4-10-2019

Identification of Bird-Associated Nonpoint Sources of Microbial Contamination in Sediments

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An Honors Thesis submitted in partial fulfillment of the requirements for Honors in Biology

By Savannah Mullins

Under the mentorship of Dr. Asli Aslan

Abstract

Fecal indicator bacteria (FIB), such as Enterococci, are commonly used to monitor the microbial contamination of recreational beach waters based on standards set by the United States Environmental Protection Agency. Sediment and sand may also harbor FIB and reintroduce these bacteria to the water column. Enterococci may be originated from various non-point sources such as humans and wildlife. Recent literature has shown that avian feces also harbor high concentrations of Enterococci. The purpose of this study is identify the relationship between Enterococci and avian-associated markers in sediments. Sediment samples were collected monthly from four sites at Kings Ferry Beach in Savannah, Georgia from October 2014 to September 2015. DNA was extracted from the sediment and the avian-associated marker, GFD, was quantified using quantitative polymerase chain reaction. The results indicate that there is a statistically significant correlation between the avian-associated marker and Enterococci. Higher concentration of Enterococci and GFD marker were detected in warmer temperatures ($p<0.001$, $p=0.009$ respectively). Currently, there are no established guidelines for monitoring microbiological contamination of the sediment. Our results suggest that Enterococci have the potential to be considered as an indicator for non-point source pollution in sediments.

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April 2019
Department of Biology
University Honors Program
Georgia Southern University

Acknowledgments

I would like to express my sincere gratitude to my thesis mentor, Dr. Aslan. Your advice and feedback were invaluable throughout the thesis project. Thank you to previous students of Dr. Aslan that collected water and sediment samples at Kings Ferry Beach.

I would also like to thank the faculty and staff of the Georgia Southern University Honors Program and the Department of Biology for their continued support and guidance.

Introduction

The health of coastal environments is important for both humans and wildlife benefiting from these environments. By 2020, it is expected that over 60% of the nation's population will be concentrated in the coastal shoreline (NOAA, 2013). Human activities causing greenhouse gas emissions, nutrient pollution due to agricultural activities, and microbial and chemical contamination through sewage and industrial effluents negatively affect coastal environmental health (Fleming et al., 2006). Fecal pollution of water also impacts human health as most of these areas are used for livelihood and recreational purposes. Exposure to contaminated water may cause gastrointestinal diseases, eye, and ear infections, primary amoebic meningoencephalitis, rashes, and wound infections (Boehm et al., 2009).

The microbiological quality of coastal waters in Georgia is particularly important to the livelihood of communities and the economy. The shoreline in Georgia is mainly used for shellfish production, recreation, and tourism; all of which are important to both state and local economies (NOEP, 2016). Most recent estimations reveal a 19% population increase in coastal counties by 2020 (NOAA, 2013) in Georgia, which means additional environmental stress in recreational waters.

The United States Environmental Protection Agency (USEPA) established guidelines to monitor the water quality at beaches based on epidemiological studies (USEPA, 2012) and states are required to monitor their recreational beaches on a routine basis. The USEPA recommends using Enterococci for beach monitoring (USEPA, 2012). These microorganisms are abundantly present in the feces of animals and humans.

Therefore, detecting these bacteria in water indicates fecal pollution (Brinkmeyer et al., 2015; NRC, 2004).

Monitoring Enterococci instead of identifying all pathogens in marine beaches has been the most reliable and economic approach for water pollution diagnostics (Griffith et al., 2009). One disadvantage of using indicator bacteria is that they may not provide information about the actual source of pollution. For example, both point (e.g. sewage leakages) and nonpoint (e.g. animal waste, farmland, boats, urban development) sources may introduce Enterococci into the aquatic environment. The recent literature has shown that while Enterococci are found the in human intestines, avian feces is known to harbor higher concentrations of this bacterium (Ahmed et al., 2016). This is particularly a problem from a beach management perspective since knowing the exact source of fecal pollution is crucial to mitigate the problem indefinitely.

One approach to overcome this issue is to apply microbial source tracking (MST). This method is a molecular method using quantitative polymerase chain reaction to identify fecal pollution sources, and relies on the relationship between certain fecal markers to have direct relationship with specific hosts (Harwood et al., 2014). A genetic marker associated with the host can be used to identify the source of microbial pollution. For example, *Helicobacter* spp. are commonly found in the intestines of avian species and GFD has been a well-studied avian-associated marker on Helicobacter 16S rRNA (Harwood et al., 2014). However, there is very limited knowledge on the distribution of this marker on beach sand and sediments and its relationship with Enterococci.

Kings Ferry Beach on Ogeechee River in Georgia has been under permanent health advisories for recreational activities due to high levels of Enterococci present in

water. The source of these high levels of Enterococci can originate from many point and nonpoint sources including, but not limited to sewage, farming, runoff, and wildlife. One non-point source can be sediment and sand at the beach which may harbor high levels of Enterococci (Phillips et al., 2011). Previous studies have shown that sand may hold pathogens and fecal indicator bacteria that are transferred to the water (Perkins et al., 2014; Shah et al., 2011; Vogel, et al., 2017). Therefore, there is a potential for sediment being another source of high Enterococci levels at Kings Ferry Beach.

Research Question

The purpose of this study is to identify the sources of high Enterococci in the sediments of Kings Ferry Beach by using an avian-associated MST marker. The findings of this study are expected to show the relations between non-point source pollution from birds and its contribution to high and persistent levels of Enterococci in sediments.

Methodology

Study Area and Sample Collection

Sediment samples were collected from four sites at Kings Ferry Beach from October 2014 to September 2015 (Figure 1). The coordinates of the locations are given in Table 1. Sediment samples were collected using a hand core. Using a sterile syringe, subsamples were collected from the top 1 cm of the sediment. All samples were transported to the laboratory on ice. The temperature and three days of total precipitation were collected from University of Georgia's www.georgiaweather.net website.

Enumeration of Enterococci in sediments

Sediment samples were weighed (10 g) and reconstituted in 90 ml of sterile deionized water. Bacteria were disassociated from sediment particles by using ultrasonicator for 30 seconds. The supernatant (10 ml) were mixed with 90 ml sterile deionized water. The Enterolert® media was added to the sample and dissolved completely. This mixture was poured into the Quanti-Tray 2000® and sealed. These trays were incubated at 41° C±0.5°C for 24 hours. The wells that fluoresce under UVB light were accepted as positive and final concentrations calculated from the MPN table provided by the manufacturer (IDEXX, IDEXX Laboratories, Inc., Westbrook, ME, USA). Sterile deionized water was used as negative control in each run for quality assurance and quality control.

Nucleic acid extraction

In the laboratory, 0.25 g of the sediment was weighed and placed in a tube. The DNA was extracted from these aliquots using MoBio PowerSoil® DNA Isolation Kit (Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, the 0.25 g of sediment was vortexed in a PowerBead Tube and 60 µl Solution C1 was added. The tube was vortexed again before being centrifuged 10,000 x g for 30 seconds at room temperature. The supernatant was collected and transferred to a clean 2 ml collection tube. After adding 250 µl Solution C2, the tube was vortexed and incubated at 4°C for 5 minutes. Following incubation, the tube was centrifuged 10,000 x g for 1 minute at room temperature and 600 µl of the supernatant was transferred to a clean 2 ml collection tube. After adding 200 µl Solution C3, the tube was vortexed and incubated again at 4°C for 5

minutes. Following incubation, the tube was centrifuged 10,000 x g for 1 minute at room temperature and 750 µl of the supernatant was transferred to a clean 2 ml collection tube. Then, 1200 µl Solution C4 was added and the tube was vortexed. Approximately 675 µl of the supernatant was transferred to a Spin Filter, centrifuged at 10,000 x g for 1 minute at room temperature, and the flow through was discarded. An additional 675 µl of supernatant was added to the Spin Filter, centrifuged at 10,000 x g for 1 minute at room temperature, and the flow through was discarded. The remaining supernatant was loaded in the Spin Filter, centrifuged 10,000 x g for 1 minute at room temperature, and the flow through was discarded. Following this process, 500 µl Solution C5 was added, the tube was centrifuged at 10,000 x g for 30 seconds at room temperature, and the flow through was discarded. The tube was centrifuged again at 10,000 x g for 1 minute at room temperature and the spin filter was placed in a clean 2 ml collection tube. Finally, 100 µl Solution C6 was added to the center of the filter, the tube was centrifuged at 10,000 x g for 30 seconds at room temperature, and the extracted DNA in the tube was stored at -20°C.

Quantitative polymerase chain reaction (qPCR)

For the quantification of an avian-associated marker (GFD), an SYBR Green assay that was previously published was used (Green et al., 2012). Briefly, genomic DNA for this marker was used for standard curve preparation (Ahmed et al., 2016). The concentrations of DNA were measured by spectrophotometry (Nanodrop 2000, Thermo Scientific, Wilmington, DE, USA). Serial dilutions of cell suspensions and plasmids (10^1-10^4 target sequences per 5 µl) were used to generate standard curves. The DNA extracts

of the sediment samples (5 µl) were amplified by Step One Plus Thermocycler (Life Technologies, Thermo Scientific, Grand Island, NY) in a total reaction volume of 25 µl. This mixture consisted of 12.5 µl Power SYBR® Green PCR Master Mix (Life Technologies, Thermo Scientific, Grand Island, NY), 2.5 µl of 2 mg/ml bovine serum albumin, 1 µM forward and reverse primers and 3 µl of the template (Green et al., 2012). Plates were sealed with an adhesive film, centrifuged for 30s, and placed into the thermocycler. The qPCR conditions for GFD were 10 min at 95 °C, followed by forty cycles of 15 s at 95 °C, 30 s at 57 °C. A melting curve analysis (the temperature was increased from 65 to 95 °C at 0.5 °C increments) was performed following the GFD assay to confirm amplification. Results were reported as copies/100 g for the GFD marker. Threshold values were set to 0.8. Filter blanks, plasmids, and no template control were used in each run for quality assurance and quality control.

Results

Occurrence of Enterococci and avian-associated marker in Kings Ferry sediments

The mean Enterococci in KF3 sediment was 243 MPN/100 g, with the highest concentration of 866 MPN/100 g, detected in June 2015. The mean Enterococci in KF4 was 10 times higher than KF3 (3336 MPN/100 g). The highest level detected at this site was in July 2015 with a concentration of 17697 MPN/100 g. The mean Enterococci in KF5 sediment was 7862 MPN/100 g, and the highest Enterococci detected at this site was in May 2015 with a concentration of 24196 MPN/100 g. This site had the highest Enterococci levels throughout the study. The mean Enterococci in KF6 sediment was 2307 MPN/100 g., and the highest Enterococci at this site was detected in December 2014 with a concentration of 6879 MPN/100 g (Table 2 & Figure 2).

GFD marker concentrations were lower than the Enterococci, but had a similar pattern in each site. The highest concentrations of GFD were also found at KF5 throughout the study; the mean was 164 copies/100 g and the highest concentration was detected as 1132 copies/100 g in November 2014. The mean GFD in KF3 sediment was 37 copies/100 g. The highest GFD level at this site was detected in September 2015 with a concentration of 83 copies/100 g. The mean GFD in KF4 sediment was 32 copies/100 g. The highest level detected at this site was in August 2015 with a concentration of 126 copies/100 g. The mean GFD in KF6 sediment was 67 copies/100 g. The highest GFD at this site was detected in June 2015 with a concentration of 109 copies/100 g (Table 2 & Figure 3).

The average Enterococci concentrations in the sediment across the sites for the year stayed above 70 CFU/100 ml, the standard level used for water quality control (GDNR, 2015) (Figure 4). Over the year, the average GFD level, for which there is not yet an established standard, was higher at the KF5 and KF6 sites than at the KF3 and KF4 sites (Figure 5).

The impact of controlling the septic leak on Kings Ferry Beach sediment

The septic leak on Kings Ferry Beach was controlled in March 2015. Prior to the control of the leak (October 2014 – March 2015), the KF5 sediment had the highest average concentrations of Enterococci and GFD marker at 3612 MPN/100 g and 228 copies/100 g, respectively. The second highest average concentrations were at KF6 with Enterococci at 2311 MPN/100 g and GFD at 61 copies/100 g. Enterococci concentrations

were 155 MPN/100 g at KF4 and 91 MPN/100 g at KF3. GFD marker concentrations were 28 copies/100 g at KF3 and 18 copies/100 g at KF4 (Table 3).

Following to the control of the leak (April 2015 – September 2015), the KF5 sediment remained the site with the highest average concentrations of GFD marker at 100 copies/100 g and Enterococci at 12113 MPN/100 g, more than three times higher than before the leak (3612 MPN/100 g). The second highest average concentration of Enterococci was 6518 MPN/100 g at KF4 followed by 2304 MPN/100 g at KF6 and 471 MPN/100 g at KF3. The second highest average concentration of GFD marker was 72 copies/100 g at KF6 followed by 50 copies/100 g at KF3 and 46 copies/100 g at KF4 (Table 4.).

Temperature and precipitation

Across the sites, average precipitation ranged between 0.71 and 0.77 inches. Precipitation levels peaked in February, April, June, and September of 2015 (Figure 6). The water temperature ranged between 6.5 to 30.5°C. (Table 5). Water temperatures were lower in the fall and winter seasons than they were in the spring and summer seasons (Figure 7). There was no significant correlation detected between precipitation and Enterococci or GFD marker. However, there was a significant correlation between water temperature and Enterococci ($p<0.001$) as well as GFD marker ($p=0.009$) (Table 6).

Relationship between bacteria and environmental factors

Using IBM SPSS Statistics 25 (Armonk, NY, USA), Pearson correlations were computed with four variables including average GFD marker concentration, Enterococci

concentration, precipitation level, and water temperature (Table 6). There was a statistically significant ($p<0.001$), moderately positive ($r=0.530$) correlation between avian-associated GFD marker and Enterococci levels (Figure 8). GFD marker and temperature were significantly correlated ($r=0.375$, $p=0.009$) (Figure 9). There was a statistically significant ($p<0.001$), moderately positive correlation between Enterococci levels and water temperature (Figure 10) as well. There were no meaningful correlations detected between any of the variables and precipitation level.

Discussion

Beach sediments can be impacted by either point sources such as sewage leaks (Mallin et al., 2007) or non-point sources such as wildlife droppings (Zhu et al., 2011). In our study, we found that both types of pollution sources were effective on the beach water quality. In the first half of the study, the sediments were influenced by a sewage leak which was controlled in March.

Across the sites sampled at Kings Ferry Beach, KF5 had the highest levels of Enterococci and concentrations of GFD marker regardless of the sewage leak. KF3 had the lowest standard deviation, indicating that, overall, there were consistently lower levels of Enterococci and concentrations of GFD marker at the site. Even though the sites are in close proximity, the composition of the sediment may have an impact on the concentrations of microorganisms. Hartz et al. (2008) found that *E. coli* and Enterococci can flourish in beach sediment and be transferred to the water by weather or tidal events. Similarly, Phillips et al.'s (2014) findings suggested that Enterococci can survive by being loosely attached to a substrate, like sand, and may persist due to biofilms which

stabilize the sand granules, protect the bacteria, and make it more difficult to remove from the sand.

KF5 is closer to the boat ramp where the avian species usually congregate and act as a potential source of fecal pollution. In a study on the management of boat head waste at a commercial marina, public and private boat docks, and a pier, secondary ruminant and avian sources were detected in 38% of samples (Mallin et al., 2010). The two highest average concentrations of GFD detected were 164.22 and 66.69 copies/100 g at KF5 and KF6, respectively. These sites are close in proximity and are located closest to the boat ramp and beach where the majority of recreational activity would occur. KF3 and KF4 are located further out where there may be less recreational activity. These results show the impact of man-made constructions on bird habitation and overall impact on water pollution.

The average Enterococci levels at all sites were above the recreational water beach action value of 70 CFU/100 ml and these concentrations increased after the control of the leak. It is well known in literature that bacteria tend to accumulate and persist in warm temperature sediments. Anderson et al. (2005) reported the tenacity of fecal indicator bacteria in sediment in a laboratory setting. More recently, Byappanahalli et al. (2011) found that *E. coli* and Enterococci can colonize and metabolize in tropical sediment. Both studies support the persistence of bacteria in sand as a source of pollution in the water column. Our findings also showed that GFD and Enterococci concentrations were significantly correlated with temperature (GFD $p=0.009$, Enterolert $p< 0.001$). The control of the sewage leak was completed in early spring. These findings indicate that the

warming temperatures during summer had a positive impact on the occurrence of fecal indicator bacteria and GFD marker, regardless of the leak.

There was a statistically significant relationship between the Enterolert levels and GFD concentrations at all of the sites. These results show that birds are a major contributor to fecal pollution in the Ogeechee River. Marion et al. (2010) found that humans are at an increased risk for gastrointestinal illness in recreational waters with increased levels of fecal indicator bacteria. In addition to an increased risk of gastrointestinal and acute febrile respiratory illnesses in recreational waters, Fleisher et al. (2010) reported evidence for a dose-response relationship between Enterococci exposure and skin illness. Bathers were 5.31 times more likely to report skin illness than non-bathers ($p=0.0001$).

Conclusion

Based on USEPA water quality standards, the level of Enterococci in sediment at Kings Ferry Beach adversely impact the quality of this inland beach. Identifying the sources of the bacteria is important for environmental, economic, and human health in the area. Strong correlations detected in this study support the evidence that non-point sources of pollution, such as avian fecal matter, can be a contributor to Enterococci occurrence in recreational waters. Establishing health risk associated with fecal pollution from non-point sources will be necessary for providing safe recreational waters.

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Tables

Table 1. Coordinates of each sample point.

Site ID	Latitude (N)	Longitude (W)
KF3	31.97728	81.28703
KF4	31.97747	81.28719
KF5	31.97814	81.28761
KF6	31.97842	81.28797

Table 2. Descriptive statistics of Enterococci and avian-associated marker (GFD) in Kings Ferry Beach sediments.

	Enterolert (MPN/100 g)				GFD (copies/100 g)			
Site ID	Min	Max	Avg	St. Dev	Min	Max	Avg	St. Dev
KF3	15	865.50	243.15	257.41	1.67	94.64	37.41	28.54
KF4	10	17697	3336.35	6439.42	1.67	144.55	32.19	33.36
KF5	10	24196	7862.40	7978.05	19.18	1409.29	164.22	66.84
KF6	20	6879	2307.15	2516.47	12.16	167.97	66.69	31.37

Table 3. Descriptive statistics of Enterococci and avian-associated marker (GFD) in Kings Ferry Beach sediments before the control of the septic tank leak (October 2014-March 2015).

	Enterolert (MPN/100 g)				GFD (copies/100 g)			
Site ID	Min	Max	Avg	St. Dev	Min	Max	Avg	St. Dev
KF3	15	176	91.08	60.12	1.67	73.10	27.78	21.69
KF4	10	373	154.58	141.67	1.67	76.06	18.44	17.51
KF5	10	12756.50	3611.92	5273.12	19.18	1409.29	228.23	424.24
KF6	20	6879	2310.58	3365.60	12.16	167.97	61.08	35.71

Table 4. Descriptive statistics of Enterococci and avian-associated marker (GFD) in Kings Ferry Beach sediments after the control of the septic tank leak (April 2015-September 2015).

	Enterolert (MPN/100 g)				GFD (copies/100 g)			
Site ID	Min	Max	Avg	St. Dev	Min	Max	Avg	St. Dev
KF3	213.5	865.50	471.25	277.73	1.67	94.64	49.78	32.15
KF4	25.25	17697	6518.13	8179.76	6.28	144.55	45.95	39.81
KF5	29.35	24196	12112.90	8298.36	48.71	164.07	100.21	31.17
KF6	26.75	4564.50	2303.71	1613.83	32.64	129.42	72.29	26.52

Table 5. Descriptive statistics of precipitation levels and temperature in Kings Ferry Beach sediments.

	Precipitation (in)				Water Temperature (°C)			
Site ID	Min	Max	Avg	St. Dev	Min	Max	Avg	St. Dev
KF3	0	3.60	0.77	1.12	6.50	30	19.01	7.79
KF4	0	3.60	0.71	1.09	8.55	30	20.23	7.77
KF5	0	3.60	0.71	1.09	8.60	30.50	20.27	7.76
KF6	0	3.60	0.71	1.09	8.70	29.90	20.25	7.71

Table 6. Relationship between Enterococci, avian-associated marker (GFD), precipitation, and water temperature.

		GFD copies/100 g	Enterolert MPN/100 g	Precipitation inches	Temperature °C
GFD copies/100 g	Pearson Correlation	1	0.530**	0.115	0.375**
	Sig. (2-tailed)		0.000	0.442	0.009
	N	47	46	0.47	47
Enterolert MPN/100 g	Pearson Correlation	0.530**	1	-0.279	0.547**
	Sig. (2-tailed)	0.000		0.061	0.000
	N	46	46	46	46
Precipitation Inches	Pearson Correlation	0.115	-0.279	1	0.215
	Sig. (2-tailed)	0.442	0.61		0.147
	N	47	46	47	47
Temperature °C	Pearson Correlation	0.375**	0.547**	0.215	1
	Sig. (2-tailed)	0.009	0.000	0.147	
	N	47	46	47	47

**. Correlation is significant at the 0.01 level (2-tailed).

Figures

Figure 1. Locations of the sampling points.

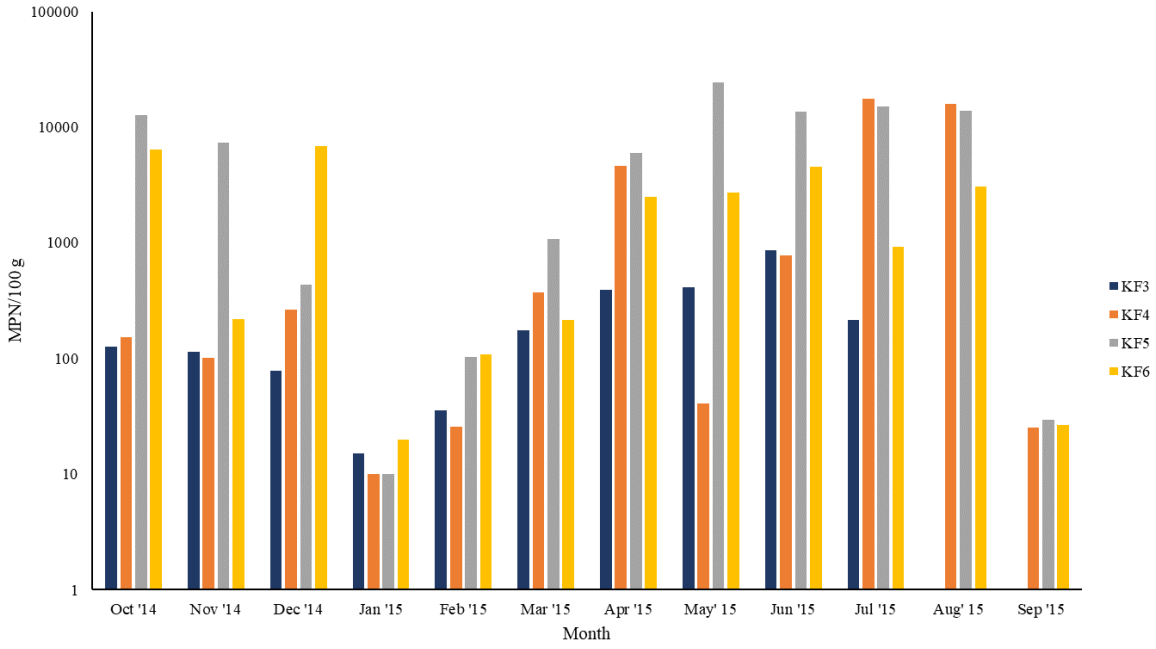

Figure 2. Monthly occurrence of Enterococci in Kings Ferry Beach sediments.

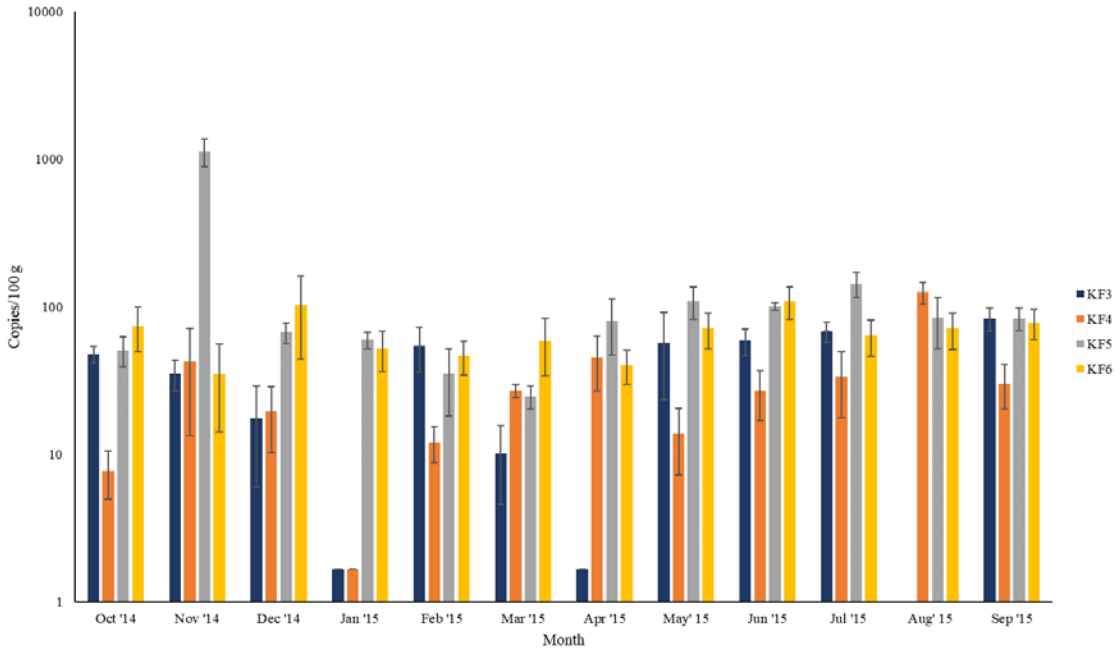

Figure 2. Monthly occurrence of avian-associated marker (GFD) in Kings Ferry Beach sediments.

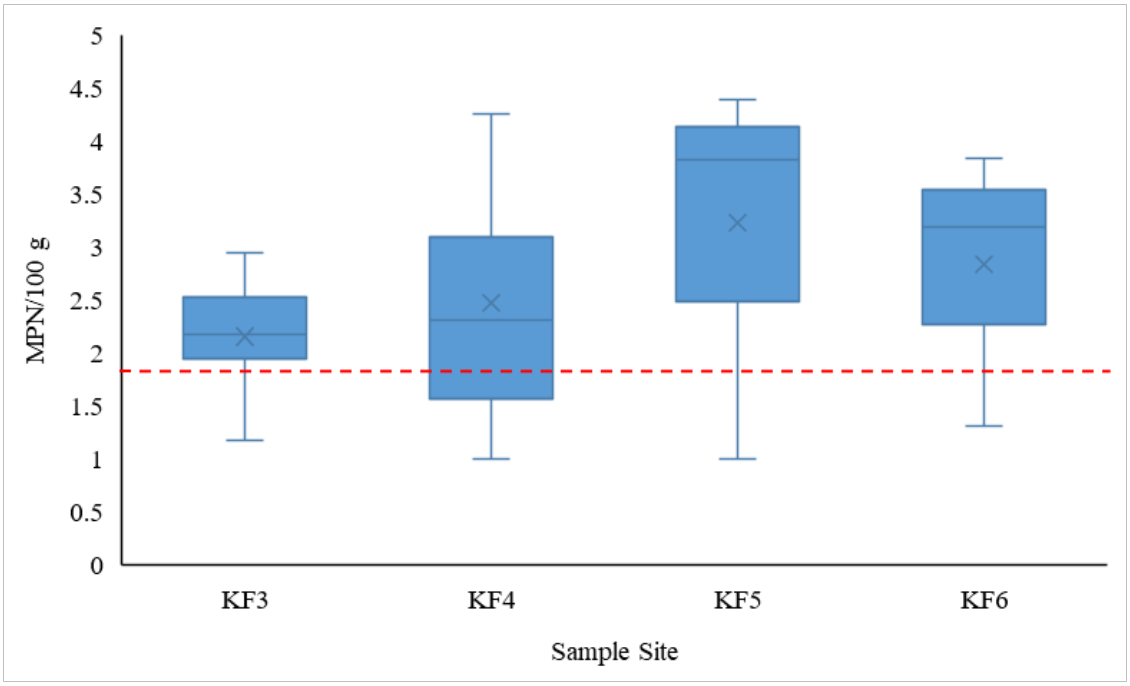

Figure 4. Box-whisker plots for Enterococci in Kings Ferry sediments. The boxes represent the ranges from the first quartile (Q1) to the third quartile (Q3) of the distribution; the line across the box indicates the median. The minimum and maximum values are illustrated as end points for the whiskers, and each outlier is represented by an individual circle. The red dashed line represents beach action value for swimming (70 CFU/100 ml).

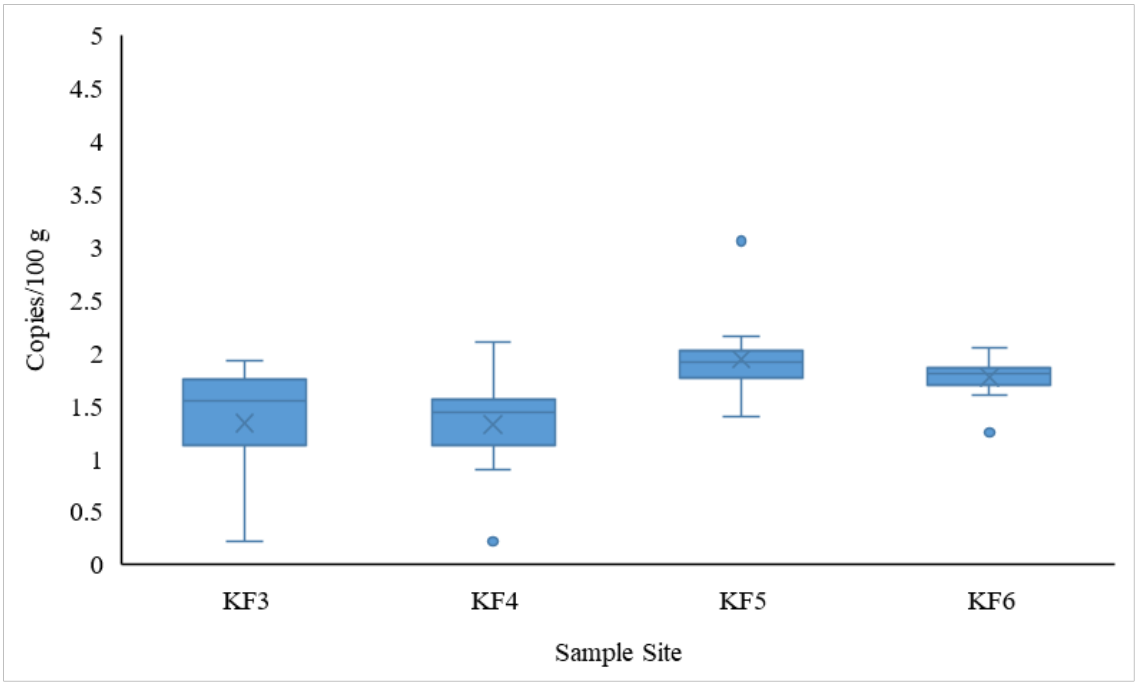

Figure 5. Box-whisker plots for avian-associated marker (GFD) in Kings Ferry sediments. The boxes represent the ranges from the first quartile (Q1) to the third quartile (Q3) of the distribution; the line across the box indicates the median. The minimum and maximum values are illustrated as end points for the whiskers, and each outlier is represented by an individual circle.

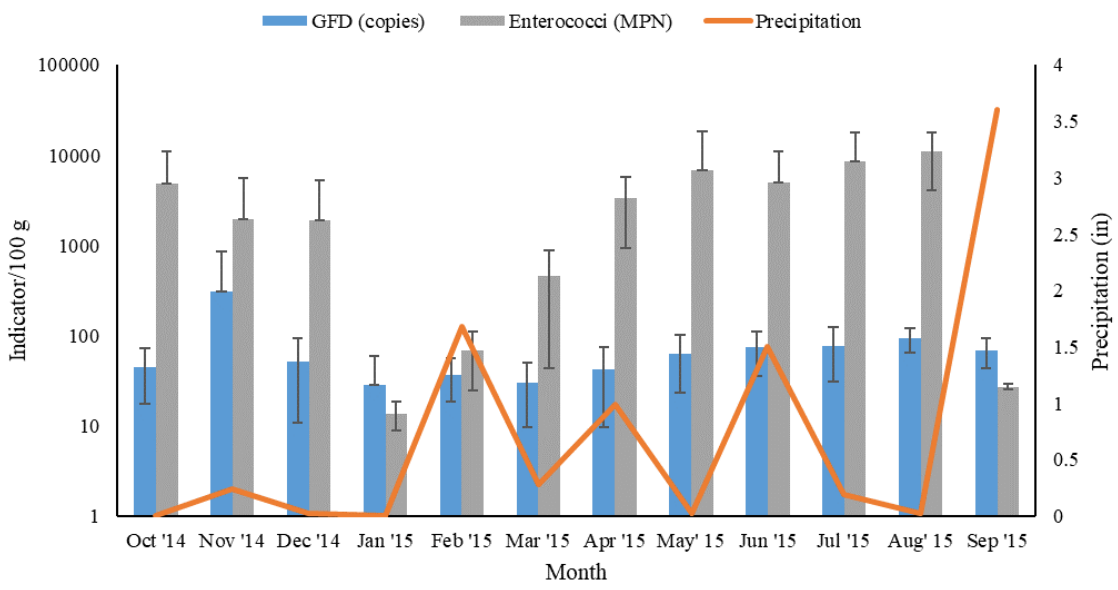

Figure 6. Mean microbiological data and precipitation over time at Kings Ferry beaches.

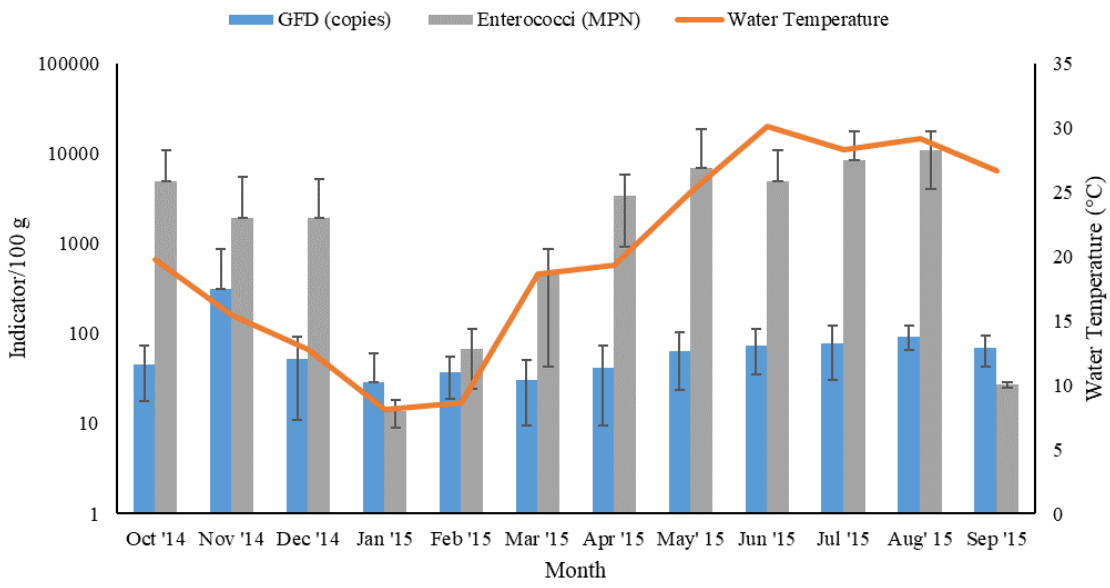

Figure 7. Mean microbiological data and water temperature over time at Kings Ferry beaches.

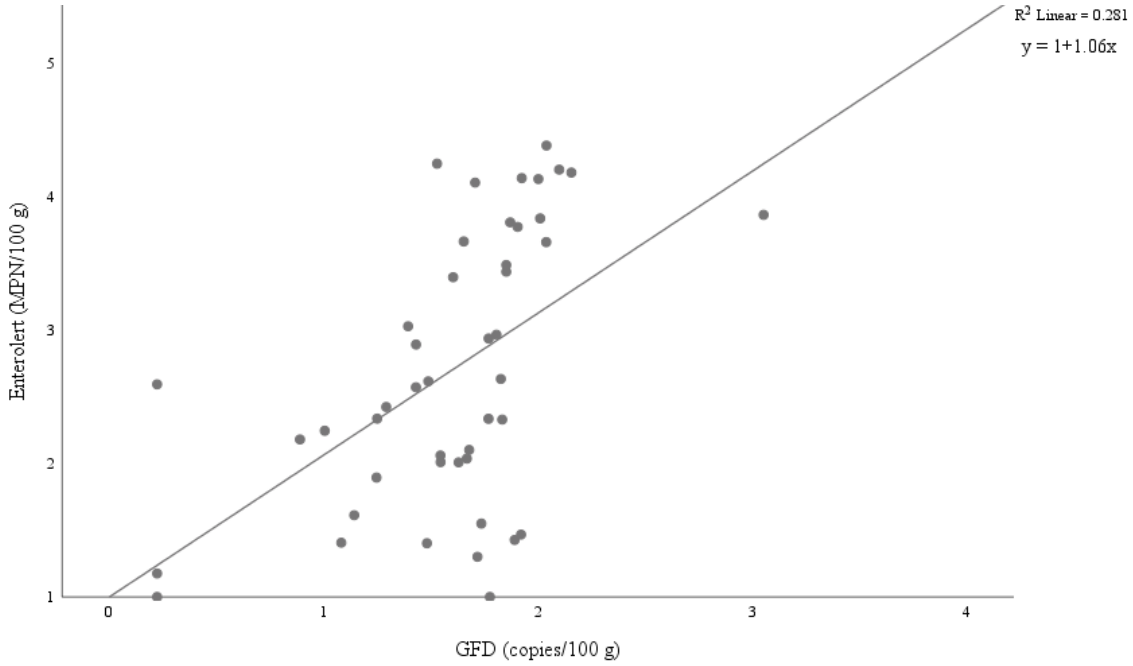

Figure 8. Relationship between Enterococci and avian-associated marker (GFD).

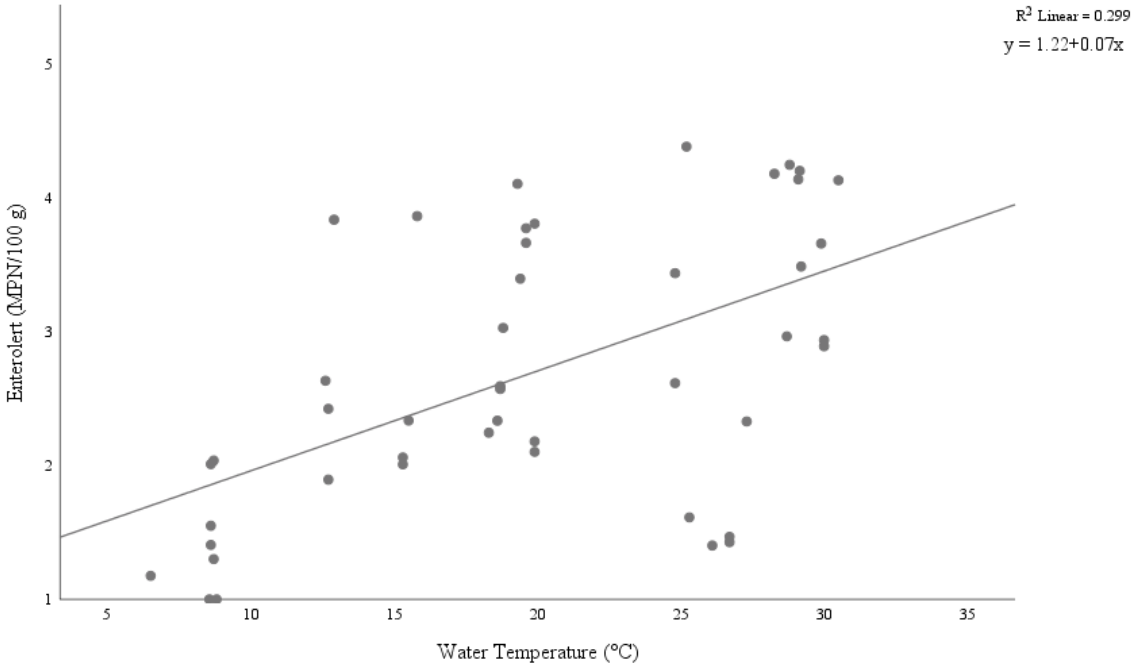

Figure 9. Relationship between Enterococci and water temperature.

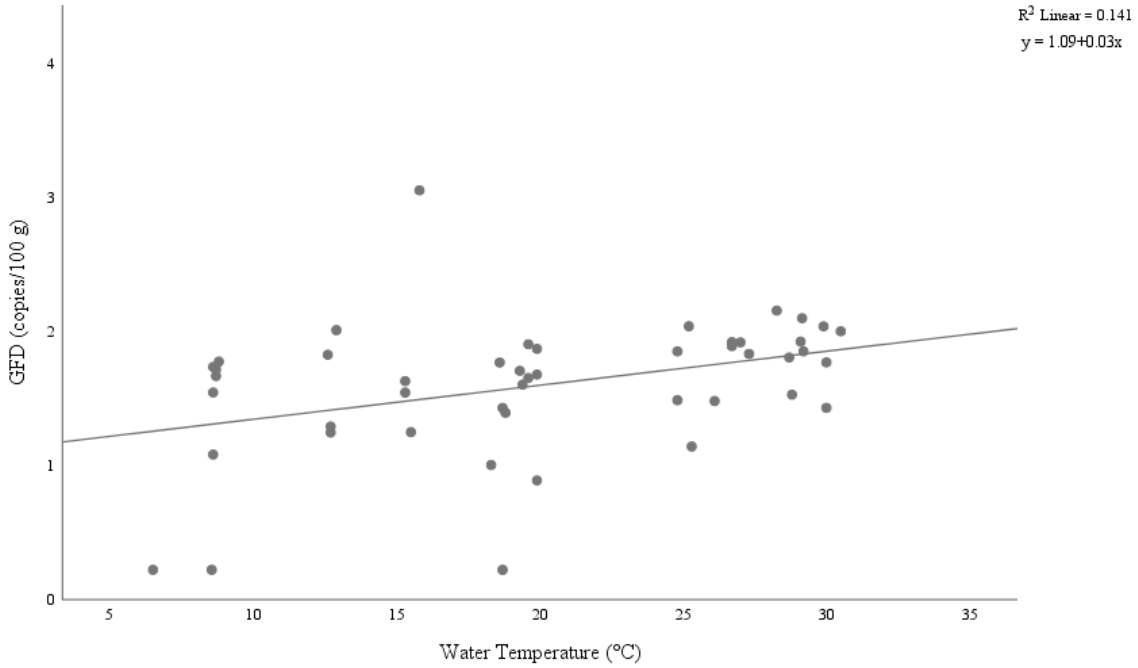

Figure 10. Relationship between avian-associated marker (GFD) and water temperature.