INVESTIGATION OF ESTUARINE SEDIMENT AS A RESERVOIR FOR SEWAGE ASSOCIATED BACTERIA

A Final Report of the Tibor T. Polgar Fellowship Program

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ABSTRACT

The Fecal Indicator Bacteria (FIB), *Escherichia. coli* and *Enterococci*, are commonly used by Hudson River monitoring programs to quantify the level of sewage pollution in estuarine water and to provide information about the health risk to recreators from sewage associated bacteria. It is generally assumed that these FIB do not persist in the water for extended periods of time and that their presence represents a recent release of sewage into the environment. However, much less is known about the abundance and environmental persistence of FIB in sediments, as opposed to the water column, of the Hudson River Estuary. In this study, FIB were quantified, using cultivation-based techniques, in water and sediment samples collected from six locations in the estuary, and the persistence of FIB in sediment was investigated in laboratory incubation experiments. FIB were found to be widely distributed in both sediment and water from the estuary. *E. coli* and *Enterococci* displayed correlated abundances in sediment, consistent with sewage pollution as a shared source for both FIB in the environment. However, the levels of FIB were not correlated in paired water and sediment samples collected simultaneously from the same sites, suggesting that environmental persistence of these FIB differs in water versus sediment. *Enterococci* concentrations were found to decrease over time in laboratory incubations of estuarine sediment but remained at detectable levels for weeks after collection. In order to confirm the presence of *Enterococci,* and rule out the possibility of false positives from the cultivation-based assay, isolated bacterial colonies were characterized using molecular genetic techniques and the vast majority (96%) were confirmed as *Enterococci*.

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INTRODUCTION

Fifty years ago, the lower Hudson River and connected waterways surrounding New York City were commonly considered to be inappropriate for any recreational contact due to extensive pollution, with the river often acting as an open sewer for the surrounding population. Enforcement of the Clean Water Act and major investment in wastewater treatment facilities since the 1970s initiated a trend of improving water quality (Steinberg et al. 2004; NYC DEP 2009). Long-term environmental monitoring data from New York Harbor clearly document the prior history of poor water quality and the resulting improvements in mean seasonal water quality in recent decades (Brosnan and O'Shea 1996; Hetling et al. 2003; Brosnan et al. 2006; NYC DEP 2009). Along with improvements in water quality, there has been a widespread increase in recreational use of the Hudson at official and "unofficial" swimming beaches (Lawler, Matusky, and Skelley Engineers 2005), and management action aimed at re-claiming the waterfront and increasing public access to the river (New York-New Jersey Harbor and Estuary Program 2013; City of New York 2013).

Despite improvement in wastewater treatment infrastructure and in seasonal water quality, raw and partially treated sewage continues to enter the river and continues to be a management challenge. It is estimated that approximately 27 billion gallons of stormwater, mixed with raw sewage, are still released each year into New York Harbor through Combined Sewer Overflow (CSO) events (NYC DEP 2010). Numerous studies from other aquatic systems have demonstrated that waterways contaminated with sewage contain potentially pathogenic strains of microbes such as *Salmonella*, *Campylobacter*, and *Escherichia coli* 0157:H7 (Obiri–Danso and Jones 2000; Walters et al. 2007).

Similarly, a recent study in the Hudson River Estuary found that the abundance of antibiotic resistant microbes was correlated to the concentration of Fecal Indicator Bacteria (FIB) and that levels of these bacteria increased following rainfall (Young et al. 2013), presumably due to sewage discharge from CSOs.

Increased public access to the waterfront and improved mean seasonal water quality has led to a widespread demand from the public for more detailed water quality data. *Enterococcus* is an Environmental Protection Agency (EPA) approved FIB used for recreational water quality management whose presence in water has been shown to correlate with the occurrence of gastrointestinal illness in recreators (US-EPA 2004). Multiple monitoring programs in the lower Hudson River (NYC DEP 2013; New York Water Trails Association 2013; Riverkeeper 2013) now collect data on the concentration of FIB, and distribute these data to the public. *Enterococci* are commonly detected in the Hudson River Estuary (HRE) at elevated levels, with 21% of water samples (from 75 locations in the lower HRE) tested from 2006 through 2010 deemed unacceptable by EPA standards for primary contact recreation (Riverkeeper 2011).

Microorganisms released into the coastal environment are subjected to numerous stressors such as temperature change (Davies et al. 1995; Thomas et al. 1998), salinity (Mezrioui et al. 1995), nutrient deficiencies (Ozkanca and Flint 1997), and sunlight (Sinton et al. 1999). As a result, FIB and other sewage-associated bacteria are generally not thought to live for very long in the water column. In fact, short environmental persistence is a desired characteristic of FIB, because their detection is intended to reflect the recent input of sewage to the environment. However, in the HRE water column a high percentage of *Enterococci* are attached to particles, (Suter et al. 2011) and settle out

of the water column to the underlying sediment more quickly than "free-living" bacteria. Environmental conditions in the sediment are quite different than in the water column, including reduced sunlight, protect against predators, increased nutrient and organic carbon availability, and increased colonizable surfaces (Brettar and Holfe 1992; Davies et al. 1995; Blumenroth and Wagner-Dobler 1998; Sinton et al. 1999). In combination, sediment conditions may lead to increased environmental persistence for FIB, as compared to the water column (Lee et al. 2006).

For the past two years, data on the persistence of sediment associated *Enterococci* and *E. coli* in the Hudson River has been collected and analyzed in the O'Mullan laboratory at Queens College and the Juhl laboratory at Columbia University. Sediment samples collected from the environment in areas impacted by sewage pollution and incubated under a range of temperatures in the laboratory suggest that cultured FIB can remain at detectable levels for weeks to months in Hudson sediment (O'Mullan and Juhl, unpublished data).

Although local monitoring programs typically only measure FIB in water, recreators may also come into contact with contaminated sediment. In addition, the turbulence from large storm events and disturbance from boats or recreators may reintroduce sediment FIB, and associated pathogens, back into the water column creating a connection between water quality and sediment quality. Therefore, studying the persistence patterns of FIB in the all compartments of the environment, including sediment, is imperative to understanding the ecology of sewage associated bacteria in the environment and to interpreting water quality patterns from local monitoring programs.

The goals of this study were to: 1) quantify FIB abundance in sediment and water from six locations in the estuary, using cultivation-based approaches; 2) to determine if FIB abundance would be correlated in paired water and sediment samples; and 3) to use DNA-based assays to confirm that FIB enumerated, with cultivation-based methods, from sediment in laboratory persistence experiments were correctly identified as *Enterococci*. The hypothesis was tested that FIB would be widely distributed in sediment samples from the estuary, but that their abundance would not be well correlated in paired water and sediment samples due to longer FIB persistence in sediment relative to water. In addition, the hypothesis was tested that the majority of isolated colonies characterized using DNA based assays would be confirmed as *Enterococci* and that FIB cultivation-based methods applied to sediment would not be prone to false positives.

METHODS

Paired Water and Sediment Field Sampling

Water and sediment samples for FIB analyses were collected from six field locations in Flushing Bay, Sparkill Creek, and the lower Hudson River (Figure 1) between late May and mid-July in 2012. Samples were collected four to six times from each of the six field sites. Approximately 40 ml of surface water was collected from along the shoreline (depth of less than 0.3 m), just above the paired sediment sample that was also collected (see below), into sterile 50ml plastic tubes that had been triple rinsed with water from the environment. A surface sediment core (approximately 20 ml from the top 3 cm of sediment) was collected using a modified sterile 60ml syringe barrel as a coring device, along with a metal putty knife to help retain the core in the syringe barrel

during collection. The sediment was then extruded into a sterile 50ml tube for storage and transport to the laboratory. Both water and sediment sampling tubes were placed into a cooler, away from light, and transported to the laboratory for processing within six hours of collection.

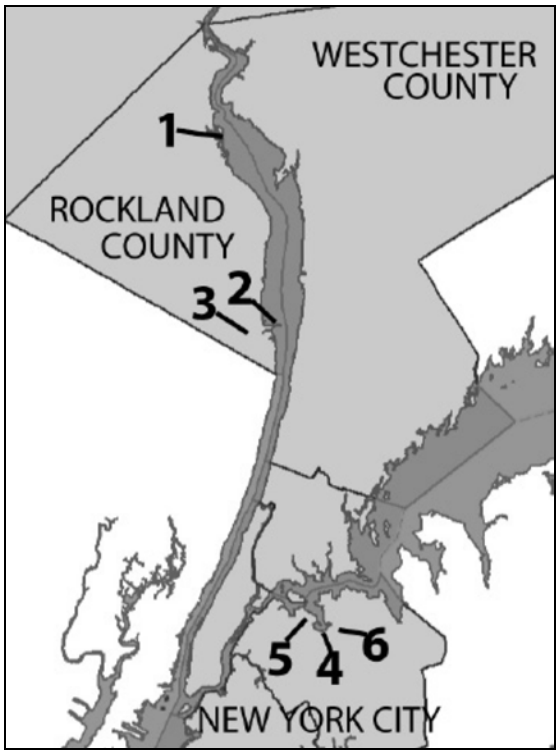

Figure 1. Map of sampling sites. Tappan Zee region stations: 1- Stony Point, 2- Piermont Pier, 3- Sparkill Creek; Flushing Bay region stations: 4- Flushing Bay Boat Launch, 5- Flushing Bay Marina, 6- Flushing River-Corona Park.

Laboratory Persistence Experiments with Sediment

Bulk samples of sediment, scraped from the top 3 cm using a cleaned trowel, were removed from the estuary and transferred to clean plastic incubation chambers (approximately 20cm long x 10cm wide x 10cm tall). Two samples were collected in late May 2012; one from a muddy, organic rich, site near the public boat launch in Flushing Bay, and one from a sandy site also in Flushing Bay but closer to Flushing Bay Marina

(Figure 1). A third sample was collected, also from the sandy Flushing Bay Marina site but in late June 2012. All samples were immediately transported to the laboratory and incubated at 4^{o}C in the dark for approximately five weeks. This incubation temperature was selected based upon prior research (O'Mullan and Juhl, unpublished) to allow for weeks of FIB persistence in order to test for false positives after an extended incubation.

Sediment samples were collected from each incubation chamber periodically over the five weeks to quantify the persistence of FIB in the sediment sample, using the same procedures for *Enterococcus*, as described below, for field sediment samples. It is worth noting that other samples from the O'Mullan and Juhl labs have been used to more completely characterize persistence rates of *Enterococci* in laboratory incubations using a variety of conditions (e.g., variable temperature). The purpose of the persistence incubations was to obtain cultured isolates of *Enterococci* that could be processed for DNA based identification using the 16S rRNA genes, to confirm that cultivation based approaches were actually quantifying *Enterococci* and were not prone to false positive results. The rates of decay are not reported quantitatively in this report, as this was not a goal of the study and is best estimated with a more complete data set spanning more persistence experiments.

FIB Enumeration Procedures

Microbes were extracted from sediment samples, using a method modified from Van Elsas et al. (2002), by mixing 10ml of sediment with 100ml of extraction buffer containing 0.1% sodium pyrophosphate and 0.1 mM EDTA in a sterile, sealed 500ml container shaken at 200 rpm for 30 minutes. Mass of replicate sediment samples was

recorded before and after drying at 60^{o}C for two days to determine wet and dry sediment mass and to allow normalization of microbial counts per gram dry weight of sediment.

After extracting sediment samples, microbial processing of sediment and water samples for FIB was identical, although only *Enterococci* was measured from water, while both *Enterococci* and *E. coli* were measured from sediment. *Enterococci* and *E. coli* were enumerated using the IDEXX Enterolert and Colilert methodology (www.Idexx.com). A 10% dilution of sample water in sterile water and growth media was sealed into a quanti-tray 2000 (IDEXX) vessel and incubating at 41^{o}C (*Enterococcus*) and 37^{o}C (*E. coli*) for 24 hours. After incubation, samples were exposed to UV light and the Most Probably Number (MPN) of *Enterococci* or *E. coli* cells was calculated per 100 ml (for water samples) or per gram of dry sediment weight (for sediment). In addition, a subset of samples were also processed using the EPA approved membrane filtration technique (US-EPA 2007) so that the isolated colonies could be used for DNA based taxonomic identification (described below) to confirm that the cultivation based technique was not prone to false positive results.

DNA Characterization of Sediment FIB and Statistical Analyses

Isolated *Enterococci* colonies from membrane filtration based enumeration of laboratory persistence samples were picked off petri dishes using sterile pipette tips and transferred into tubes with 40 µl of sterile water for molecular analysis. Colonies were then heated to 95^{o}C for 5 minutes using an Eppendorf mastercycler to lysis cells and the 16S rRNA gene was amplified from the released DNA using universal primers 8F and 1492R, followed by gene sequencing by SeqWright Inc. using the conditions described

by Young et al. (2013). The resulting gene sequences were taxonomically classified using the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/) and searched against the Genbank database (www.ncbi.nlm.nih.gov/genbank/) to confirm species identification. Non-parametric tests, including the Spearman's coefficient, were preformed using the GraphPad Prism (Version 4C, May 2005) statistical analysis software.

RESULTS

Paired Water and Sediment Field Sampling

Enterococci were detected in both water and sediment at all of the six sampling sites (Table 1). Only one water sample, out of 30 total samples enumerated, was found to be below detection for *Enterococci* (Stony Point, MPN <10/100ml). All 30 sediment samples were found to have detectable levels of FIB. Stony Point, in the Tappan Zee, had by far the lowest water and sediment FIB concentrations compared to all other sites (Table 1, Figure 2) with a maximum *Enterococci* MPN of only 113/100ml in the water, more than four times lower than any other site; and a maximum *Enterococci* MPN of only 19.7/g in sediment, more than an order of magnitude lower than any other site.

Levels of *Enterococci* and *E. coli*, the two common FIB used in water quality monitoring programs, were positively correlated (Spearman $r = 0.622$; $p < 0.001$) when sediment samples were compared among all sites (Figure 3). In contrast, levels of *Enterococci* in paired water and sediment samples, collected at the same site and at the same time, were not correlated (Spearman $r = 0.124$; $p = 0.515$) (Figure 4).

Site	# of samples	*Enterococci* water MPN/100ml		*Enterococci* sediment MPN/gram dry wt.		*E. coli* sediment MPN/gram dry wt	
		Minimum	maximum	minimum	maximum	minimum	maximum
1) Stony Point	4	<10	113	5	20	2	12
2) Piermont Pier	4	63	471	46	3788	274	3170
3) Sparkill Creek	4	121	>24196	12	2377	188	2378
4) Flushing Bay Boat Launch, muddy	6	10	>24196	134	4327	484	4327
5) Flushing Bay Marina, sandy	6	20	>24196	97	2866	143	2296
6) Flushing River-Corona Park	6	20	>24196	76	2818	112	2818

Table 1. FIB samples processed from the Hudson River Estuary.

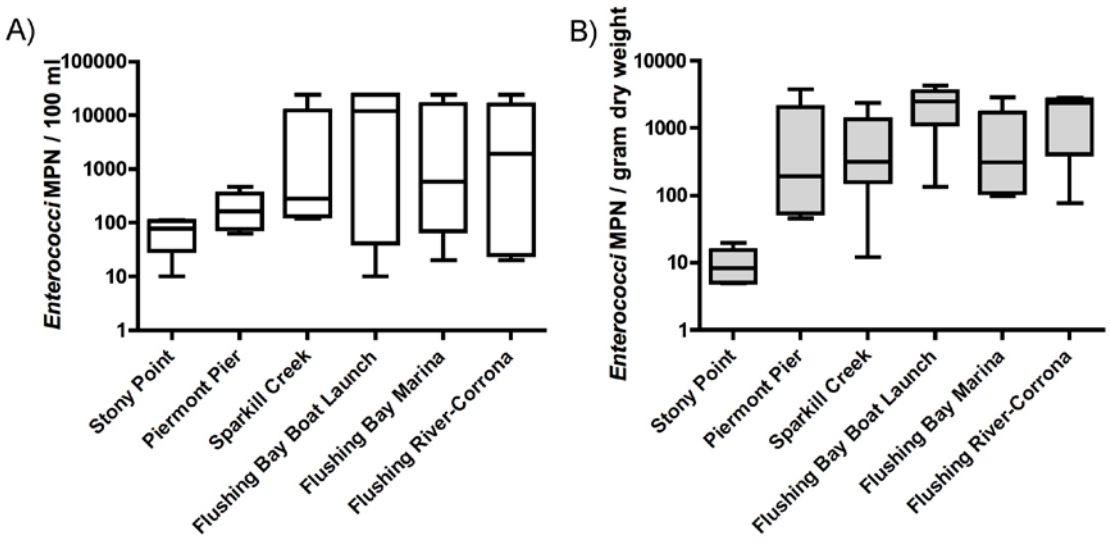

Figure 2. *Enterococci* Concentrations in Water and Sediment. A) water concentration and B) sediment concentration from the six spatial sampling sites. Stony Point, in the Tappan Zee, had the lowest concentrations of *Enterococci* for both water and sediment.

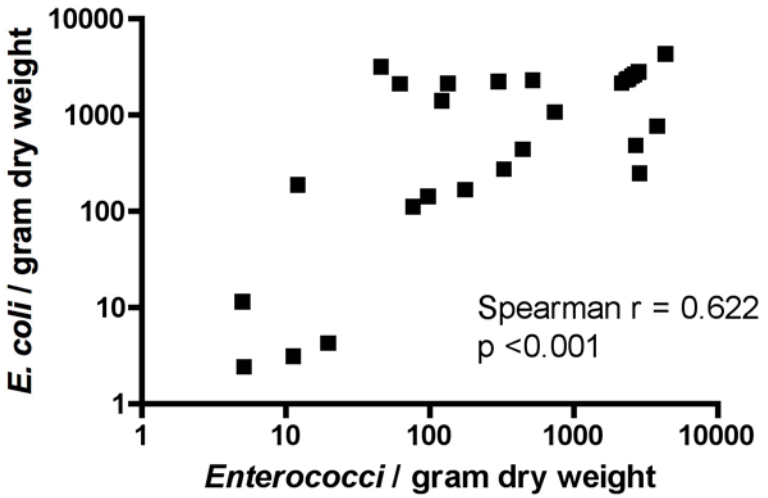

Figure 3. ***Enterococci*** **and** ***E. coli*** **Correlation in Sediment. Concentrations of** ***Enterococci*** **and** ***E. coli*** **measured from the same sediment samples were found to have a significant positive correlation.**

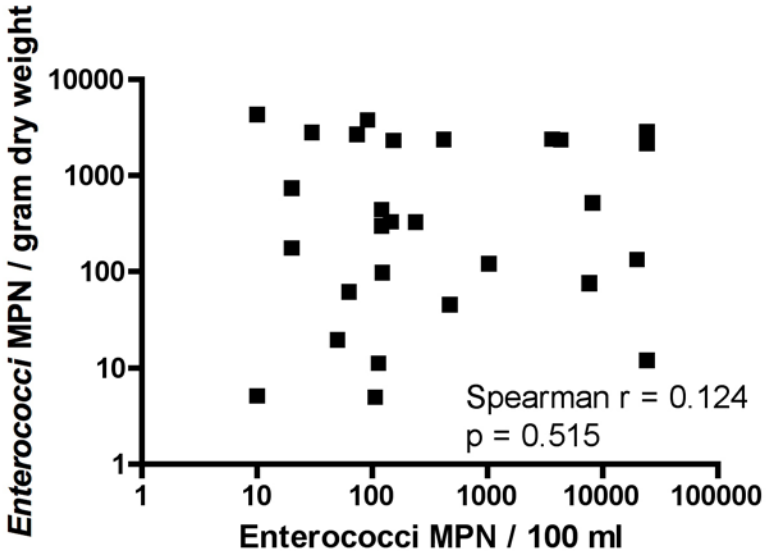

Figure 4. ***Enterococci*** **in Paired Water and Sediment.** ***Enterococci*** **concentrations in paired water and sediment samples were not significantly correlated.**

Sediment Persistence Experiments and Molecular Identification of *Enterococci*

Enterococci concentrations were found to decrease over time in laboratory incubations of estuarine sediment, based on cultivation-dependent techniques, but all three samples still had detectable FIB levels even five weeks after collection (Figure 5). In order to confirm the presence of *Enterococci,* and rule out the possibility of false positives from the cultivation-based assay, isolated bacterial colonies from the sediment persistence experiment were characterized using 16S rRNA gene sequencing. Of 113 colonies identified, the vast majority (96%) were classified as belonging to the genus *Enterococcus*, with most (72 sequences) classified as *Enterococcus faecium,* a bacterium commonly found in the intestine of humans. Four percent of sequences obtained from cultured isolates were false positives and classified as either *Lactobacillales*, *Desemzia* or *Klebsiella,* closely related enteric organisms.

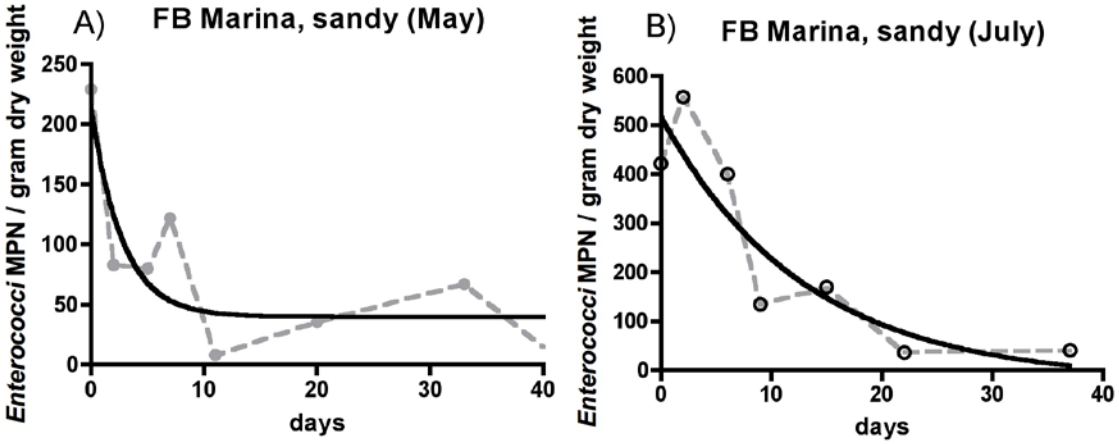

Figure 5. Sediment FIB Decay Curves. FIB decay curves from laboratory incubation experiments conducted at 4°C with Flushing Bay sediment. Dotted lines connect observed data and solid line represents a calculated exponential decay curve.

DISCUSSION

Paired Water and Sediment Field Sampling

Despite the improving water quality of Hudson River, FIB were widely distributed, detected in both water and sediment at every sampling site and within every individual sediment sample analyzed (Table 1, and Figure 2). These data suggest that sediment in the HRE act as a significant reservoir for FIB, as has been found in other similar systems (Anderson et al. 2005; Boehm et al. 2005; Bonilla et al. 2007). The correlated abundances of *Enterococci* and *E. coli* in sediment samples provides added confidence in the use of each indicator and would indicate that both FIB share a common delivery mechanism (Nobel et al. 2003), consistent with sewage as a source for both FIB to the Hudson sediment. Given the correlations of FIB to known pathogens (e.g. Walters et al. 2007), recreator illness (e.g. Haile et al. 1999), and antibiotic resistant bacteria (Young et al. 2013) from prior studies, the current FIB results are also strong evidence that other microbial agents of concern, beyond the FIB themselves, are likely to be widely distributed in Hudson sediment.

Recreators could be exposed to sewage-associated pathogens residing in sediment through activities along the shoreline, for example wading (Phillip et al. 2009), when sediment is directly contacted, but also through other activities when sediment becomes resuspended into the water column. Boat traffic (Pettibone et al. 1996), wave-shore interactions (LeFevre and Lewis 2003), high flow conditions in rivers and estuaries (Jamieson et al. 2005; Wilkinson et al. 2006), and even high winds (Roslev et al. 2008) can cause sediment re-suspension events that may negatively impact water quality. Some

water quality models are now attempting to incorporate FIB resuspension as a central factor controlling water quality (Liu et al. 2006).

Sediment Persistence Experiments and Molecular Identification of *Enterococci*

FIB in Hudson sediments from this study were found to persist for more than five weeks at detectable levels (Figure 5), a similar time scale found in some other aquatic systems (e.g. Haller et al. 2009). Extended persistence is not only expected in sediments, but there is also some evidence that particle-associated microbes in the water column may persist for longer periods than free-living FIB (Fries et al. 2008). This may be significant in the Hudson, where a high percentage of FIB are found to be particle associated (Suter et al. 2011).

A recent review (Jamieson et al. 2005) of water quality modeling studies identified gaps in the understanding of FIB and enteric ecological behavior within the environment as a significant obstacle to the generation of improved prediction systems. Variable persistence in high versus low nutrient environments, the significance of particle attachment for transport, and interactions between sediment and water column associated microbes are all important areas of continued research toward the goal of next generation water quality models (US-EPA 2007; Surbeck 2009; Kim et al. 2010).

Finally, the molecular genetic characterization of FIB isolates suggest that the vast majority (96%) of isolates obtained using cultivation based approaches were *Enterococci*. This finding is significant because it supports the use of cultivation-based approaches to quantify FIB in estuarine sediments and it confirms the long persistence of FIB in estuarine sediment, as suggested by cultivation based approaches.

CONCLUSION

The results from this study demonstrated that FIB are widely distributed in Hudson River sediment and appear to act as a reservoir for sewage associated pathogens. The microbes within this reservoir can persist for weeks, complicating the interpretation of FIB monitoring data. The high FIB content in sediments and attached to particles suggestions that additional research is required to understand the ecology of FIB in the Hudson and to allow improved approaches to water quality monitoring and modeling. Finally, the molecular genetic results from this study confirm that cultivation based approaches can be confidently used to enumerate FIB from sediments, supporting this method for studying FIB ecology in the environment.

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