EXHIBIT "H"

Investigation on Fecal Indicator Bacteria, Estero River, 2019-2021

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Florida Gulf Coast University

The Water School

FGCU Estero River study July 2019 – March 2021

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Final Report to Village of Estero

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Student participation

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Fecal Indicator Bacteria in Three Tidal Streams in Southwest Florida: Temporal and Spatial Variation

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In the U.S., surface water is subject to regulatory limits for facal indicator bactaria (FIBs) including facal coliforms, E. coli, and Exterococci, which are intended to serve as indicators of cossible contamination with wastes and the negative health effects that can accompany it (USEPA, 2002). Sources include those known to cause regative health effects (leaking sewage lines or septic systems) and others not believed to affect human health (runoff from animal husbandry, facul matter from wild warm-blooded species). Research has established that FIB concentration varies by an order of magnitude or more, over time frames as short as 1 hour, distances as short as 100 meters, and storage of live Fits in soils of tropical waterbodies for months (boshim, 2007). Data from 20 years of sampling (Availone at al., 2020) show that tidal streams in urbanized parts of SW Florida have repeatedly, and by large magnitudes. averaged and the factorial standards of E cold and Enterior or in threatanet streams: Estero River, Spring Greek, and Imperial River (F.A.C. 602-304.800, 2019; Lapointe et al., 2018).

Objectives

- · Characterize spatial and temporal variability of two Fibs in tidal streams to improve undestanding of how to identify sources.
- Investigate whether source locations, variation due to environmental conditions, and long-term variability can be can be identified or nerowed down using infrequent sampling with high spatial resolution.

Methods

- · Sites selected for sampling allowed for greater residution, more closely spaced, than ever before on these waters: 10 stations within Sriver kilomaters. Several sites were directly downstream of accorded sources loackase plants or residences with servic systems).
- Temporal campling was not high resolution. Fills you on time frames lass. than one hour, and this research collected only 3 to 8 times during the 1-year research period.
- Samples included (wetseason) and 'dry season' events for each waterbody. recognizing the strong seasonal variation in Florida's aquatic systems.
- · Sample collection protocols were rigorous to avoid contamination by human hands, rapid bacteria de-activation by storing on ice, and delivery for lab analysis within 6 hours of collection to accurately quantify becteria.



Figure 1. Imperial River sampling sites. All three taget streams were samples with similar spatial resolution: 10 sites, along a river reach approximately 8km.



2.5 . 3.5 . 4.1 Figure 2. Estero River, one sample day, dry season: 4/8/20. All graphs are by river mile, increasing upstream from arbitrary datum at mouth.



Figure 3. Estero Kiver, one sample day, wet season: 7/28/20

Estero River graph is consistent with moderate upstream sources. declining downstream during wet weather as inflows dilute. It both seasons concentration is relatively high near the mouth at Estero Bay. Sites near small wastewater treatment plants on Estero River (miles 3.5-4.5) reached extremely high FI8 concentration, with both enterococci and a coli exceeding the method quantitation limit - but only during dry season, and not in every dry-season sample.

All target waterbodies showed high FIB concentrations near their mouths (sometimes exceeded near major upstream sources) suggesting. Estero Bay - the estuarine receiving water for all three - is a FIB reservoir and tidal action moves FIBs up into the streams. Therefied was mild or abant on some days, presumably varying with tide direction, magnitude, and timing.

Concentration of enterocoasi and e. and/correlate poorly with one another, to varying degrees. For example: similar in Spring Greek August 11 2020, ooke dialmiler in Imperial River December 18 2019.



the river was at high tide throughout the sample session.



Figure 5. Imperial River, one sample day, dry season, 11/10/19 - when the river was at low tide throughout the sample session.

In Imperial River, FIB concentration was much higher during the low tide sampled day, though that does not conclusively show it would behave that way at all low tides.



Figure 6. Spring Geak, one sample day, wet season: 8/11/20

Spatial variation in Spring Creak is consident with Fills originating with danse urban land uses (in uppermost reaches), then diluted by runoff and groundwater entering the stream in the middle reaches, and near the mouth receiving additional Fills from tidal action in the asturary.

Discussion

RB concentration variability due to tidal mixing and transport is believed to be powerful, but shown to be highly complex in these streams of low freshwater How that experience two tides daily of variable timing and magnitude. The limited sampling did not quantify tidal effects but did identify tidal vs upstream maches.

Infrequent sampling with 16 km spatial resolution was sufficient for initial identification of source regions. H8 variability occurs on even finar scalas so location information was not precise erough to verify discharge sources.

Temporal variation is such that sources were evident during some samples but not others - sufficient to trigger further investigations but not to support statistical analyses that would quantify loads or predict daily conditions.

Known and suspected sources of FBs on these streams are very small in magnitude but created some occasions of high in-stream concentration, well in excess of regulatory standards, some of them exceeding the method ovariation limit.

Conclusions

- Routinely low FIS concentration in an undeveloped upgrown watershed (Estero River) but high in developed upstream watersheds (Spring Creak, Imparial River) strongly suggests residential land uses are sources of Fits.
- High concentration downstream of suspected discharges on one stream (Estaro River), during dry weather, were strong evidence that they are FIB SHOWING ST.
- As expected, PID concentration patterns were different between wet-weather and dry-weather sensors. High Row appears to discte discharges, megating their effect, but makes inte difference to dispensed land-use sources or influence of tidal mixing from high-concentration estuary receiving waters.
- Data support numerous previous researchers in documenting decoupled variation between different species of Fills: EcoF and Enterococci varied independently in maily all samples

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Fecal indicator bacteria: FIB

- Designated by USEPA and FL DEP for water quality standards, two species:
 - Enterococcus
 - E. coli
- Surrogates for species that can cause human health effects
- Relatively easy to measure while potentially harmful species are very difficult to measure
- Highly variable in the environment, by time and place

Key findings

- High FIB numbers in upstream locations are evidence that human activities produce and/or enhance sources
- High FIB variability, temporally and spatially, confirms sources are episodic, short-term, varied in action/inputs: in aggregate, sources are substantial
- Variability within and between sampling events, affected by many factors, masks specific sources
- Frequency of high-FIB events, with presence of sucralose and microbe species found in humans, confirms presence of human waste AND other sources
- Groundwater does not appear to convey waste in soils
- Small, steady surface flows of groundwater may convey human waste into the Estero River

Outline: Evidence for Findings

- Historic data from Lee County monitoring
- Estero River "length of river" samples
- Groundwater sampled near river
- Surface stormwater backup, Sherrill Lane, one morning
- Sucralose, genetic sequencing: additional evidence
- Summary of key findings / weight of evidence



Frequency of high-MPN (high abundance) points: greater downstream than upstream *Enterococcus,* Three Oaks (upstream - top) and **Riverwoods** (downstream – bottom) Lee County Natural Resources, March 2015 – January 2020

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*



Ten sites on Estero River used for length-of-river sampling

- Lee County "Riverwoods" site is G10
- Lee County "Three Oaks" is upstream (right) of G06



E. coli also <u>greater</u> <u>downstream</u> <u>than upstream</u>

E. coli, Three Oaks (upstream – top) and Riverwoods (downstream – bottom) Lee County Natural Resources, June 2016-January 2020

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.*

Summary of 5 years historical data, 2 sites

		Enterocod	cci		E. coli				
	3 Oaks	Rt 41	Riverwoods		3 Oaks	Rt 41	Riverwoods		
n	58	58	58		42	42	42		
Median	40	345	214		59	236	350		
TPV = 90 th %ile	146	1,203	2,420		238	770	2,420		
TPTV	130	130	130		410	410	410		
Exceed- ences	8	28	39		2	12	20		
>1000 MPN	0	7	15		0	2	8		
> 2420 MPN	0	2	9		0	1	6		

Lee County Natural Resources, March 2015 – January 2020 TPV: ten percent value, i.e. 90%ile of data at a location TPTV: ten percent threshold value, regulatory numeric target for ten percent value at a location TPTV = 130 MPN/100mL for *Enterococci*, 410 MPN/100mL for *E. coli*

Surface water sampling, Estero River









Two dry-season "length of river" sampling days on the Estero River: 11/13/19, 4/8/20

Estero Bay Village is about mile 3.5 Sunny Groves is about mile 4.2



Ten sites on Estero River used for length-of-river sampling

- Lee County "Riverwoods" site is G10
- Lee County "Three Oaks" is upstream (right) of G06



Two wet-season "length of river" sampling days on the Estero River: 7/14/20, 7/28/20

Estero Bay Village is about mile 3.5 Sunny Groves is about mile 4.2



Groundwater sampling near Estero River



Groundwater samples



Groundwater at 5 piezometers and 2 surface flows



A01 through A05: near-surface groundwater (piezometers) – almost no *Enterococci* except A01 (believed erroneous)



Small surface drainages conveying groundwater – the "ditches"

Groundwater at 5 piezometers and 2 surface flows



G07, Estero Bay Village "ditch"; G09, Charing Cross "ditch:" convey groundwater year-round, routinely high *Enterococci*

Sample, stormwater ponding, Sherrill Lane north of Broadway, September 4, 2020: Very high FIB, other factors not surprising

<u>Constituent</u>	<u>Measurement</u>	
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%	
рН	7.58	
Electric conductivity	255 µS/cm	
Turbidity	24.1 NTU	

Sucralose

- Sucralose is a chemical tracer: originates in the environment almost exclusively with human waste
- Can be measured in ng/L, 10⁻¹² (parts per trillion)

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

	Electric																
			Sucralose		DO			cond	Turbidity	ORP	enterococci	E. coli (MPN/100	Ratio		NH4+	NOx (ug-	SRP (ug-
Date	FGCU ID	Sample type	(ng/L)	Temp (C)	(mg/L)	DO (%)	рН	(µS/cm)	(NTU)	(mV)	(MPN/100 ml)	ml)	(ent/ecoli)	TIN (ug-N/L)	(ug-N/L)	N/L)	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 <u>6</u> .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	з	.4 42	.0 6.4	6	2.7	30.6	2	0	2	4517	3823	694	932
10/30/2019	A02	Groundwater	<u>2231</u> 0.33	27.6	2	.430	.2 <u>6.</u> 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

Sucralose

- High concentration in A02 and A03: Estero Bay Village About 15 - 35 μg/L, or 15 - 35 x 10⁻⁹, parts per billion
- Expected to be high in groundwater near human use
- Not present at A01, A04, or A05 in soils (35 45 ng/L, i.e. 35 x 10⁻¹²) little human waste in those locations
- Tested 'ditch' at G07 also: surprised to find high concentrations (35 – 45 μg/L)
- Unusual in surface water evidence that flow in the 'ditches' is mostly groundwater recently reaching surface

DNA sequencing

- A biological tracer: Compare DNA in samples to a database, and identify the <u>species of microbes present</u> in samples of Estero River water
- Captures only those species of microbes present in sufficiently high numbers to be detected in the lab – i.e. presence means something; absence means nothing
- Species of microbes <u>in some cases might</u> correspond to the gut biomes of some species of warm-blooded animals
- When certain species of microbes are present, it might suggest a higher than random probability that waste from certain warm-blooded animals are present

DNA sequencing: Species of microbes that the literature identifies as "markers" that were present in Estero River samples, 2019-2020

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
<i>Rikenella</i> sp.	Chicken, Japanese quail, other birds

DNA sequencing

- Human waste is present in Estero River
- Waste from other species is present in Estero River birds, rodents, other mammals

Key findings

- High FIB numbers in upstream locations are evidence that human activities produce and/or enhance sources
- High FIB variability, temporally and spatially, confirms sources are episodic, short-term, varied in action/inputs: in aggregate, sources are substantial
- Variability within and between sampling events, affected by many factors, masks specific sources
- Frequency of high-FIB events, with presence of sucralose and microbe species found in humans, confirms presence of human waste AND other sources
- Groundwater does not appear to convey waste in soils
- Small, steady surface flows of groundwater may convey human waste into the Estero River

Key findings: advances in knowledge

- High variability masks sources: not able to pinpoint without intensive studies. Typical in US waters, perhaps more so in S FL tidal waters
- Seasonal variability (wet-weather vs dry-weather) is not so powerful as to be visible among other factors: tides, source variability, precipitation/mobilization, human activities, animal activities, sediment disturbances, etc.
- Variation affected by factors not discernible here: tides, precipitation patterns, lawn activities, sediment disturbances – including very small scale
- Presence of FIB in some soils and in riverbed sediments suggests sediments may be proximate sources for some sampling events, so may confound ability to identify originating sources
- The two regulatory FIB (*Enterococcus, E. coli*) do not track one another – need to monitor both to verify condition of Estero River



Additional slides follow – in case of questions



Intermediate location: Estero River at Rt 41 bridge *Enterococci* (top) and *E. coli* (bottom)

Lee County Natural Resources, June 2016-January 2020

Dashed line is TPTV, "ten percent threshold value," numeric target not to be exceeded by more than 10% of samples, 130 MPN/100mL *Enterococcus* and 410 MPN/100mL *E. coli*

Site name	River mile	FGCU unique identifier
Armada Ct below canal	2.31	G12
Estero Ct above tributary	2.56	G11
At boat launch near Broadway (Lee County 47A- 4GR)	3.17	G10
Below Tahiti	3.51	G01
At Koreshan boat launch	3.96	G02
At Sunny Grove	4.23	G03
Below Rt 41 bridge		
(Lee County 47A-15GR)	4.58	G04
At Sandy Lane bridge	4.95	G08
S Branch – Country Ck Dr bridge	5.52	G05
N Branch – Country Ck nr Candlewood Hollow	5.74	G06

Site name	River mile	FGCU unique identifier
Estero Bay Village near retaining wall	3.70	A01
Estero Bay Village near "ditch"	3.78	A02
Estero Bay Village near lagoon	3.70	A03
Koreshan near boat launch	3.96	A04
Charing Cross	2.90	A05
Estero Bay Village "ditch"	3.78	G07
Charing Cross "ditch"	2.90	G09

Groundwater at 5 piezometers and 2 surface flows



A01 through A05: near-surface groundwater (piezometers) – almost no *E. coli*

G07, Estero Bay Village "ditch", and G09, Charing Cross "ditch:" convey groundwater year-round, routinely high in *E. coli*

- 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.

- 1. 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.
- 2. 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.
- 3. It was expected that FIB concentration patterns would be different between wetweather 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.

Key findings

- 1. 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.
- 2. 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.
- 3. 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.

Image of a field sheet