2022 PARSONS CREEK PHYLOCHIP | MEMORANDUM

TO: Kim Reed, Town of Rye; Deb Loiselle, NHDES
FROM: Luke Frankel, FB Environmental Associates (FBE)
SUBJECT: 2022 Parsons Creek PhyloChip Memorandum
DATE: May 26, 2023
CC: Matt Scruton, Becky Bergeron, Jason Rucker, Chuck Marsden, Town of Rye, NH
Sally Soule, Dennis Greene, NHDES
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EXECUTIVE SUMMARY

To identify sources of fecal contamination, water samples were collected throughout the Parsons Creek watershed in 2022 and analyzed using PhyloChip® technology. PhyloChip® is a new and innovative fecal source tracking method that determines the complete microbial composition of environmental samples and compares it to the microbial composition of known fecal sources to determine the likelihood that fecal contamination from that source is occurring. This approach provides much more information about the microbial community present than other fecal source tracking methods that typically only target a specific subset of the community. Although this was the first time that PhyloChip® technology was used within New Hampshire, the method has been employed in neighboring states (ME and MA) with success. The nine animal groups that the PhyloChip® method can currently identify fecal waste from are birds, dogs/cats, horses, humans, pigs, pinnipeds, raccoons, rats, and ruminants (deer).

For all five sample dates (with two during wet weather and three during dry weather conditions), the five sites that were sampled in Parsons Creek showed **raccoon and rat as potential sources of fecal waste, with no evidence of human fecal waste contamination.** This result was surprising given that previous fecal source tracking efforts in the watershed, including canine scent detection and DNA ribotyping, have indicated that human fecal waste is a diffuse source throughout the watershed. After thoroughly reviewing all field and laboratory protocols with Veracet and confidently confirming that proper collection, handling, and analysis of the samples were followed by all parties, we identified four possible explanations as to why the recent PhyloChip® analysis produced results that are outliers among historic fecal source tracking efforts. **The most probable explanation for the lack of human fecal sources detected by PhyloChip® is related to method sensitivity rather than a true indication of environmental conditions.** After reviewing the use and results of PhyloChip® in other New England coastal waterbodies, we discovered that **PhyloChip® may not be a suitable technology for source tracking in brackish, salt marsh environments**. There is scientific evidence supporting the notion that salt marsh systems can alter microbial communities in unique ways, and in this case, ways that could deviate from the microbial communities associated with known fecal sources as part of the PhyloChip® technology's source library. Results like this are common when novel technologies are employed in studies and are useful in guiding potential applications for future projects. These results are helpful for other New England communities to understand when and where PhyloChip® technology might be most advantageous and provide the most useful results. There are many other source tracking methods that we can and should use in Parsons Creek, including those that have been used successfully in the watershed before.

The PhyloChip® results did yield other information that is helpful for public health awareness in Parsons Creek. Because PhyloChip® technology characterizes the entire microbial community profile within samples, we can determine the presence and relative abundance of hundreds of potentially harmful strains of bacteria in each sample. **Two potentially harmful strains of bacteria were found in Parsons Creek: Streptococcus and Staphylococcus.** This information is useful from a public health perspective and can help diagnose potential illnesses of people who have encountered water from

the Creek. It can also be used to better inform public outreach campaigns in the watershed that are aimed at promoting awareness of the potential harms of fecal contaminated waters.

BACKGROUND

In 2022, through the Restoring Parsons Creek Clean Water State Revolving Fund (CWSRF) project, FB Environmental Associates (FBE) collected water samples for fecal source tracking in Parsons Creek using PhyloChip® technology. Parsons Creek has a long history of elevated fecal indicator bacteria counts, with the waterbody listed as impaired for primary contact recreation since 2008 (NHDES, 2010). As the 2011 Watershed Based Plan for Parsons Creek outlines, roughly 34% of the annual fecal load to the creek is estimated to come from septic systems in the watershed (FBE, 2011).

Historical fecal source tracking efforts using canines and DNA analyses have suggested that human fecal contamination is a diffuse problem throughout the watershed that is likely caused by the low-lying topography and high groundwater table allowing septic system leachfields to become inundated on a regular basis during storm events and/or spring tides. Substantial progress has been made over the past decade to address fecal pollution from septic, including identifying and replacing failed systems and the implementation of a town ordinance requiring that residents in the watershed pump out their systems every three years. Despite these efforts, bacteria concentrations in Parsons Creek remain high, prompting the need for additional fecal source tracking efforts.

METHODS

To assist in identifying the cause of high bacteria concentrations, FBE collected water samples at five locations in Parsons Creek on five dates for PhyloChip® analysis (Figure 1). PhyloChip® is a novel microbial source identification method that determines the likelihood that individual fecal source types, including human, dog, bird, horse, pig, and ruminant (deer), are present in environmental samples. It is typically more effective than traditional fecal source tracking methods because it uses a series of diagnostic probes that represent groups of bacteria known to be associated with individual fecal source types to determine whether a source is present or not. Three of the five PhyloChip® sample dates were characterized as dry weather events (7/5/2022, 7/21/2022, and 10/4/2022), while the other two were characterized as wet weather events (9/6/2022 and 9/20/2022; Table 1). Each PhyloChip® sample was accompanied by an enterococcus grab sample and field measurements of temperature, dissolved oxygen, specific conductivity, salinity, and pH. After collection, PhyloChip® samples were stored on ice until they were transported back to the FBE office in Dover, NH for filtration. For each sample, 200 mL of water was passed through a 0.45 µm filter; then that filter was sealed in aluminum foil, placed in a plastic bag, and stored in a deep freezer until all samples had been collected. All sampling and filtering procedures were performed in accordance with FBE's Veracet-approved PhyloChip® Standard Operating Procedure (SOP; FBE, 2021). Once Veracet's laboratory was ready for receipt, the filters were packaged with dry ice and shipped on 10/31/2022 via overnight air to Berkley, CA for PhyloChip® analysis.

Figure 1. Locations of 2022 PhyloChip® sample sites in the Parsons Creek watershed.

Table 1. Environmental conditions for the five PhyloChip® sample dates in Parsons Creek.

Sample Date	24-hr Precip. (in)	48-hr Precip. (in)	96-hr Precip. (in)	Dry/Wet	Low Tide Time
7/5/2022	0.00	0.00	0.05	Dry	9:57
7/21/2022	0.00	0.00	0.32	Dry	12:29
9/6/2022	1.05	2.34	2.34	Wet	13:39
9/20/2022	0.62	0.98	0.98	Wet	13:45
10/4/2022	0.00	0.00	0.00	Dry	12:23

RESULTS

PhyloChip® results for Parsons Creek did not match any human sources of fecal contamination within Veracet's SourceTracker database during the time of the study (Attachment 1). Out of the nine animal groups represented in the SourceTracker database (bird, dog/cat, horse, human, pig, pinniped, raccoon, rat, and ruminant), only two, raccoon and rat, emerged as potential sources of fecal waste to Parsons Creek (predicted contributions greater than 0.1 but less than 0.2; Table 2). These potential sources were present at sites PC08 (7/21/2022 and 10/4/2022), PC07 (7/21/2022 and 9/20/2022 [raccoon only]), BCH11 (9/6/2022), and ACPS005-U35 (9/20/2022 and 10/4/2022). To be considered a clear source of bacteria, the predicted contribution for an individual sample and source should be greater than 0.2, which was not achieved for any animal group at any site. Besides raccoon and rat, all other animal groups had predicted contributions less than 0.1, indicating that they were not likely sources of bacteria to Parsons Creek. After reviewing both sample collection, storage, and shipping procedures with FBE staff and DNA preparation and chip scanning procedures with Veracet, both FBE and Veracet are confident that all protocols were followed appropriately and that the "microbial community composition of each sample is reflective of the DNA that was isolated from the filters" (Attachment 1).

The microbial communities characterized via PhyloChip® also allow for the identification of potentially harmful bacterial genera that may be associated with fecal contamination from human and animal sources. In this case, the five potentially harmful bacterial genera discovered were Streptococcus, Staphylococcus, Serratia, Salmonella, and Legionella, with Streptococcus and Staphylococcus being of greatest concern due to the greater health risk if infection were to occur. Streptococcus was found in the greatest numbers in Parsons Creek and was present at all sites, particularly at PC07 (Table 3). From a public health perspective, harmful strains of Streptococcus can most commonly cause streptococcal sore throat (strep throat), pink eye, meningitis, impetigo (blisters on the skin), and bacterial pneumonia; however, in severe cases, infections can cause life-threatening illnesses such as toxic shock syndrome, endocarditis, and necrotizing fasciitis (flesh eating disease). The other potentially harmful bacterial genera found at all sites in Parsons Creek were Staphylococcus and Serratia (Table 3). Harmful strains of Staphylococcus most commonly cause skin infections, while some strains of Serratia can cause respiratory and urinary infections. **Overall, although it may be difficult to determine the source of these potentially harmful bacterial genera, their presence in high concentrations (>> 100 mpn/100mL) in Parsons Creek may pose a risk to public health and should be taken into consideration for future management of the creek.**

Site BCH11 on July 5, 2022. © FBE.

Site PC07 on August 18, 2022. © FBE.

Table 2. PhyloChip® predicted contributions from animal groups and relevant water quality parameters for all 25 samples collected in Parsons Creek. Bolded red text indicates exceedances of state water quality criteria for water quality parameters (DO < 75%, DO < 5 mg/L, Entero > 104 mpn/100mL) or potential fecal sources via PhyloChip® showing predicted contributions greater than 0.1 but less than 0.2. Rows highlighted in blue represent wet weather samples.

Sample Information			Water Quality Parameters							PhyloChip® Predicted Contributions								
Site ID	Date	Time	Temp. (°C)	DO (%)	DO (mg/L)	SPC (µS/cm)	Sal. (ppt)	pH	Entero. (mpn/100mL)	Bird	Dog/ Cat	Horse	Human	Pig	Pinniped	Raccoon	Rat	Ruminant
PC-OUT	7/5/2022	9:40	18.9	95.0	7.55	40,950	26.27	7.81	20	0.008	0.022	0.004	0.015	0.006	0.005	0.072	0.073	0.008
BCH11	7/5/2022	10:15	20.6	***1.2***	***0.09***	34,205	21.52	6.55	***211***	0.005	0.020	0.003	0.010	0.008	0.005	0.058	0.072	0.010
PC08	7/5/2022	10:45	23.0	***10.5***	***0.78***	35,590	22.46	6.85	***14,126***	0.006	0.015	0.004	0.010	0.009	0.004	0.052	0.062	0.011
PC07	7/5/2022	10:55	23.6	***1.2***	***0.09***	32,285	20.16	7.00	***92,080***	0.004	0.021	0.003	0.010	0.008	0.004	0.059	0.069	0.009
ACPS005-U35	7/5/2022	11:25	18.8	***2.1***	***0.19***	369	0.18	6.40	***4,360***	0.004	0.017	0.004	0.010	0.016	0.004	0.072	0.065	0.013
PC-OUT	7/21/2022	11:20	21.7	99.2	7.29	47,147	30.71	7.66	97	0.009	0.020	0.004	0.014	0.006	0.006	0.062	0.066	0.009
BCH11	7/21/2022	11:45	22.0	***2.2***	***0.17***	40,353	25.83	6.56	***425***	0.005	0.016	0.004	0.009	0.013	0.004	0.047	0.064	0.011
PC08	7/21/2022	12:10	27.6	***65.9***	***4.49***	39,600	25.18	6.71	***>24,196***	0.020	0.026	0.009	0.024	0.010	0.009	***0.115***	***0.112***	0.011
PC07	7/21/2022	12:25	26.1	281.5	19.72	40,683	25.99	6.89	***>24,196***	0.034	0.030	0.012	0.033	0.011	0.014	***0.127***	***0.107***	0.012
ACPS005-U35	7/21/2022	13:10	18.9	***1.5***	***0.14***	439	0.21	6.39	***2,187***	0.004	0.019	0.003	0.009	0.018	0.005	0.079	0.070	0.011
PC-OUT	9/6/2022	11:40	17.0	***69.6***	5.71	42,144	27.11	7.44	***809***	0.006	0.025	0.004	0.014	0.009	0.006	0.071	0.081	0.010
BCH11	9/6/2022	12:05	20.7	***0.5***	***0.04***	36,076	22.83	6.76	***11,199***	0.022	0.030	0.009	0.022	0.011	0.014	***0.144***	***0.110***	0.011
PC08	9/6/2022	12:20	18.4	***9.3***	***0.77***	31,123	19.41	6.82	***19,863***	0.004	0.015	0.003	0.010	0.008	0.003	0.046	0.056	0.009
PC07	9/6/2022	12:30	17.2	***70.7***	6.55	11,155	6.37	7.05	***9,804***	0.004	0.015	0.003	0.011	0.007	0.003	0.052	0.054	0.009
ACPS005-U35	9/6/2022	12:55	18.0	***2.0***	***0.19***	419	0.20	6.80	***107***	0.005	0.019	0.004	0.011	0.012	0.005	0.087	0.081	0.013
PC-OUT	9/20/2022	11:50	16.6	78.5	6.37	46,654	30.35	7.69	***134***	0.008	0.028	0.005	0.016	0.006	0.006	0.076	0.075	0.010
BCH11	9/20/2022	12:05	19.0	***1.9***	***0.15***	39,050	24.92	6.82	72	0.005	0.019	0.004	0.011	0.009	0.004	0.051	0.065	0.013
PC08	9/20/2022	12:20	17.4	***37.3***	***3.09***	37,625	23.92	6.92	***24,196***	0.004	0.018	0.003	0.009	0.009	0.004	0.059	0.069	0.010
PC07	9/20/2022	12:30	16.9	75.9	6.94	16,390	9.66	7.10	***7,270***	0.009	0.024	0.006	0.018	0.012	0.006	***0.105***	0.094	0.011
ACPS005-U35	9/20/2022	12:50	16.0	***1.0***	***0.10***	395	0.19	6.85	***115***	0.027	0.029	0.009	0.037	0.010	0.007	***0.128***	***0.109***	0.009
PC-OUT*	10/4/2022	11:00	12.3	95.4	8.59	43,180	27.77	7.77	***121***	0.003	0.003	0.005	0.002	0.002	0.008	0.004	0.002	0.002
BCH11	10/4/2022	11:20	13.4	***6.4***	***0.56***	43,551	28.06	6.86	***402***	0.003	0.020	0.003	0.009	0.009	0.003	0.048	0.065	0.008
PC08	10/4/2022	11:30	13.3	***66.0***	5.86	41,384	26.52	6.93	***8,664***	0.028	0.033	0.011	0.031	0.008	0.021	***0.117***	***0.104***	0.014
PC07	10/4/2022	11:40	12.3	132.5	12.16	38,676	24.59	7.03	***1,785***	0.009	0.019	0.005	0.016	0.005	0.006	0.070	0.077	0.010
ACPS005-U35	10/4/2022	11:50	11.0	***3.9***	***0.43***	384	0.19	7.02	20	0.058	0.039	0.013	0.045	0.007	0.013	***0.116***	***0.118***	0.008

*Low DNA in the sample suggestive of DNA extraction or sample preparation issues; interpret this sample result with caution.

Table 3. The occurrence of potentially harmful bacterial genera that may be associated with fecal contamination from human and animal sources as identified via PhyloChip® in all 25 samples collected in Parsons Creek. Rows highlighted in blue represent wet weather samples.

Sample Information			Number of Bacterial Genera Present				
Site ID	Date	Time	Streptococcus	Staphylococcus	Serratia	Salmonella	Legionella
PC-OUT	7/5/2022	9:40	10	2	1	1	
BCH11	7/5/2022	10:15	5	3			
PC08	7/5/2022	10:45	1	2	1	1	
PC07	7/5/2022	10:55	3	1	1	1	
ACPS005-U35	7/5/2022	11:25	1	1	1		
PC-OUT	7/21/2022	11:20	2	2	1	1	
BCH11	7/21/2022	11:45	2	9	1		
PC08	7/21/2022	12:10	1	1	1		
PC07	7/21/2022	12:25	1	1	1		
ACPS005-U35	7/21/2022	13:10	4		1	1	
PC-OUT	9/6/2022	11:40	10	1	1		1
BCH11	9/6/2022	12:05		1	1		
PC08	9/6/2022	12:20	2	1	2		
PC07	9/6/2022	12:30	22	3	1		
ACPS005-U35	9/6/2022	12:55	3	1	1	1	
PC-OUT	9/20/2022	11:50	8		2		
BCH11	9/20/2022	12:05	1	2	4		
PC08	9/20/2022	12:20	6	3	1		
PC07	9/20/2022	12:30	16	1	1		
ACPS005-U35	9/20/2022	12:50	11	6			
PC-OUT*	10/4/2022	11:00					
BCH11	10/4/2022	11:20	11	3	4		1
PC08	10/4/2022	11:30	1	1			
PC07	10/4/2022	11:40		1		1	2
ACPS005-U35	10/4/2022	11:50	2	1			
		Total	**123**	**47**	**27**	**7**	**4**

*Low DNA in the sample suggestive of DNA extraction or sample preparation issues; interpret this sample result with caution.

DISCUSSION

There are four primary explanations that were developed by FBE and Vercet as to why human fecal contamination was not identified as a source of bacteria to Parsons Creek in the PhyloChip® results despite previous results indicating otherwise (e.g., positive results from canine scent detection, DNA ribotyping, historical evidence of high Enterococcus levels in the creek, documented issues with septic systems in the watershed, etc.).

1. The microbial community in Parsons Creek was not a good match for any microbial community in Veracet's database of known fecal sources. Veracet suggested that "it is possible that compositional differences between local fecal sources and the source samples comprising the categories in the source library used to train the SourceTracker classifier resulted in lower predicted contributions of source categories to samples of unknown composition" (Attachment 1). In other words, it is possible that the microbial community associated with human fecal waste in Parsons Creek was different enough from the microbial communities that PhyloChip® uses to identify human fecal waste that it did not appear as a source during analysis. Veracet mentions that the "inclusion of locally derived source samples into the source library can improve SourceTracker predictions;" however, they also add that "the SourceTracker relies on such a large set of bird and human samples in its library that local samples would provide, at

most, only minor refinement of results in these cases" (Attachment 1). PhyloChip® analysis performed in nearby watersheds (the Ogunquit River in Ogunquit, ME) has successfully identified human fecal contamination in samples using the same SourceTracker library (FBE, 2022), suggesting that the methodology is applicable to coastal watersheds in northern New England. Note that despite two wet-weather sampling dates, the watershed was experiencing an exceptional drought in 2022 when samples were collected (D1 in July and October and D2 for August and September according to the U.S. Drought Monitor; NOAA, 2023), which may have resulted in an atypical transport pattern for fecal waste (for human, pets, and wildlife).

2. The high concentrations of particulates in the samples could have caused matrix interference that impacted the results. As is typical for tidal creeks in salt marshes, the water samples collected in Parsons Creek contained a high level of particulates that were retained on the water filters along with the DNA material of interest after filtration. At ACPS005-U35 there was so much particulate matter in the samples that filtering took substantially longer than the other sites, and in two cases, only 100 mL could be filtered (Attachment 2). The presence of this additional substrate could have impacted laboratory processes or the DNA on the filter directly; however, Veracet confirmed that there was only one potential issue with DNA preparation (PC-OUT on 10/4/2022 had low DNA after multiple extractions; Attachment 1) and that all other samples had "thousands of bacterial species that would be commonly found in environmental water" (Attachment 1). Together, this suggests that although possible, matrix interference is unlikely to have impacted the results.

3. It is possible that, because of watershed management efforts such as the replacement of failed septic systems and the implementation of the town pump out ordinance, human fecal contamination in Parsons Creek has been reduced to the point where the human signature is undetectable at the five sampling sites. If these results were interpreted without the knowledge of previous water quality data and trends in Parsons Creek, this explanation would seem like the most likely scenario. However, given that human fecal contamination has been identified as a diffuse source throughout the watershed via other methods (canine scent detection and DNA ribotyping) and that Enterococci levels at the five sites have increased or remained high in recent years, it would be reasonable to doubt this conclusion. To better resolve these conflicting results, it is recommended that additional source tracking data be collected using alternative methods (e.g., mitochondrial DNA markers).

4. The sensitivity of the PhyloChip® method may not be high enough to detect the diffuse levels of human fecal contamination in Parsons Creek. Since the method is centered around matching the microbial composition of known fecal sources to the microbial community found in water samples, it is possible that results indicate no human sources if the microbial community is altered in transit from its source(s) to the sampling site in such a way that it becomes unrecognizable among the fecal source assemblages listed in Veracet's SourceTracker database. Both the degree to which the microbial community changes as it travels through the environment and the sensitivity of the PhyloChip® method to these changes are difficult to quantify. One study in New England has shown that habitat characteristics have a greater impact on microbial community composition in salt marshes than external, human-derived inputs such as nutrients (Bowen et al., 2009). This conclusion aligns well with the microbiological paradigm that "everything is everywhere, but the environment selects" (Bass-Becking, 1934), and suggests that the salt marsh area surrounding Parsons Creek could be actively dampening any human waste signal from septic systems by amplifying or reducing specific bacterial taxa based on environmental conditions.

   Additionally, there is precedent for the PhyloChip® method appearing less sensitive than other fecal source tracking methods, as recent sampling conducted by FBE in Rocky Neck State Park in East Lyme, CT yielded multiple positive hits for human waste via canine scent detection but no human sources via

PhyloChip® (FBE, 2023). Like Parsons Creek, the primary waterbody of interest in Rocky Neck State Park was surrounded by expansive brackish wetlands. In addition, the successful identification of human fecal contamination in samples from the Ogunquit River in Ogunquit, ME occurred from a freshwater tributary to the tidal portion of the Ogunquit River.

CONCLUSIONS AND RECOMMENDATIONS

Enterococcus is the New Hampshire state water quality standard for recreational waters and is based on federal recommendations. Many health studies at bathing beaches have found that high levels of Enterococcus are associated with illnesses after swimming, and therefore Enterococcus has a strong scientific basis as an indicator of unsafe recreational waters; however, Enterococcus are not specific to any single source of pollution. They may come from untreated wastewater, pets, wildlife including mammals and birds, and may occur in soils, aquatic sediments, decaying plants, and groundwater where a fecal source is not clear (Boehm and Sassoubre, 2014). For this reason, Enterococcus sampling must be paired with other fecal source tracking methods to understand the pollution sources and risks to beach swimmers. Human fecal contamination was not detected in the current tracking round using PhyloChip®, contrary to prior source tracking results in Parsons Creek. In fact, no "clear" animal sources were detected at all. Birds were not detected, and only two animal categories were found at "grey area" possible detection levels (rats and raccoons). Many potentially harmful bacterial genera which are transmissible through contaminated water were detected, including high numbers of Streptococcus and Staphylococcus genera. This information is important for the public to be made aware of as vulnerable populations should avoid coming into direct contact with water from Parsons Creek.

We outline four possible explanations for these results. When examined together, we believe that the fourth and final scenario (low sensitivity) is the most likely explanation as to why human fecal contamination was not identified in Parsons Creek during PhyloChip® analysis, although we do not rule out some degree of water quality improvement due to years of sustained action by the Town which may have reduced human fecal contamination. We are skeptical of the claim that PhyloChip® results indicate *no* human fecal contamination in Parsons Creek. That claim contradicts multiple fecal source tracking efforts that have been performed in the past and appears inconsistent with the elevated Enterococci levels observed in the watershed in recent years. Showing substantially reduced (or no) fecal contamination requires additional confirmation from multiple investigative approaches to be credible. The contradictory results found in a concurrent study in East Lyme, CT where PhyloChip® and other source tracking methods were employed simultaneously in a similar salt marsh dominated watershed provide some evidence that PhyloChip® may have insufficient sensitivity for fecal contamination in this type of environment. **Thus, we conclude that the PhyloChip® results for Parsons Creek reflect poor method sensitivity in brackish, salt marsh environments rather than a true indication of environmental conditions characterized by a lack of human fecal sources.** We further highlight that the high per sample cost of PhyloChip® is particularly limiting in a case such as this, where additional sampling would ordinarily be warranted to better support the hypothesis of reduced human fecal contamination. We recommend that other communities in New England carefully consider the type of environment intended for source tracking before using PhyloChip® technology, which may be more suitable for freshwater systems or estuaries with minimal salt marsh.

To better interpret these PhyloChip® results, especially given previous fecal source tracking efforts showing human sources and the continued high levels of Enterococcus at some sites in the watershed, it is recommended that the following actions be taken. The Town is planning on applying for a second Clean Water State Revolving Fund (CWSRF) project to secure funding, along with using the Town's annual water quality monitoring budget, to implement these recommendations.

- Collect additional fecal source tracking data in Parsons Creek using an alternative method. A lower-cost method that has a proven track record of success locally is analysis for mitochondrial DNA markers of known fecal sources by Dr. John Bucci's Laboratory at the University of New Hampshire.
- Continue monitoring Enterococci levels at multiple stations to establish a time series for trend analysis.
- Consider adding or adjusting monitoring stations to be closer in proximity to suspected fecal sources (e.g., failing septic systems, highly trafficked pedestrian areas, etc.).
- Map septic system pump out ordinance compliance against Enterococcus data to better understand potential positive impacts on Parsons Creek water quality.
- Conduct additional analyses for harmful bacterial genera.
- Convene water quality experts to review Parsons Creek data and discuss management approaches.

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ATTACHMENT 1: VERACET REPORT

Veracet Report for Project RYE

2023-03-22

METHODS

Sampling

RYE samples (25 total) were collected from 5 sites (ACPS005-U35, BCH11, PC07, PC08 and PC-OUT) on 5 dates over 4 months: 7/5/2022, 7/21/2022, 9/6/2022, 9/20/2022, and 10/4/2022. Samples were processed on two different plates of G4-PhyloChip arrays; negative controls prepared using nuclease-free water (NFW) were processed alongside samples.

DNA Extraction, amplification, and hybridization

DNA was extracted from water filters using the DNA-EZ extraction kit (Generite, New Brunswick, NJ). The bacterial 16S rRNA genes were amplified using the degenerate forward primer: 27F.1 5'-AGRGTTTGATCMTGGCTCAG-3' and the non-degenerate reverse primer: 1492R.jgi 5'-GGTTACCTTGTTACGACTT-3'. For each sample, amplified products were concentrated using a solid-phase reversible immobilization method for the purification of PCR products and quantified by electrophoresis using an Agilent 2100 Bioanalyzer®. PhyloChip Control MixTM was added to each amplified product. Thirty-five cycles of bacterial 16S rRNA gene PCR amplification were performed. Labelled bacterial products were fragmented, biotin labelled, and hybridized to the PhyloChipTM Array, version G4. PhyloChip arrays were washed, stained, and scanned using a GeneArray® scanner (Affymetrix). Each scan is captured using standard Affymetrix software (GeneChip® Microarray Analysis Suite). Samples are processed in a Good Laboratory Practices (GLP) compliant service laboratory running Quality Management Systems for sample and data tracking. The laboratory implements detailed SOPs, equipment and process validation, training, audits, and document control measures. QC and QA metrics are maintained for all sample handling, processing, and storage procedures.

PhyloChip analysis

Two approaches were used to analyze the fluorescent image files following array scanning. In the first approach, the presence of 59,316 different bacterial OTUs was determined by positive hybridization of multiple probes that correspond to distinguishing 16S rRNA gene polymorphisms (average of 37 probes/OTU). The hybridization score (HybScore) for a taxon was calculated as the mean intensity of the perfectly matching probes exclusive of the maximum and minimum. This approach yields an inventory of detected taxa that compose the microbial community. The second analysis approach considered probe quartet data. The probe-based approach uses each of the PhyloChip's 1,015,124 probe features to determine diagnostic sequences for specific fecal sources and detect these targets in environmental samples. In this study, we analyzed quartets of probes that target the sense, anti-sense, and corresponding mismatch probes of each targeted sequence. This approach controls for non-specific hybridization and relies on the detection of both complementary DNA strands to increase the performance of the assay. PhyloChip analysis was conducted using Sinfonietta software in the R Environment. SourceTracker (v2) was used to determine the proportion of samples that could be attributed to animal sources.

RESULTS

General Overview of Fecal Sources (SourceTracker)

The potential source pools were various animal groups: Bird, Dog/Cat, Horse, Human, Pig, Pinniped, Raccoon, Rat, and Ruminant. Source pools were constructed using PhyloChip scanning data from 80 samples with each source category representing 3–20 samples (average of 8.89 samples per category[1]). The values for the source groups across the water samples represent the proportion of the bacterial community in that sample that likely came from the indicated source group; the standard deviation of 10 independent runs of the SourceTracker tool. The share of the bacterial community in the sample that cannot be associated with a source group in the data set is classed as unidentified.

Previous research has shown that source pools with values greater than 0.20 for a particular sample are a clear source of bacteria for that sample. A value between 0.2 and 0.10 is a grey area, where the potential source pool may be a source for that sample.

Table 1 shows that for the samples analyzed (rows in the table), humans are not a clear source of bacteria. Fecal material from **Raccoon** is a potential source of bacteria in samples BCH11 12:05 9/6/2022, ACPS005-U35 12:50 9/20/2022, PC07 12:55 7/21, PC08 11:30 10/4/2022, ACPS005-U35 11:50 10/4/2022, PC08 12:10 7/21/2022, and PC07 12:30 9/20/2022. Fecal material from **Rat** is a potential source of bacteria in samples ACPS005-U35 11:50 10/4/2022, PC08 12:10 7/21/2022, BCH11 12:05 9/6/2022, ACPS005-U35 12:50 9/20/2022, PC07 12:55 7/21/2022, and PC08 11:30 10/4/2022. Other non-human animals do not appear to be a source of bacteria in the water samples.

We are confident that the microbial community composition of each sample is reflective of the DNA that was isolated from the filters. The samples that we analyzed had thousands of bacterial species that would be commonly found in environmental water but had very few of the bacterial species that would be found in fecal contamination. It is possible that compositional differences between local fecal sources and the source samples comprising the categories in the source library used to train the SourceTracker classifier resulted in lower predicted contributions of source categories to samples of unknown composition[2]. Inclusion of locally derived source samples into the source library can improve SourceTracker predictions. Indicative thresholds for SourceTracker predicted contributions can also be established empirically if known positive samples are included in the data set. However, the SourceTracker relies on such a large set of bird and human samples in its library that local samples would provide, at most, only minor refinement of results in these cases.

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[1] Number of source samples by source: Bird = 24; Dog/Cat = 7; Horse = 5; Human = 20; Pig = 4; Pinniped = 4; Raccoon = 3; Rat = 3; Ruminant = 10. The source samples were collected in California and Ohio and are mixtures of composite samples (i.e., the sample used for the PhyloChip is a mixture of DNA from 2 or more different fecal samples). Generally, but not in all cases, the source samples are composites and the human source samples are a combination of 2 or more individuals. The source sample library was collected from 2016-2019.

[2] The "unknown" category is how SourceTracker classes microbial community compositional patterns that cannot be assigned to any of the source categories provided in the training data set. Essentially, where the microbial community 'fingerprint' of the 'sink' (the sample being queried, more commonly referred to as the 'test' set by machine learning classification algorithms) cannot be ascribed to any of the 'source' categories (commonly referred to as the 'training' set by machine learning classification algorithms), that fraction of the sink is classed as "unknown". This should not be interpreted as the sample containing an "unknown" fecal source. Rather, it should be interpreted more along the lines of "An unknown microbial community has contributed [SourceTracker predicted "Unknown" value] to the microbial community of this sample." For complex environmental samples, collecting samples from similar microbial ecosystems that are known to be unimpaired by fecal sources of interest can dramatically reduce the degree of SourceTracker predicted "unknown" contributions in samples. This strategy effectively introduces a 'pristine' control into the training data set, providing SourceTracker with a viable alternative to the "unknown" category.

Table 1. Predicted contributions of different source categories to environmental samples. Data are mean of 10 SourceTracker iterations calculated using PhyloChip responsive probe incidence data. Previous research has shown that source pools with values greater than 0.20 (red) for a particular sample are a clear source of bacteria for that sample. A value between 0.2 and 0.10 (yellow) is a grey area, where the potential source pool may be a source for that sample.

Site Name / Time / Date / Sampler	Bird	Dog/Cat	Horse	Human	Pig	Pinniped	Raccoon	Rat	Ruminant
ACPS005-U35 11:25 7/5 MJK	0.004	0.017	0.004	0.010	0.016	0.004	0.072	0.065	0.013
ACPS005-U35 11:50 10/4/2022 MJK	0.058	0.039	0.013	0.045	0.007	0.013	0.116	0.118	0.008
ACPS005-U35 12:50 9/20/2022 MJK	0.027	0.029	0.009	0.037	0.010	0.007	0.128	0.109	0.009
ACPS005-U35 12:55 9/6/2022	0.005	0.019	0.004	0.011	0.012	0.005	0.087	0.081	0.013
ACPS005-U35 13:10 7/21 MJK	0.004	0.019	0.003	0.009	0.018	0.005	0.079	0.070	0.011
BCH11 10:15 7/5 MJK	0.005	0.020	0.003	0.010	0.008	0.005	0.058	0.072	0.010
BCH11 11:20 10/4/2022 MJK	0.003	0.020	0.003	0.009	0.009	0.003	0.048	0.065	0.008
BCH11 11:45 7/21 MJK	0.005	0.016	0.004	0.009	0.013	0.004	0.047	0.064	0.011
BCH11 12:05 9/20/2022 MJK	0.005	0.019	0.004	0.011	0.009	0.004	0.051	0.065	0.013
BCH11 12:05 9/6/2022	0.022	0.030	0.009	0.022	0.011	0.014	0.144	0.110	0.011
PC07 10:55 7/5 MJK	0.004	0.021	0.003	0.010	0.008	0.004	0.059	0.069	0.009
PC07 11:40 10/4/2022 MJK	0.009	0.019	0.005	0.016	0.005	0.006	0.070	0.077	0.010
PC07 12:30 9/20/2022 MJK	0.009	0.024	0.006	0.018	0.012	0.006	0.105	0.094	0.011
PC07 12:30 9/6/2022	0.004	0.015	0.003	0.011	0.007	0.003	0.052	0.054	0.009
PC07 12:55 7/21 MJK	0.034	0.030	0.012	0.033	0.011	0.014	0.127	0.107	0.012
PC08 10:45 7/5 MJK	0.006	0.015	0.004	0.010	0.009	0.004	0.052	0.062	0.011
PC08 11:30 10/4/2022 MJK	0.028	0.033	0.011	0.031	0.008	0.021	0.117	0.104	0.014
PC08 12:10 7/21 MJK	0.020	0.026	0.009	0.024	0.010	0.009	0.115	0.112	0.011
PC08 12:20 9/20/2022 MJK	0.004	0.018	0.003	0.009	0.009	0.004	0.059	0.069	0.010
PC08 12:20 9/6/2022	0.004	0.015	0.003	0.010	0.008	0.003	0.046	0.056	0.009
PC-OUT 11:00 10/4/2022 MJK	0.003	0.003	0.005	0.002	0.002	0.008	0.004	0.002	0.002
PC-OUT 11:20 7/21 MJK	0.009	0.020	0.004	0.014	0.006	0.006	0.062	0.066	0.009
PC-OUT 11:40 9/6/2022	0.006	0.025	0.004	0.014	0.009	0.006	0.071	0.081	0.010
PC-OUT 11:50 9/20/2022 MJK	0.008	0.028	0.005	0.016	0.006	0.006	0.076	0.075	0.010
PC-OUT 9:40 7/5 MJK	0.008	0.022	0.004	0.015	0.006	0.005	0.072	0.073	0.008
Neg Control Plate_1420070001	0.008	0.005	0.008	0.003	0.002	0.005	0.004	0.002	0.003
Neg Control Plate_1420090001	0.004	0.003	0.004	0.002	0.003	0.004	0.003	0.004	0.003

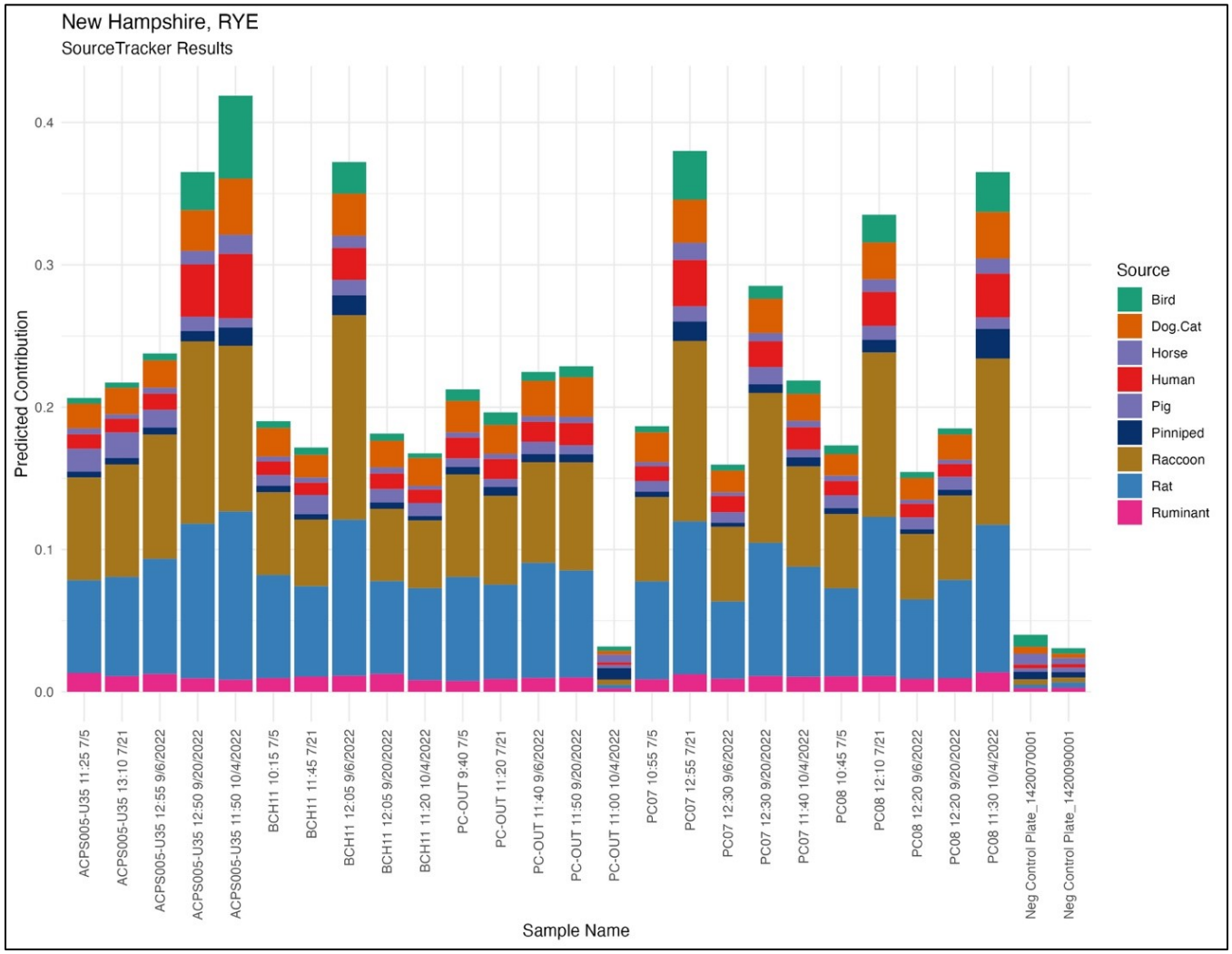

Figure 1. Predicted contributions of different source categories to environmental samples. Data are the mean of 10 SourceTracker iterations calculated using PhyloChip responsive probe incidence data.

The SourceTracker profile of sample PC-OUT 11:00 10/4/2022 is markedly different to those of samples collected from PC-OUT at different time points but is largely similar to those of the two negative controls, suggesting a possible issue with sample collection, DNA preparation, or chip scanning (Figure 1).

Evaluation of DNA extraction and amplification quality control metrics shows a low yield of DNA from sample PC-OUT 11:00 10/4/2022. DNA was extracted from this sample twice, with the extractions yielding 246.55 and 373.75 ng, respectively. Both yields are lower than is typically expected from environmental samples (typical range 700-1000 ng). In addition, the purity and fluorescence concentration of DNA from this sample were both abnormal. The purity of the DNA was low (A260/280 = 4.1), likely indicating carryover proteins or other contaminants from the sample during preparation. This type of carryover often results when there is poor yield of DNA. Fluorescent analysis of DNA concentrations, which is less sensitive to contaminants than spectroscopic determination, found a very low concentration of DNA in the sample (1.59 ng, far lower than the other PC-OUT samples at 21.26±9.02 ng). Together, these metrics suggest an issue with DNA extraction or preparation. Whether that issue is related to sample collection, sample preparation, or an actual lack of DNA sources in the sample is difficult to determine. Measured

environmental metrics for which sample PC-OUT 11:00 10/4/2022 differs from other PC-OUT samples are 1-,2- and 4-day precipitation levels, nitrate-nitrite, total phosphate, and temperature (Figure 2), and may or may not have been related to the low yield of DNA for PC-OUT 11:00 10/4/2022.

The chip manufacturer, Affymetrix ThermoFisher, has rigorous controls in place and destructively tests several of the microarrays before they leave their manufacturing facility. The service provider requires one of the chips of each set of 16, 24 or 96 to be reserved for their internal control test. We have an internal control spike mix that goes on every microarray that we process. This has synthetic and PCR-derived DNA of specific concentrations that is added to the sample after PCR amplification. The spike mix has corresponding control probes that we designed for analysis of chip-to-chip variability both between and within a specific microarray hybridization run. Finally, positioning probes are used on all Affymetrix microarrays to ensure proper alignment when laser scanning the microarray, since each probe is less than 2 microns in size, as a final control.

Many of the filters were dark clay-brown and caked over with sediment due to high particulates in the water samples. We cannot say for certain whether or not the high particulate composition changed the results in any significant way.

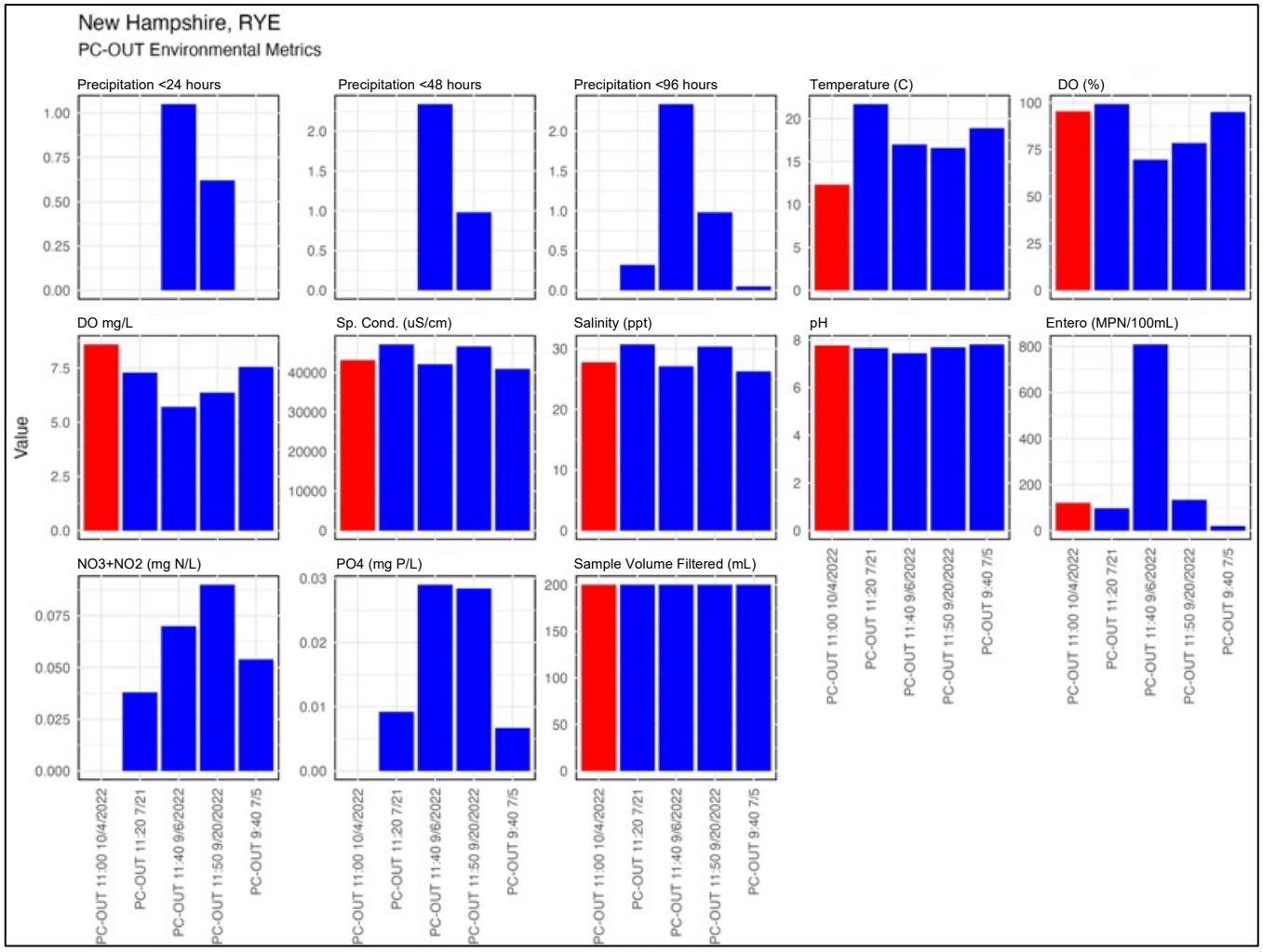

Figure 2. Numeric environmental metrics for PC-OUT samples; PC-OUT 11:00 10/4/2022 is shown in red, other PC-OUT samples are shown in blue.

Comparison of PhyloChip and Culturing

Notably, sample PC-OUT 11:40 9/6/2022 had an abnormally high count of Enterococcus (MPN /100 mL = 809; more than quadruple the Enterococcus MPN /100 mL of the other PC-OUT samples). The MPN /100 mL count for this sample is not an outlier among all samples (Figure 3), suggesting that the Enterococcus MPN /100 mL counts for this sample are reasonable.

We explored the relationship between the percent of bacterial sources traced to humans and the Enterococcus count (MPN/100mL). Excluding sample PC07 10:55 7/5/2022, which had an abnormally high Enterococcus count but relatively low proportion of human, there is a weak correlation between the two measurements (Figure 4). This correlation should be interpreted with caution due to the low value of the predicted contributions.

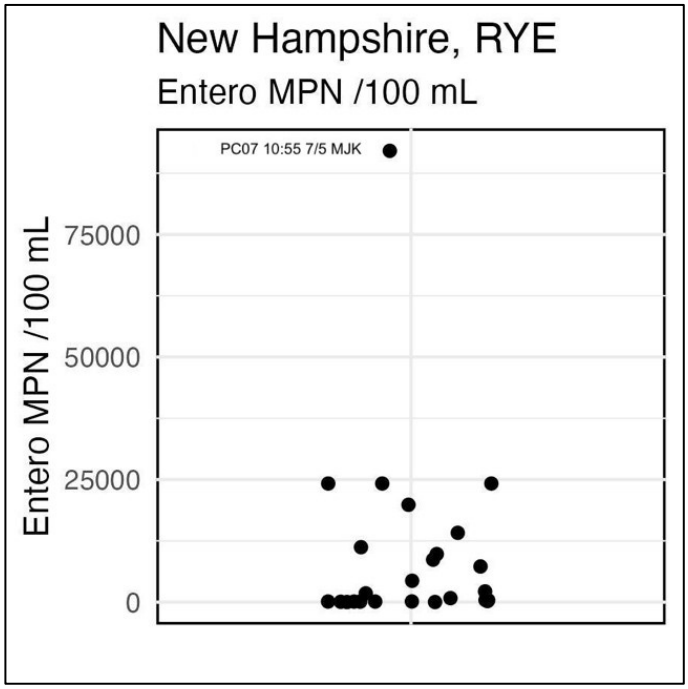

Figure 3. Measured Entero MPN/100mL.

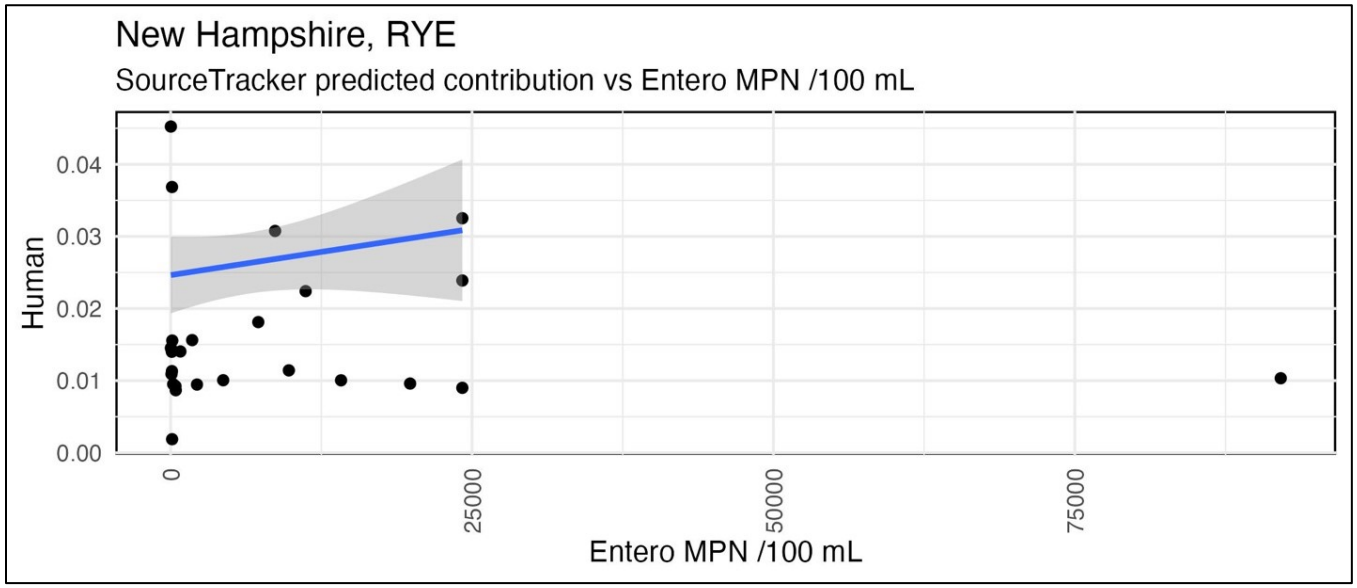

Figure 4. Relationship between Entero MPN/100mL and predicted contribution from Human sources. Entero MPN/100mL from sample PC07 10:55 7/5/2022 has been excluded from the calculation of the linear fit.

Ordination & Hierarchical Clustering

Another way to look at PhyloChip results is to compare the make-up of the different bacterial communities in the samples. In an ordination, each sample is represented by a dot. The samples that share many bacteria in common are close together in the plot; in other words, the dots are close together. On the other hand, those samples that do not share species are far apart - the dots are more distant (Figures 5 and 6, top panel). Hierarchical clustering is another way to look at relationships between samples (Figure 7). Both plots further indicate that PC-OUT 11:00 10/4/2022 is an outlier from the other PC-OUT samples (Figures 5-7). These data also provide additional evidence that the high Enterococcus MPN/100mL counts for sample PC07 10:55 7/5/2022 are likely not an indication of high fecal contamination, since the microbial community composition of this sample is similar to other samples with low Enterococci MPN/100mL counts and low evidence of fecal contamination as determined by PhyloChip. The clustering on both the dendrogram and NMDS plot show that the microbial community of some samples (PC-OUT, PC-07, PC-08, and BCH11) are largely governed by sample site, but that the community in samples from site ASPS005-U35 is more variable over time (Figures 5-7).

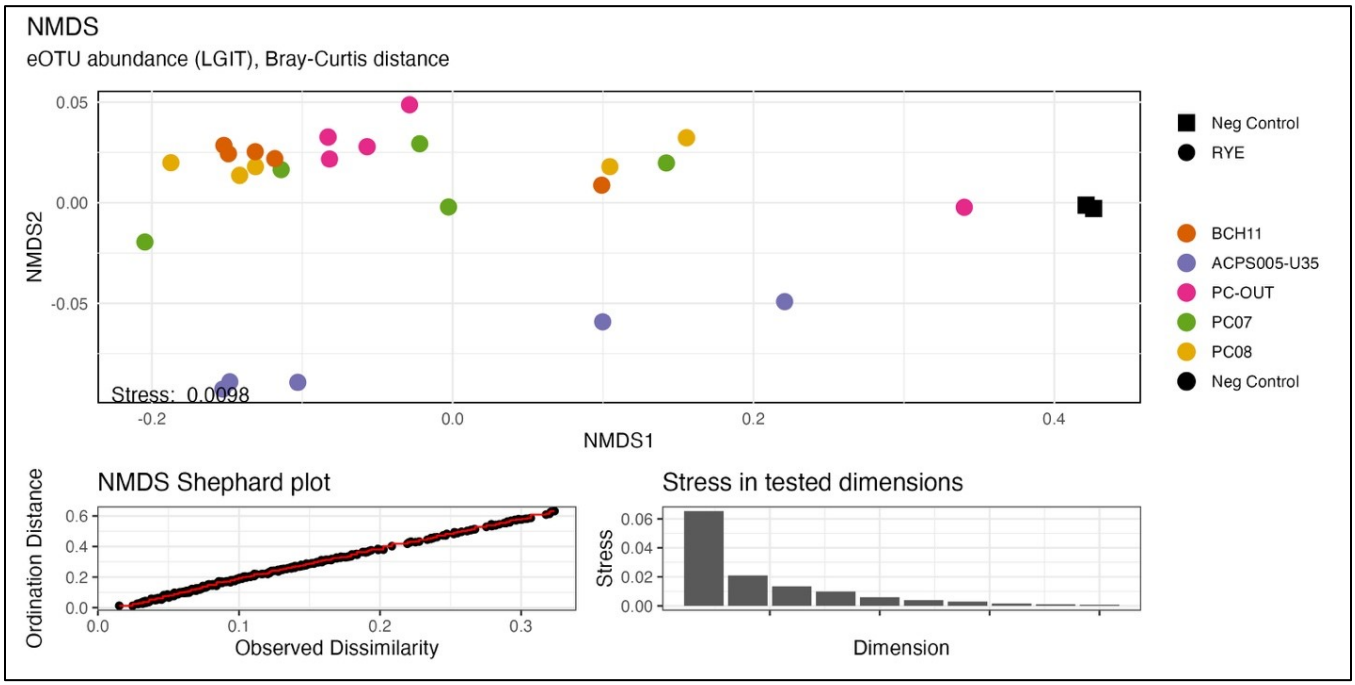

Figure 5. NMDS ordination of community composition; samples are colored by sample group (top panel). Fit and stress metrics used to evaluate the reliability of the NMDS (bottom left and right panels, respectively)

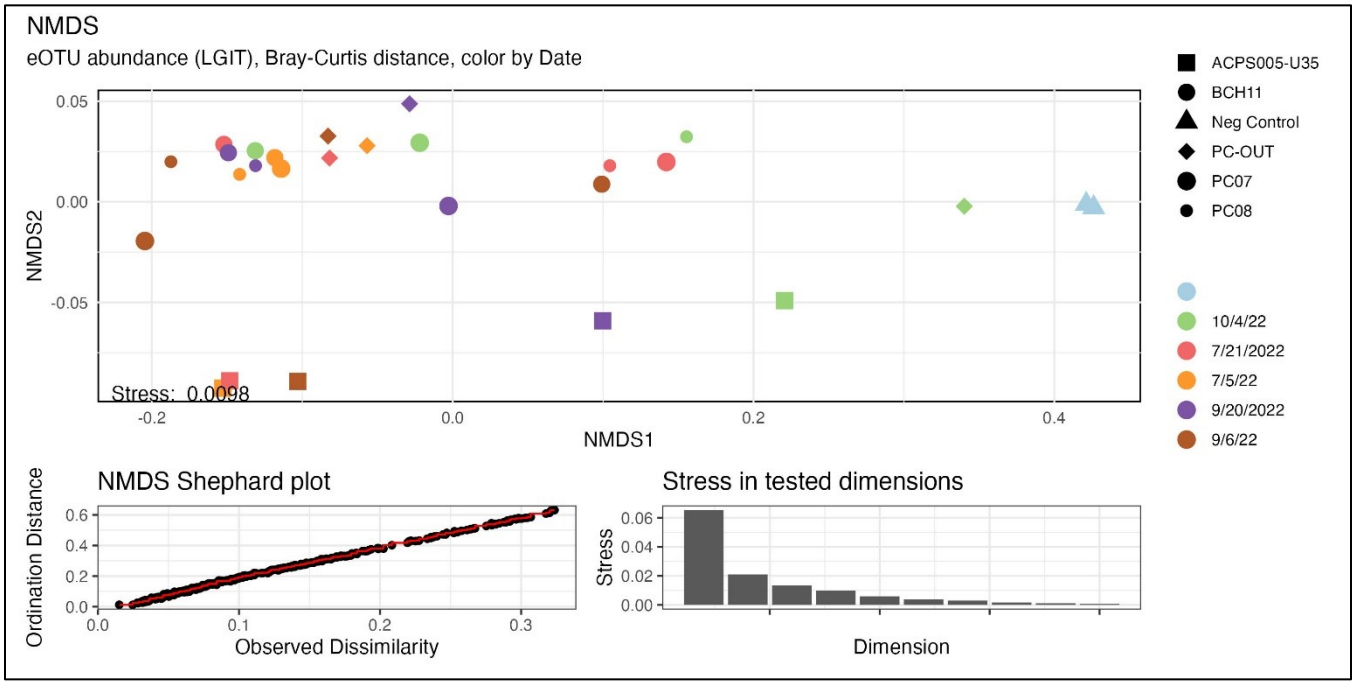

Figure 6. NMDS ordination of community composition; samples are colored by sample date and symbolized by sample group (top panel). Fit and stress metrics used to evaluate the reliability of the NMDS (bottom left and right panels, respectively)

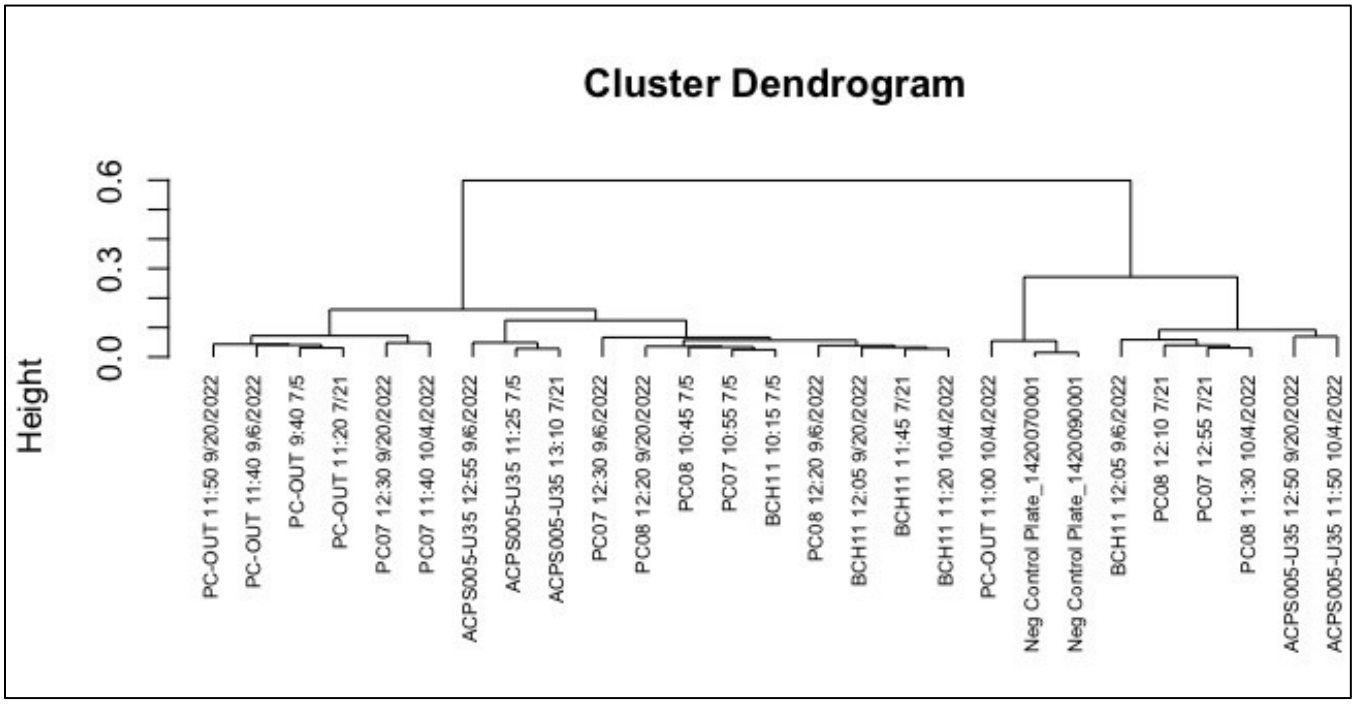

Figure 7. Hierarchical clustering of community composition.

Abundance of Specific Bacterial Genera

The presence of specific bacterial genera can also be an indication of fecal contamination in water samples. Taxonomic assignment of groups of responsive probes on the PhyloChip provides insight into the number of different bacterial populations from specific genera that are present above a defined threshold in samples (Figure 8). Streptococcus was most abundant across sites.

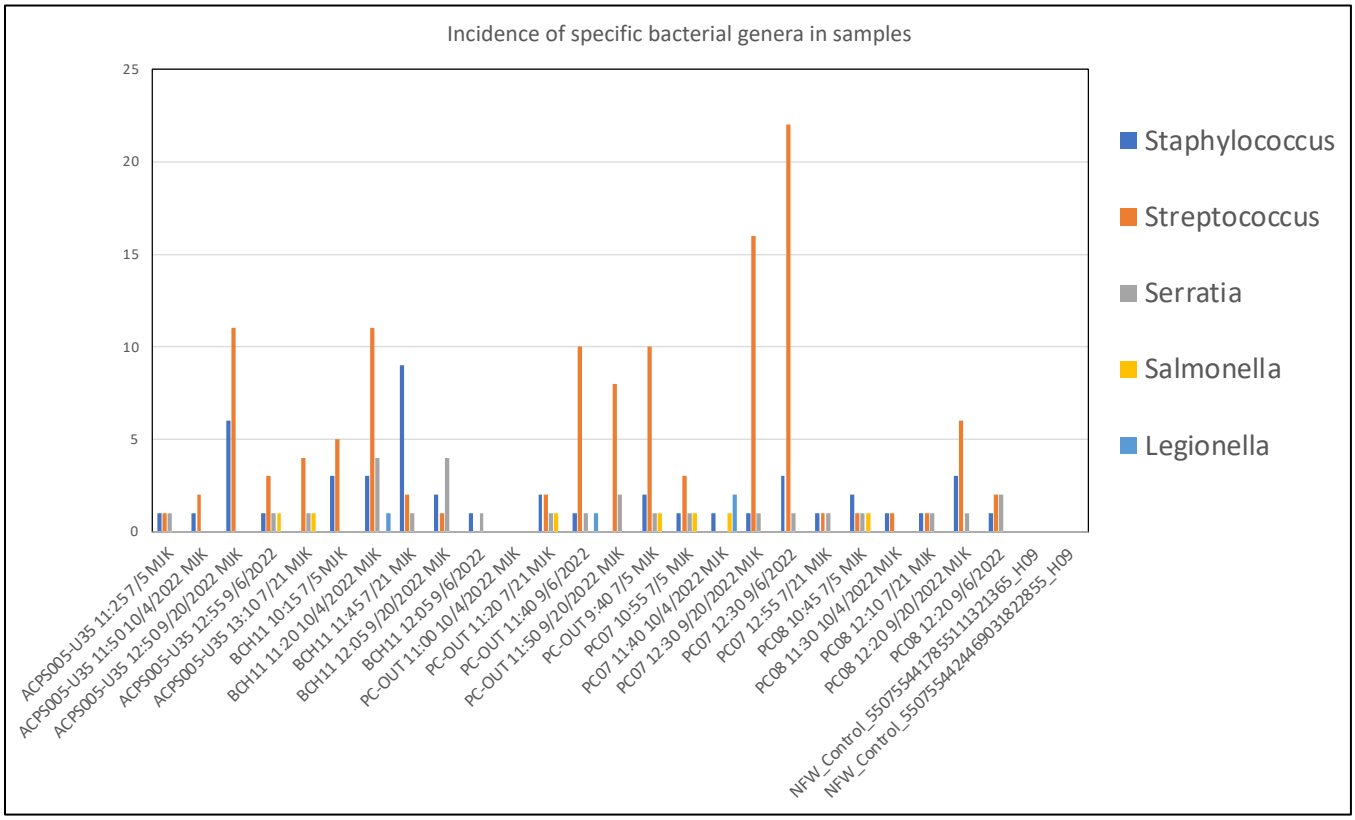

Figure 8. Occurrence of bacterial populations identified as belonging to specific genera sometimes associated with fecal impairment. Values shown are the total number of different OTUs belonging to the indicated genera in samples.

Overall Conclusions

We examined potential sources of contamination in the RYE samples using the whole water microbiome as the basis for the analysis. This contrasts with a single bacterial indicator of the presence of fecal contamination approach (e.g., Enterococci culture counts). Although indicators of fecal contamination may be correlated with actual contamination, in some cases they are not. This has been documented in several well-cited scientific publications.

From our analysis, there does not appear to be any human-associated fecal contamination present. The sources of contamination that had the highest proportion (Racoon, Rat) were still below the level of statistical confidence that these sources of contamination were present. Because there was no definitive fecal contamination in these samples, we looked at the metadata that was provided to see if anything from the analysis of the entire microbial community from each sample could provide any useful information. Adding all the proportions from each potential source as shown in Figure 1 is a way to visualize the entire number for fecal-like microbes that are present. This is sometimes useful if there is a source of contamination that is from a yet unidentified source. Although that does not appear to be the case in this instance, it does give a sense for where there is absolutely no chance of fecal contamination and where a sample would merit further study. An examination of specific bacterial genera associated with fecal contamination was also visualized in Figure 8. Several samples had higher than expected values for Streptococcus. This may occur from environmental sources, as well as fecal sources, but is included for reference.

Overall, even though some samples were shown to have high levels of Enterococcus, we have high confidence that this is not related to human-associated fecal contamination. In fact, we demonstrate that the bacterial community present in each sample is consistent with very low levels of fecal contamination by statistically significant analytic methods and would be considered non-impaired during the time of the study if PhyloChip were used rather than the EPA recommended culture-based Enterococcus water quality standards.

ATTACHMENT 2: QUALITY ASSURANCE / QUALITY CONTROL REVIEW

All standard field sampling and filtering protocols for PhyloChip® analysis were followed by FBE staff with one minor exception. Due to low filtration rates caused by high particulates in the water, two of the five PhyloChip® samples collected at ACPS005-U35 only had 100 mL of water filtered (Table A1). This smaller filtration volume was at the recommendation of Veracet due to low filtration rates and was determined to not impact the results (Attachment 1). All other samples received the full 200 mL filtration. All samples were filtered within the eight hours of collection in accordance with the PhyloChip® hold time (Table A1). To ensure that the water collected was representative of upland sources in the watershed, all samples were collected within two hours of low tide (Table A2). For a quality control review of PhyloChip® laboratory procedures, see Attachment 1.

Table A1. Summary of filtration information for all PhyloChip® samples.

Sample Collection Information			Filtration Information			
SiteID	Date	Collection Time	Filter Time	Difference Between Collection Time and Filter Time (hh:mm)	Volume Filtered (mg/L)	Notes
PC-OUT	7/5/2022	9:40	14:35	4:55	200	Quick filter
BCH11	7/5/2022	10:15	14:25	4:10	200	Quick filter
PC08	7/5/2022	10:45	14:40	3:55	200	Quick filter
PC07	7/5/2022	10:55	14:50	3:55	200	Quick filter
ACPS005-U35	7/5/2022	11:25	14:55	3:30	200	Took ~30 minutes to filter. There is lots of small floating plant material at this site
PC-OUT	7/21/2022	11:20	16:00	4:40	200	Quick filter
BCH11	7/21/2022	11:45	16:05	4:20	200	Quick filter
PC08	7/21/2022	12:10	16:10	4:00	200	Quick filter
PC07	7/21/2022	12:25	16:15	3:50	200	Quick filter
ACPS005-U35	7/21/2022	13:10	16:20	3:10	200	Took ~20 minutes to filter. There is lots of small floating plant material at this site
PC-OUT	9/6/2022	11:40	17:37	5:57	200	Quick filter
BCH11	9/6/2022	12:05	17:50	5:45	200	Quick filter
PC08	9/6/2022	12:20	17:57	5:37	200	Quick filter
PC07	9/6/2022	12:30	18:05	5:35	200	Quick filter
ACPS005-U35	9/6/2022	12:55	18:12	5:17	100	Filter rate was slow at the end of first 100 mL so only 100 mL was filtered
PC-OUT	9/20/2022	11:50	18:40	6:50	200	Quick filter
BCH11	9/20/2022	12:05	18:45	6:40	200	Quick filter
PC08	9/20/2022	12:20	18:50	6:30	200	Quick filter
PC07	9/20/2022	12:30	18:55	6:25	200	Quick filter
ACPS005-U35	9/20/2022	12:50	19:00	6:10	100	Filter rate was slow at the end of first 100 mL so only 100 mL was filtered
PC-OUT*	10/4/2022	11:00	16:00	5:00	200	Quick filter
BCH11	10/4/2022	11:20	16:05	4:45	200	Quick filter
PC08	10/4/2022	11:30	16:12	4:42	200	Quick filter
PC07	10/4/2022	11:40	16:16	4:36	200	Quick filter
ACPS005-U35	10/4/2022	11:50	16:25	4:35	200	Took ~10 mins to filter

Table A2. Summary of PhyloChip® sample times in relation to low tide.

Sample Date	Low Tide Time	Time of First Tidal Sample	Time of Last Tidal Sample	Time to Low Tide (First Tidal Sample) (hh:mm)	Time to Low Tide (Last Tidal Sample) (hh:mm)
7/5/2022	9:57	9:40	11:25	0:17	1:28
7/21/2022	12:29	11:20	13:10	1:09	0:41
9/6/2022	13:39	11:40	12:55	1:59	0:44
9/20/2022	13:45	11:50	12:50	1:55	0:55
10/4/2022	12:23	11:00	11:50	1:23	0:33