Microbial Source Tracking Survey of Sparkill Creek: Confirmation of methods and initial evidence supporting widespread human sewage contamination following rainfall.

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Project Summary:
Over the last decade, citizen scientist monitoring has demonstrated widespread fecal contamination in Sparkill Creek using EPA approved cultivation-based enumeration of the Fecal Indicator Bacteria (FIB), enterococci. The level of FIB have consistently exceeded EPA Recreational Water Quality Criteria at all monitored sites above tidal influence of the Hudson and increase following rainfall. The widespread patterns of FIB in monitoring data have been interpreted to suggest substantial non-point source fecal pollution but have been unable to determine if human fecal waste contributes to these patterns due to the inability of FIB assays to differentiate host-specific FIB sources. This project examined DNA-based microbial source tracking (MST) assays to confirm the usefulness of these assays for identifying human specific fecal waste in Sparkill Creek and to examine the change in distribution of this waste in the creek following rainfall. Evidence for the presence of human fecal waste was detected in sewage (positive control) and in some Sparkill Creek samples, but not in negative control samples, utilizing two independent assays based on EPA approved MST methods. Human fecal contamination was detected from four of six sites tested during dry weather periods, but near the minimum detection limit of the assay in all cases. Following rainfall, human fecal contamination was found at five of the six sites tested, and in significantly higher concentrations than in dry weather. The only site that showed no evidence of human fecal contamination under any condition tested was within Tackamack Park, the study site least impacted by human development. This initial MST survey suggests that human fecal contamination does contribute to the FIB signal observed in Sparkill Creek, especially following rainfall, and that additional use of MST tools could help to identify locations within the creek and watershed (e.g. pipes, culverts, drainage areas), with the highest concentration of human fecal contamination, as targets to investigate future mitigation activities.

Introduction:

Increasing population density and aging sewer infrastructure create concerns for the potential contamination of streams and rivers in many urban and suburban watersheds across the United States. Fecal pollution from human and animal sources represents the most common source of waterway impairment in surface water systems (US-EPA, 2020). Assessing the level of fecal contamination in water typically relies upon enumeration of US-EPA approved fecal indicator bacteria (FIB), including enterococci (ENT) and *E. coli* (US-EPA, 2012). Elevated levels of these FIB in recreational water have generally been found to correlate with increased rates of gastrointestinal infection in recreators (Cabelli et al. 1982; Prüss 1998; Yau et al. 2009, US-EPA,

2012), supporting the continued use of these pollution assessment indicators. Although the majority of infections resulting from contact with contaminated water go unreported, the rates of these infections in recreators have continued to rise across the United States (Dorfman and Haren, 2014), highlighting the need for mitigation of fecal contamination throughout our watersheds.

Traditional cultivation-based enumeration of FIB is a useful tool in waterway management and the basis for most regulatory decision making but interpreting patterns in these data can be complicated by the diverse sources and extra-entric ecology these indicators (O'Mullan et al 2017). For example, FIB are known to originate from both human and diverse animal sources including birds, dogs, deer, and cattle (e.g. Alderisio et al 1999; Guber et al 2015; Silkie and Nelson, 2009; Topp et al 2009; Wright et al 2009). A major limitation to traditional FIB monitoring is the inability to differentiate human versus animal sources. It is well documented that there is risk of illness from contact with both human and animal feces (US-EPA, 2012), but the level of risk differs among sources (Soller et al 2014; Soller et al 2017) and the design of mitigation efforts to reduce fecal contamination relies upon understanding the source of fecal input.

In recent years there has been substantial effort to develop and validate DNA based microbial source tracking (MST) assays to identify specific sources of fecal bacteria in water (e.g. Bernhard and Field, 2000; McLellan and Eren, 2014). The most common type of MST assay involves quantitative Polymerase Chain Reaction (qPCR) which allows amplification and enumeration of host-specific DNA fragments. The US-EPA has recently released approved methodologies for two human fecal pollution specific qPCR MST assays based on prior scientific literature that demonstrates the ability of these methods to amplify DNA from human fecal bacteria with very limited cross-reactivity to bacteria from other sources. Method 1696 targets *Bacteroides* gene sequences that are present in human feces, as well as sewage and septic discharge, using the HF183/BacR287 primer set (US-EPA 2019A). Method 1697 targets *Bacteroides-like* gene sequences that are present in human feces, as well as sewage and septic discharge, using the

HumM2 primer set (US-EPA 2019B). These methods represent the first EPA approved tools for MST to characterize human specific fecal pollution in recreational waters.

Riverkeeper and the Sparkill Creek Watershed Alliance (SCWA) have been monitoring enterococci concentrations with cultivation-based methods at twelve to sixteen sites in Sparkill Creek since 2011 (Vail, 2015; Riverkeeper 2019). These data demonstrate the presence of FIB throughout the watershed, with geometric means exceeding the EPA Recreational Water Quality Criteria geometric mean guideline of 30 colony forming units per 100 ml at all sites. The concentration of enterococci at all sites increase significantly following rainfall. Although similar patterns of FIB contamination are known to occur in many other tributaries for the lower Hudson River, Sparkill Creek has the highest geometric means and greatest frequency of exceeding EPA single sample guidelines of all the tributary systems monitored by Riverkeeper (Riverkeeper, 2015), indicating the need for additional management action in this system. The widespread pattern of contamination and increased levels in wet weather suggest that non-point sources of pollution are likely the major contributors to FIB in Sparkill Creek. Although FIB are known to be present at unacceptable levels, prior monitoring efforts have been unable to determine if human fecal contamination contributes to these patterns. The overarching objectives of this study were to evaluate the use of molecular source tracking methods to determine if human fecal pollution contributes to FIB levels observed in Sparkill Creek and to determine how this contribution changed under dry vs wet weather conditions.

Brief Description of Sampling and Analytical Methods:

The Sparkill Creek watershed is located in southeastern Rockland County, NY and a small portion of Bergen County, NJ. The creek flows through a twelve square mile watershed of parkland, suburban and low density industrial/commercial landscapes before entering the Hudson River via a tidal wetland at Piermont NY. The creek is listed on the New York State Priority Waterbody List of stressed streams (NYS-DEC, 2013). Riverkeeper and SCWA have monitored enterococci concentrations, utilizing EPA approved IDEXX Enterolert cultivation-based methods, at twelve to sixteen sites since 2011 (Vail, 2015; Riverkeeper 2019). This MST study collected samples at 6 of

these longer term monitoring stations (sites 1-6), in addition to two storm sewer outfalls (Oaktree Road outfall and Walnut outfall), and one culvert drainage that only flowed following rainfall in our study period (Spruce) but generally flows for most of the year based on prior monitoring (Table 1; Figure 1). Sampling occurred on three dates, including two following at least 48 hours of dry weather (7/28/20, 9/17/20), and one immediately after heavy rainfall (9/30/20) (Table 1).

Sparkill Creek water samples were collected, using gloved hand or sampling pole, into autoclave sterilized 250 or 1000 ml polyproplene bottles, triple rinsed with creek water before final sample collection, and immediately placed into an opaque ice filled cooler until processing. One negative control sample was included for each sampling date and consisted of an autoclaved sterile water sample that was transferred into a sample bottle in the field and handled in parallel to creek water samples. A positive control sample consisting of untreated human waste from the Orangetown sewage plant was collected on 7/28/20 and processed in parallel with other samples with the exception that a larger (1 in 100) sample dilution with sterile water was included for FIB enterococci enumeration from this sample. FIB enumeration and filtration for MST occurred within six hours of collection for all samples. Enterococci were enumerated using the IDEXX Enterolert variant of EPA method 1600 (US-EPA, 2009), including a 1/10 dilution in sterile water of each creek sample and a negative (sterile water only) control with each sampling date, as previously described in Young et al (2013). The MST samples (100-200ml) were vacuum filtered onto sterile 0.45 um polycarbonate membranes, using sterile technique to handle samples, filtration funnels and membranes, and immediately following filtration membranes were transferred into 2ml sterile cryotubes and frozen before overnight shipping for DNA extraction and qPCR analysis.

DNA extraction and MST qPCR were performed at Source Molecular Corp (Miami Lakes, FL), an ISO 17025 accredited testing laboratory, using assays based on EPA Method 1696 (HF183; EPA, 2019A; all samples) and EPA Method 1697 (HumM2; EPA, 2019B; only samples from 7/28/20). For each sample, DNA was extracted from filters using the Generite DNA-EZ ST1 extraction kit (GeneRite, NJ), eluted in 100µl of sterile water. MST qPCR assays were run on duplicate reactions

using 2µl of extract as template and a third reaction using 2µl of a 1 in 10 sterile water dilution of the extract. An Applied Biosystems StepOnePlus real time thermocycler (Applied Biosystems, Foster City, CA) was used for qPCR assays with a final reaction volume of 20µl. For each batch of qPCR results assay controls including negative (no template), positive (positive control plasmid added), and a dilution series of calibration curve samples (to determine limits of detection and amplification efficiency) were included at Source Molecular. Samples with none of the replicates positive (positive meaning fluorescence signal above background in the qPCR assay) are reported as "*No Detection" (ND)*. For the purposes of this preliminary MST data set, samples with only 1 of 2 undiluted replicates found to be positive are reported as "*Partial Detection" (PD)*, samples with both undiluted replicates positive but outside the range of quantification (generally meaning a quantitative cycle (Cq) above 34) are reported as "*Detected, Not Quantified" (DNQ)*, while samples with both non-diluted and diluted replicates positive and non-diluted replicates within the range of quantification (generally a Cq below 34) are reported as "*Detected and Quantified" (DQ)* and the number of gene copies per 100ml of creek water is reported based on extrapolation from the calibration curve. Samples in the "partial detection" and "detected, not quantified" categories are considered to be low level detection near the minimum detection level of the assay.

Results and Discussion:

Confirmation of Traditional Fecal Indicator patterns-

Negative control FIB samples all had an MPN of <1/100ml, as expected, and the positive control untreated waste from the Orangetown sewage plant (collected on 7/28/20) had an MPN of <241,960/100ml (1/100 dilution) consistent with expectations for untreated sewage. Patterns of enterococci from field samples were generally consistent with previously described monitoring data from Sparkill Creek (prior data in Table 1). Enterococci values across sites from dry sampling dates (7/28/20 and 9/17/20) ranged from an MPN of 20 to 1782 per 100ml, with nine of the ten enterococci samples exceeding the US-EPA Beach Action Value (BAV) guideline indicating fecal contamination at a level of concern for primary contact (Table 2). The one sample with an enterococci value consistent with primary contact (MPN of 20/100ml) occurred in Tackamack

Park (Site 2, on 9/17/20), the Sparkill Creek site with the lowest frequency of exceedance of the BAV in prior monitoring (Riverkeeper, 2019). The enterococci values in wet weather (9/30/20) were all higher than values from the same sites during dry weather, consistent with increased fecal contamination following rainfall. The wet weather values ranged from MPNs of 1,223 to >24,196 per 100ml, with the lowest value collected at Tackamack. Values of contamination in the lower Sparkill (sites 3-6) where increased human development occurs were all maximum detection levels for the FIB assay with 1 in 10 dilution (>24,196 per 100ml) on the wet weather sampling date. The consistency of FIB values from this study (Table 2) with prior monitoring data from the last decade (Table 1) suggest that the samples analyzed for MST are likely to be generally representative of conditions commonly experienced in Sparkill Creek.

The Two Human Specific MST Assays Provided Consistent Patterns-

Two independent human specific MST assays (HF183 and HumM2) were processed for seven samples on 7/28/20 including an untreated sewage sample from the Orangetown treatment plant (positive control), a sterile water sample transferred and handed in parallel with field samples (negative control), four dry weather creek samples and one duplicate creek sample (Moturis had duplicate field samples). **The two independent MST methods had consistent results across samples (Table 3) providing confidence in the use of EPA approved human specific MST assays.** The untreated sewage from the Orangetown treatment plant was detectable with a strong signal within the quantifiable range for both markers, as would be expected for sewage. The sterile water (negative control) samples did not have detectable human fecal signal for either MST assay. The samples from three (Marsico, Tackamack, Clausland Arm) of the four sites were negative for both MST assays, while one site (Moturis) and the duplicated field sample from this site (Moturis duplicate) had low level detection, below quantifiable levels, for both MST assays. Given the consistency of these two human specific MST assays, and the reputation for stronger signal from HF183 (consistent with levels detected in the positive control sample), the later sample dates (9/17 and 9/30) were only processed for the HF183 assay.

Human Fecal Contamination was Detectable in Sparkill Creek and Increased Following Rainfall-
When examining the HF183 results across sites, **human fecal contamination was detected from four of six sites tested in dry weather (7/28 and 9/17; Table 4), but near the minimum detection limit of the assay in all cases. Following rainfall (9/30), human fecal contamination was found at five of the six sites tested, and in significantly higher concentrations than in dry weather (Table 4).** The lower creek sites (Moturis, Stateline, and Rockleigh) had higher frequency of detection across dates and weather conditions, suggesting more consistent human fecal contamination- however the number of samples included in this study do not allow a robust conclusion about temporal patterns. The only site that showed no evidence of human fecal contamination under any condition tested was Tackamack, the study site least impacted by human development and located within a forested park. It is interesting to note that the Marsico Ct was the creek sample with the highest gene copy concentration (during wet weather) and had some detection in both dry and wet weather, but was not the site with the highest cultivation based FIB in our samples or in prior monitoring. This may suggest (but can only be cautiously suggested with this preliminary data) that there is a higher proportion of human to animal fecal waste at this site. The lower Sparkill sites had higher cultivation based FIB levels in our wet weather sample date, which is generally consistent with prior monitoring data, and these sites all had quantifiable human fecal signal in dry weather but with lower concentrations than in the Marsico Ct sample. This likely suggests significant contributions from human and animal fecal contamination at these sites. These hypotheses require additional study to investigate in more detail.

Evidence for Human Fecal Contamination was Found in Outfall Pipes-
Three samples (Sites 7-9; Table 1, Figure 1) were collected following wet weather on 9/30/20 from inputs to Sparkill Creek that were not flowing during dry weather sampling. Two of the sites were from stormwater outfall pipes and one from a culvert that dries up in periods of prolonged dry weather (Spruce). All three of these samples had evidence for human fecal contamination, but one site (Oaktree Road pipe) had only a very low level detection that should be confirmed with future sampling. Given the success of this preliminary effort in using MST assays, these tools appear well suited to future human sewage trackdown efforts in Sparkill Creek.

Future Steps:

These data provide very useful information in building upon the FIB monitoring work of the last decade, but they are only a first step in applying MST methodologies to the study of Sparkill Creek. These data suggest that qPCR assays based on EPA approved MST methods can provide useful information about the presence of human fecal contamination in Sparkill Creek. Expanded sample collection for human specific assays could be used to identify outfalls and regions of the creek that have the highest relative concentration of human contamination to prioritize areas for trackdown of illicit connections or consideration of sewer upgrades. Now that initial evidence for human fecal contamination in Sparkill Creek exists, building upon prior FIB data, in order to have additional confidence in these results a series of local dog and bird fecal samples could be examined to rule out any low level cross-reactivity with the human MST assay. While these cross-reactivity experiments have been completed in other systems during EPA validation of the methods, they are also suggested to be conducted with local fecal sources to provide added validation of the approach.

The routes of delivery to Sparkill Creek and the storage of fecal microbes within Sparkill Creek are still not fully understood. For example, sediment is known to contain high levels of FIB throughout the region in fecal impacted waterways (O'Mullan et al 2019) and in small volume systems like Sparkill Creek there is extensive water and sediment interaction that has the potential to influence FIB dynamics in the creek water. MST approaches could be used to examine whether human fecal signal is retained in the sediment, which could then act as a reservoir (potentially resuspended during the higher flow following rainfall). Other components of the system, such as groundwater could also be tested to better understand the routes of contaminant delivery to Sparkill Creek. Finally, it is very likely that FIB signal is only partially due to human fecal contamination and additional qPCR MST assays targeting specific animal sources should also be examined to determine areas where other management actions (e.g. focused on control of dog and bird waste, or infiltration of suburban stormwater) may be productive to reduce the overall level of FIB in Sparkill Creek. These initial data suggest that MST tools could

be very helpful in better understanding pollutant delivery and storage in Sparkill Creek and can therefore better inform management actions to improve water quality in this stressed stream.

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Figures and Tables:

Table 1: Sampling locations and dates from MST study along with SCWA historical monitoring (2012-2019) enterococci data from Riverkeeper (2019) provided as context for contamination.

Sampling Site	Sample type	Latitude/ Longitude	Dates sampled	% of ENT exceeding BAV	ENT geometric mean/ 100ml (2011-2019)
1- Marsico Ct (Blauvelt)	Creek	41.066399° -73.940746°	7/28/20 (dry), 9/17/20 (dry), 9/30/20 (wet)	90%	552
2- Tackamack (Blauvelt)	Creek	41.061141° -73.938688°	7/28/20 (dry), 9/17/20 (dry), 9/30/20 (wet)	77%	159
3- Clausland Arm (Blauvelt)	Creek	41.054444° -73.945064°	7/28/20 (dry), 9/17/20 (dry), 9/30/20 (wet)	96%	681
4- Moturis (Tappan)	Creek	41.017773° -73.935189°	7/28/20 (dry), 9/17/20 (dry), 9/30/20 (wet)	98%	1368
5- Stateline (Tappan)	Creek	41.016375° -73.944856°	9/17/20 (dry), 9/30/20 (wet)	96%	980
6- Rockleigh (Sparkill Brook)	Creek	41.007646° -73.939978°	9/17/20 (dry), 9/30/20 (wet)	96%	976
7- Spruce (Blauvelt)	Culvert, leading to Creek	41.058629° -73.945322°	9/30/20 (wet)	100%	1081
8- Walnut	Stormwater Outfall into Spruce	41.058613° -73.945346°	9/30/20 (wet)	NA	NA
9- Tappan Library Oaktree Rd	Stormwater Outfall into Sparkill	41.020726° -73.947591°	9/30/20 (wet)	NA	NA

Table 2: FIB (enterococciMPN/100ml) values for each site and date. The number of sampling sites increased throughout the study.

Sample site	Sample type	Dry 7/28/20	Dry 9/17/20	Wet 9/30/20
1- Marsico Ct	creek	723	63	6867
2- Tackamack	Creek	336	20	1223
3- Clausland Arm	Creek	1782	131	>24196
4- Moturis (duplicate on 7/28)	Creek	644 (839)	148	>24196
5- Stateline	Creek		121	>24196
6- Rockleigh (duplicate on 9/17)	Creek		259 (350)	>24196
7- Spruce (duplicate on 9/30)	Culvert			15531 (14136)
8- Walnut Outfall	Outfall			17329
9- Oaktree Rd Outfall	Outfall			>24196

Table 3: Comparison of the two independent human specific MST assays (HF183 and HumM2) from 7/28/20. ND (green)= No Detection; PD (yellow)= Partial Detect; DNQ (orange)= Detected, Not Quantified; DQ (red)= Detected and Quantified.

Sample site	HF183	HumM2	HF183 gene copies/100ml	HumM2 gene copies/100ml
Orangetown sewage (Positive control)	DQ	DQ	1.2×10^8	5.8×10^6
Negative control	ND	ND	-	-
Marsico Ct	ND	ND	-	-
Tackamack	ND	ND	-	-
Clausland Arm	ND	ND	-	-
Moturis	DNQ	DNQ	-	-
Moturis, duplicate sample	DNQ	PD	-	-

Table 4: Comparison of the human specific MST HF183 assays from dry (7/28/20 and 9/17/20) and wet (9/30/20) weather. ND (green)= No Detection; PD (yellow)= Partial Detect; DNQ (orange)= Detected, Not Quantified; DQ (red)= Detected and Quantified. Empty cell indicate no sample collected at this site on this date. Sites 7-9 were only flowing in wet weather.

Sample site	**Sample type**	**Dry 7/28/20**	**Dry 9/17/20**	**Wet 9/30/20**	**Wet 9/30/20 HF183 gene copies/100ml**
1- Marsico Ct	creek	ND	PD	DQ	2.2×10^5
2- Tackamack	Creek	ND	ND	ND	ND
3- Clausland Arm	Creek	ND	ND	DQ	1.7×10^4
4- Moturis (duplicated on 7/28)	Creek	DNQ (DNQ in dup)	DNQ	DQ	1.7×10^3
5- Stateline	Creek		DNQ	DQ	2.8×10^3
6- Rockleigh (duplicated on 9/17)	Creek		ND (PD in dup)	DQ	2.7×10^3
7- Spruce	Culvert			DQ	1.9×10^3
8- Walnut Outfall	Outfall			DQ	2.4×10^3
9- Oaktree Rd Outfall	Outfall			PD	PD

Figure 1: Map of the Sparkill watershed showing surface water in aqua, locations of samples 1-9 (see Table 1) with white labels, stormwater lines in blue and sanitary sewer lines in red.