non-human organisms, compounded by the fact that many kinds of everyday activities can produce short-term, highly-localized, high-concentration conditions, severely confounds the ability of snapshot sampling such as this to

identify locations of sources. But the data do persuasively document the recurring presence of extremely high concentrations of FIB in the Estero River.

- Historical data from the period 2015 through 2020 indicates that samples with high concentrations of FIB are found in increasing frequency moving downstream in the river, though highly variable over time. This is persuasive evidence that some portion of the FIBs originate with urban / human activities, because as the river flows downstream it increases its potential encounters with short-term, episodic source events, so the probability of high-concentration samples increases. Multiple lines of evidence suggest that FIB source events are highly episodic: temporary, short-term, and varying in intensity in time and place.

- The effect of several suspected source activities (small wastewater treatment facilities, septic systems, residential lawns used by pets extending directly to river's edge, and others) on surface water in the Estero River could not be reliably differentiated from other land uses, as there were no locations where persistent high concentrations were co-located with any of the suspected sources. The findings are consistent with all those sources, and more, contributing to the periodically very-high FIB concentrations on the Estero River.

- The high-concentration conditions in the Estero River do not correspond in any obvious way with known high-precipitation events, seasonal changes in population, season-long changes in rainfall or water table changes, or identifiable tidal conditions. It is clear that multiple sources to the river affect those high bacteria concentrations, possibly including but not necessarily limited to: the neighborhood-maintained wastewater treatment facilities; densely-clustered or improperly-maintained septic systems; and runoff from community lawns.

- Groundwater sampling revealed routinely low concentrations of FIB in samples collected from surficial groundwater 1m to 2m below the surface. That was true even though several groundwater sampling sites showed high concentration of sucralose, which indicates presence of either treated or untreated human wastewater. This suggests that, when human wastes in those vicinities are transported with groundwater flowing slowly through the soils, those wastes are being effectively biodegraded by treatment systems and/or microbes in the soils, and that sub-surface groundwater flows may not be contributing FIB to environmental systems such as Estero River, if these two locations are typical of other locations in the watershed.

- On the other hand, two small surface flows, believed to convey mostly groundwater from two neighborhoods, both were extremely high in FIB, and sucralose studies showed them to be strongly affected by human wastewater. One ditch drains a neighborhood, Charing Cross Circle, that is served by septic systems; the other

drains a neighborhood, Estero Bay Village (formerly Tahiti), that is served by a small 'package' wastewater treatment plant. It is believed that in both cases, the two ditches carry groundwater that has 'short-circuited' the intended mechanisms, i.e. the wastes do not have a sufficiently long residence time in the soils for microbes to biodegrade FIB, and presumably also do not break down any pathogens that may be in those wastes. It is not clear whether these two very small flows are sufficient to affect FIB in the Estero River, but it is conceivable that, if many such flows exist along the River, in aggregate they could be a substantial source of the high FIB that are detected in some locations at some times in the Estero River.

- This "short circuiting" of the wastewater's movement through the soil has produced a condition where FIB enter the Estero River, which in turn indicates the potential presence of other potentially harmful substances originating with human wastes.
- It was expected that FIB concentration patterns would be different between wet-weather and dry-weather seasons. Instead, concentration patterns varied substantially among sampling events in each season, and no discernible pattern shows more variability between seasons than within seasons. Those effects may be present, but they do not influence the concentration at a given site or a given time to a discernible extent.
- Data support numerous previous researchers in documenting decoupled variation between different species of FIB: *E.coli* and enterococci varied independently of one another in nearly all samples.
- Genetic sequencing analysis of the samples indicates presence of bacteria known to populate the human enteric system, and other bacteria identified as markers of non-human animals. The genetic data definitively indicate presence of waste originating from both human and non-human species, but are not capable of quantifying the extent to which FIB and other bacteria originate with human vs. non-human species.

2.0 Introduction

2.1 Purpose and design of this project

This research consisted of a monitoring program for bacteria and related water quality parameters within the Estero River by FGCU personnel under contract with the Village of Estero. The research analyzed data collected for this purpose by the FGCU research team, and compared those results with historical data collected at two sampling stations by Lee County Natural Resources, focusing on the period from 2015 through 2019.

The sampling period was designed to span both wet and dry seasons, with field sampling beginning August 2019 and concluding in August 2020. Water samples were collected, tested in the field for some basic water quality constituents, and delivered to FGCU's laboratory for chemical testing to determine the concentration of the two target bacteria species and some related water quality constituents. The monitoring program consisted of five groundwater sampling locations along the Estero River; two locations in very small tributaries, also considered to be groundwater because their flow clearly originates with subsurface discharge; and "length-of-the-river" monitoring with samples collected by watercraft at about 10 sites on a single day, conducted seven times in total on the target waterbody between August 2019 and August 2020.

The project period encompassed 18 months, with the research consisting of a field sampling and laboratory analysis period of about 13 months (August 19, 2019 – September 15, 2020) and a period of data analysis, report preparation, review, and revision from April 1, 2020 through January 15, 2021.

2.2 Objectives

The primary objective of the research was to gather preliminary information about temporal and spatial distribution of two types of fecal bacteria associated with human origins (*Enterococcus* and *Escherichia coli*) in and near the Estero River, a tidal waterbody located in Lee County, Florida. The research is intended to give preliminary information about distribution and potential sources in the river, as well as to begin to characterize other watershed factors that might affect the temporal and spatial distribution of the bacteria in a way that could interfere with identifying relationships between sources and measured concentration in the environment. Those factors might include origins with non-human species (evaluated in this report); tidal conditions; precipitation volume, intensity, and timing; activities in the waterbodies such as boating; mobilization of bacteria deposits in benthic and riparian sediments; and others. The purpose of that analysis is ultimately to distinguish the influence of those environmental factors from the signature of source activities in the two watersheds. The ultimate goal is to use this information, and additional research, to identify the sources of these bacteria and consequently develop a course of

action that would control source activities and reduce the amount of bacteria in the environment.

2.3 Analyses

The analyses in this report are described in terms of "frequency of high-concentration events" at particular locations, and for the most part the report does not compute averages over time or location. Samples found to have high concentration of FIBs are termed "high-concentration occurrences," defined purely for the purposes of this report as greater than about 800 MPN/100 mL. That is not related to any regulatory standard but derives entirely from the data we are observing. The report also occasionally refers to "very high-concentration occurrences" which we define as greater than the maximum quantitation limit of the lab method applied, which is about 2,420 MPN/100mL.

The frequency of high-concentration occurrences is more meaningful to this analysis. Averages are not meaningful because a) FIB appear to originate with highly localized, episodic, short-term sources or events; b) FIB's transport in the river does not thoroughly mix them into the environmental system, but instead they are found in samples in localized patches of varying magnitude, location, and event (or species) of origin; and c) the ways in which humans may experience negative health effects do not result from long-term, average conditions but short-term exposure to pathogens, which is governed by the presence or absence of bacteria in high amounts at any one time, not the average conditions in time or place. (Negative effects of course are not experienced by all humans who happen to contact FIB at any one time, but some statistically variable proportion of exposed persons.)

3.0 Current State of Knowledge about Fecal Indicator Bacteria Sampling, Variability, and Source Identification

3.1 Fecal Indicator Bacteria (FIB)

Potential for illness from human fecal contamination of recreational waters is commonly approximated by measuring the presence of fecal indicator bacteria (FIB) found in surface water (USEPA, 2012). The effectiveness of FIB is categorized by their relationship to the presence of illness-causing pathogens, an imperfect and incomplete comparison but an implementable method in place of the impracticality of testing for hundreds or thousands of bacteria species that could be harmful to humans. Fleisher (1996) identifies gastroenteritis and nasal/respiratory illnesses as directly linked to human sewage contamination to recreational waters, and finds that gastroenteritis is the single disease of which there is a direct mathematical correlation between exposure to increasing bacterial content related to sewage pollution and risk of subsequent illness.

Analyses published in a 1986 report (USEPA 1986) identified two bacteria, enterococci and *E. coli*, as the most useful FIB, documenting a better relationship than other species with epidemiological evidence of negative effects on humans and therefore presumably with human pathogens in general. Therefore, in the US these two are most widely used as the basis for water quality criteria. Both enterococci and *E. coli* are considered useful indicators in freshwater systems, but enterococci is the only FIB that yielded a good correlation to the illness-causing pathogens in marine systems (USEPA 1986), largely because they are shown to survive better in saline conditions than *E. coli* (Borrego et al 2002; Geldreich 2002). Due to the tidal influences and brackish environment in most reaches of the Estero River suspected to potentially be contaminated by human fecal bacteria, enterococci are expected to be the target of Florida state standards for those waters.

3.2 Effectively Determining Sources: Human Sources

The ultimate purpose of this project, and ongoing studies, is to identify sources of FIB and therefore of potential fecal bacteria in the two target waterbodies. The near-term goal is to characterize variability in time and space of the FIB in the waterbodies, with the intent of using that variability to help identify locations and times of greatest FIB presence and thus deduce potential sources of FIB in the waters.

Scientists have identified and quantified fecal and sewage contamination as existing in both soil and water (Luna et al. 2016; He et al. 2017). Luna et al. (2016) state that there are many anthropogenic pollutants in aquatic sediments and the pollutants come from a variety of sources. Further, Luna et al. (2016) asserts that aquatic soil contamination is highest in urban areas and reduces to the point of being nearly non-existent as sampling moves towards the open ocean.

Lipp et al. (2001) reported results that indicate that the greatest risk of human enteric pathogens and fecal pollution is in the areas that are proximal to the areas with high densities of onsite sewage disposal systems, i.e. individual or community septic systems. Lipp et al. (2001) also cite Griffin et al. (1999) who state that groundwater is at significant risk for microbial fecal contamination in concentrated areas of septic systems. Additionally, Griffin et al. (1999) discussed research from the Florida Keys which indicates widespread contamination in surface waters that are proximal to septic systems.

3.3 Confounding Factors and Reasons Sources are Not Always Discernible: Non-Human Sources

One of the limitations of the two widely used FIB is that they originate in the colons of any warm-blooded animal, and the traditional laboratory methods have been unable to distinguish bacteria of human origin – which would be effective indicators of presence of human waste – from bacteria originating in other warm-blooded species (Borrego et al.

2002; Geldreich 2002). It has been widely assumed that human fecal bacteria are the primary, or only, sources of negative health effects, though the current EPA guidance recognizes that some negative health effects may originate with the wastes of non-human sources and those potential impacts should not be overlooked (USEPA, 2002). It should be noted that the kinds of potential health effects do not include the most virulent pathogens, those that wastewater treatment is designed to eliminate, but other risks are less well understood and vary widely from the health effects known to be possible when recreational waters have been contaminated with human sources (USEPA, 2012). At present, the analysis of FIB continues to be focused on identifying potential sources of fecal bacteria of human origin, so the potential presence of bacteria originating with non-human organisms is an important confounder.

It is valuable to be able to distinguish human and non-human sources, and Microbial source tracking (MST) has the potential to be a useful tool in helping to understand potential sources of fecal contamination in recreational waters.

Ahmed et al. (2015) discuss how microbial source tracking methods are able to differentiate between human and non-human sources of fecal pollution within environmental waters and even identify specific hosts. Ahmed et al. (2015) cite Unno et al. (2010) as having created a method for identifying sources of fecal pollution within South Korean waterways and specifically differentiate between human and bovine fecal sources.

One method of source tracking that has been proposed and tested is species differentiation within *Enterococcus* using multiplex PCR methods (Layton et al. 2010). This method uses several fecal samples from various sources, human, dog, gull, etc. and amplified targeted *Enterococcus* species to examine which species of *Enterococcus* are present in the different samples. This method effectively created species fingerprints, however there is variability with this as the samples are regionally based and as environmental variability can affect the ability of certain species of *Enterococcus* to be amplified. Another detail to consider is that almost all species targeted appeared in the samples from each type of host, making it impossible to name one single species of *Enterococcus* as an effective way to track human sources (Layton et al. 2010). The method can identify presence or absence of human-originating FIB among the multiple species of sources in a given environmental system, but when other species are also present, the method typically cannot determine the relative amounts of waste originating with each species.

3.4 Confounding Factors and Reasons Sources are Not Always Discernible: Variability in the Environment

Identifying sources is complicated by the known high variability of concentrations of FIB in the environment. A study of near-shore waters at bathing beaches by Boehm

(2007) determined that concentration of enterococci can change hourly, including changing within one hour from concentration less than the recommended Federal criterion of 130 cfu/100 ml to well in exceedance of that criterion. Other studies including Clemente (2018) document that nearshore marine waters are highly variable because they are subject to mechanical mixing by tides, rip currents, wave action, varying runoff, varying wildlife activity, and others - including the potential for wave and tidal action to resuspend bacteria in sand, soils, and sediments, so the complex causes for variation have not been fully understood or modeled.

Flowing streams appear to have similar variability, as described by Crosby et al (2019), to an extent that appears comparable to the variation in beach waters. The tidal riverine and estuarine systems of southwest Florida experience some of these same factors, complicated further by changing tides in the constricted channels, resuspension of sediments by human recreational and boating uses, and varying activities such as lawn care, pet activities, and wildlife on the riverbanks. These too are not fully understood, not predictable with information that agencies are in a position to collect, and not readily subject to detailed models that can quantify their effects on the number of bacteria observed at a given moment in a grab sample from the aquatic environment. Therefore the results of this analysis are necessarily highly approximate and subject to unforeseen variability, though they would be sufficient to identify any powerful, sustained sources, and are useful to develop a general idea of the long-term conditions in various reaches of the waters.

3.4.1 Sediments

Luna et al. (2016) pointed out that "sediments are environmental reservoirs of fecal bacteria" and that is necessary to use the more modern methods in order to ensure that bacteria are related and traceable to a current pollution source, rather than being of a persistent nature in the soil (2016). Yamahara et al (2007) described presence of FIB in beach sands and identify those sediments as diffuse sources of bacteria to coastal waters. Ahmed et al. (2015) discussed using a variety of methods to determine the source and viability of the bacterial population.

3.4.2 Weather and Tidal Influences in Coastal Waters

Weather and natural factors' influences on coastal water bacteria levels from tidal cycles and other weathering conditions have been a concern for varying levels of FIB. Storm surges and runoff are known contributors to FIB in coastal waters. Tidal influences on bacteria have shown mixed results in previous studies and have been analyzed in a number of different ways. Tides modulate water with land and can potentially mix polluted land surfaces with natural waters. A study conducted in tropical waters in California showed a tidal influence on only half of their sampling stations, where they identified FIB in higher abundance during low tide (Santoro and Boehm, 2007). Other studies conducted in

tidally muted areas, like wetlands controlled by flow gates that would only have tidal changes during storm surges, also showed diminished concentrations of FIB during high tide as the wetlands act as a natural sink for bacteria (Johnston et al., 2015). Past studies have shown statistical correlation of FIB and tidal associated process, dilution and salinity having a significant effect on rainfall and temperature impacts on bacteria concentrations. Tides have the ability to change the properties of a water body like salinity and dilution which can affect FIB concentrations. Evaluating FIB in tidal estuary systems can be very complicated due to the mixing of marine and freshwater and the effects that each has on FIB concentrations (Johnston et al, 2015).

3.5 Estimating Sources using Multiple Lines of Evidence

Analyzing FIB is a well-established method, available to most environmental laboratories, and less costly per sample than other methods in the attempt to determine the potential presence of human waste in recreational waters. But that approach can be improved. The best currently available means is to consider multiple lines of evidence, that is, FIB in combination with one or more of three emerging methods: microbial source tracking (MST); chemical tracers; and stable isotopes of nitrogen. These are further explained below. None of these by itself is definitive, but the three taken together can give reasonable confidence in an assessment of presence or absence of human waste in a target environmental system. A recent study conducted for the Caloosahatchee River and small tributaries at North Fort Myers demonstrated assessment using multiple lines of evidence. (Lapointe et al., 2018). The approach used in the current study was to rely primarily on FIB analysis, quantifying FIB at as many times and places as fuinding allowed, then adding a limited number of combined approaches to increase confidence in our assessment of the presence or absence of human waste in the Estero River. Characterizing variability of FIB and of nutrients in the target waters is a means to identify locations and times of greatest concentration and thus potential sources, while the other three methods give information as to whether the sources are human in origin.

3.6 Chemical Tracers: Sucralose

FIB such as *E. coli* and enterococci are used for regulatory purposes because they are believed to associate with human fecal pollution, which is a health threat because it potentially contains pathogens harmful to humans. However, both those FIB species are also found in the fecal matter of other warm-blooded animals. Non-human fecal matter is not believed to be strongly associated with pathogens harmful to humans. Waters of the U.S. are believed to commonly have FIB that originate with various domestic animals and wildlife (e.g., cows, chickens, gulls, other avian species, and others) (Byappanahalli et al. 2012). The counting of these bacteria (as in Sections 7 and 8 of this report) does not indicate the organism that was the source of the bacteria.

One method to determine whether human fecal matter may be present is to test for chemical tracers in the environment. Chemical tracers are any substances that originate with human waste and that are persistent in the environment; that is, they are not broken down during wastewater treatment and do not biodegrade in the environment; and that are not known to originate with any source other than human waste. Typically these are complex synthetic organic substances consumed by humans in processed food, beverages, or pharmaceuticals that pass through the human body and are excreted in urine and/or fecal matter.

Several tracers have been tried and found effective to varying degrees as indicators of potential presence of human waste and its attendant health effects. These include sucralose, an artificial sweetener; acetaminophen, a pain relief medication; caffeine; and others. None of these tracers in themselves is potentially harmful to human health in the minute quantities found in environmental systems, so they are not the subject of any numeric standards. The advantages of the chemical tracer approach are that they are much more stable than FIB, which may biodegrade or change form in a matter of days; they reliably indicate presence of human waste, as they do not originate in any other ways (except in rare cases of spillage into the environment); and they are source specific, that is, they are relied on to originate only with humans, while all known enteric bacteria that originate with humans also are known to originate with some, or many, other warm-blooded animals.

This project tested for sucralose. Sucralose is highly water-soluble and resistant to microbial degradation but does not bioaccumulate in the body of aquatic organisms. Advantages in using sucralose include that it persists in environments for up to 4 years and is highly resistant to degradation in the human body and in a typical biological wastewater treatment plant (Soh et al. 2011). But it is much more mobile in the environment than FIB because sucralose is highly soluble (Mawhinney et al. 2011), so sucralose is effective at indicating whether human waste was present in a multi-year period, well beyond the persistence of the two target FIB, which is a maximum of 30 to 60 days in soils and even less in aquatic environments (Anderson et al. 2005). Sucralose may easily be more widespread (Mawhinney et al. 2011) compared to any FIB or other human-originating bacteria that may cause health effects. No toxicity or other adverse effects have been reported from sucralose in aquatic plants, algae, crustaceans, or fish (Tollefsen et al. 2012).

Sucralose is carried into Florida waters along with discharge originating with any treated or untreated waste. That includes any centralized wastewater treatment system or high-density septic systems operated at individual residences. Sucralose enters a waterbody in treated effluent from treatment plants; by moving through groundwater from a septic system; or conveyed by stormwater runoff from soils or surfaces where wastes have accumulated.

Typically analyses for sucralose focus on groundwater, because it may be concentrated in soils or groundwater in the vicinity of a septic system, and it moves more slowly in groundwater than surface water – even while it moves much more rapidly than FIB or a substance that sorbs to soil particles, because sucralose is highly soluble in water. Once groundwater carrying sucralose enters a surface waterbody, the sucralose is highly diluted based on the size of the water body. The concentration of sucralose in groundwater typically is found to be one order higher than the river water, as in the study by Lapointe et al. (2017) in the watershed of the Caloosahatchee River in North Fort Myers. In that study, sucralose ranged between 215 and 790 ng/L in the Hancock Creek and 673 and 968 ng/L (Lapointe et al. 2017). In another study, a much higher concentration of greater than 5,000 ng/L of sucralose was been documented from multiple samples collected from the St. Lucie Estuary watershed (Lapointe et al. 2018). That finding suggests we should expect sucralose to be found in the upper part of that range in the Estero River.

3.7 Biological Tracers: Genetic Sequencing

Biological tracers are another method used to help determine whether any FIB identified in the environment originated with human or non-human warm-blooded animals. *E. coli* and *Enterococcus,* which are used as FIB for regulatory purposes because they can be readily identified and quantified by existing laboratory methods, but they are known to populate the gut of many warm-blooded animals, so their presence and magnitude do not show evidence of whether their source was human or non-human. As noted in Section 3.6 above, non-human fecal organisms are not believed to be widespread sources of health effects in humans, so we would like to be able to determine whether any fecal bacteria found in a sample identified with humans or other animals.

Source-specific tracking methods using a molecular identification platform (Chern et al. 2009; Kreader 1995) analyze the DNA of a wide range of bacteria found in the environment, not limited to enterococci and *E. Coli*. Rapid advances in the DNA sequencing methods, over the past decade continuing through today, allow us to use DNA sequencing to identify many more species of bacteria than previously recognized. The method could be an important supplement to the conventional laboratory analysis for *E. coli* and enterococci. However, the current state of knowledge has not identified any species of bacteria that are routinely, exclusively found in any particular organism. Therefore DNA identification of bacteria species, as a biological tracer, supplies important evidence about whether non-human animals may be present in a given environment, but not definitive evidence.

Some gut microbes are found more commonly in some animals than others. For instance, the gut microbial flora of birds is characterized by a lower abundance of Firmicutes and Bacteroidetes and a higher abundance of Actinobacteria and Proteobacteria in comparison with non-flying mammals (Grond et al. 2018). Species found to be present in

high amounts in southwest Florida tidal and brackish waters include the class Gammaproteobacteria, which includes a variety of pathogenic bacteria (e.g., *Aeromonas*, *Escherichia*, *Legionella*, *Pseudomonas*, *Salmonella*, *Vibrio*) and ecologically important bacteria (e.g., *Alcanivorax*, *Beggiatoa*, *Methylomonas*, *Nitrosococcus*). Alphaproteobacteria widely inhabit natural environments, particularly saline environments. Alphaproteobacteria are a good indicator of the presence of seawater (Garcia et al. 2015; Urakawa and Bernhard 2017).

A great advantage of the direct DNA sequencing method over traditional cultivation methods is that it is possible to detect and identify multiple functionally different

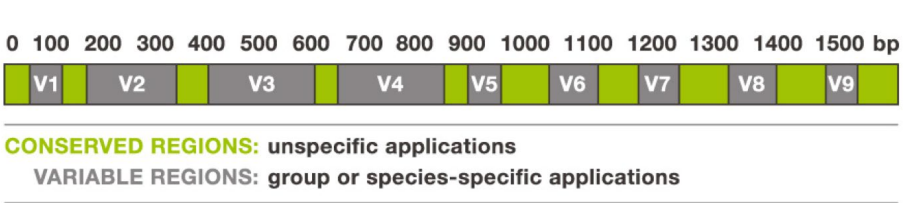

Figure 3.1. Location of conserved and variable regions of 16S rRNA gene. In this research, we use V3 and V4 regions to identify microorganisms (credit, alimetrics.net).

microorganisms in a single analysis. There is no need to prepare each cultivation medium for each group of bacteria; for example, using traditional methods separate media would be needed for cyanobacteria and *E. coli* because cyanobacteria do not grow in the medium used to culture *E. coli*.

A method using 16S rRNA gene sequences has been widely used for classification and identification of Bacteria and Archaea. This conserved gene marker is considered the "gold standard" of recent microbiome studies. The length of the gene is approximately 1,500 bp and the gene contains multiple conserved and variable regions (V1 to V9 regions) (Figure 3.1). This project used high-throughput DNA sequencing (amplicon sequencing) using the 16S rRNA gene, which has been extensively used in recent microbiome studies and has a potential to be used for FIB tracking.

4.0 Historical Data, Lee County Natural Resources

Samples have been collected by Lee County Natural Resources on a once-monthly basis since about 2000 at several sites on or near the Estero River. Two of those same sites were used also by the FGCU project to collect our data, and the sites appear on the maps of FGCU sampling locations. The Lee County Environmental Laboratory has conducted analysis of samples for a number of chemical constituents and for two types of bacteria, *Enterococcus* and *E. coli.* The laboratory method for enterococci has been modified periodically as USEPA had updated its guidance about preferred laboratory methods. The latest date when the Lee County labs changed their analysis method occurred in 2015. The methods are sufficiently different from one another that it is questionable to compare data developed by one method to data developed by another, and so this summary uses Lee County enterococci data only for the period January 2015 through December 2019. The data for *E. coli.* were graphed for a similar period, June 2016 through December 2019, to allow for a consistent comparison.

4.1 Historical Data for Enterococcus and E. coli

The graphs in Figures 4.1 through 4.6 were prepared by FGCU researchers using data provided by Lee County Environmental Laboratory in December 2019. The graphs show the concentration of *Enterococcus* and *E. coli* at three locations on the Estero River.

The data in Figures 4.1 through 4.6 show the extreme variability of FIB in Estero River samples across the period of record 2015-2019. In 60 samples collected once per month over the period January 2015 through December 2019, at the Riverwoods site (mile 3.2), on 11 occasions the concentration of enterococci exceeded the laboratory maximum of about 2,400 MPN/100mL. On 22 occasions the concentration of enterococci was less than 250 MPN/100mL, and on a total of 40 occasions the concentration of enterococci was less than 500 MPN/100mL.

Further upstream, in 60 samples over the same period at the Route 41 site (mile 4.6), on only 2 occasions the concentration of enterococci exceeded the laboratory maximum of about 2,400 MPN/100mL, but on 5 other occasions the concentration was between 1,000 and 1,500 MPN/100mL. At the furthest upstream site for which samples were collected, the Three Oaks bridge over the North branch of Estero River (mile 6.2), there were no samples in which *Enterococcus* were as high as 600 MPN/100mL - only one sample at 550 MPN/100mL, one other at 310 MPN/100mL, and all others less than 200 MPN/100mL. Even at this site the Estero River would not meet the numeric TPTV (ten percent threshold value) target for enterococci, because 8 of the 60 samples were measured at greater than 130 MPN/100mL. The samples for *E. coli* at Three Oaks do meet the TPTV at that site, which is 410 MPN/100mL for *E. coli.*

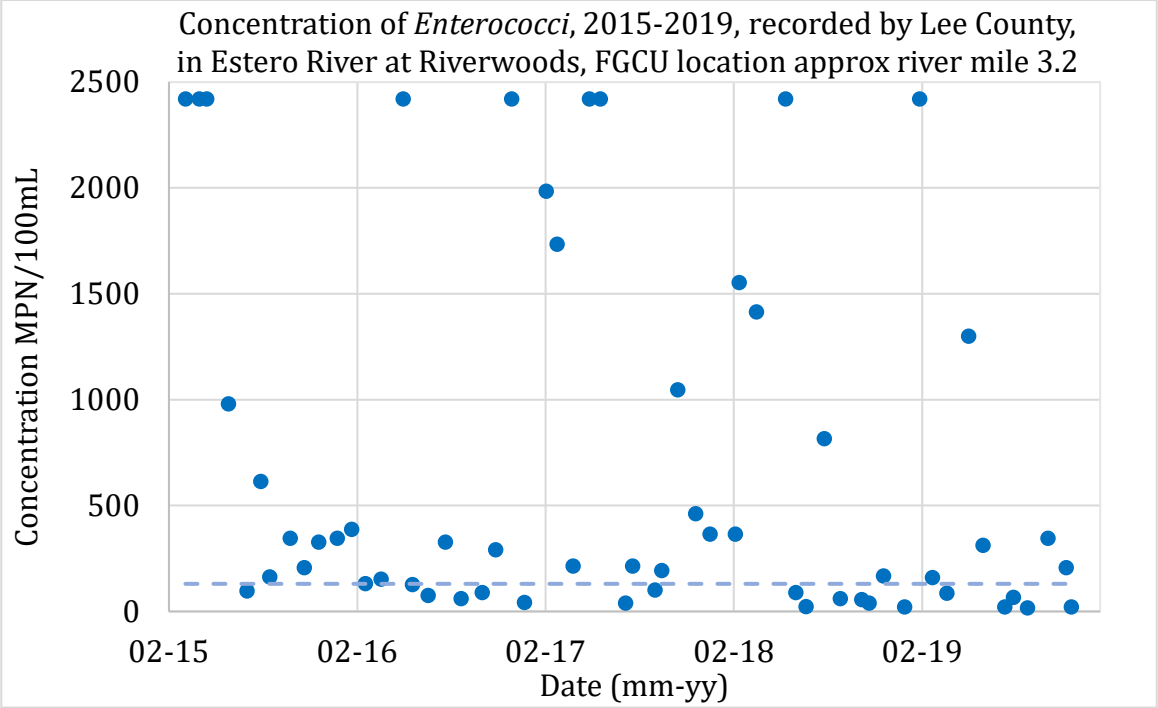

Figure 4.1. Enterococci concentration reported by Lee County Natural Resources at Riverwoods sampling site, near FGCU sampling site G10, January 2015-December 2019. Dashed line is TPTV, "ten percent threshold value," numeric target not to be exceeded by more than 10% of samples, at 130 MPN/100mL for enterococci.

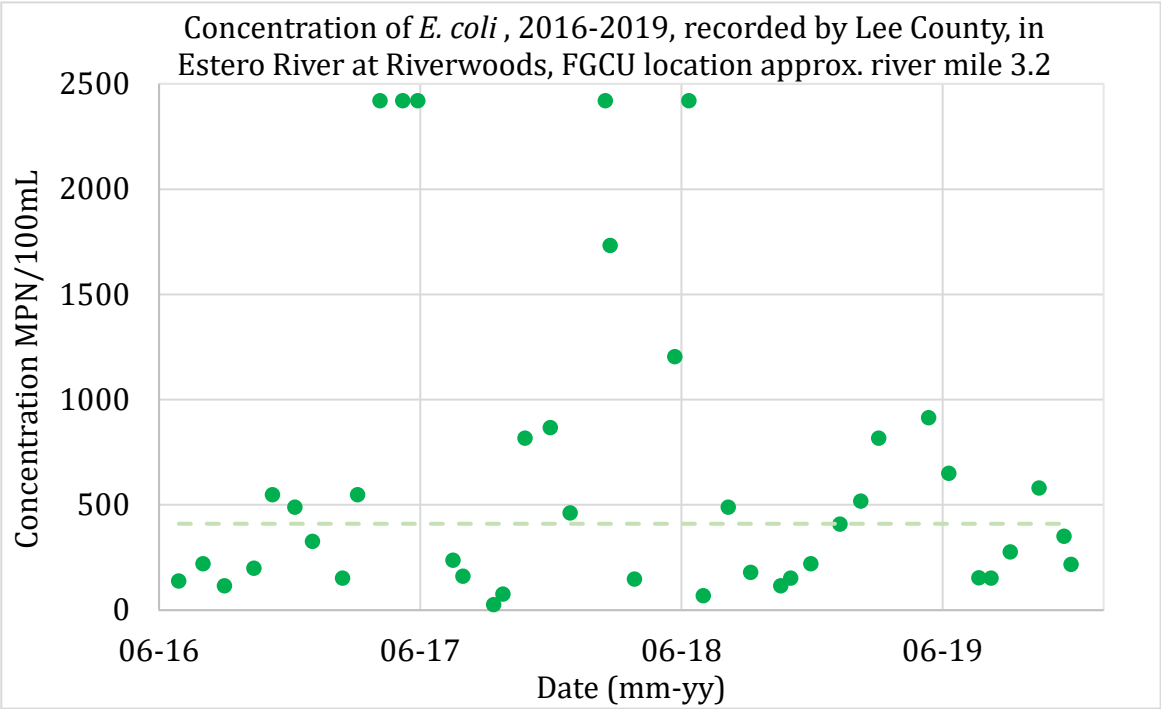

Figure 4.2. *E. coli* concentration reported by Lee County Natural Resources at the Riverwoods sampling site, near FGCU sampling site G10, June 2016-December 2019. Dashed line is TPTV, "ten percent threshold value," numeric target not to be exceeded by more than 10% of samples, at 410 MPN/100mL for *E. coli.*

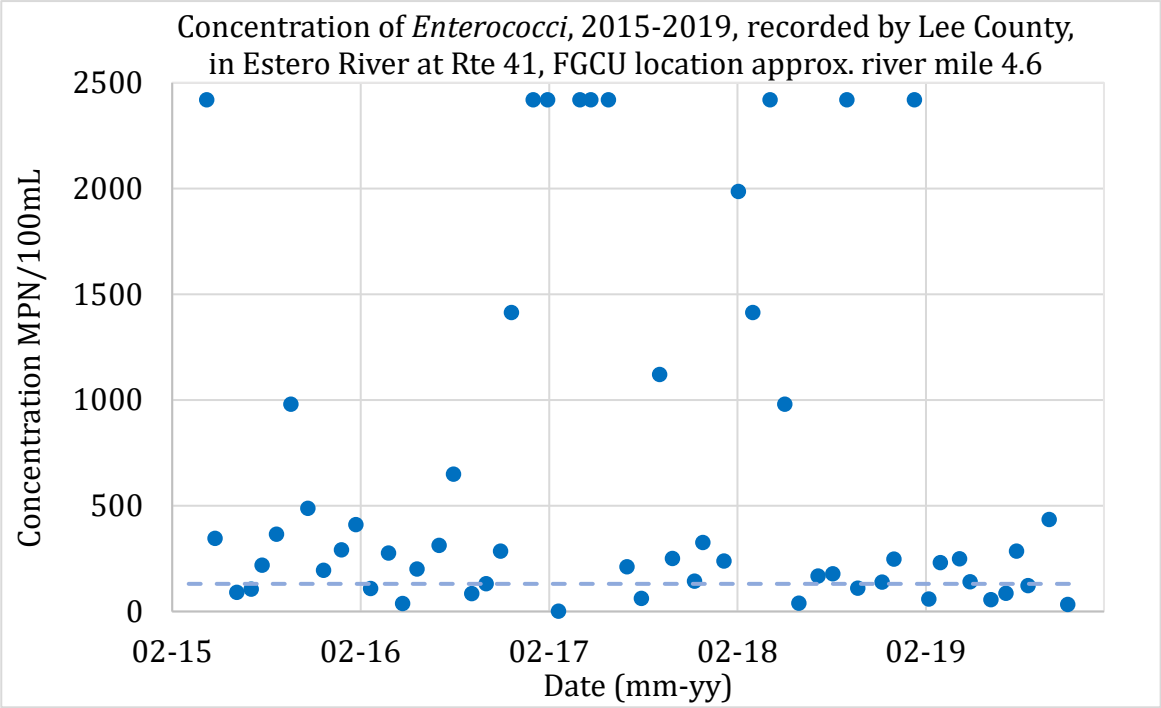

Figure 4.3. Enterococci concentration reported by Lee County Natural Resources at the Route 41 Bridge sampling site, near FGCU sampling site G04, January 2015-December 2019. Dashed line is TPTV, "ten percent threshold value," numeric target not to be exceeded by more than 10% of samples, at 130 MPN/100mL for enterococci.

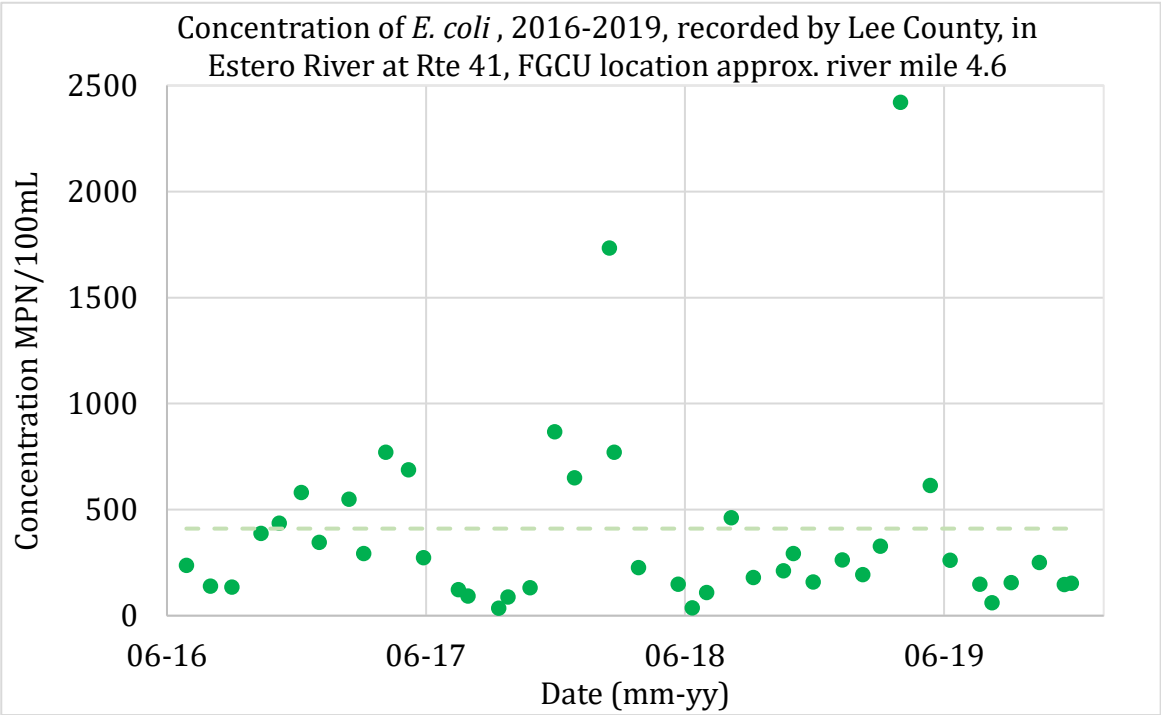

Figure 4.4. *E. coli* concentration reported by Lee County Natural Resources at the Route 41 Bridge sampling site, near FGCU sampling site G04, June 2016-December 2019. Dashed line is TPTV, "ten percent threshold value," numeric target not to be exceeded by more than 10% of samples, at 410 MPN/100mL for *E. coli.*

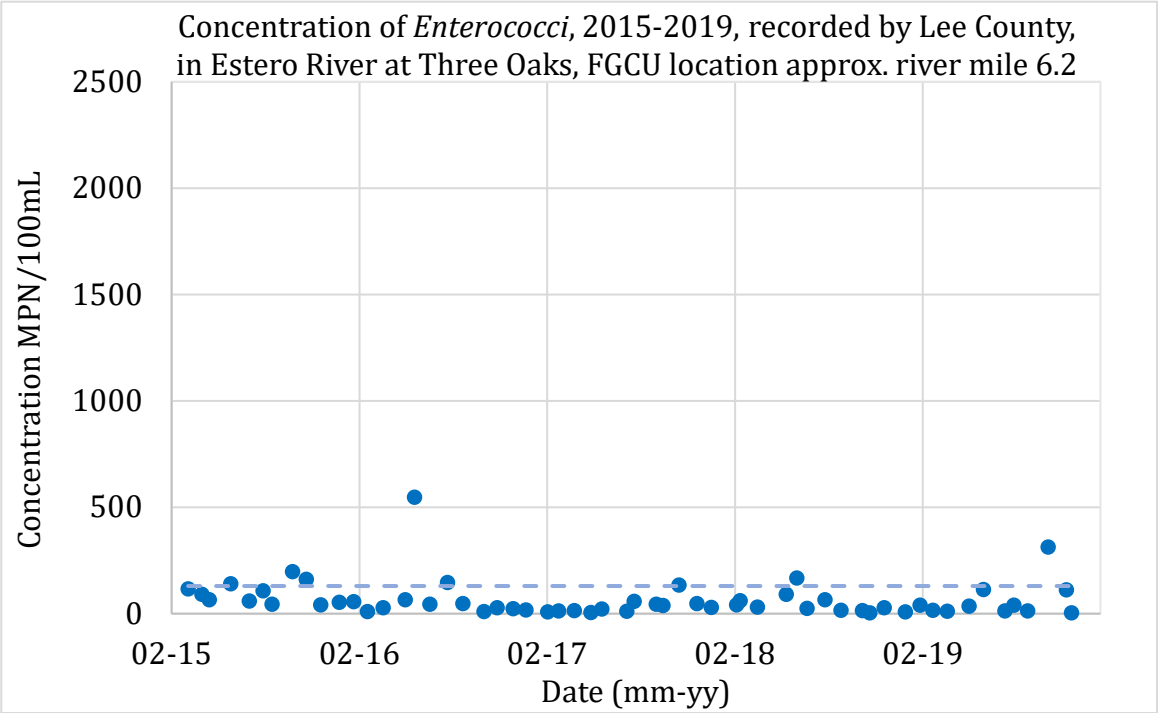

Figure 4.5. Enterococci concentration reported by Lee County Natural Resources at the Three Oaks Parkway sampling site, January 2015-December 2019. Dashed line is TPTV, "ten percent threshold value," numeric target not to be exceeded by more than 10% of samples, at 130 MPN/100mL for enterococci.

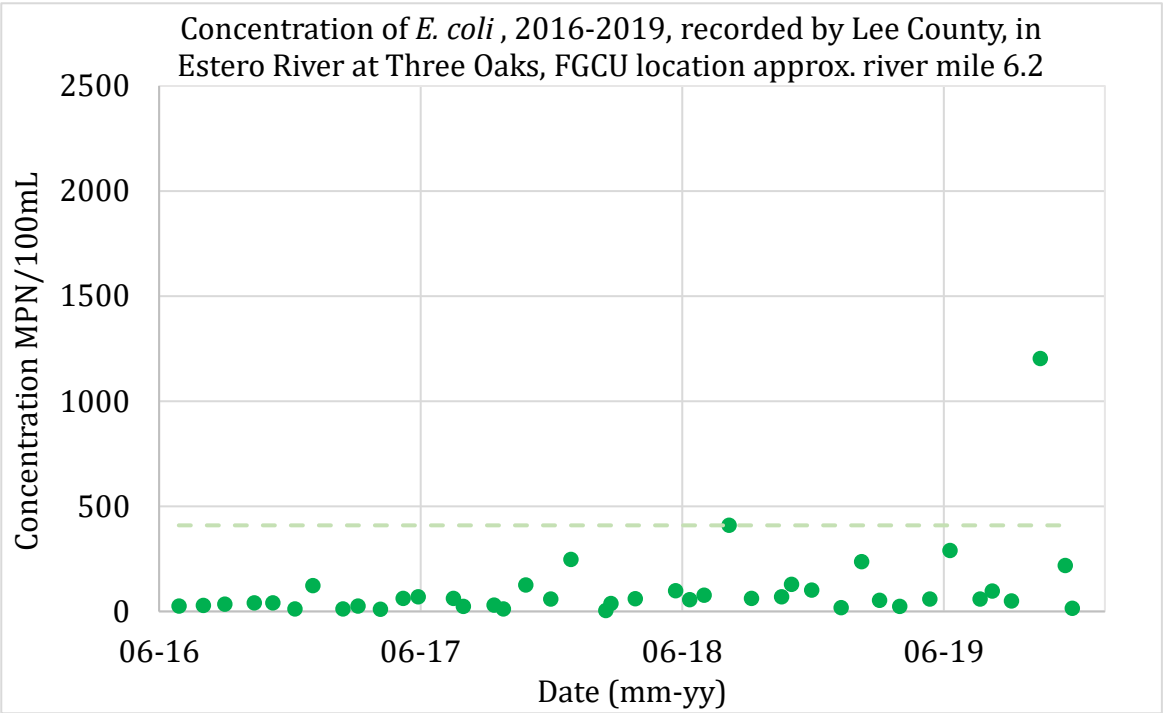

Figure 4.6. *E. coli* concentration reported by Lee County Natural Resources at the Three Oaks Parkway sampling site, June 2016-December 2019. Dashed line is TPTV, "ten percent threshold value," numeric target not to be exceeded by more than 10% of samples, at 410 MPN/100mL for *E. coli.*

4.2 Lack of Seasonal Trend in Historical Enterococci Data from Lee County

The graph in Figure 4.7 groups the MPN data for *Enterococcus* collected by Lee County Environmental Laboratory for one site, the Route 41 Bridge, in divided into wet and dry seasons. The graph displays the mean and the 95% confidence limit for the mean. The data show no statistically significant trend for difference between the two seasons. While the mean enterococci concentration is visibly less during wet season than dry, the fact that the confidence limit does not overlap demonstrates the two means are not different from one another within the target of 5% significance. Visual inspection shows that the 2 extremely high events (higher than laboratory maximum of 2,400 MPN/100mL) both occurred during dry season, but the other 5 high events were divided between dry (2 events) and wet (3 events) seasons. The graph also displays how the low-concentration events of less than 250 MPN/100mL were also divided between seasons, with 7 occurring during dry season and 15 during wet season.

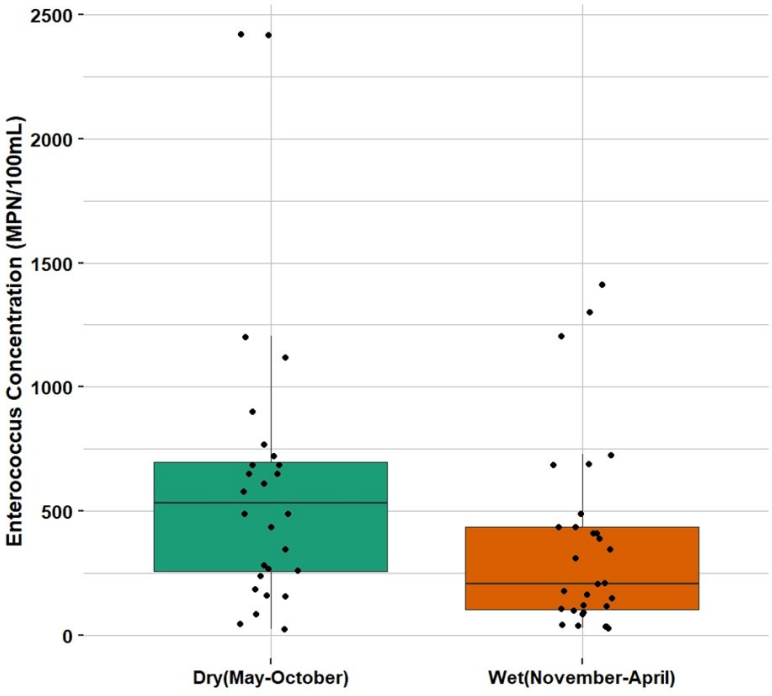

Figure 4.7. Enterococci concentration reported by Lee County Natural Resources at the Route 41 Bridge sampling site, near site G04 for this project, January 2015-December 2019, disaggregated by wet weather season vs. dry weather season.

5.0 Project Sampling: Locations and Frequency

Data were collected by two monitoring approaches. Surface water samples were collected from 10 sites on the Estero River, seven by boat and three from the riverbank at upstream, narrow-access sites, collected on seven occasions within a 4-hour sampling time ("length-of-the-river" sampling); and 7 groundwater sites, five from fixed devices installed in the surface soils and two from small surface flows that are confidently assumed to be carrying groundwater discharge. Surface water sampling events were conducted from August 2019 through August 2020; and groundwater sites were assessed with 9 sampling events completed from September 2019 through August 2020.

5.1 Length of River Surface Water Sampling Sites

The purpose of this part of the sampling strategy was to collect data for dry and wet weather conditions at multiple locations on the Estero River. The intent was to provide data on a finer resolution than that of the long-running, once-monthly Lee County sampling program, and to determine whether bacteria concentration varies on a shorter spatial scale than the existing data could detect.

Executing this strategy required mobilizing a boat and collecting samples from as large a section of the river as could be completed in one day while still delivering all samples to the laboratory for analysis within 6 hours of the first sample collection, in order to meet the 6-hour holding time specified by the EPA-approved laboratory method for bacteria testing.

The design was to collect data at between 8 and 10 locations within a reach of approximately 5 miles, extending from near the mouth on Estero Bay (where conditions would be dominated by mixing from tidal and wave actions) upstream through the populated areas, or as far as the FGCU watercraft could access. The data are expected to determine whether potential sources of bacteria on the waterbodies can be detected using this kind of targeted sampling, or if not, to deliver some information on any locations in the waterbodies that routinely or occasionally show high bacteria concentrations that may relate to locations or activities that contribute bacteria to the waterbodies. The sampling sites are more closely-spaced, and thus the data are at higher spatial resolution, than any previous study on those target waterbodies; and as far as we know, higher resolution than any study of bacteria in our region other than the comparably-spaced sampling on the Imperial River and Spring Creek, conducted by this research team through the same approximately 12-month period.

The sampling events were designed to capture about 3 to 4 samples each during the wet season and the dry season, based on the hypothesis that source activities, bacterial transport, and bacterial survival in the environment may be different between wet and dry seasons. The sampling period was intended to be August 2019 to March 2020. The plan was modified to extend through August 2020, accommodating an unexpectedly early end to the wet weather season in fall 2019, when so little rainfall occurred after September 1,

2019 that the waterbodies were essentially experiencing dry weather conditions after that day. One length-of-the-river sampling event was conducted during the 2019 wet weather season, and two others during the wet season in July and August 2020. Sampling was conducted on dates shown in Table 5.1.

Table 5.1. Sampling dates of length-of-the-river sampling.

Wet Season		Dry Season	
Date sampled	Number of sites	Date sampled	Number of sites
August 19, 2019	7	September 18, 2019	7
July 14, 2020	10	November 13, 2020	10
July 28, 2020	10	April 8, 2020	10
August 10, 2020	10		

The project team used a motorized Jon boat owned by FGCU to collect samples at various points along the Estero River. The sampling locations were selected during the first boat trip, with the goal of approximately 2 locations per river mile, and using professional judgment about the potential influence on the waterbody of adjacent land uses and tributaries. Sites were selected at greater density in areas of more intense land use and in reaches where more tributaries entered the main stream. The locations are shown in Figure 5.1, and the coordinates, unique identifiers, and 'river mile' are shown in Table 5.2. The table shows the location name assigned for this project; the unique project identifier, a term assigned by the FGCU laboratory and field crews; and the 'river mile' for each site. The 'river mile' was measured using coordinates collected in the field during the first sampling event and mapped by FGCU researchers using GIS methods, assigning an arbitrary 'mile 0' representing a point at or near the mouth of the Estero River.

Table 5.2. Sampling sites on Estero River

Site name	River mile	FGCU unique identifier	Coordinates	
Armada Ct below canal	2.31	G12	26.43574	-81.8372
Estero Ct above tributary	2.56	G11	26.43411	-81.8338
At boat launch near Broadway (Lee County 47A-4GR)	3.17	G10	26.44	-81.8278
Below Tahiti	3.51	G01	26.43745	-81.8233
At Koreshan boat launch	3.96	G02	26.43802	-81.8187
At Sunny Grove	4.23	G03	26.43698	-81.8149
Below Rt 41 bridge (Lee County 47A-15GR)	4.58	G04	26.43492	-81.8106
At Sandy Lane bridge	4.95	G08	26.43474	-81.8049
S Branch – Country Ck Dr bridge	5.52	G05	26.43334	-81.7966
N Branch – Country Ck nr Candlewood Hollow	5.74	G06	26.4412	-81.7956

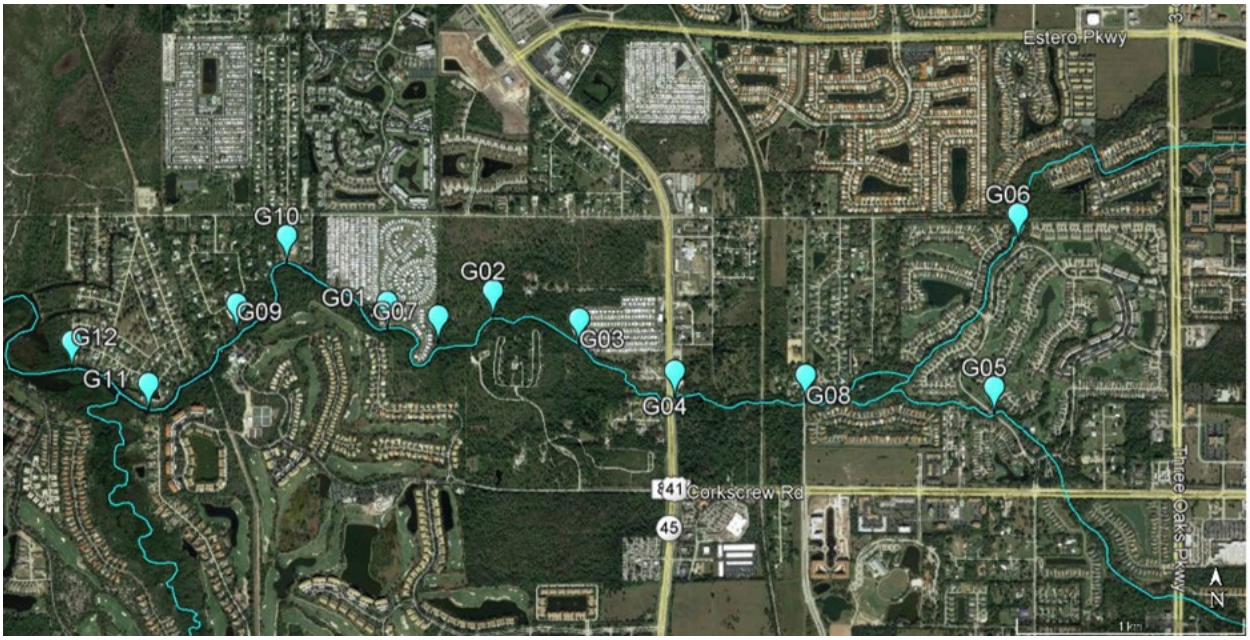

Figure 5.1. Aerial image showing ten sampling sites on the Estero River used for length-of-the-river sampling. Courtesy of Google Earth imaging.

5.2 Groundwater Sampling Sites

Groundwater samples were collected from five piezometers, each about 2 m deep. These were installed for purposes of this project, with permission of three landowners (the Koreshan State Park, the Estero Bay Village development of manufactured homes (known as Tahiti at the time of our sampling), and an individual landowner in the Charing Cross development). Additional samples, considered to be groundwater indicators also, were collected from two small flowing drainage conveyances, one found on the Estero Bay Village neighborhood and the other on the Charing Cross property. Groundwater sampling locations are displayed on Figure 5.2.

The seven sites were:

- At Koreshan State Park, site A04, on the south side of the Estero River approximately 5 m inland of the river's edge. This was selected as a control site because there is no development or permanent residence in the State Park. The site is more than one kilometer from any other developments on that side of the river, though potentially affected by the State Park's public restrooms which are about 300 m from the site. All other groundwater sampling sites are on the north side of the river: this site is about 200 m upstream (east) of the nearest other site, at the eastern edge of the Estero Bay Village neighborhood.

- In the Estero Bay Village neighborhood: site A01, at a point about 5 m from the river's edge, separated by from the river by a concrete retaining wall.

Figure 5.2. Aerial image showing five water-table sampling sites near the Estero River used for groundwater sampling. Courtesy of Google Earth imaging. Site G07, the "Tahiti Ditch," was at the same location as groundwater site A02. Site G09, the "Charing Cross Ditch," was at the same location as groundwater site A05.

Table 5.3. Sampling dates of groundwater sampling

Wet Season		Dry Season	
Date sampled	Number of sites	Date sampled	Number of sites
June 29, 2020	7	September 25, 2019	4
July 29, 2020	7	October 9, 2019	4
August 4, 2020	7	October 30, 2019	6
		November 25, 2019	7*
		January 15, 2020	6
		April 1, 2020	7**

*includes site G09 sampled on Nov. 13
**includes site G09 sampled on April 8

- In the Estero Bay Village neighborhood: site A02, less than 5 m from the edge of the Estero River at the eastern edge of the development adjacent to land managed as an open preserve by Koreshan State Park, separated from the preserve land by a small flowing ditch that conveyed water throughout the year, both wet weather and dry weather seasons. Upstream of the sampling site, the ditch runs parallel to the western edge of two wastewater treatment lagoons that form part of a treatment system, installed in approximately the 1950s, that collects and treats domestic wastewater from

the approximately 150 residences in the Estero Bay Village neighborhood. The ditch is about 5m from the edge of the nearer lagoon, separated by a raised embankment.

- In the Estero Bay Village neighborhood: site A03, about 20 m from the southwest corner of the treatment lagoon, about 100 m inland from the Estero River.
- In the Estero Bay Village neighborhood: site G07, immediately adjacent to the shallow groundwater sampling device, the project also collected samples from the flowing surface water in the small drainage ditch.
- In the Charing Cross Circle neighborhood: site A05, within 5 m of the river's edge, immediately adjacent to another small conveyance, on the property of an individual who gave permission to install and access this device for purposes of this project. This site was selected because the approximately 28 homes that front Charing Cross Circle are served by septic systems, which can be considered a potential source of bacteria to the Estero River of the kind this project was intended to assess.
- In the Charing Cross Circle neighborhood: site G09, immediately adjacent to the shallow groundwater sampling device, the project also collected samples from the flowing surface water in the small drainage ditch.

The two small drainage systems were sampled as part of the groundwater analysis because it is clear they convey groundwater in addition to their intended purpose of conveying stormwater in the immediate aftermath of a precipitation event. Both ditches were observed to continue flowing year-round, even through the driest weather, which is strong evidence to suggest they convey groundwater discharge. The two channels are both in areas where year-long groundwater flows can be expected: one as groundwater contributed from the standing water of the Estero Bay Village treatment lagoon, and the other as routine flows from the septic systems in the Charing Cross neighborhood.

Table 5.4. Groundwater sampling sites near the Estero River

Site name	River mile	FGCU unique identifier	Coordinates	
Estero Bay Village near retaining wall	3.70	A01	26.43667	-81.8219
Estero Bay Village near "ditch"	3.78	A02	26.43693	-81.8211
Estero Bay Village near lagoon	3.70	A03	26.4375	-81.8215
Koreshan near boat launch	3.96	A04	26.4367	-81.8201
Charing Cross	2.90	A05	26.43729	-81.8299
Estero Bay Village "ditch"	3.78	G07	26.43693	-81.8211
Charing Cross "ditch"	2.90	G09	26.43729	-81.8299

6.0 Project Sampling: Field Methods

Sampling for this project was conducted under carefully controlled conditions to avoid contamination to the maximum extent possible. FIB can be difficult to separate from other bacteria commonly found on human skin, on clothing, and in human breath and perspiration. Sampling was performed by personnel wearing nitrile gloves. Samples for FIB in surface waters (0 to 30 cm) were directly collected using a 50-ml syringe to avoid contamination from a sampling gear. Syringes that remained within sterile factory packaging were used to fill surface water into sterile coliform water sample bottles (100-mL) contain sodium thiosulfate, with the inside of the bottle carefully prevented from contacting gloves, breath, or the exterior of the syringe. The coliform water sample bottles were immediately sealed in a newly-opened plastic food bag to avoid further direct contact with ice. The ice-filled cooler used for transportation was maintained at less than 8°C.

The samples were handled in such a way as to inactivate bacteria, using preservatives in every bottle and burying each bottle in ice for transport to the lab. That is done because FIB that may remain active after removal from the environment may reproduce; or may be digested by other bacteria that may be present. Either would lead to inaccurate estimates of their presence in the environmental compartment being sampled. The samples were delivered to the laboratory so that analysis could begin within 6 hours of the sample being removed from the environment, in conformance with USEPA guidance for testing for FIB.

Surface water samples for nutrient analyses were collected in 1-L acid-washed brown plastic bottles. Nutrient samples were collected by personnel wearing nitrile gloves, but because no preservative was used within the bottle, the sample was collected by inserting the bottle into the water. Bottles were double-rinsed before sampling, then the sample was collected elbow-deep in the waterbody and capped underwater, to avoid contact with the water surface layer which is populated by different organisms than water below the surface. Bottles from each site were sealed in a ziplocked plastic bag immediately buried in ice in a large cooler. Because bacteria may remain active after removal from the environment, which may include consumption or modification of some nutrient substances, all bottles were immediately cooled by burying in ice in an insulated cooler, to inactivate any bacteria in the sample.

Groundwater sampling was accomplished using the same sample handling, cooling, and laboratory analysis methods, but with additional field techniques to extract water from the groundwater sampling devices, or piezometers. The piezometers were installed during the early dry season in September 2019. They consisted of 2-inch diameter PVC Schedule 40 pipe (2.5 foot long of 2”-diameter screened pipe coupled with an adequate length of 2”-diameter riser), inserted into a 3” diameter borehole about 1.5 m to 2 m deep dug with a hand auger. A 4”-diameter casing was used to aid in the digging of the borehole to prevent the collapse of the wall of the hole. The piezometer was fitted into the 4” casing with the screened section encapsulated in a 3”-diameter sock filled with silica sand 20-30 (Standard

Sand & Silica Co.) topped with a 6-inch length of bentonite pellets, also encapsulated in the sock. After installing the pipe and sock, the casing was removed and the bore hole was refilled, to the top of the sock, with the excavated materials. Then bentonite gravel (enviroplugs) was poured into the hole, forming a seamless plug along with the bentonite inside the sock, preventing surface water from penetrating into the sampling device. The hole was then filled to the top with the excavated materials. The pipe extended above the surface by 6 inches to 2 feet for easy access. The devices were sealed with a cap atop the protruding pipe to prevent precipitation, debris, or organisms from entering from the top.

The exact installation depth was selected separately for each piezometer, chosen to ensure that the screened section of pipe extended 50 cm or more into the water table when installed (early in the dry season when the water table was still declining). Sampling events found water within the pipes, to a depth of 20 cm or more, during all sampling events, which verified that the devices were drawing groundwater, and thus remained within the water table, at all times of the project period.

During groundwater sampling, water was extracted through a ¼" I.D. polytetrafluoroethylene (PTFE) tube using a peristaltic pump (Solinst™ 410 pump) with sterile silicone tubing. Project staff wore nitrile gloves to handle the tubing and sample bottles to avoid contamination by any bacteria that might have been present on personnel's skin. The piezometers were first pumped to evacuate about 2 to 3 well volumes of standing water, after which the samples were drawn. In all cases, the devices were rapidly refilled with water, so that samples were readily drawn immediately after the devices were evacuated. The rapidity of refilling demonstrated a rapid flow of groundwater in the surficial soils, which ensures that the samples consisted of groundwater that was in situ in the soils at the time of sampling, not water that had been standing in the devices where bacteria might have multiplied in ways they might not in the natural soils. Inserting the tubes into the 2 m sampling pipes required some precautions to avoid the tubes' scraping materials from the interior surfaces of the tubes, including the bottom cap, which might have led to the samples' containing bacteria that grew on those surfaces. Because it is impossible to see into the 2 m sampling devices, and the plastic tubes needed to be inserted by hand at each sampling event (as leaving them in place would have allowed them to promote bacterial and cyanobacterial growth within the tubes), it is possible that the measures taken to avoid contamination may not always be fully successful.

7.0 Project Data and Analysis: *Enterococcus* and *E. coli* in Estero River Surface Water

7.1 Project Data: Wet Weather Monitoring Results

Figure 7.1 shows the concentration results for two fecal indicator bacteria, *Enterococcus* and *E. coli*, for four sampling events conducted on Estero River during wet weather conditions, August 2019 and July – August 2020.

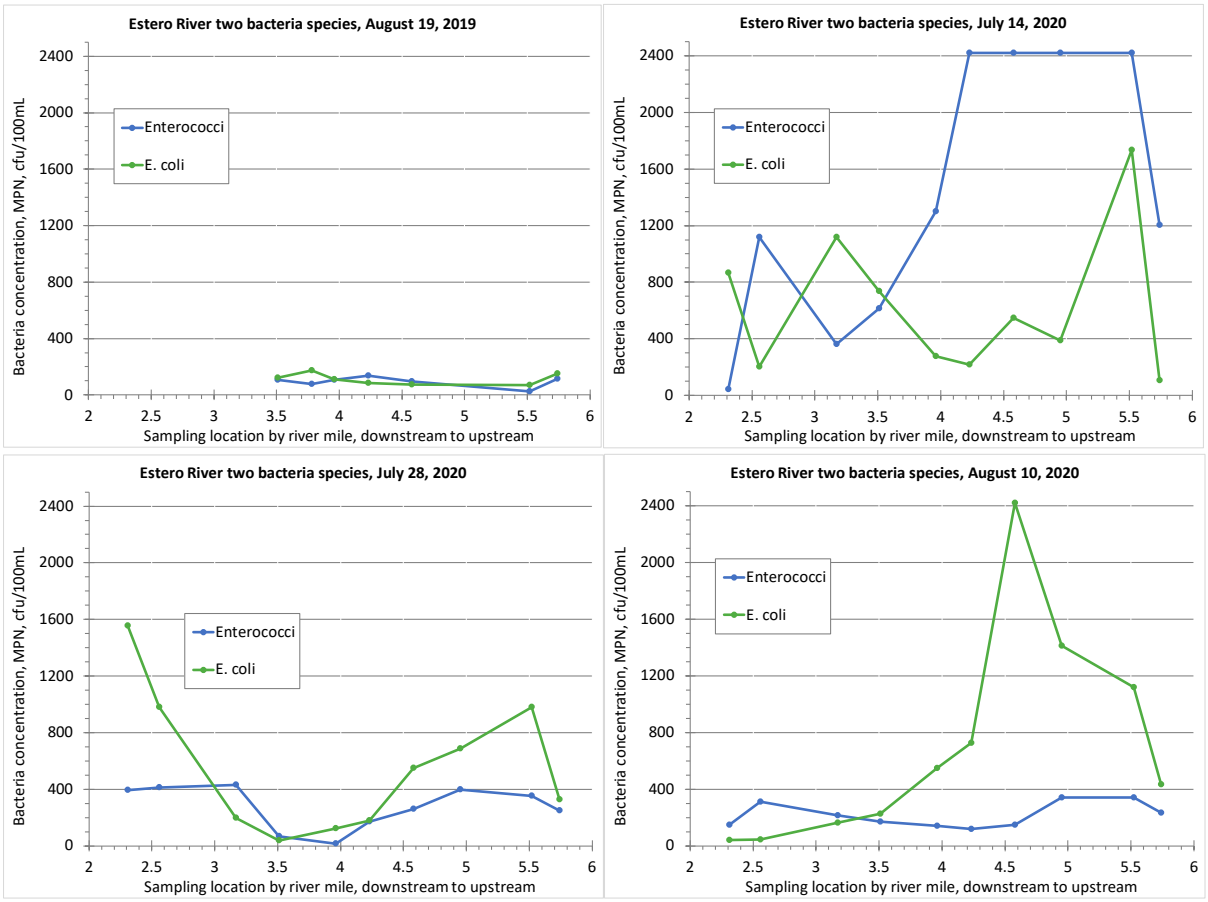

Figure 7.1. Monitoring results for four run-of-the-river sampling events conducted during two wet seasons: August 2019 (top left), July 14, 2020 (top right), July 28, 2020 (bottom left) and August 10, 2020 (bottom right).

7.2 Project Data: Dry Weather Monitoring Results

Figure 7.2 shows the concentration results for two fecal indicator bacteria, *Enterococcus* and *E. coli*, for the three sampling events conducted on Estero River during dry weather conditions, September 2019 – April 2020.

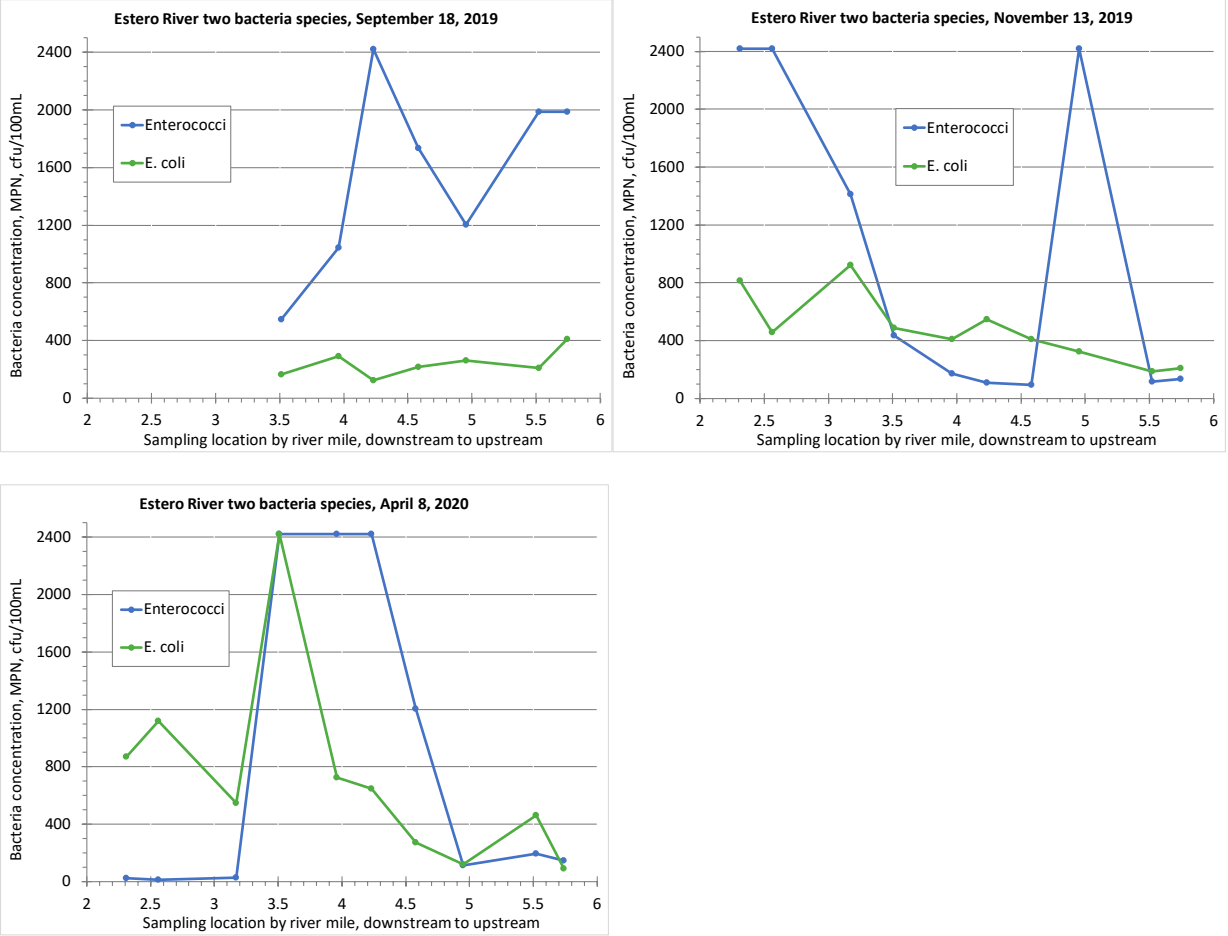

Figure 7.2. Monitoring results for four run-of-the-river sampling events conducted during one dry season, 2019-2020.

7.3 Project Data Analysis: Length-of-River, High Spatial Resolution Sampling

The results demonstrate the Estero River does, on some occasions and in some locations, experience high or very high concentrations of FIB. The results do not pinpoint particular activities or land uses that are demonstrably sources of FIB. The MPN results demonstrate that FIB are a problem not of a limited location, a limited time, or a specific set of conditions, but that high and varying concentrations may recur at unpredictable times and locations on the river.

Essentially every surface water location that was sampled showed "very high" concentration (the maximum laboratory detection capability of over 2,400 MPN/100mL) for one of the two target FIB for at least one sample results. Very high results were found at some locations during both wet and dry weather conditions, and very low results (less than about 300 MPN/100mL) were detected at some Estero River locations during every sampling event. Only one sampling event, on August 19, 2019, did not identify any locations with very high concentration – in fact, no locations of greater than the regulatory target of 300 MPN/mL for either of the targeted FIB.

Suspected sources of FIB in the Estero River included a) package wastewater treatment plants in the Estero Bay Village neighborhood (approximately river mile 3.7) and Sunny Grove neighborhood (approximately river mile 4.2); b) septic systems treating wastewater in a number of locations, including but not limited to the Charing Cross development at approximately river mile 3; c) other residential activities, including but not limited to pet wastes in residential developments near the waterbody as in developments at and near river miles 2.5 through 3.7, 4 through 4.6, and 5 through 6; d) golf courses with associated fertilized vegetation, and potential bacteria colonies from bird and other warm-blooded animals that may congregate to feed on high-nutrient vegetation in those land uses, approximately river miles 5 through 6; e) wild warm-blooded organisms in the large undeveloped upstream parts of the waterbody, upstream of river mile 6; and f) tidal action that may resuspend FIB in sediment and/or may push outgoing flows, and bacteria, back upstream leading to high-concentration conditions, in the reaches downstream from approximately river mile 3. The data do not show that any of those locations, or any others, routinely and replicably contained FIB at very high concentrations throughout either wet or dry season conditions.

8.0 Project Data and Analysis: *Enterococcus* and *E. coli* in Near-Surface Groundwater

As stated in the Methods section 6.0, groundwater samples were collected from five devices installed on three different properties: one a control site, in Koreshan State Park on the south side of the Estero River, approximately 5 m inland of the river's edge; three in the Estero Bay Village neighborhood, two of them within 5 m of the river's edge and the other adjacent to the wastewater treatment lagoon, approximately 100 m inland; and one in the Charing Cross development, within 5 m of the river's edge. The Charing Cross site and one of the three Estero Bay Village sites were immediately adjacent to two small conveyances, which can be assumed to be conveying groundwater discharge because they were observed to continue flowing year-round, even through the driest weather. Those two 'ditches' were included in the groundwater sampling scheme as it is clear they convey groundwater in addition to their intended purpose of conveying stormwater in the immediate aftermath of a precipitation event.

Sampling of the devices was conducted on nine occasions. On the first two occasions only three devices had been installed, so the other two locations were sampled only seven times. The surface groundwater flows were sampled on most of these occasions, and on some other occasions they were sampled during the run-of-the-river sampling days but not during the groundwater sampling days. In those cases the data are included on these two graphs only for those surface-sampling events that were collected within 7 days of the groundwater sampling event. In total the surface-groundwater drain G07 was sampled eight times, and G09 was sampled seven times, at times that were near enough to the subsurface-groundwater sampling events to be included on the two graphs below.

8.1 Data from Groundwater Sampling

Samples from the subsurface-groundwater sampling sites found little or no FIB for the four target groundwater sites (A01, A02, A03, and A05), and for the control site A04 (Figures 8-1 and 8-2), with one exception. The exception is three samples measured with high MPN for enterococci at A01, and these are believed to be erroneous for reasons described below, so they are not considered in this analysis. Of the valid results, only two of the samples were as high as 100 MPN/mL: enterococci at A02 was measured at 164 MPN/mL on September 25, and *E. coli* at A04 was measured at 112 MPN/mL on November 25. One other sample, at A03 on 11/25, was measured at 42 MPN/100mL. All other samples were measured at less than 25 MPN/100 mL for both enterococci and *E. coli* on all occasions and at all sites, except for the erroneous A01 data.

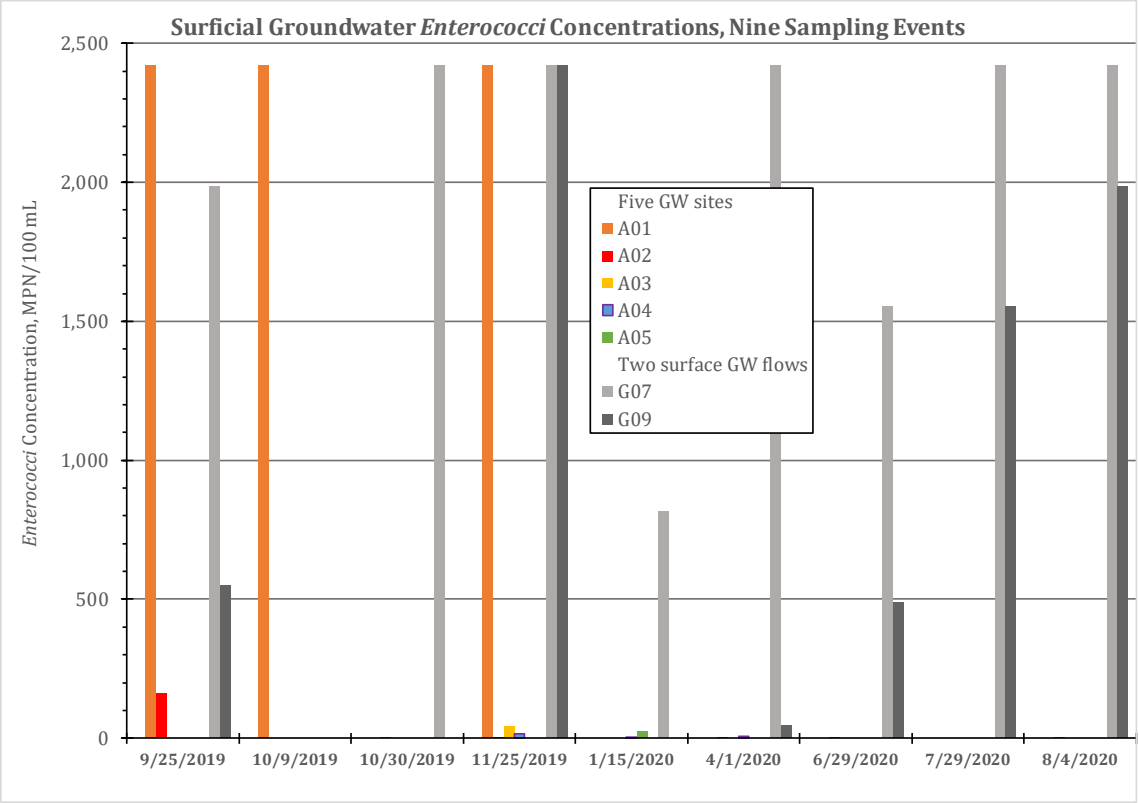

Figure 8-1. Enterococcus concentration from 9 sample events, for five subsurface-groundwater sampling sites (colors) and two surface-groundwater flows (grey tones).

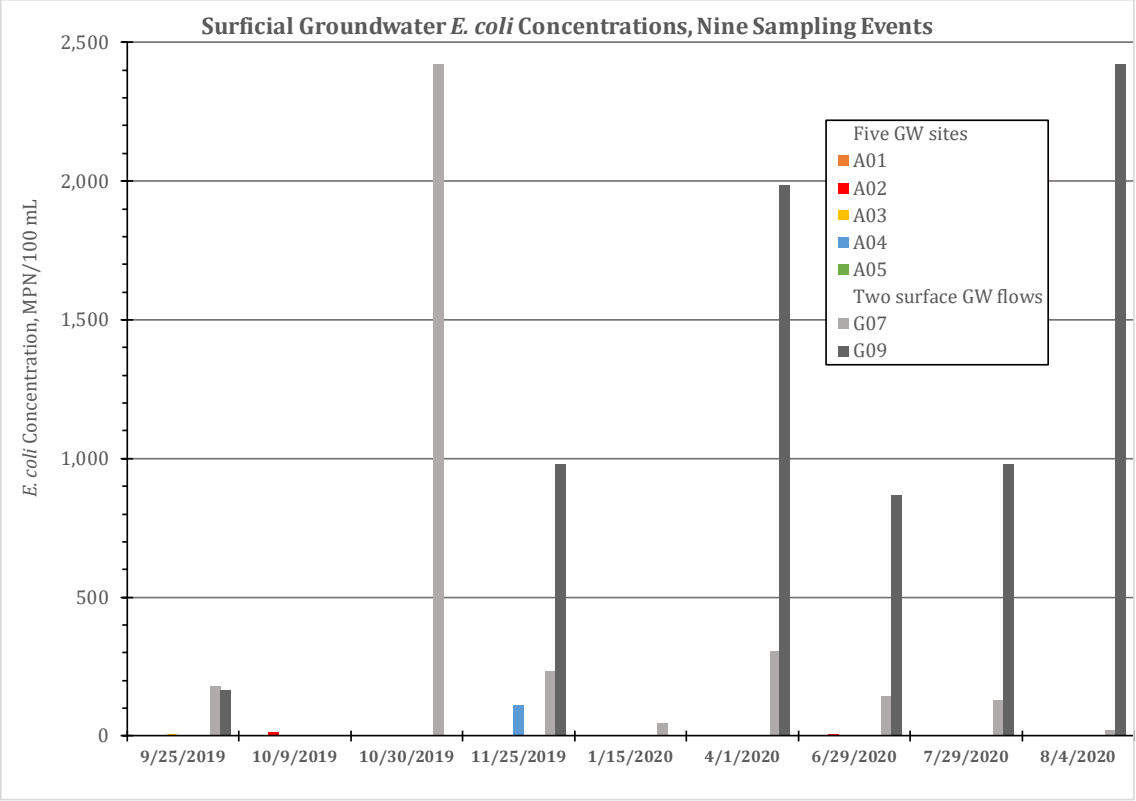

Figure 8-2. *E. coli* concentration from 9 sample events, for five subsurface-groundwater sampling sites (colors) and two surface-groundwater flows (grey tones).

On the other hand, the samples from the two surface-groundwater drains were quite high in FIB on several occasions. As Figure 8-1 shows, *Enterococcus* was found in the Estero Bay Village surface-groundwater drain, Site G07, in almost every sample: in five of the nine samples, at the laboratory-method maximum; and on three other occasions, between 700 and 2,000 MPN/100 mL. On one occasion enterococci were not detected at that location. Four of those samples were tested for sucralose, and sucralose concentrations were quite high, more than in most other samples tested for this research: between 17 ug/L and 32 ug/L. That is persuasive evidence that the surface-groundwater flow at Estero Bay Village was heavily affected by wastewater discharges. The Charing Cross surface-groundwater drain (Site G09) also had *Enterococcus* at high concentrations on some occasions: three times between 1,500 and 2,500 MPN/100 mL; and twice about 500 MPN/100mL. On two other occasions *Enterococcus* were much lower at Site G09 (< 50 MPN/mL). The site was not sampled on two occasions. As Figure 8-1 shows, the results for *E. coli* were high in several of the surface-groundwater drain samples: only once in eight samples from Site G07, the Estero Bay Village neighborhood, but in five out of seven samples from Site G09, the Charing Cross neighborhood site. That result suggests the Charing Cross surface-groundwater drain is also affected by wastewater discharges, but none of those samples were tested for sucralose.

For the two ditches together, the mean concentration of enterococci was 1,622 MPN/100mL with a standard deviation of 934 MPN/100 mL, highly variable but with a mean well in excess of the Florida recommended target of a geometric mean of 300 MPN/mL. The mean concentration of *E. coli* was 725 MPN/100mL with a standard deviation of 807 MPN/100 mL, even more variable because of its long upper tail and several results of 0 MPN/mL, but also well in excess of the Florida recommended TPTV for surface waters (a standard that does not apply in a discharge ditch of this kind, but the most nearly relevant numeric target by which to judge this flow).

In this study, the two FIB were present at different concentrations, which is a pattern that has been widely shown in the literature. That lack of correlation is among the reasons that neither *E. coli* nor *Enterococcus* is a perfect indicator of presence of human waste: the differences in results are affected by different source organisms, different human populations, and differences in survivability at different locations and different times. In this study, it is believed that *E. coli* may survive better in the soils of the Charing Cross ditch while *Enterococcus* survives better in the Estero Bay Village ditch – such that the soils themselves may be the proximate source of the FIB that are measured in any one sample, with human wastewater being at least part of the ultimate source – but that survivability may well be different on separate sample days, depending on factors such as sunlight, depth of inundation, volume of flow, dilution by other flow sources, and others.

The three high-enterococci results for Site A01, a site in the Estero Bay Village neighborhood adjacent to the Estero River at the midpoint of the Estero Bay Village shoreline, 100 m downstream of the mouth of the drainage ditch, are believed to be erroneous. All three of those results occurred during the first four sampling events. In all

three cases, the concentration of *E. coli* was measured as below the detection limit (reported as 0 MPN/mL). It is believed that these were the contamination of sediment particles attached to the wall or settled on the bottom of the well through the sampling operation. Such contamination has been observed by other researchers, for example Byappanahalli et al. (2012). Sampling from the surficial groundwater devices was accomplished with a pump and 5-mm hose, and inserting the hose into the 2-in diameter vinyl sampling device, which can result in scraping the sides or the bottom of the device in a way that stirs sediment and microorganisms inhabiting the sediment.

The assumption that the three high-enterococci results from A01 are in error is supported by the fact that no other samples drawn from the shallow sampling devices show FIB greater than 200 MPN/mL – including on other occasions from the A01 location. The three high-enterococci samples contained low or non-detectable amounts of *E. coli* in any of the samples from that location, including in the three samples with very high enterococci results. Furthermore, Site A01 was tested for sucralose on three occasions, including two of the three that reported very high FIB, and on all three of those occasions sucralose was undetected. Nearly all other groundwater samples showed non-zero sucralose, and some of them were quite high, as reported in Section 10 below. Altogether the evidence supports the interpretation that those three samples were contaminated and not reflective of FIB presence in groundwater.

8.2 Findings about FIB in Groundwater

Groundwater analysis demonstrates two main findings: 1) there appears to be little conveyance of FIB through the soils into the Estero River, as little to no bacteria were detected in any of the shallow groundwater sampling devices on essentially every occasion; and 2) substantial to very high FIB concentrations are contributed by surface flows of groundwater in the vicinity of potential FIB sources, in this case one small channel receiving groundwater discharge from a treatment lagoon of a small package wastewater treatment plant (G07) and another in a neighborhood where homes' wastewater is treated in individual septic systems (G09).

The overall finding is that FIB do not appear to be conveyed in the groundwater from potential sources into the Estero River, and that groundwater entering the Estero River is likely not contaminated with any human-associated wastewater contamination at these locations. Presence of sucralose in high concentrations in the groundwater at Site A02 documents that water originating as wastewater does flow with groundwater at that site, and potentially at other sites near the river. But the absence of FIB in those same samples shows that the wastewater reaching that site – after flowing through the soil in groundwater – has experienced microbial attenuation, which is the intent of a treatment lagoon. The site at A03, much nearer to the lagoon, was high in both sucralose and FIB, indicating that groundwater in that vicinity has not been in the soil long enough to experience similar biodegradation – but that site is not near to the river. Site A01, near the river, showed low concentration of both sucralose and FIB, so does not appear to be affected by discharge from the treatment lagoon. The pattern, at both the Estero Bay Village

and Charing Cross neighborhoods, suggests the soils are acting as intended to delay subsurface flow from septic systems and the treatment lagoons while organisms in the soil break down FIB, and presumably other potentially harmful bacteria originating with human wastes, before they can reach the river.

On the other hand, the small surface drainages could be important conveyance mechanisms for FIB to reach the Estero River. The fact that the two 'ditches' were measured on multiple occasions with high concentration of both FIB, and also as shown in Section 9 below with very high concentration of sucralose, is strong evidence that they are receiving discharges that have been in contact with human waste that is not adequately treated to destroy FIB. That is probably because the water has been in the soil for too short a time (i.e. flowed over too short a distance) for microbes in the soil to have digested the FIB, in the mechanism that is intended for septic systems and treatment lagoons. This "short-circuiting" of the wastewater's movement through the soil has produced a condition where FIB enter the Estero River, which in turn indicates the potential presence of other potentially harmful substances originating with human wastes. The flows in these channels are small but constant, so it is not known to what extent they contribute FIB, and whether that is in sufficient quantity to affect the concentration of FIB in the Estero River.

9.0 Project Data and Analysis: Sherrill Lane Short-term Stormwater Inundation

The street surface of Sherril Lane extending from the intersection with Broadway northward for about two to three blocks is known historically to experience inundation after periods of intense rainfall. This project included a task to collect and test a sample of the standing water if possible.

On the night of September 3-4, an overnight rainfall of about 2.5 inches produced storm runoff inundation. A sample was collected from standing surface water in Sherril Lane, about 10 m north of the intersection with Broadway, at about 9:30 AM. Table 9.1 shows the results.

Table 9.1 Analysis of sample of standing surface water at Sherrill Lane, one-time grab sample September 4, 2020.

Constituent	Measurement
enterococci	2420 MPN/100 mL
E. coli	2420 MPN/100 mL
Dissolved oxygen concentration	7.33 mg/L
Dissolved oxygen proportion of saturation	93.5%
pH	7.58
Electric conductivity	255 µS/cm
Turbidity	24.1 NTU

The data show that the standing water was of near-zero salinity and well oxygenated, as would be expected of stormwater runoff. Turbidity was measured as quite low, i.e. very clear water, but the sample was visibly high in large particulate matter – particles of mulch and sediments that rapidly settled – which the sampling procedure was unable to avoid because the water was constantly churned by passing vehicles at the time of sampling. The samples were very high in FIB concentration, with both *Enterococcus* and *E. coli* measured as greater than the laboratory method maximum of 2420 MPN/100mL. The sample was not tested for sucralose or for DNA sequencing.

There is no way to know the source of FIB in the standing water. It could be contributed by releases from septic systems produced by inundation by the storm runoff, or by mobilization of FIB from the soil surrounding septic systems before they were able to be biodegraded by microbes in the soils. In either of those two cases, the presence of FIB could indicate a potential health threat to humans who contact the standing water.

On the other hand, the FIB could originate with pet wastes or wastes from wild birds that might have been present in the yards of the neighborhood, which if present would

certainly have been mobilized by the runoff and conveyed to the standing water in the street. Waste from even a few pets could potentially produce the high concentrations measured because solid wastes would be extremely high in bacteria, and those bacteria would have been mixed throughout the water by the very high mechanical mixing produced by vehicles driving through the standing water. If the FIB in the standing water originated with wastes from non-human animals, then the standing water is not expected to contain pathogens harmful to humans.

The particulates that could not be screened out of the samples may have contributed large amounts of nutrients and FIB to the samples, which might have moved from the particulates into the sample water in the sample containers before they were filtered out from the sample. The findings might be measuring bacteria from the soil and mulch in the neighborhood rather than in the flowing water (Ishii et al. 2006; Jang et al. 2017). The consequence of that unknown partitioning relates to how the substances might be mobilized: as the runoff moves downstream through the storm drain system into the Estero River, FIB and nutrients present in the water column would be moved very rapidly and completely into the river, while FIB and nutrients bound to the particulates might settle into the soils and/or be taken up by vegetation of the drainage ditches and never reach the river. Regardless, the very high concentrations of both FIB identified in this one sample suggests that this kind of infrequent, high-precipitation, inundation event may be conveying short-term, high-volume pulses of bacteria into the Estero River.

10.0 Project Data and Analysis: Sucralose

As described in Section 3.7 above, the presence of FIB such as *E. coli* and *Enterococcus* are assessed because they are believed to indicate the presence of human fecal pollution. However, they are also widely found in natural environments (Anderson et al. 2005). Waters of the U.S. are believed to commonly have FIB that originate with various domestic animals and wildlife (e.g., cows, chickens, gulls, other avian species, and others) (Byappanahalli et al. 2012). The counting of these bacteria (as in Sections 7 and 8 of this report) does not indicate the organism that was the source of the bacteria.

Sucralose can be an indicator that wastewater – treated or untreated – is present, but does not indicate whether FIB or pathogens might be present. The location of centralized municipal treatment facilities is well known, so the waters into which they discharge may contain sucralose (even though sucralose is quickly diluted in large waterbodies). In smaller waterbodies such as the Estero River, and the groundwater in its vicinity, presence of sucralose typically indicates either presence of septic systems in a community or the kind of smaller, neighborhood-scale 'package' treatment facilities of the kind known to be operated at the Sunny Groves and Estero Bay Village neighborhoods adjacent to the Estero River. All those systems will convey sucralose to the receiving waters, regardless of whether the wastewater has been completely treated to eliminate pathogens, nutrients, and other pollutants.

Sucralose can reach receiving waters if it originates in septic tank by flowing through shallow groundwater, and reaches receiving waters directly from treatment facilities in their routine discharges. At the Estero Bay Village site, the treatment system discharges into two treatment lagoons, which appear to be connected in series, and does not have a surface discharge – all treated wastewater exits via the groundwater or evaporation – so sucralose from that facility enters the Estero River through the groundwater (including drainage ditches that may carry the groundwater). Sucralose in the receiving water is diluted to varying extents based on the flow volume of the receiving waterbody. Since the receiving surface water typically has volume flow rates that are orders of magnitude greater than groundwater or the small drainage ditches such as those observed at Estero Bay Village and Charing Cross neighborhoods, the concentration of sucralose in the groundwater typically is one or more orders of magnitude greater the river water, as found by Lapointe et al. (2017) in their studies of small tributaries to the Caloosahatchee River in North Fort Myers.

Samples tested for sucralose included one surface water location (on two occasions), five shallow groundwater sites (three on four occasions, two on two occasions), and one surface drainage ditch known to carry groundwater discharge (on four occasions). Results are shown in Table 10.1. Sucralose was detected in essentially all samples, typical of developed residential areas where essentially all water has been in contact with human waste, but the samples showed very large differences: some locations with zero or near-zero sucralose, indicating little or no contact with human wastewater; and some locations with very large concentrations, considered a definitive indicator of contact with human wastes. Each tested location was notably consistent over time in its sucralose concentration across the four sample collection dates, from September 2019 to January 2020. Throughout the 4-month sampling period, the detected concentration of sucralose in the groundwater was remarkably consistent at each groundwater site.

Sucralose was primarily used to assess groundwater in this study, with just two samples tested for sucralose at only one site, G04, the Route 41 Bridge site, and only on two occasions, in August and September 2019. In both cases it was present, but at only small concentrations. The mean (± SD) sucralose concentration in the surface water was 320 ± 52 ng/L (n = 2). That low concentration in the surface water is consistent with other studies, where presence or absence of sucralose in surface water is not expected to be an effective indicator of presence or absence of human wastes. That is because the rapidly-flowing surface waters do not allow for accumulation of sucralose as groundwater does, and the highly attenuated concentrations typical of surface water are not generally meaningful.

The separate groundwater samples were consistent within the locations across all the sample dates, suggesting these findings are good indicators of the long-term conditions at the sites. Three groundwater locations were extremely high in sucralose: the shallow-groundwater site A03, located just 30 m from the treatment lagoon at the Estero Bay Village site; the shallow-groundwater site A02, located at the bank of the Estero River and

adjacent to a shallow drainage ditch (never more than about 5 cm depth) that paralleled the entire eastern edge of the treatment lagoon about 50 m upstream of the point where it flowed into the river; and the samples from G07, which were collected from that flowing surface ditch, considered to be groundwater originating from the lagoon because the ditch was dry upstream of the lagoon but flowed continuously year-round downstream of the lagoon. The mean (± SD) sucralose concentration in the ditch water was 33,000 ± 11,000 ng/L and ranged between 17,400 and 42,700 ng/L. The extremely high concentration of sucralose at those three sites is persuasive evidence that the groundwater flow, and the discharge from the drainage ditch, was routinely conveying treated human wastewater exiting the lagoon through the groundwater. As described in Section 8 above, that ditch was also found to have a very high, but variable, concentration of both FIB. Together, the evidence of high FIB and high sucralose concentration are persuasive indicators that the small area contributing subsurface flow into the ditches is contaminated with human wastewater that has not been fully treated to remove FIB and other potentially harmful bacteria.

No sucralose was detected from A01, the groundwater site adjacent to Estero River at about the midpoint of its bank in the Estero Bay Village neighborhood. That location is quite near the two sites, A02 and A03, where sucralose was quite high, showing that the exact direction of groundwater flow strongly affects the presence of fecal contaminants. That flow direction is often difficult or impossible to predict prior to sampling, but these

Table 10.1. Sucralose concentration, MPN and other environmental factors.

Date	FGCU ID	Sample type	Sucralose (ng/L)	DO Temp (C)	DO (mg/L)	DO (%)	pH	Electric cond (µS/cm)	Turbidity (NTU)	ORP (mV)	enterococci (MPN/100 ml)	E. coli (MPN/100 ml)	Ratio (ent/ecoli)	TIN (ug-N/L)	NH4+ (ug-N/L)	NOx (ug-N/L)	SRP (ug-P/L)
8/19/2019	G04	Surface	285.56	29.2	3.5	45.1	7.5	430	2.7	491.7	96	75	1.28	197	138	60	16
9/18/2019	G04	Surface	358.83	29.3	3.2	42.2	7.5	626	2.9	442.8	1986	411	4.83	224	75	149	11
8/19/2019	G07	Ditch	17418.41	28.3	2.1	26.5	7.3	454	2.3	81.3	78	172	0.45	1270	1257	13	22
10/30/2019	G07	Ditch	35568.04	26.2	2.1	25.8	7.1	532	1.1	163.7	2420	2420	1	1018	512	506	1690
11/25/2019	G07	Ditch	42710.33	22.0	4.9	55.0	7.8	495	0.0	495.0	2420	236	10.26	517	104	413	1711
1/15/2020	G07	Ditch	36647.11	24.1	4.8	51.7	7.7	449	1.0	313.7	816	45	18.14	2117	950	1167	1989
9/25/2019	A01	Groundwater	0	28.1	1.8	23.4	6.3	652	45.1	-71.1	2420	0	2420	5206	5157	49	1773
9/25/2019	A02	Groundwater	17310.35	27.5	4.2	52.4	6.9	535	3.6	-22.7	164	0	164	890	852	38	1511
9/25/2019	A03	Groundwater	20287.92	27.2	2.2	27.1	6.8	517	6.0	-92.4	0	7	0.14	1164	1120	44	1589
10/30/2019	A01	Groundwater	0	27.8	3.4	42.0	6.4	6	2.7	30.6	2	0	2	4517	3823	694	932
10/30/2019	A02	Groundwater	22310.33	27.6	2.4	30.2	6.9	680	1.0	78.9	2	1	2	579	486	93	1387
10/30/2019	A03	Groundwater	19675.39	28.9	2.7	34.3	7.0	446	14.0	21.4	0	0	0	1705	1545	161	1678
11/25/2019	A01	Groundwater	0	24.2	3.3	38.4	7.0	434	5.6	169.3	2420	0	2419.6	3179	2745	434	143
11/25/2019	A02	Groundwater	32891.69	28.7	4.3	49.0	7.2	585		-48.4	4	0	4.1	1710	225	1484	1272
11/25/2019	A03	Groundwater	27708.41	25.1	2.2	26.0	7.1	542	10.3	-20.5	42	0	42.2	2951	2211	740	1885
11/25/2019	A04	Groundwater	940.87	21.1	3.9	45.4	7.1	3517	6.2	-97.8	17	112	0.15	620	313	308	117
11/25/2019	A05	Groundwater	953.16	23.0	3.4	40.3	7.0	6502	4.7		0	1	0	2083	668	1415	52
1/15/2020	A01	Groundwater	0	23.9	3.7	43.1	6.6	443	2.2	51.7	2	0	2	1614	356	1258	2013
1/15/2020	A02	Groundwater	31287.88	22.4	2.2	25.8	7.0	445	2.2	-114.4	0	1	0	387	303	84	1991
1/15/2020	A03	Groundwater	30540.15	24.7	1.6	19.3	6.9	560	10.0	-105.0	0	0	0	3332	2214	1118	1780
1/15/2020	A04	Groundwater	2568.72	21.7	3.9	43.2	7.0	874	3.7	29.3	2	0	2	647	84	564	109
1/15/2020	A05	Groundwater	649.48	25.1	3.0	31.2	6.9	7592	1.5	-198.4	25	0	24.9	1579	552	1027	164

results show that the site A02 is down-gradient from the source of FIB but A01 is not. The site A03, much nearer to the treatment lagoon, was also found to be high in sucralose, demonstrating that lagoon as a likely source of the fecal contamination. The finding of very low sucralose presence at A01, along with the very low or undetectable presence of FIB as reported in Section 8, suggests the A01 site is unaffected by the treatment lagoon even though it is nearer to the lagoon than A02. (That observation assumes we should ignore three samples with high enterococci believed to have been contaminated by sediment in the sample, as in the high turbidity event on Sep 25, 2020 shown in Table 10.1.)

The A01 site can be seen as indicative of groundwater conditions in that region near the river and uninfluenced by discharges of wastewater from either septic tanks (there are none in the community) or the treatment lagoon but likely influenced by permeated rainwater, which is suggested by high total inorganic nitrogen level (Table 10.1). The overall matrix of the groundwater in the immediate vicinity may be concluded to have a minimal influence of FIB or other fecal contaminants from wastewater. That may be true of the Estero River basin generally, but this single data point is insufficient evidence to draw conclusions about the entire watershed.

On the other hand, there was moderate concentration of sucralose detected in both samples that were tested from site that was selected with the intent to serve as a control site – site A04, on the property of Koreshan State Park, on the south bank of the Estero River where presumably it was isolated from the known wastewater treatment facilities at Sunny Grove and Estero Bay Village. The presence of sucralose, which was higher than the background level (i.e. riverwater sucralose concentrations) in the Koreshan location was unexpected, as there is no permanent residence at that location, only campsites (including a small restroom facility some 100 m upstream from the A04 site). It may be speculated that human wastes from the temporary visitors to the State Park have found their way into the shallow groundwater, though in only moderate amounts. High conductivity of A04 groundwater suggested the influence of saltwater from the river (Table 10.1).

The final groundwater site tested, at A05, is a shallow sampling device located in the Charing Cross neighborhood. That site too was adjacent to a surface drainage ditch, which was observed to flow in small volume throughout the sampling year, and is believed to be conveying discharges from shallow groundwater in the neighborhood, where the residences are all served by septic systems. There were modest concentrations of sucralose (about 650 and 950 ng/L) found at that location from the two samples collected there, in November 2019 and January 2020. Those low concentrations indicate presence of human waste, but they are orders of magnitude less than the concentration consistently identified at the Estero Bay Village location. This suggests that septic systems in the Charing Cross neighborhood, while presumably contributing FIB and other pollutants to the river, are much less of a source than the treatment lagoon at the Estero Bay Village neighborhood. High conductivity of A05 groundwater suggested the influence of saltwater from the river (Table 10.1).

There were no correlations between sucralose concentrations and the MPN counts of *Enterococcus*, *E. coli* and the ratio of *Enterococcus*/*E. coli* (Table 10.2). The sucralose also did not correlate with inorganic nitrogen, NOx (nitrite + nitrate), and ammonium, but showed a correlation with Soluble reactive phosphorus (SRP) and pH (Table 10.2). Nitrogen species are unstable in groundwater because they are quickly transformed by microbial activities. On the other hand, phosphate is more stable. Thus, the source of sucralose and the high level of nutrients was identical and identified as wastewater. On the contrary, both the MPN counts of enterococci and *E. coli* did not match this pattern, suggesting that the sources of these FIB differ from wastewater through the groundwater.

Table 10.2. Pearson correlation between sucralose, MPN and other environmental factors.

Variables	Sucralose (ng/L)	Temp (C)	DO (mg/L)	DO (%)	pH	Electric cond	Turbidity (NTU)	ORP (mV)	enteroco cci	E. coli (MPN/10	Ratio (ent/ecol	TIN (ug-N/L)	NH4+ (ug-N/L)	NOx (ug-N/L)	SRP (ug-P/L)
Sucralose (ng/L)	**0**	0.738	0.985	0.756	**0.044**	0.096	0.362	0.717	0.715	0.193	0.138	0.422	0.333	0.725	**0.001**
Temp (C)	0.738	**0**	0.186	0.489	0.591	0.147	0.295	0.568	0.959	0.778	0.802	0.570	0.263	0.159	0.944
DO (mg/L)	0.985	0.186	**0**	**<0.0001**	**0.029**	0.744	0.062	**0.049**	0.859	0.402	0.424	0.197	0.054	0.149	0.511
DO (%)	0.756	0.489	**<0.0001**	**0**	**0.035**	0.981	0.079	**0.021**	0.850	0.416	0.432	0.156	0.066	0.375	0.399
pH	**0.044**	0.591	**0.029**	**0.035**	**0**	0.799	**0.027**	**0.000**	0.308	0.480	0.129	**0.004**	**0.003**	0.932	0.439
Electric cond (µS/cm)	0.096	0.147	0.744	0.981	0.799	**0**	0.705	0.096	0.339	0.664	0.612	0.797	0.367	0.083	**0.031**
Turbidity (NTU)	0.362	0.295	0.062	0.079	**0.027**	0.705	**0**	0.198	0.216	0.490	**0.001**	**0.002**	**0.000**	0.385	0.321
ORP (mV)	0.717	0.568	**0.049**	**0.021**	**0.000**	0.096	0.198	**0**	**0.022**	0.324	0.829	0.149	0.217	0.480	0.415
enterococci (MPN/100 ml)	0.715	0.959	0.859	0.850	0.308	0.339	0.216	**0.022**	**0**	**0.020**	**0.003**	0.471	0.266	0.340	0.820
E. coli (MPN/100 ml)	0.193	0.778	0.402	0.416	0.480	0.664	0.490	0.324	**0.020**	**0**	0.643	0.393	0.447	0.749	0.669
Ratio (ent/ecoli)	0.138	0.802	0.424	0.432	0.129	0.612	**0.001**	0.829	**0.003**	0.643	**0**	**0.004**	**0.001**	0.374	0.852
TIN (ug-N/L)	0.422	0.570	0.197	0.156	**0.004**	0.797	**0.002**	0.149	0.471	0.393	**0.004**	**0**	**<0.0001**	0.220	0.326
NH4+ (ug-N/L)	0.333	0.263	0.054	0.066	**0.003**	0.367	**0.000**	0.217	0.266	0.447	**0.001**	**<0.0001**	**0**	0.678	0.382
NOx (ug-N/L)	0.725	0.159	0.149	0.375	0.932	0.083	0.385	0.480	0.340	0.749	0.374	0.220	0.678	**0**	0.711
SRP (ug-P/L)	**0.001**	0.944	0.511	0.399	0.439	**0.031**	0.321	0.415	0.820	0.669	0.852	0.326	0.382	0.711	**0**

Values in bold are different from 0 with a significance level alpha=0.05.

In Florida, where sucralose is present, generally it indicates not a leak of raw sewage but the discharge of wastewater from a treatment process. Wastewater that has been fully and properly treated conveys sucralose to receiving waters because sucralose is resistant to degradation in treatment systems and in the environment. If the wastewater is not properly or fully treated, either in a treatment process or in the soils surrounding a septic system, then it can convey high concentrations of nutrients, FIB, and potentially pathogenic organisms.

The small flow in the Estero Bay Village ditch at site G07 is similar in sucralose concentration to the groundwater directly adjacent to the lagoon, not the order of magnitude less that we might expect. That suggests the ditch water receives little if any dilution, and is essentially the same as the treated wastewater that exits the lagoon into the shallow groundwater. The concentration of sucralose in the Estero River in our data was two orders of magnitude less than in the two ditches, but greater than in the groundwater sampled at locations distant from the G07 and G09 ditches. The treatment system is shown to be a source of flow into the Estero River, but the data do not indicate whether that system is a source of the occasional high concentration of FIB that this study identified in the Estero River. It does suggest that wastewater, treated or otherwise, at areas served by septic systems such as the Charing Cross neighborhood are a smaller source of sucralose, but that does not indicate whether those septic systems are a greater or lesser source of FIB that reach the Estero River.

11.0 Project Data and Analysis: Genetic Sequencing and Species of Origin

As described in Section 3.7 above, the presence and magnitude of *E. coli* and enterococci are used to indicate potential influence of human waste in an environmental system, because they do originate in the human gut and can be readily identified and quantified by existing laboratory methods. However, they are known to also populate the gut of many warm-blooded animals, so their presence and counts (number of microbes per volume of water) do not correlate in any known way to the animals that are the bacteria's source.

Table 11.1. Overview of the samples used for high-throughput DNA sequencing analysis

Date	FGCU ID	Sample type	HT seq(515yF-926pfR)	Enterolert HT seq(27F-515R)
8/19/2019	G01	Surface	6213	
8/19/2019	G07(D07)	Ditch	6213	
8/19/2019	G02	Surface	6213	
8/19/2019	G03	Surface	6213	
8/19/2019	G04	Surface	6213	
8/19/2019	G05	Surface	6213	
8/19/2019	G06	Surface	6213	
9/18/2019	G01	Surface	6262	9505
9/18/2019	G02	Surface	6262	
9/18/2019	G03	Surface	6262	9505
9/18/2019	G04	Surface	6262	9505
9/18/2019	G05	Surface	6262	
9/18/2019	G06	Surface	6262	9505
9/18/2019	G07(D07)	Ditch	6262	
9/18/2019	G08	Surface	6262	
9/18/2019	G09(D09)	Ditch	6262	
9/18/2019	S02	Sediment		9505
9/25/2019	A01	Groundwater	6262	9505
9/25/2019	A02	Groundwater	6262	
9/25/2019	A03	Groundwater	6262	
9/25/2019	G07(D07)	Ditch	6262	
4/1/2020	A02	Groundwater	6507	
4/1/2020	A03	Groundwater	6507	
4/8/2020	G01	Surface	6507	
4/8/2020	G02	Surface	6507	
4/8/2020	G03	Surface	6507	
4/8/2020	G04	Surface	6507	
4/8/2020	G05	Surface	6507	
4/8/2020	G06	Surface	6507	
4/8/2020	G07(D07)	Ditch	6507	
4/8/2020	G08	Surface	6507	
4/8/2020	G09(D09)	Ditch	6507	

The numbers shown in the columns of DNA sequences are assigned numbers for each operational process. Orange color identifies a groundwater sample; brighter orange is a sample of surface groundwater flow from the "ditches."

Rapid advances in DNA sequencing technology makes it possible to identify many species of bacteria in aquatic systems. Some microbes are found more commonly in some animals than others. For instance, the gut microbial flora of birds is characterized by a lower abundance of Firmicutes and Bacteroidetes and a higher abundance of Actinobacteria and Proteobacteria in comparison with non-flying mammals (Grond et al. 2018). Therefore DNA identification of bacteria species, as a biological tracer, supplies important evidence about whether non-human animals may be present in a given environment, but not definitive evidence.

As described in Section 3.7 above, high-throughput DNA sequencing using 16S rRNA gene was determined for 31 samples. (Table 11.1). This project conducted DNA sequencing of a subset of the samples because of the high cost of this kind of analysis compared to other laboratory analyses used.

11.1 DNA extraction and DNA sequencing

Surface water samples were aseptically collected using Nalgene wide-mouth high-density polyethylene bottles (1 L) and transferred to the laboratory on ice. Each water sample (200 ml) was filtered through a 0.2 µm polysulfone-cellulose nitrate membrane filter and stored at -20°C until DNA extraction. A half-sized filter was used for DNA extraction with MagAttract PowerSoil DNA KF kit (Qiagen) according to the manufacturer's instructions.

Archaeal and bacterial 16S rRNA genes were amplified using the primer set, 515yF and 926pfR (Parada et al., 2016) tagged with the Illumina i5 forward and i7 reverse sequencing primer. After polymerase chain reaction (PCR), amplicons were visualized with eGels (Life Technologies) and products were pooled equimolar with each size selected quantified using the Quibit 2.0 fluorometer (Life Technologies). Amplicons were then loaded on an Illumina MiSeq (Illumina) 2 x 300 flow cell at 10 pM.

For DNA data analysis, FASTQ formatted files were merged using the PEAR Illumina paired-end read merger. Prefix dereplication and clustering at a 3% divergence level were conducted using the USEARCH. After operational taxonomic unit (OTU) selection and chimera (i.e., sequencing artifacts) checking, representative OTUs were used to determine taxonomic information. The microbial community analysis was conducted based on the relative abundance at the phylum and genus level. We used a high-quality rRNA database, Silva, which provides quality checked and regularly updated datasets of aligned small (16S/18S, SSU) and large subunit (23S/28S, LSU) rRNA sequences for all three domains of life (Bacteria, Archaea and Eukarya). In total 585,435 DNA sequence reads were obtained after these processing. The mean (± standard error) DNA sequence count in each sample was 18,885 ± 3392 reads (n = 31).

11.2 Microbial Community Analysis for Estero River Samples In Aggregate

A majority of 16S rRNA gene sequences obtained belonged to the domain Bacteria. A total of 36 phyla were found in the Bacteria domain (Figure 11.1). The data in Figure 11.1 also describes relative magnitude of taxa broken down by their location, by sample site, and that information is discussed in Section 11.3 below; this section describes only the relative counts of taxa in the aggregate data. Within the Bacteria domain, some "Candidatus" groups such as '*Candidatus* Atribacteria' and '*Candidatus* Parcubacteria' were found. In prokaryote nomenclature, this Candidatus (abbreviated as *Ca*.) is used to name

prokaryotic lineages that are well characterized (e.g., phylogenetic position, genome sequences, host organisms) but uncultured.

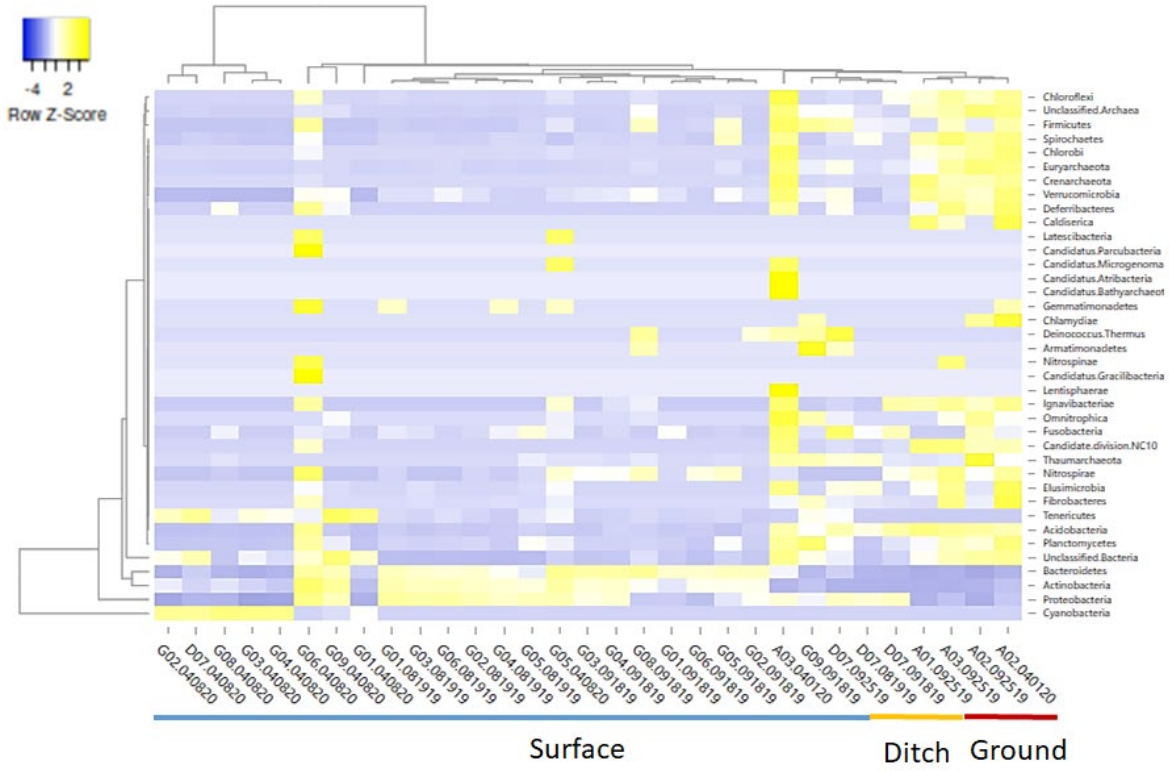

Figure 11.1. Heat map of the relative abundance of prokaryotic taxa at the phylum level. Euclidean method was used to make a distance matrix and the average linkage method was used for clustering. Many anaerobic/microaerobic taxa were found at the top-right, in the groundwater samples. Cyanobacteria dominated in surface water samples collected on April 8th, 2020 and shown at the bottom-left corner. It made April water samples distinguishable from other samples (see the dendrogram above).

Archaea were found in much lower abundance than Bacteria (less than 2.5% of the total communities in all the cases). Their importance in this analysis is because they were much more abundant in groundwater than surface water, and somewhat more abundant in samples from the two small drainage ditches than in Estero River samples (Figure 11.2). The ditch water is established in Sections 9 and 10 above to consist largely or entirely of discharges of groundwater originating with the wastewater treatment lagoon at the Estero Bay Village neighborhood (the D07 samples) or septic systems at the Charing Cross neighborhood (the D09 samples). The heat map data showed that the microbial communities in the ditch water were greatly different between the April 8 sampling (located in left and mixed in the surface waters) and the other cases (concentrated and located nearby the groundwater cluster), indicating a potential shift of major water sources of the ditches.

Because the majority of Archaea are anaerobic or microaerobic, groundwater is a suitable habitat for this lineage of prokaryotes. Our observation fitted the previously

known habitat ranges of Archaea. In those cases where Archaea are found in samples of surface water from the Estero River, Archaea, particularly Crenarchaeota, could be a good microbial indicator of groundwater influence. Archaea embrace some functionally imperative groups of organisms. Crenarchaeota includes a variety of sulfur-oxidizing bacteria. Euryarchaeota embraces methanogens.

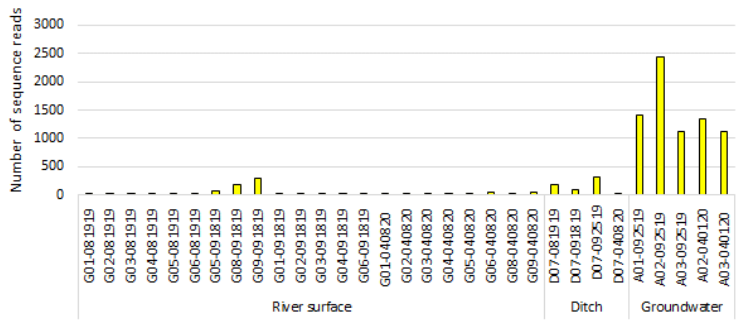

Figure 11.2. The relative abundance of Archaea. The data are shown as the sum of Crenarchaeota, Euryarchaeota, Thaumarchaeota, '*Ca*. Bathyarchaeota', and unclassified Archaea. The data are normalized to be 10,000 sequence reads per sample (1,000 reads correspond to 1% of the total community).

The entire biological methane production on Earth is mediated by methanogenic archaea. Some ammonia-oxidizing microorganisms, which play a fundamental role in the nitrogen cycle, are included in Thaumarchaeaota. '*Ca*. Bathyarchaeota' is a recently characterized lineage of the domain Archaea (Harris et al. 2018). Its function is genomically elucidated as a denitrifying anaerobic methanotroph.

The microbial communities in the Estero River were strongly influenced by four large groups of bacteria: Actinobacteria, Bacteroidetes, Proteobacteria, and Cyanobacteria.

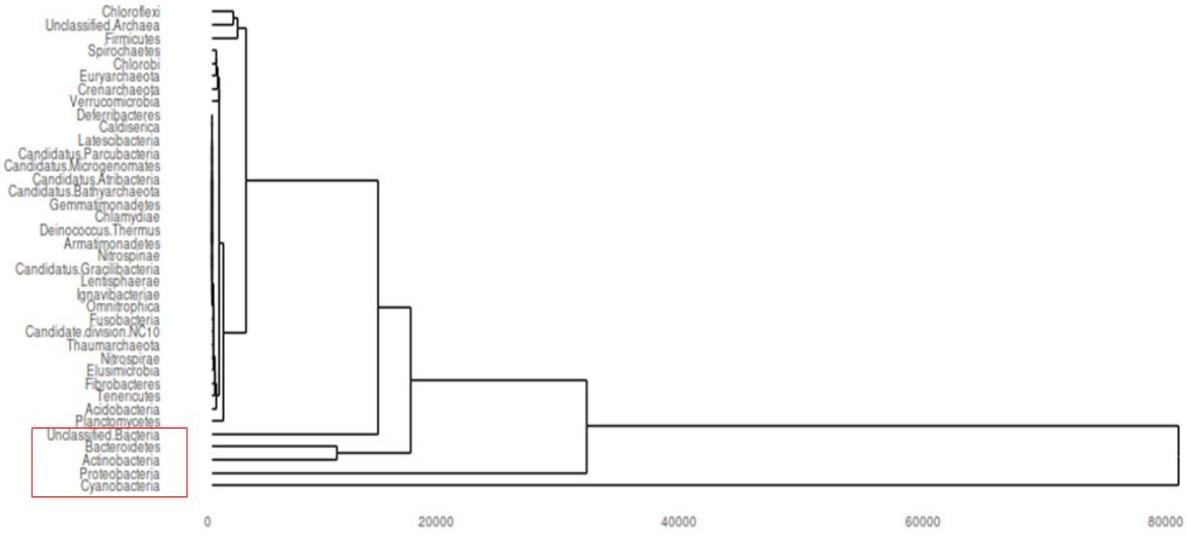

Figure 11.3. Dendrogram at the phylum level. The Euclidean method was used to make a distance matrix and the average linkage method was used for clustering. The data show which microbial taxa are more abundant in the microbial communities of the samples. The data show the importance of four phyla in the communities: Actinobacteria, Bacteroidetes, Proteobacteria, and Cyanobacteria (marked with a red box in the figure).

(Figure 11.3). The dominance of these four groups of bacteria in freshwater environments is common. For example, this pattern resembles a previous research from the Caloosahatchee River (Garcia et al. 2015). When the water discharge from Lake Okeechobee was low, the abundance of Proteobacteria increased. On the contrary, when the water discharge was high, the relative abundance of Cyanobacteria and Actinobacteria increased.

Proteobacteria is a major phylum of Gram-negative bacteria and contains five well-recognized groups (Alpha-, Beta-, Gamma, Delta-, and Epsilonproteobacteria) and three newer groups (Zetaproteobacteria, Oligoflexia, and Acidithiobacillia). They include a wide variety of functional groups such as nitrification, denitrification, nitrogen fixation, methane oxidation, sulfur oxidation, and several other functions. Proteobacteria is a well-studied bacterial taxon because it includes many human, animal and plant pathogenic groups, such as *Salmonella*, *Acinetobacter*, *Vibrio*, *Pseudomonas*, *Yersinia*, *Legionellales*, and many others. *E. coli* belongs to Gammaproteobacteria. An avian fecal indicator, *Helicobacter*, is included in Epsilonproteobacteria.

The relative abundance of different species of Proteobacteria in a tidal system like the Estero River is strongly influenced by the extent to which seawater is present. Betaproteobacteria are a typical freshwater bacterial lineage while Alphaproteobacteria are highly abundant in the sea. The data (Figure 11.4) show that during the wet season, freshwater from the watershed influenced the microbial communities, such that Betaproteobacteria predominated in the entire river. That means that the *Enterococcus* and *E. coli* identified during the wet season, as discussed in Section 8 above, likely originated with runoff and with groundwater feeding the river, not with any transport of seawater or sediment from the estuary upward into the river via tidal action. That is to be expected of the high concentrations identified upstream of about mile 4, which are more distant from the estuarine regions; but the presence of Betaproteobacteria throughout the Estero River suggests that the occasional relatively high *Enterococcus* and *E. coli* in the samples nearer the estuary also originate with freshwater, perhaps backed up into the channel by tidal action near the mouth, but do not originate with more saline water or sediment from Estero Bay. In the dry season, the increased fraction of Alphaproteobacteria throughout the Estero River reaches suggests the influence of saltwater incursion, particularly in the samples downstream of about river mile 3 in the samples collected November 13 2019 and April 8 2020.

Many members of the class Deltaproteobacteria adapt to anaerobic/microaerobic environments, so they are abundant in sediment and groundwater. Presence of those species in the samples would indicate a disturbance of river sediment, perhaps by tidal action or the churning action of boats in the river, and/or of enhanced groundwater discharges as would be expected during the wet season. Epsilonproteobacteria and other Proteobacteria were less abundant.

As described in Sections 3.6 and 3.7, the literature clearly shows that presence and magnitude of *E. coli* and enterococci, identified as FIB for regulatory purposes, do indicate the presence of fecal matter from warm-blooded organisms in a given waterbody, but fail to distinguish between human and non-human sources. The methods discussed in this

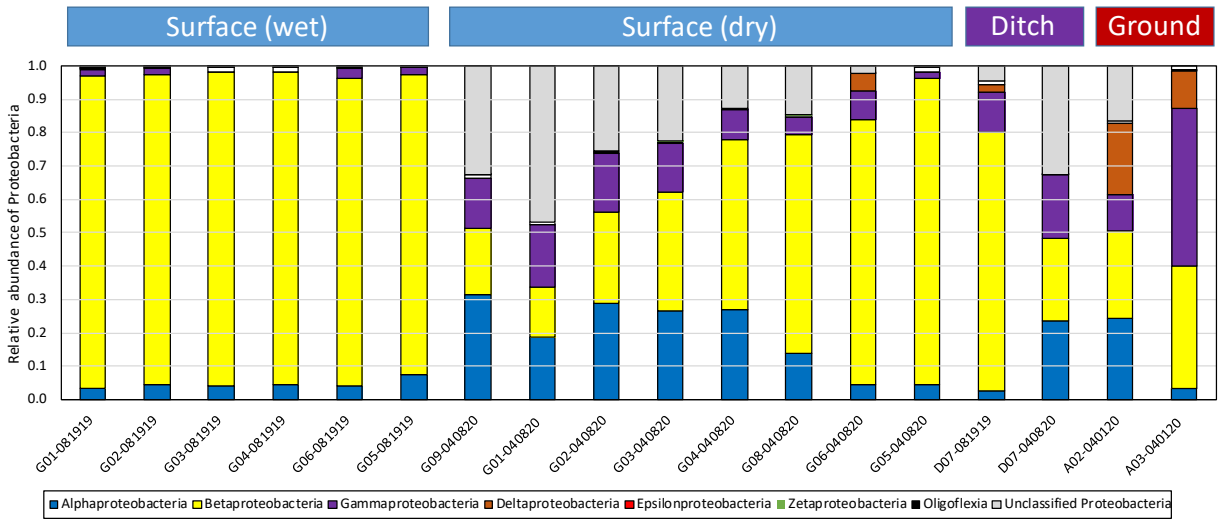

Figure 11.4. Proteobacterial microbial community profiles at the class level. The data show that during a wet season, freshwater from the watershed influenced the microbial communities and Betaproteobacteria predominated in the entire river. In a dry season, saltwater incursion expanded the fraction of Alphaproteobacteria, particularly in a lower river mile zone. Betaproteobacteria are a typical freshwater bacterial lineage while Alphaproteobacteria are highly abundant in the sea. Many Deltaproteobacteria adapt anaerobic/microaerobic environments. They are abundant in sediment and groundwater. Epsilonproteobacteria and other Proteobacteria were less abundant. G09 was a ditch water sample and wrongly labeled.

section succeed in identifying a great many additional species of bacteria in environmental samples. However, as discussed in Section 3.7, while some bacteria may be found more commonly in the gut of some warm-blooded animals than others, the current state of knowledge does not include any species that can be definitively identified as originating with any one specific warm-blooded organism. The presence of particular species of bacteria in the Estero River does not either definitively indicate, or rule out, presence of human waste.

As with any Florida watershed with residential development, the Estero River watershed can be expected to house modest to large populations of various domestic animals and wildlife, primarily dogs as pets; cows, chickens, and other livestock; and gulls and a wide variety of other avian species.

No guidelines have been established for interpreting gene copy numbers at this point. The gene copy numbers determined could be changed by various environmental factors and the size of water bodies. Normally, a quantitative PCR (qPCR) method is employed for this purpose (e.g., specific genetic markers contain HF183 (human), GFD

(avian), and Gull2 (avian)), we used a high-throughput DNA sequencing method to detect these genes. The advantage of this sequencing method is that we can simultaneously detect multiple specific genetic markers from a single water sample. This approach is still in the infancy stage but can potentially replace the traditional qPCR methods.

In qPCR assays, the lower detection limit is usually 10^2 gene copies/100 mL. Our cell counting method showed that 10^6 cells of bacteria per mL of surface water. Our average DNA sequence reads were 20,000 reads per sample. Thus, one read corresponds to 50 cells/mL. This means that if 50 cells exist in 1 mL of the water sample, the method can detect the target bacteria. If one uses 100 mL for analysis, the detection limit can be changed to be 0.5 cells/mL. Thus, the detection limit of the qPCR and 16S rRNA gene amplicon sequencing method used in this study (20,000 reads per sample) are theoretically equivalent.

Table 11.2. The potential sources of specific Bacteroidales genetic markers found in this study

Specific genetic marker	Source
Bacteroides barnesiae	Chickens, other birds
Bacteroides fragilis	Human
Bacteroides intestinalis	Human
Bacteroides massiliensis	Human
Bacteroides sp.	Human, mammal, bird
Barnesiella sp.	Human
Dysgonomonas gadei	Human
Dysgonomonas sp.	Animal
Dysgonomonas termitidis	Termites, other insects
Paludibacter sp.	Human, cattle, other mammals
Parabacteroides chinchillae	Rodents, esp. chinchilla (*Chinchilla lanigera*)
Prevotella sp.	Human
Alistipes sp.	Human
Rikenella sp.	Chicken, Japanese quail, other birds

In summary, Table 11.2 lists those genetic markers found in the Estero River samples in sufficient abundance to reasonably support a conclusion that wastes from the listed species are present in the waterbody. Multiple markers for human waste were identified, demonstrating with reasonable assurance that human waste was present in the Estero River at the time of one or more of these samples. Also present were markers the correspond to other species: *Bacteriodes barnesiae* and *Rikenella* sp. populate the gut of chickens or other birds, and not of humans, documenting that fecal matter from birds was also present in some abundance in the samples. Presence of *Parabacteriodes chinchillae* demonstrates that waste from non-human mammals – most commonly, rodents – is also present in some abundance. And presence of *Dysgonomonas termitidis* demonstrates that

waste from non-human insects was also present in some abundance. The weight of evidence suggests that human wastes are present so that the exceedance of regulatory numeric targets for *Enterococcus* and *E. coli* documented in Sections 7 and 8 above is due at least in part to the presence of human waste. The data also show that it is reasonable to conclude that wastes from other species also contribute the counts, and the frequency of high-concentration events, of *Enterococcus* and *E. coli* in the Estero River. It is not possible to determine the relative contribution of those different species to the presence of the regulated *Enterococcus* and *E. coli.*

The same 16S rRNA gene amplicon sequencing method was applied to these samples in an attempt to detect the regulated FIB *Enterococcus.* This new approach is expected in the future to be a powerful supplement to the traditional counting methods; if a genetic sequencing method could also detect enterococci, the analysis could be more precise. However, this method cannot detect species unless they are at high abundance. This method was first used in Australia, where Schang et al. (2016) documented that the *Enterococcus* and *Escherichia* genera comprised less than 0.01% of the total bacterial community and the detection of these enterobacteria were difficult for high-throughput sequencing. The authors had to lower the resolution of analysis to get several "rare" fecal organisms.

Our Estero River high-throughput sequencing analysis showed the same results, in which the abundance of enterococci was less than 0.01% and *E. coli* was not detectable. Among the water samples subjected to the 16S rRNA gene amplicon sequencing, enterococci were found in only two samples: the D09 site (Charing Cross ditch) on Sep 18, 2019, and the D07 site (Estero Bay Village ditch) on Sep 25, 2019. *Enterococcus* was not detected from the surface river water and groundwater samples. No sample contained *E. coli* at high enough counts to be detectable by this method. These results indicate that the high-throughput DNA sequencing method used in this study (20,000 reads per sample) was not the best method to directly detect FIB. Therefore, a much deeper depth of reading or a more advanced method is required. That is why the standard laboratory methods to detect and quantify FIB, as described in Section 8, were employed.

11.3 Species of Microbes Found in This Study and their Locations; Tentative Evidence for Presence of Human and Non-Human Fecal Matter in the Estero River

The phylum Bacteroidetes was one of the major groups found in this study (Figure 11.4). Bacteroidales is one of the orders mainly formed by obligate anaerobes with some facultative anaerobes. They are found in various anaerobic environments. Some of these bacteria are known as inhabitants of animal guts and could be used as specific genetic markers. *Bacteroidetes* spp. were highly abundant in groundwater samples.

In the present study, 14 genera formed by 25 taxa were found. Among them, specific genetic markers were selected (Table 11.1) and the distribution pattern was examined

(Figure 11. 6). Similar to other coliform data, the gene markers were more frequently found in groundwater and ditch water sites than the surface water samples, so the soil-groundwater and the surface conveyance of groundwater both are shown to be contaminated with bacteria species that populate the gut of warm-blooded animals, though it is not able to discern whether these are humans or other animals.

The results can be examined in relation to the separate reaches of the river, as described in Section 12 below: Upstream Reaches (G08, G05, and G06, at Sandy Lane mile 4.95, South Branch, and North Branch, respectively) as compared to the Middle Reach (from G10, Riverwoods, mile 3.17 at the downstream end to the Route 41 Bridge, G04, mile

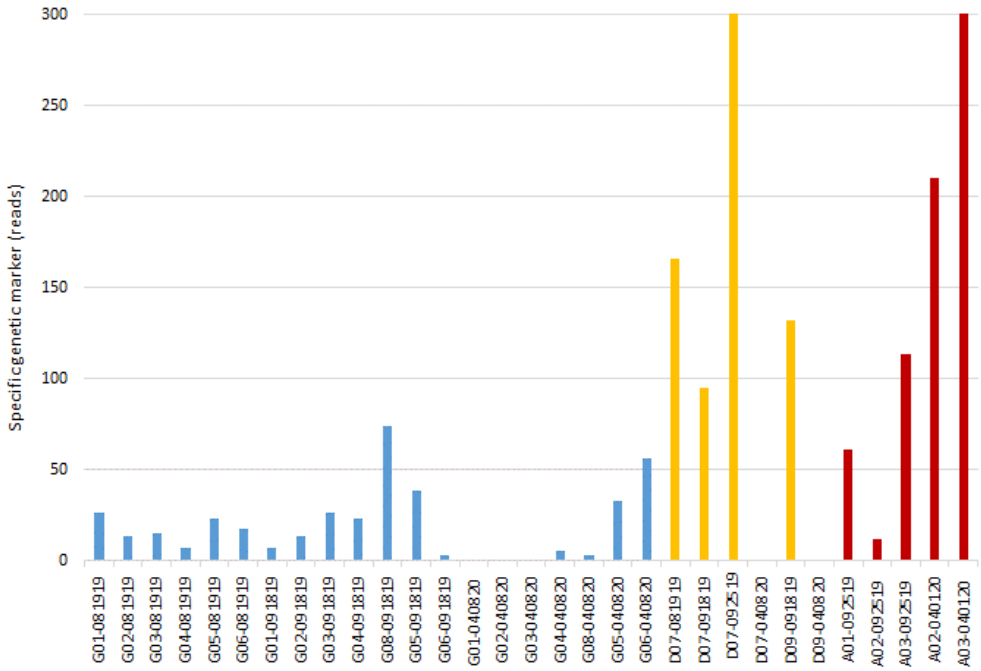

Figure 11.6. The distribution of specific Bacteroidales genetic markers found in the surface river water (blue), ditch (yellow) and groundwater (red) samples. The data were shown as the sum of 14 markers shown in Table 11.1. Two analyses ranged higher than the scale of the y-axis: D07-092519 (924) and A03-040120 (1,196).

4.58 at the upstream end).

The three sites in the Upstream reach had higher numbers of gene markers in a different distribution pattern than the downstream sites. One sample from one location, at Sandy Lane (G08, mile 4.95) in April 2020, contained a much larger number of identified bacteria species than the other five Upstream Reach samples (including the one other Sandy Lane sample). That sample identified *Parabacteroides chinchilla*, a specific gene marker for rodents, which was present on the same day at Route 41, mile 4.58, less than half a mile away, and not identified in any other of the 31 samples on which genetic sequencing was conducted. The upstream sites were different than other sites in other ways as well: for example, *Bacteroides barnesiae*, a specific gene marker for birds, was

detected on multiple occasions in the Upstream Reaches but was only once detected in the Middle Reach, at the G01 site (mile 3.51) on August 19, 2019. The fact that the times, frequencies, and relative abundance of markers were different between locations is further evidence of the finding that bacteria in the Estero River originate from multiple and temporary sources.

The infrequent identification in abundance of a wide variety of bacteria, especially those with genetic markers linked to particular species, further documents the variability of conditions in the Estero River and the wide range of circumstances that contribute at various times to bacteria in the river – including regulated bacteria *Enterococcus* and *E. coli;* unregulated bacteria that originate with non-human species, identified here; and bacteria of concern regarding human health, not named in the regulations but the overarching reason for regulating bacteria in environmental waters.

12.0 Summary and Conclusions: Geographic Localities and the Estero River

This section presents brief summaries of the findings for five separate localities in this study, recapitulating the evidence presented in the sections above to arrive at the best available judgment about the presence of FIB and thus of human fecal bacteria. This section is divided into five sub-sections for these localities: three separate reaches of the Estero River, and two neighborhoods where groundwater was studied. The reaches were chosen specifically for this study, not based on any other agency's demarcations, and were selected to consist of portions of the Estero River where tidal action mixes the water in different ways, and where the data on FIB were reasonably similar in frequency and distribution. Each is about 1.5 miles long. The neighborhoods identified for groundwater studies are smaller in scale, and results apply more locally – findings may indicate how groundwater may behave in other localities but the findings apply strictly to these two very small parts of the watershed. The five localities are:

- Upper reaches, from Sandy Lane upstream about 1.5 miles, including one sampling site on each of the North and South Branches (3 sites);
- Middle Reach, from Charing Cross upstream about 1.5 miles to Route 41 (5 sites);
- Lower / Estuarine Reach, from Armada Court upstream about 1.5 miles to the Charing Cross vicinity (2 sites);
- Estero Bay Village groundwater locality (4 sites);
- Charing Cross neighborhood groundwater locality (2 sites).

As described in Section 2, the data analyses here are conducted this section describes data in terms of "frequency of high-concentration events" at particular locations, and does not compute averages over time or location. The analyses are on the basis of the

number of samples found to have high concentration of FIBs, or "high-concentration occurrences," defined purely for the purposes of this report as greater than about 800 MPN/100 mL. The frequency of high-concentration occurrences is more meaningful to this analysis because FIB appear to originate with highly localized, episodic, short-term events; and their transport in the river does not thoroughly mix them into the environmental system, but instead they are found in samples in localized patches of varying magnitude, location, and event (or species) of origin.

12.1 Estero River upstream of Sandy Lane

For purposes of this study the "upper Estero River" constitutes the portion from Sandy Lane, at about mile 5, upstream on the two branches, to the site G05 about 0.5 miles upstream on the South branch and, on the North branch, to site G06 about 0.8 miles upstream on the North branch, and for purposes of historical data to about 1.2 miles upstream of Sandy Lane at the Three Oaks Parkway bridge. These reaches are affected by tides, but indirectly: the water level in the channels rises and falls with the tides because tidal action 'backs up' freshwater flow, to a variable extent, on a daily or twice-daily (semi-diurnal) basis; the waterbodies do not under normal circumstances receive any saltwater, so are free from any bacteria or substances that have been in contact with the estuary.

Historical data from Three Oaks is somewhat upstream of these locations and is on the North Branch of the Estero River. Those data (Figure 4.3 in Section 4 above) show very infrequent high-concentration events: a total of eight times in the 5-year period January 2015 – January 2020 for *Enterococcus*, and only twice in the 4.5-year period June 2016 through January 2020 for *E. coli*. In fact, even at this upstream location, the concentration of enterococci exceed the regulatory target (the TPTV, or "ten percent threshold value") that specifies no more than 10% of the samples are greater than 130 MPN/100 mL for enterococci. The historical samples for *E. coli* at this location do not exceed the regulatory target of 410 MPN/100 mL for *E. coli*.

Data from the "length of the river" analyses in Section 7 above, as presented in Figures 7.1 and 7.2, show that the FIB found by this study in the North Branch (site G06, at mile 5.74) is similar to the historic data for Three Oaks bridge as summarized in Section 4, Figure 4.3, above: all samples were lower than other parts of the river for both nterococci and *E. coli,* though two of the seven samples found enterococci concentrations well in excess of the TPTV numeric target, once during the wet season (July 2020) and once during the dry season (September 2019). The same graphs show this upstream reach main stem (Sandy Lane site, G08, at mile 4.95) and South Branch (G05) is not much different in the magnitude and frequency of high-concentration conditions from what is found in the Middle Reach and is similar to historic data for the Route 41 bridge. On four occasions out of 14 possible occurrences, one or the other of the two FIB were present in greater concentration at either Sandy Lane or the South Branch than in any samples collected

further downstream. This highlights the fact that FIB enters the river at varying times from varying locations, and the upstream reaches are not immune to fecal contamination.

In summary the findings suggest that, even in this area that receives runoff from only a small area of urban development, FIB do occasionally enter the Estero River.

This upstream reach of the Estero River has fewer cases of high-bacteria conditions, with those conditions occurring more often below the confluence of the two branches. It is the furthest upstream reach studied in this project, but is not so far upstream as to be above the sources of bacteria, as was the case for the Three Oaks site where data was acquired over a longer period by Lee County monitoring. The Estero River in these locations commonly has bacteria conditions exceeding standards for protection of human health, but not as frequently as other parts of the Estero River. That finding, that high-concentration events overall increase in frequency moving downstream in the river, is powerful evidence that the FIBs originate with urban/human activities. In the case of a pollutant that enters a river in steady, small amounts from urban land uses, we would expect to see a gradual increase in pollutant concentration as the river flows downstream, passing more and more contributing sources. In the case of FIBs, as the river flows downstream it increases its potential encounters with short-term, episodic source events, so the probability of high-concentration samples increases, and the frequency of high-MPN samples over time increases at sampling sites proportional to the distance the river covers – thus increased frequency at downstream locations. That pattern is observed in the Estero River, evidence that FIBs originate with residential land uses in the watershed.

There were no samples tested for sucralose in this reach, so there is no chemical tracer that could help indicate the extent to which the detected FIB originate with human waste as opposed to other warm-blooded animals. However, two samples were tested for sucralose collected at a site just below the downstream end of this reach – and upstream of the suspected sources of wastewater in the Middle Reach – at the Route 41 bridge (site G04). Both samples were found to have very small concentrations of sucralose compared to the amount found in groundwater. As discussed in Section 12.2 below, that is typical for surface water samples: sucralose is generally an effective indicator in groundwater, and not in flowing surface water, because surface waters have much greater volumetric flow per unit time and any sucralose is heavily diluted, frequently below the laboratory detection limit. The very low concentration detected at G04 supports the assumption that the FIB in these “upstream” reaches likely originate in part with human waste, but the low concentrations indicate there is unlikely to be a continuing, sizable source in this vicinity.

Results of the biological tracer analysis – genetic sequencing of the bacteria in the samples, searching for genetic markers that might indicate the warm-blooded animal from which the bacteria originated – also suggest some human, and some non-human, sources in the upstream reaches. As noted in Section 11 above, these methods succeed only where

bacteria counts are sufficiently high to produce results in the laboratory test, so the presence of a result is definitive, while the absence of result is not definitive (as demonstrated by the fact that enterococci were identified in only two samples out of 31 analyzed, whereas were found in nearly all of the more than 150 samples tested using the conventional laboratory methods).

The data in Section 11.3 conclusively show multiple species of bacteria known to originate in the human gut in the Upstream Reaches (which is true of all other localities as well). In addition, in the Upstream reaches, several of the 31 tested samples also showed the presence of species originating in various avian species; and one sample showed the presence of species considered to be a genetic marker for rodents. The weight of the evidence strongly suggests that some of the bacteria originate with non-human animals – including, most likely, the regulated bacteria *Enterococcus* and *E. coli.* The state of the art is not sufficient to estimate the relative contribution of human and non-human sources to the high concentrations of the regulated bacteria, but is sufficient to document that humans contribute. If that is true here, at the furthest-upstream locality evaluated in this study, it can be reasonably concluded for the downstream reaches also.

12.2 Estero River Middle Reach

This study designated the Middle Reach as the Estero extending from about mile 3.0, near the Charing Cross neighborhood, upstream to the Route 41 bridge, at about river mile 4.6. The Route 41 site was chosen because it is the same location as sampled by the Lee County Department of Environmental Management, at its site numbered 47A-15GR. The downstream end (at the Charing Cross neighborhood) is just downstream of the boat launch at Riverwoods, at about river mile 3.2, which is also a Lee County sampling site, numbered 47A-4GR.

This constitutes a distinctive reach for two reasons. The first reason is the influence of tides, which may strongly affect the input, mixing, transport, and sample concentration of FIB. The reach experiences some mixing by semi-diurnal tides moving water upstream from the estuary, so that the FIB concentration measured at any one time may not be the result of FIB discharged “upstream” of the point where the sample is collected. Water flows in either direction, and with markedly varying velocity, at different times on different days. It is not known whether FIB may survive in the environment long enough to accumulate in the estuary and then be moved back up the channel, but if that is the case, then those organisms will be present in samples and add to the count of any bacteria that may be entering the river from the watershed. Typically the estuarine, salty, water moves upstream beneath the flowing freshwater, whose lower density keeps it afloat atop the saltwater, but there may be sufficient turbulence that a sample at any depth might contain FIB from upstream, downstream, or both. The second reason this reach of the river is considered separately is that there are suspected FIB sources within the reach: FIB might

reach the Estero River if there are incompletely treated discharges from two small-scale 'package' wastewater treatment facilities, at the Estero Bay Village neighborhood (G07, about mile 3.6) and Sunny Groves neighborhood (G06, about mile 4.25). FIB might also reach the Estero River from septic systems if those systems are too close to the river, too near to one another, or improperly maintained and operated, or any combination of those factors. It is known that there are septic systems in this reach, including but probably not limited to the Charing Cross neighborhood.

Historic data show that the frequency of high-concentration events, and especially of very-high-concentration events, is much greater at Riverwoods, near the downstream end of this segment, than at the Route 41 at the upstream end. Figures 4-1 and 4-2, in Section 4 above, show that the Riverwoods site recorded 9 occasions of enterococci at the method-quantitation maximum of 2,420 MPN/100 mL between January 2015 and December 2019, and 9 other occasions between 800 and 2,000 MPN/100 mL. For the same period, the upstream Route 41 site detected only 2 occasions of *Enterococcus* 2,420 MPN/100 mL and 6 occasions above 800 MPN/100 mL. This is consistent with the pattern of increasing frequency of high- and very-high-concentration events as the river moves downstream, documenting that FIB enter the waterbody in increasing frequency as it flows further through areas of developed land uses where sources might be found. This 1.5-mile reach of the river clearly is such an area of FIB sources.

That finding is supported by data from the present study. Of the seven sampling events conducted, in most samples, the highest concentrations for either or both FIB were found in this Middle Reach, with the exception of some that suggest the influence of tides when the lowest reach was higher (July 28 2020, November 2019). Several samples (July 14, August 10, September 13, April 8), showed "off-the-chart" extremely-high-concentration results for one or both FIB at one or more of the stations between mile 3.0 and mile 4.6, mostly during samples when no other sites were nearly so high. Those four occasions included two wet season and two dry season samples. Considering the three reaches together, it appears that the Middle Reach is the source of some of the FIB in the Estero River. The repeated finding of FIB at greater concentration in this reach than either upstream or downstream – not on every sample, but most – is reasonably strong evidence.

However, the data from the current study are not able to definitively document that any one location or activity is a FIB source. The exact locations of highest-concentration findings within the reach vary between samples. There was one sampling event, September 2019, when no locations within the Middle Reach displayed high FIB concentration (though this event did not collect samples in the lower reach). This is consistent with the finding that FIB sources enter the Estero River as discrete, short-term, statistically-varying events, not from a continuous condition or source. These are not continuous discharges as from a polluting factory or an improperly-treated waste, but intermittent pulses carried by varying conditions of precipitation, groundwater flows, seasonality, tidal flushing and other

unknown factors. But the highest frequency of such events appears to occur in the Middle Reach of the Estero River, which is known to contain potential sources including neighborhoods with septic systems and small-scale, privately-operated treatment facilities.

The high-concentration conditions in the Estero River do not correspond in any obvious way with known high-precipitation events, seasonal changes in population, season-long changes in rainfall or water table changes, or identifiable tidal conditions. The fact that the high-concentration events predominate in this section of the river suggest that multiple sources to the river affect those high bacteria concentrations, possibly including but not necessarily limited to the neighborhood-maintained wastewater treatment facilities; densely-clustered or improperly-maintained septic systems; and runoff from community lawns.

Evidence suggests the FIB in this reach originates, at least in large part, with human wastes. Sucralose was tested at only one site, G04, the Route 41 bridge, and only on two occasions. In both cases it was present, but at only small concentrations. That is typical of surface water when it has in the past been tested for sucralose, and the sucralose data does not contribute to our understanding of bacteria in this section of the Estero River.

Genetic sequencing for this reach identified a large number of various bacteria, as expected in an environmental system. However, the results did not find quantifiable amounts of any of those few species identified as genetic markers for particular warm-blooded organisms. That is, all the species identified are known to originate with either a) humans or b) humans and other warm-blooded species. There is no evidence as to whether bacteria in the surface water of the Middle Reach originates predominantly with humans, predominantly with other species, or some mix of multiple species.

12.3 Estero River Lower/Estuarine Reach

The lower reach of the Estero River is so near to Estero Bay that it may be fully estuarine, not only mixing with saltwater but routinely flushed by tides. This study assumes that in this reach fresh water from the Estero River watershed is fully intermingled with saline or brackish water from the Estero Bay estuarine system. That assumption is not fully documented by any hydrologic studies but is believed to be a reasonable explanation for the behavior identified in our data. The research did not include any study of data from Estero Bay, although it is known that Lee County has conducted sampling at various points in the Bay, because that waterbody is so variegated and its mixing regime so complex that understanding it is beyond the scope of this study. The concentration of FIB measured in any one sample is expected to be affected by mixing, varying upstream sources, and potential downstream sources to an extent that the source of any one sample, or the dominant influence at any one location, is indeterminate.

The sampling points in this reach of the Estero River were G12, at approximately mile 2.31, and G11, at approximately mile 2.56. Only five of the seven sampling events included those two sites. Results showed FIB concentration that varied from extremely high to extremely low. These data also exhibit the known lack of correlation between the two FIB species. In one wet season day's sample, November 2019, the concentration of *Enterococcus* (at > 2,420 MPN/100 mL) was much greater than the concentration of *E. coli* (between 400 and 800 MPN/100 mL) at both G11 and G12 sites. In another wet season day's sample, July 28, 2020, the concentration *E. coli* (1,600 MPN/100mL at G12 and 1,000 MPN/100 mL at G11) was much greater than the concentration of *Enterococcus* (400 MPN/100 mL at both sites). In a dry season day's sample, April 2020, again the concentration *E. coli* (900 and 1,100 MPN/100 mL) was much greater at both sites than the concentration of *Enterococcus* (less than 50 MPN/mL at both). In the July 14 2020 sample, *E. coli* was much higher at G12 and *Enterococcus* was much higher at G11. The chaotic mixing within this reach renders it impossible to make any determination about presence of sources in this reach but implies the existence of multiple FIB sources, and the small number of samples possible under this study does not give sufficient information to understand the effect of tide stage, direction, and magnitude on the sample results.

No surface water samples from this reach were tested for sucralose, so there is no chemical tracer information about the Lower / Estuarine Reach. The samples were not tested because it is expected that sucralose, known to be present at low concentration in surface waters generally, is unlikely to be detectable in the high-volume, chaotic mixing regime of this reach.

As in the Middle Reach described above, genetic sequencing for this reach identified a large number of various bacteria, as expected in an environmental system. However, the results did not find quantifiable amounts of any of those few species identified as genetic markers for particular warm-blooded organisms. That is, all the species identified are known to originate with either a) humans or b) humans and other warm-blooded species. There is no evidence as to whether bacteria in the surface water of the Lower / Estuarine Reach originates predominantly with humans, predominantly with other species, or some mix of multiple species.

12.4 Groundwater at the Estero Bay Village neighborhood

The Estero Bay Village neighborhood was surveyed with three sampling sites that collected water from the subsurface, and one site of flowing water at small volume (roughly 1 to 3 ft^3/second) that observations demonstrated consisted of groundwater discharged into the channel less than 200 m upstream from its discharge into the Estero River. That small channel runs parallel to the two treatment lagoons that serve as a final "polishing" treatment step for wastewater from the privately-operated "package" wastewater treatment facility at Estero Bay Village. The lagoons do not appear to have a piped outlet

for the wastewater, and instead deplete their standing water through evaporation and infiltration into the groundwater. The intent of this design is that microorganisms in the groundwater will biodegrade any waste or organisms that may remain in the wastewater thus rendering the discharge safe for the environment and for human contact.

The channel is about 5 m from the parallel edges of the lagoons, separated from the standing water of the lagoon by a raised embankment. The channel was observed to contain flow on every occasion the site was visited, including the dry-season samples in January and June 2020. Inspection on foot revealed that the channel was dry upstream of the uppermost treatment lagoon, including during the wet season, but that flowing or standing water was visible beginning at the upstream edge of the first lagoon, and flowing water was visible from the downstream end of the second lagoon through the discharge into Estero River some 100 m downstream. It is unambiguous that the flow originates as groundwater discharging from the vicinity of the lagoons. That does not mean that the flow is, or is not, satisfactorily treated before entering the Estero River, either in the package treatment operations, or by biodegradation in the soils, or some combination of the two.

Evidence is persuasive that FIB are not conveyed to the Estero River in subsurface groundwater, at least in the two neighborhoods sampled fo this study. The evidence is shown in Figures 8.1 and 8.2 above, where data from the subsurface groundwater (sites A01, A02, A03) for the most part do not show presence of FIB in large amounts, excluding samples believed to be contaminated (for reasons explained in Section 8). One persuasive feature is the site A02, drawing groundwater from about 2 m beneath the surface, where samples routinely were found to be less than 200 MPN/100 mL for enterococci. That site is directly adjacent to the flowing surface-groundwater drain Site G07 (about ½ meter away), which on multiple occasions exceeded the laboratory-maximum of 2,420 MPN/100 mL for enterococci. The high concentration of sucralose at those three sites is persuasive evidence that the groundwater flow, and the discharge from the drainage ditch, was routinely conveying treated human wastewater exiting the lagoon through the groundwater. The low concentration of FIB, at the same time as high sucralose, in A02 and A03 show that biodegradation in the soil is satisfactorily removing FIB as intended in the design of a treatment lagoon such as these.

On the other hand, the surface flows of discharging groundwater at Site G07 and Site G09 do appear to carry high concentration of FIB. At Site G07, in five of eight tested samples, *Enterococcus* were present at the laboratory-method maximum 2,420 MPN/100 mL. The high concentration of sucralose in four samples tested from Site G09 is strong evidence that the Estero Bay Village treatment lagoon is the ultimate source of FIB in that surface-groundwater drain, though the sporadic nature of the FIB and the fact that E. coli were absent in most samples suggests that the proximate source on most occasions may be enterococci that survive for some time in the soils of the ditch. That suggestion is supported by genetic sequencing data that showed surface-groundwater flow at G07 contains numerous species of sulfur bacteria, which originate from the sediment. The

oxygen-rich flowing water continuously carries sediment particles and sediment bacteria into the river, and in this location, it appears to be mostly enterococci that survive long enough to reach the Estero River. The G07 water was high in organic matter and nutrients, which may support survival of bacteria in the ditch sediments. Evidence from previous research suggests that both *Enterococcus* and *E. coli* can survive in these environments (Byappanahalli et al. 2012; Ishii et al. 2006; Jang et al. 2017). The high sucralose in G07, combined with the high *Enterococcus,* is strong evidence that the flow in that ditch does originate with the treatment lagoons; is not being satisfactorily treated either in the treatment system or in the soils; and the survival of enterococci in the soils of the ditch allows this to be a source of FIB to the Estero River.

Testing for the biological marker, via genetic sequencing, was conducted for the groundwater samples of this locality. There was high abundance of multiple anaerobic and microaerobic species of bacteria in all three soil-groundwater sample sites (A01, A02, and A03), in samples from at all times in which they were tested. That includes both the A03 site, adjacent to the treatment lagoon, which was uniformly found to be high in the target FIB enterococci and *E. coli*; and also of the A01 site, which had no examples of high concentrations of the two target FIB and essentially zero sucralose on each occasion when it was tested. That means that robust presence of multiple species of bacteria in the soil groundwater does not indicate either presence, or absence, of FIB or human waste, and may also not be associated with presence or absence of potentially harmful bacteria. On two occasions the surface-groundwater flow, from the ditch at D07, was also tested for the biological marker; the abundance of species in that flow was less than in the soil-groundwater samples, but greater than in the surface samples from the Estero River.

12.5 Groundwater at the Charing Cross Circle neighborhood

The Charing Cross neighborhood groundwater was investigated with one shallow-groundwater sampling site, at A05, directly adjacent (about ½ m) to a surface drainage ditch, sampled as site G09. That ditch was to flow in very small volume (about ½ to 2 cfs) on every occasion when the study team visited, including the dry weather visits in January and April 2020. That visual observation suggests the channel is conveying discharges from shallow groundwater in the neighborhood, where the residences are all served by septic systems.

At Site G09 in the Charing Cross neighborhood, *E. coli* were present in much greater amounts than *Enterococcus*, suggesting that human wastewater may be the ultimate source in that drain also, likely from septic systems in that neighborhood. This could suggest that in those particular soils, *E. coli* survive better than *enterococcus,* and succeed in reaching the Estero River. The presence of the two FIB in different amounts at the same location over different times – and at different locations in different amounts at the same time – originate from a complex interrelationship of differing sources, different survibability, and varying conditions in the environment. In both locations, it is suggested that FIB reach the two surface-groundwater flows or “ditches” because those “ditches” are so near the

wastewater sources – septic systems at Charing Cross, treatment lagoons at Estero Bay Village – that the flows can "short-circuit" the soils and reach the surface drainages in such a short time the FIB are not attenuated by biological and physical process in the soils.

However, data from the flowing channel sampled as G09 suggests that flow does convey FIBs. The site G09 on the graphs had *Enterococcus* at high concentrations on three occasions (between 1,500 and 2,420 MPN/100mL, out of seven occasions sampled. The same site was high in *E. coli* on five occasions – two of them at 2,000 MPN/100mL or greater, and three of them between 800 and 1,000 MPN/100mL. This is different than the results from the similar small channel studied in the Estero Bay Village neighborhood, where nearly every occasion was high in enterococci but only once in *E. coli*. No samples from G09 were tested for sucralose.

The overall pattern where more than half of the sampling events contained high MPN of one or both of the FIB demonstrates the small surface flow at Charing Cross is a source of FIB to the Estero River. Those findings are consistent with the channel conveying wastewater, which could originate with septic systems in the neighborhood if they should be "short-circuiting" the soils by reaching the small surface channel after spending too little time in the soil for FIB to be biodegraded by organisms in the soil.

Because these sites were added relatively late in the study period, no chemical tracer (sucralose) analysis was conducted for either the soil groundwater samples from site A05 or the surface-groundwater samples from the ditch site G09.

Two samples from G09 (September 18, 2019 and April 8, 2020) were analyzed for the biological tracer (genetic sequencing). The results do not definitively indicate either the presence or absence of non-human sources but do definitively indicate the presence of human sources. The two samples found a different relative abundance of various species during the two separate sampling times. This is further evidence for the finding that source events are episodic, short-term, and temporary: even in this location where the bacteria is believed to originate predominantly with septic systems, the ways in which those bacteria reach the surface appear to be episodic, such that septic facilities contribute flow the surface in different amounts at different times. That would argue against the likelihood of one or a few septic systems having failed or being operated improperly, and suggest instead that varying conditions of usage, precipitation, water table height, and other factors govern the transport of fecal bacteria in complex ways at different times.

12.6 Findings from This Study

A main goal of the project was to determine whether sampling of this type, with high spatial resolution (10 sites for one 5-mile reach) and limited snapshot occurrences (3 during dry weather and 4 during wet weather within one 13-month period) were capable of identifying locations where FIB might be entering the waterbody from source activities or conditions on or near the waterbody. The data succeeded in documenting that FIB

within the Estero River at various locations, at various times, do reach extremely high concentrations, which documents that some source activities or conditions do contribute FIB to the river. The extreme variability of FIB within the environment and the extreme variability of potential source activities and conditions preclude the possibility of identifying river-mile locations of sources, but the project did succeed in documenting that conditions on the Estero River do on multiple occasions lead to FIB concentrations well in excess of the regulatory standard.

The following findings accrue from the quantitative results.

1. Data support numerous previous researchers in documenting decoupled variation between different species of FIB: *E.coli* and *Enterococci* varied in ways that did not correspond to one another in nearly all samples. This finding supports the conventional wisdom that no one species is an ideal indicator of potential presence of bacteria originating with human waste. Our data suggested that both bacterial species had multiple sources, which likely vary both temporally and spatially. As both FIB species are present to varying extent in humans and in other organisms, and any group of humans or other species will have both of these, and other organisms, present in their wastes in ways that vary between individuals, and between groups, over time both within the digestive track and in environmental systems affected by the wastes of warm-blooded species.
2. Data on FIB in the waters of the Estero River varied spatially and temporally. In almost no cases – wet or dry seasons, or in any run-of-the-river sample – was the MPN either high (above 1000 MPN/mL) or low (below 200 MPN/mL) in all locations sampled. The data thus show that spatial variability within the stream at a given time is greater than variability between times. That finding indicates that high MPN counts can be triggered by highly local and short-term events, and it is not clear if those events endure for hours, days, or weeks or whether they may have dissipated within hours after the sample was collected.
3. FIB concentration variability due to tidal mixing and transport is believed to be powerful, but known to be highly complex in a southwest Florida water such as Estero River with low freshwater flow that experiences semi-diurnal tides (two tides daily, on most days) of variable timing and magnitude. Two wet weather samples, and one dry weather sample, appear to show higher concentrations in the downstream portion (approximately river miles 2 through 3) where we would expect tidal action to produce conditions of resuspension of deposited sediment, or of tides 'piling up' freshwater discharges in a way that might concentrate suspended sediments, or both. That portion of the river was sampled only three times during wet weather and twice during dry weather. The results suggest that one or the other of those mechanisms, or both, might contribute to high FIB concentration under some conditions but not all. It is not possible to attribute those results to either high or low tide, or incoming or outgoing

tide, because tidal conditions changed over the course of every 4-hour sampling event. Future research might further investigate that mechanism.

4. Routinely low FIB concentration in the upstream portion of the watershed strongly suggests there is little or no source from wild warm-blooded non-human animals in that undeveloped area. Increased (though highly variable) FIB concentration as the Estero River moves downstream through residential land uses indicates that either human activities, or animals coexisting with human activities, are the sources of FIB in the waterbody. As a point of comparison, data from two waterbodies studied by FGCU during this same time period in a nearby municipality (Spring Creek, Imperial River) showed that FIB concentrations were higher, though moderate, under most conditions in the upstream portions of the watershed, which have substantially higher development density than the Estero River reaches above river mile 6. Those two other waterbodies showed routinely increasing FIB concentrations as the streams moved downstream through developed residential areas. Those observations together with the Estero River data strongly suggest that dense residential land use corresponds to areas where bacteria enter the river.
5. The effect of several suspected source activities (small wastewater treatment facilities, septic systems, residential lawns used by pets extending directly to river's edge, and others) could not be reliably differentiated from other land uses, as there were no locations where persistent high concentrations were co-located with any of the suspected sources. The findings are consistent with all those sources, and more, contributing to the periodically very-high FIB concentrations on the Estero River.
6. It was expected that FIB concentration patterns would be different between wet-weather and dry-weather seasons. Instead, concentration patterns varied substantially among sampling events in each season, and no discernible pattern shows more variability between seasons than within seasons. The high variability of FIB concentration in the environment, and the high variability of source activities, outweighs any differences that may be produced by high or low in-stream flow diluting discrete discharges, or source-mobilizing action of precipitation events, in the samples collected for this study. Those effects may be present, but they do not influence the concentration at a given site or a given time to a discernible extent.
7. Although tested numbers of samples were small, our data showed river bed sediment, river bank soil, ditch water and road standing water harboured a large numbers of FIB and demonstrated that these could be potential sources of FIB input to the Estero River. Those sediments are not believed to be the point of origin of those FIB – they receive FIB from biological sources such as fecal matter originating with human wastewater or other warm-blooded animals – but short-term disturbance of river sediments, riverbank soils, or soil from the watershed mobilized by heavy precipitation – can theoretically trigger local and temporal high FIB events, and could be the proximal source of FIB measured in any one water sample.

8. Groundwater, in the areas studied, does not appear to convey large quantities of FIB to the Estero River, even though it does receive some human wastewater. That human wastewater appears to have any FIB satisfactorily attenuated by biological and physical activity in the soils before it reaches the river, and it is not likely that direct groundwater flows into the river are a major source of high MPN counts of FIB.
9. However, surface flows of discharging groundwater that has "short-circuit" the preferred underground path do appear to convey FIB to the Estero River. Surface flows in the "ditches" does not receive the same attenuation as groundwater; rather, the soil beneath the ditches appears to provide a stable environment for FIB, so that flowing water can re-suspend FIB and convey them to the river. It is not clear how large these contributions may be, or how many neighborhoods are drained by this kind of small surface discharge, but it could potentially be a significant source of FIB to the Estero River.

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Appendix A. Field and Laboratory Data