Testing the Appropriateness of USEPA's Proposed Enterococci Bacteria Fecal Indicator Criteria in the Near Shore Marine Waters of Apalachee Bay, Florida

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Introduction

As a result of the Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000, the U.S. Environmental Protection Agency (USEPA) mandated that state governments, authorized tribes, and U.S. territories adopt enterococci bacteria, a subgroup of the fecal streptococci group, as an indicator of human health risk in coastal recreational waters. The proposed saltwater enterococci standards are based on criteria citing epidemiological studies (USEPA 1986) that concluded enterococci counts of 35 colony-forming units (CFU)/100ml (mean of at least five samples over a 30-day period) and 104 CFU/100ml (single sample maximum) would result in a risk that 19 of 1,000 people would develop gastrointestinal illness (GI). Since the publication of the study, enterococci bacteria have been used by some states as an indicator for human health risk. However, the indicator has not been adopted by all states due to concerns with the design of the epidemiological studies and the application of the standard in untested environmental conditions.

The incidence of GI has been shown to positively correlate with the presence of enterococci in waters under the influence of storm water outfalls (Haile 1999), municipal wastewater (Cabelli 1982), and waters of poor quality (Fattal 1987). However, there are reasons to believe that this correlation may not be applicable to certain recreational waters, specifically, waters that do not receive direct inputs of anthropogenic pollution sources and waters that are located in tropical and subtropical climates (Fujioka et al 2003). In a study of the interstitial swash zone on bathing beaches in Pensacola, Florida results showed that enterococci counts in

sediments were greater than counts in the water column suggesting bacteria may accumulate, and possibly reproduce, in sediments (Boraski 2001).

Although elevated levels of fecal bacteria should warrant some level of caution, the actual risk to human health may differ depending on the source. Determining the source, however, is a complicated task. Early methods for determining source were developed with the goal of targeting specific indicators and species of bacteria including *Escherichia coli*, *'Enterococcus' faecalis*, and *Clostridium perfringens* (Cabelli 1938). At one time, *'E'. faecalis* and *'E'. faecium*, were considered to be more representative of human fecal contamination than other species of streptococci (Standard Methods 1992). When methods were developed to quantify these bacterial indicators, the results were not species-specific and the methods allowed other non-targeted bacteria to be quantified. These non-targeted bacteria are more commonly found as a part of wildlife and environmental sources although they are not exclusive to any one group.

This pilot study had three objectives: 1) to determine if concentrations of enterococci could be found that exceeded the proposed criteria in near shore coastal waters of Florida not under direct influence of anthropogenic sources, 2) to compare concentrations of enterococci in sediments and overlying water to examine the potential of sediment to act as a reservoir for fecal bacteria, and 3) to speciate randomly selected enterococci isolates to determine which species were being quantified.

Materials and Methods

The study included the estuary, tidal creeks, and sea grass beds of Apalachee Bay in the Saint Marks National Wildlife Refuge in the central panhandle of North Florida. The refuge has a total landmass of approximately 68,000 acres covering portions of Jefferson, Wakulla, and Taylor counties. The study location took place within the Saint Marks unit of the refuge in Wakulla County, Florida. The area in the vicinity of the sampling locations has no residential, agricultural, or commercial development. Recreational activities in the sampling area include wading, snorkeling, fishing, and boating, but the area is not considered a bathing beach. The nearest community is the town of Saint Marks located approximately eight miles north at the confluence of the Saint Marks and Wakulla rivers (Map 1).

Samples were collected in March, May, and September of 2003. Dissolved oxygen (mg/L), specific conductivity (µmhos/cm), pH (S.U.), and temperature (ºC) were measured

before collecting surface water and sediment samples. During each sampling event, three replicate samples from the water and sediment were taken for enterococci analyses at each sampling location (Map 2). Fecal coliform analyses were performed in parallel as a reference to Florida's current fecal indicator standards. At every replicate sampling location, surface water samples were collected before sediment samples to avoid capturing suspended sediment. Each water sample was collected in two pre-sterilized 100ml Whirl-Pak® bags. The total depth of the water was typically between 0.3 and 0.6 meters.

Sediment samples were collected using cellulose acetate butyrate (CAB) core sleeves. The cores and caps were cleaned with a 3% solution of Liqui-nox® detergent followed by a deionized water rinse prior to use. Sediment cores were collected from undisturbed areas of the bottom by manually pushing a 50.8 cm-long by a 5 cm-wide (OD) core sleeve to a depth of approximately 13 cm. Core samples were then transferred to large mouth jars for transport to the laboratory in the following manner. A polyethylene cap was placed on the upper end of the core sleeve and the core was extracted from the bottom. The lower end of the core sleeve was covered with a latex-gloved hand to hold the core in place while the upper cap was removed. The overlying water was slowly poured off with care taken to retain the surficial floc layer. Next, the core was extruded from the bottom of the core sleeve until only 2.5 cm of sediment remained in the core sleeve. This portion of sediment was collected into a sterilized 500ml wide-mouth glass jar, and the sampling process repeated, until approximately three-quarters of the jar contained sediment. The samples were capped with a screw top lid and placed in a cooler on wet ice and transported back to the laboratory for analysis. This same routine was performed at each replicate location.

In the laboratory, water samples were analyzed by the membrane-filter technique using mFC media for fecal coliform (DEP-SOP MB1.1) and mEA media for enterococci (DEP-SOP MB1.3). The sample filtration volumes were 50, 10, and 1 ml. Culture dishes for fecal coliform were incubated in a waterproof bag in a water bath at 44.5ºC for 24 hours. Enterococci culture dishes were incubated at 41ºC for 48 hours. Membrane-filters from the enterococci culture dishes were transferred to esculin iron agar (EIA) plates for 20 minutes at 41ºC and colonies displaying a brown-black halo were counted.

Sediment samples were analyzed by a modified multiple-tube technique. The methods used were based on the Most Probable Number (MPN) for enterococci (Standard Methods 9230 B) and for fecal coliform (DEP-SOP MB1.9). The procedure consisted of homogenizing the

sediment sample with a sterile metal spatula then adding gram-weight amounts directly to tared glass test tubes. A total of 15 tubes were incubated per sample with the following amount of sediment: Five tubes with 10 g, five tubes with 1 g, and five tubes with 0.1 g.

Enterococci sediment MPN tubes were incubated in azide-dextrose broth at 35ºC. Presumptive positive tubes were confirmed by streaking positive cultures on to a Pfizer Selective Enterococcus (PSE) agar plate and incubated at 35ºC. Gram stain and catalase tests were done for colonies showing a brown-black halo and were transferred to brain-heart infusion (BHI) broth and BHI with 6.5% NaCl incubated at 45ºC and 35ºC, respectively. Colonies from confirmed enterococci tubes were transferred to a BHI slant and allowed to grow out at 35ºC for 24 hours. The grow out culture was then suspended and homogenized in 0.3 ml of sterile dilution water. The suspension was then flooded on a Columbia sheep blood agar plate by swabbing the entire surface of the agar with the suspension. The blood agar plate was incubated at 35ºC for 24 prior to colony selection and speciation. (API 20 Strep, bioMérieux, Inc.). Due to limited lab resources, only sediment enterococci colonies were speciated. Fecal coliform sediment MPN tubes were incubated at 35ºC in lauryl tryptose broth (LTB). Presumptive positive tubes were confirmed after incubation in EC media at 44.5ºC for 24 hours. The Most Probable Number (MPN) was determined from the combinations of positive tubes based on the MPN index (Standard Methods 1992, p. 9-50).

Results and Discussion

To determine if surface water enterococci concentrations exceeded USEPA's criteria, sample results were compared to the proposed marine enterococci standard of 104 CFU/100ml. Of the eleven surface water samples that were analyzed during the project, two of the samples exceeded the proposed standard (Table 1). The highest surface water enterococci counts were found during the March sampling event. Sample #2 and Sample #3 had counts of 136 and 122 CFU/100ml, respectively. These samples were taken following a period of rain prior to sampling that caused some flooding within the refuge. The amount of rainfall recorded 24 hours prior to sampling was between 0.75" to 1.0" (Table 4). During periods of little or no rainfall, surface water enterococci counts dropped to well below the USEPA standard. The surface water concentrations during the months of May and September ranged from less than one colony to 8 CFU/100ml.

It is widely known that elevated bacterial counts are commonly found in surface waters following a rain event. The source of the elevated counts can be attributed to anthropogenic or non-anthropogenic sources or a combination of the two. In this case, the elevated counts are believed to have originated from the increased flow from the Saint Marks Refuge estuary. Due to the remote location of the refuge, water draining from the Saint Marks Refuge is not expected to have high bacterial counts from anthropogenic sources; therefore, elevated counts in this area may not be indicative of an increase in risk to human health. The current Florida standard for fecal coliforms in Class III surface waters states that no more than ten percent of the samples can exceed 400 CFU/100ml. Surface water fecal coliform counts sampled at the same time and location as the enterococci samples showed a consistently low recovery for all three sampling events (Table 2). None of the fecal coliform counts in surface water exceeded 40 CFU/100ml. Under current Florida rule, these waters were not in violation of water quality standards for fecal coliform bacteria even though the enterococci did show that, on at least two occasions, the enterococci standard was exceeded.

It is suspected that enterococci introduced into the bay during rain events may persist within the sediments. To examine the potential of sediment to act as a reservoir for fecal bacteria, sediment counts for enterococci and fecal coliform were compared with water counts taken at the same time and location. Sediment enterococci counts ranged from 14 to greater than 1,609 MPN/100g over the course of the study (Table 1). Enterococci counts in all but one of the sediment samples were greater than the counts found in the corresponding water samples. High sediment enterococci counts were found in both March and May, whereas high water counts were only observed in March. Fecal coliform counts in sediment showed initially high levels in March but did not maintain high levels through May as shown with enterococci. It is believed that the fecal coliform levels dropped because fecal coliform bacteria are not as robust in saltwater as are enterococci.

The analytical method upon which the USEPA criteria are based was developed to isolate bacterial indicators thought to be most closely associated with human fecal contamination (*E. faecalis* and *E. faecium*). The speciation results show that seven species were isolated, including one unknown, from the sediment samples during the course of the study (Table 3). A total of 40 isolates were speciated for the March sampling event, 63 for May, and 39 for September. The species most frequently isolated during March and May was *Enterococcus gallinarum*. The bacterium *E. gallinarum* is most commonly associated with avian wildlife, not human fecal sources. This is significant because if we are to apply human health risk factors to total

enterococci values that include non-human sources of enterococci, there is a potential to overestimate the risk to recreational users.

Due to the cost and time required to do the speciation, no attempt was made to quantify the relative proportions of the enterococci recovered from the sediments. Therefore, the reported identifications can suggest only that, at times, wildlife sources of enterococci may dominate the enterococci count.

Conclusions

Enterococci concentrations can exceed USEPA's proposed single-sample maximum saltwater criteria of 104 CFU/100ml in coastal waters not under the direct influence of anthropogenic pollution sources. In this investigation, the samples exceeding the proposed criteria were found following a rainfall event. The predominant input of the enterococci bacteria is believed to have originated from wildlife sources due to the rain-induced flushing in the Saint Marks estuary. Although some risk must be attributed to pathogens associated with bacteria from wildlife sources, the level of risk is assumed to be less than that of fecal pollution from human sources. None of the samples tested for fecal coliform exceeded Florida's current bacteria water quality standard of 400 CFU/100ml.

There were notably higher concentrations of enterococci detected in sediments as compared to the concentrations in water in March and in May. For fecal coliforms, sediment concentrations largely surpassed water concentrations in March only. It is reasonable to suggest that the potential exists for bacteria to re-enter the water column during storm events, confounding the interpretation of enterococci and fecal coliform results as indicators of recent human fecal contamination.

Speciation of enterococci isolates from sediments collected as a part of this study determined that at least seven species (*E. gallinarum, E. casseliflavus, E. durans, E. faecalis, E. faecium, E. avium,* unidentified spp.) of enterococci were present. Several of these species are predominantly from wildlife sources (e.g., *E. casseliflavus*, *E. avium*, *E. gallinarium*, *E. durans*). Of the isolates that were speciated, E*. gallinarum* was the most frequently identified. Since the USEPA's enterococci criteria are based on a correlation between human pathogens and human-

associated enterococci, the fact that other species of enterococci were found besides *E. faecalis* and *E. faecium* leads us to believe that the application of the enterococci criterion in near-shore, wildlife-influenced marine waters may overestimate the illness risk to recreational users. Additional study is needed to determine the health risk associated with wildlife bacterial sources and the importance of other environmental variables such as tide, sediment composition and nutrient concentrations, on the ability of enterococci to persist in the near-shore marine environment.

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Appendix A: Tables

Table 1. Summary of Enterococci Concentrations

Sample Date	Sample Type	Units	Sample 1	Sample 2	Sample 3	Sample 3 Field Dup
10Mar2003	Water	CFU/100ml [1]	28	136	122	-NA-
	Sediment	MPN/100g [2]	920 (300-3200) [3]	>1609 -NA-	34 (12-93)	-NA-
5May2003	Water	CFU/100ml	4	4	ND [4]	ND
	Sediment	MPN/100g	350 (120-1000)	>1609 -NA-	540 (120-1000)	1609 (640-5800)
15Sep2003	Water	CFU/100ml	8	4	8	6
	Sediment	MPN/100g	14 -NA-	33 (11-93)	17 (5-46)	17 (5-46)

1. CFU = Colony Forming Units
2. Most probably number (wet weight)
3. 95% confidence interval
4. Not detected

Table 2. Summary of Fecal Coliform Concentrations

Sample Date	Sample Type	Units	Sample 1	Sample 2	Sample 3	Sample 3 Field Dup
10Mar2003	Water	CFU/100ml [1]	10	20	40	-NA-
	Sediment	MPN/100g [2]	920 (300-3200) [3]	>1609 -NA-	350 (120-1000)	-NA-
5May2003	Water	CFU/100ml	4	16	2	2
	Sediment	MPN/100g	23 (7-70)	23 (7-70)	ND [4]	ND
15Sep2003	Water	CFU/100ml	12	4	26	16
	Sediment	MPN/100g	140 (37-340)	280 (90-150)	34 (12-93)	26 (9-78)

1. CFU = Colony Forming Units
2. MPN = Most probably number (wet weight)
3. 95% confidence interval
4. Not detected

Table 3. Summary of Enterococci Speciation Results in Sediment

Sample Date	Sample 1 Species (# isolates)	Sample 2 Species (# isolates)	Sample 3 Species (# isolates)	Sample 3 Field Dup Species (# isolates)
10Mar03	E. gallinarum (5) E. casseliflavus (3) E. durans (3) E. faecalis (2) E. faecium (1) E. avium (1)	E. faecalis (4) E. faecium (3) E. durans (3) E. gallinarum (1) E. avium (1)	E. gallinarum (4) E. durans (3) E. faecalis (2) E. casseliflavus (2) E. faecium (1) unknown (1)	-NA-
5May03	E. gallinarum (12) E. faecium (3)	E. gallinarum (11) E. avium (2) E. faecalis (2)	E. gallinarum (8) E. faecium (8)	E. gallinarum (11) E. faecium (5) E. faecalis (1)
15Sep03	E. faecalis (7) E. avium (1)	E. avium (6) E. faecalis (3) E. faecium (2)	E. avium (5) E. faecalis (5)	E. faecalis (9) E. gallinarum (1)

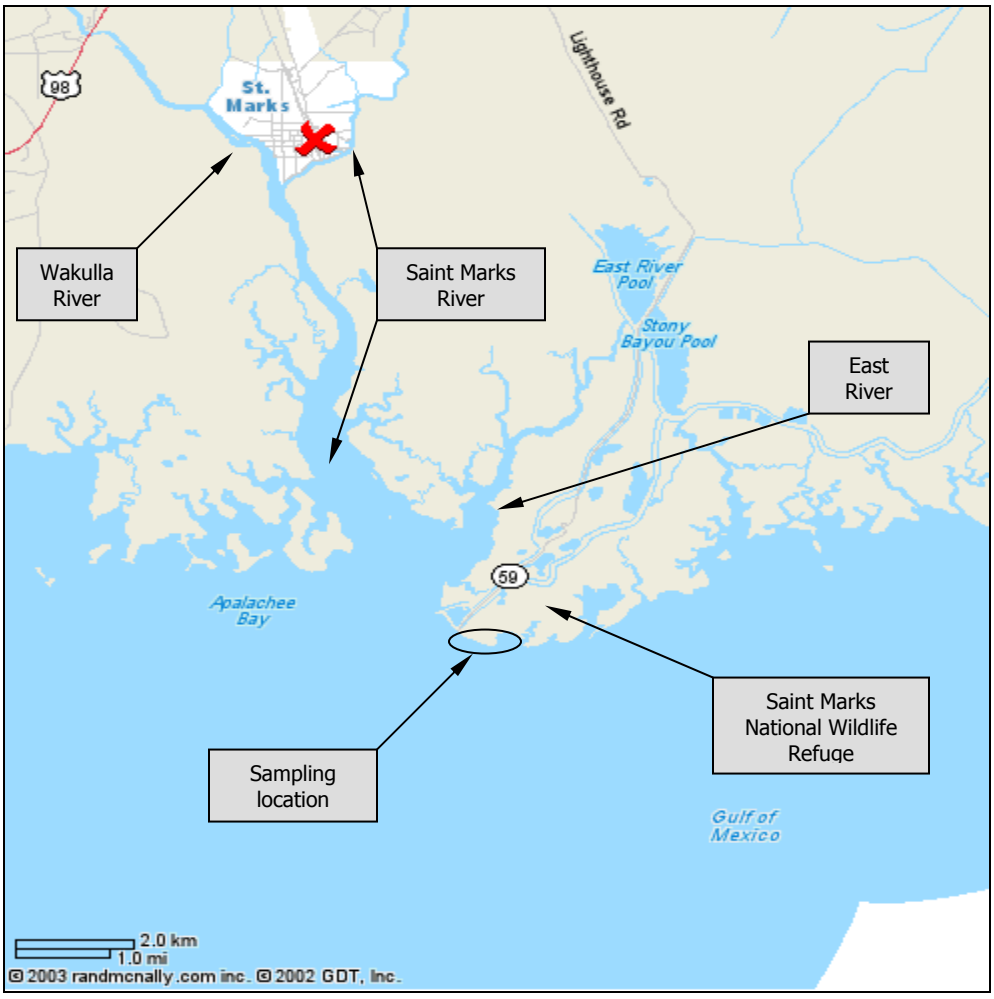
Table 4. Physical-Chemical Data

Sample Date & Time	pH	Water Temp (°C)	Diss. Oxygen (mg/L)	Specific Cond. (µmhos/cm)	Salinity (‰)	Tide (time)		Tide (stage)	24-hr Rainfall
						High	Low		
10Mar03 10:05a	7.46	18.4	6.98	26338	18.7	1715	2419	Slack high (1.5')	0.75-1.0"
5May03 10:00a	7.83	25.4	6.07	35132	21.9	1616	2340	Slack high (1.5')	NR [1]
15Sep03 10:53a	7.50	27.8	7.06	29934	17.4	0446	1130	Low (0.7')	NR

1. No rainfall

Appendix B: Maps

Map 1. Regional map of Apalachee Bay showing town of Saint Marks



Map 2. Map of sampling area in Saint Marks Wildlife Refuge

