Using Microbial Source Tracking to Quantify Human Fecal Contamination at the Santa Ana Watershed

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Acknowledgment

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Executive Summary

Water bodies are essential to monitoring for public health purposes because they provide an indispensable resource for the communities it supplies, such as for recreation or as a water source for drinking water. The Santa Ana Watershed transverses a large section of southern California, from its origin in Big Bear, CA, to its outlet in Huntington Beach, CA. This Watershed thus affects many communities along the way, and ensuring its health by monitoring its microbial activity is essential in keeping the public safe. Microbial communities are diverse and can shift when they experience external influences; this can cause it to pose little harm or, conversely, a grave danger to society. The purpose of this project is to continue the monitoring of the river using molecular biology techniques to determine if there is any impact on the waterway's health and the growing homeless presence in the watershed. The method heavily implemented was microbial source tracking (MST) using qPCR to determine the presence of host-specific human fecal indicators in relation to total bacteria concentrations. Unfortunately, due to the current COVID-19 pandemic and material failures, the project was unable to attain a definite conclusion. The continued monitoring of the Santa Ana Watershed when times allow would be vital for ensuring that all that use the water as a resource is protected.

Project Objectives

The initial objective of this project was to gather sufficient water sampling points of the Santa Ana Watershed to understand how HF183 concentrations are affected by different parameters such as dissolved oxygen, conductivity, water temperature, water flow (rain and dry seasons) and pH. From this understanding, one could use a statistical analysis test, with this project intending to use the principal component analysis, to determine the strongest influencing parameters. This information would allow a follow-up on teasing out how the parameters themselves are being affected and if there are mitigation controls to prevent further impact on the watershed.

Unfortunately, there were material failures involving the DNA extraction kit that cost loss to samples and poor controls on the qPCR analysis. So, the second objective was to begin to implement vigorous aseptic methods to minimize any possible cross-contamination that could be affecting the qPCR control wells. This included using a set of micropipettes exclusively for qPCR, ensuring that the portable fume hood was operational and using Elimase to sanitize the workspace and equipment. All new DNA extraction kits would go through a trial run before being used on a sample collection to ensure the kit was fully operational.

In the middle of processing samples from a sampling event, the COVID-19 pandemic forced California State University – Fullerton, like many other universities, to completely shut down and cease all operations, including ongoing research on campus. This impacted the project directly because a majority of the work was being done at the university. Thus, under the guidance of Dr. Gedalanga, the shift in project objective was to comb through primary literature in search of new MST techniques that can be utilized in the future.

Project Approach

Field Sampling

A stretch of the Santa Ana River, which runs through the City of Riverside, was of particular interest in this study. In Table 1, the locations are outlined with their corresponding geographic coordinates, in which sampling took place. Figure 1 also displays a map indicating the location of sampling for extra reference. These sites were selected since they are profoundly impacted by human activity, such as homeless encampments and recreational activity, but are downstream from a wastewater treatment plant; thus, theoretically, the water should be clean.

Sampling was done by collecting water at the center of the river, donning waders to reach the location, for each specified area. Care was taken to ensure that sediment was not disturbed so that only the water was filled into sterile 1L Nalgene bottles. While there, quantitative environmental water parameters were measured utilizing Orion Star™ A321 pH Portable Meter (ThermoFisher, Waltham MA) for pH, dissolved oxygen (DO), water temperature, conductivity, and oxidation-reduction potential. Qualitative parameters were also recorded, including human activity, sky conditions, and water turbidity.

Sample Processing

Water samples were transported from the river to California State University – Fullerton's environmental health laboratory inside a cooler with ice packs to preserve the specimens. These samples were then filtered following the United States Environmental Protection Agency (USEPA) Method 1600 with modifications. Briefly, water samples were poured through a 6-station stainless steel vacuum manifold (Advantec MFS, Dublin, CA) equipped with glass filtration apparatus with each device housing an Isopore Polycarbonate Membrane Filter (47mm diameter, 0.45 micrometer pore size; Millepore Sigma, St. Louis, MO)

while ensuring to follow aseptic technique, sterilizing all equipment and using good sterile handling practices. Filters were then placed on a sterile metal tray and cut into fine pieces that were transferred into a 2mL microcentrifuge tube. Tubes were stored in a 4C refrigerator until the next DNA extraction process was ready to commence.

After samples went through filtration, the next step was to extract the DNA for any captured cells. Using the ZymoBIOMICS DNA Miniprep kit (Zymo Research, Irvine, CA), the samples were processed using the manufactures instructions and solutions provided. In this task, the tubes containing lysing solution and the filter paper with cell mass were placed in a Tissuelyer LT bead basher (Quigen, Hilden, Germany) for 5 minutes. Furthermore, the tubes were placed in a Centrifuge 5430R (Eppendorf, Hamburg, Germany) and spun for one minute at 10,000 g-force. The resulting supernatant was then extracted into a collection tube, which was followed by a series of washing with buffer solution and binding steps as directed by the manufacturing instructions until the final DNA product was left with minimal contaminants and resuspended in 75µL DNAase/RNAase Free Water. The purity of the final product was determined by analyzing the solution with a Nanodrop Spectrophotometer (ThermoFisher, Waltham MA). This DNA would then be labeled and marked accordingly to be stored in the lab freezer at -18°C.

Extracted DNA solution would undergo quantitative polymerase chain reaction (qPCR), so that the amplified product could be detected, thus giving relative concentrations of the target gene marker. To minimize contamination concerns, all work was done in an Aura 250e Recirculating Ductless Fume Hood (Misonix, Farmingdale, NY), and pipettes were sterilized with ELIMINase™ Decontaminant (Decon Laboratories, King of Prussia, PA). In order to run the qPCR a master mix solution was prepared that contained 2x PowerUp SYBR Green Master

Mix (ThermoFisher, Waltham MA), 0.5 μM forward and reverse primer set (Table 3), and molecular grad biological water. A serial dilution was performed to create a standard curve for which the samples would be compared to. For the total bacteria amplification, *Escherichia coli* pure culture was used with the original DNA concentration being measure with the Nanodrop and then diluting by a factor of 10 from 10-2 until 10-4.

Similarly, for the human fecal marker, HF183, *Bacteroides dorei* pure culture set the standard curve, with the serial dilution starting at 10-4 and ending at 10-7. Each well contained 18 μL of prepared master mix solution and 2 μL of DNA sample. Plates were placed into a StepOnePlus Real-Time PCR System (ThermoFisher, Waltham, MA) for amplification. The run method was the same for both target genes except for annealing temperature, which was 61.5 ℃ for total bacteria and 59.0 ℃ for the human fecal marker (Figures 2 and 3).

Literature Research

The primary source for journal articles was through the OneSearch engine that is provided by CSUF. Utilizing this resource, many articles were analyzed for any new techniques in microbial source tracking (MST) that could be implemented on campus with current equipment or if a meaningful approach is found, a new device would need to be acquired. The search was focused on MST journals that focused on urban water bodies but would occasionally come across a noteworthy article that deviated from this narrow investigation. Essential elements that were after were seeking different primer sets that could target other host-specific organisms, as well as a comparison on how the current HF183 target measured up against emerging gene targets. All these journals were complied with in a synopsis that could gear future research endeavors with more recent approaches.

Project Outcomes

Unfortunately, the original purpose was unable to be continued due to substantial obstacles that occurred throughout the semester. Trouble began shortly after the collection of the February sampling event. Samples went through filtration, and then the DNA was extracted using the Zymo DNA extra kit. This DNA product was analyzed for concentration using a Nanodrop, and the results were perplexing. Concentrations read negative values with the absorption slope, not having a typical characteristic. The apparatus also kept breaking the sample column that forms between the pedestal and arm, signifying that reconditioning of the Nanodrop was needed. Upon reconditioning, results did not improve; thus, the protocol was analyzed, and it was determined that the wrong reagent was used. Instead of using DNase/RNase Free Water, RNA shield was accidentally being used. A qPCR reaction was ran targeting HF183, and no amplification occurred, to ensure that it was not due to concentration being below the limit of detection, a reaction for total bacteria was conducted with it resulting negative as well.

In a trial with a grown culture, DNA was extracted with the same kit, and concentration was measured with the Nanodrop. Results were different than when the RNA shield was used, but it not in line with typical results, with the absorbance curve not displaying typical characteristics. Thus, another extraction was conducted with a new DNA extraction kit, and Nanodrop analyze ran. Nanodrop data revealed no abnormalities, indicating that the extraction was successful. A qPCR reaction confirmed this when there was the amplification of the total bacteria marker. Through a series of troubleshooting activities, it was ultimately shown that a faulty DNA extraction kit was the culprit of a significant amount of time lost as well as the loss of samples from a sampling event.

Once the kit was switched out, extractions began proceeding as usual. The second sampling event of the year occurred in March, and the processing of these sampling commenced immediately afterward. Nanodrop was used on the samples to obtain concentration, and qPCR reactions targeting HF183 and total bacteria were done. The total bacteria assay returned viable results; meanwhile, the HF183 assay resulted in an amplification of the negative control compromising the results. Before the HF183 assay could be conducted, the campus of California State University – Fullerton, suspended all activities due to the COVID-19 pandemic. All faculty and students were ordered to forgo returning to school until the pandemic had eased and returning would not pose a public health risk. These unfortunate events caused Nanodrop data and lab notebooks to be unobtainable and left out a large portion of information for this research paper. This semester has shown how research can be hindered by varying factors that cannot be accounted for. Yet, with all these setbacks, some data was added to the growing monitoring information that has been constructed involving the Santa Ana Watershed's water quality. The data reveals that there could be a relationship between total bacteria and HF183 concentration (Figure 4 and 5).

The literature research component of the project discovered emerging techniques that this project can adapt to further its future goals. There has been significant development in gene targets for human fecal contamination. Of note is the use of mitochondrial DNA (mtDNA) as a gene target with great success or a bacteriophage, crAssphage, with varying success (1, 2, 3). Furthermore, there has been research on how to distinguish dead cells that pose little health risk versus live cell that could be potentially pathogenic (4). Constant literature review is crucial for ongoing projects to ensure that new approaches that are introduced can be used to aid the goals of the research further.

With the homeless population continuing to grow, it would still be worthwhile to pursue this research to gather data and determine if there is a relationship between the growing homeless community and water quality nearby. Whether water quality remains the same or it degrades can have huge implications on how policies move forward on managing the ongoing crises of the homeless community (5, 6). This research would have to be put on hold until the COVID-19 pandemic has been dealt with, and safety measures can be taken to conduct research in an appropriate manner.

Reflection

My time spent at the environmental health laboratory under Dr. Gedalanga has been undeniable a period of growth during my undergraduate experience. Starting back in the Spring of 2019 as a novice bright-eyed assistant researcher, I could not have predicted how impactful it would turn out to be. The ability to learn applicable laboratory techniques in parallel with my ongoing biology education helped me apply my research experience in the classroom as well as use my biology courses to understand the research I was conducting at a fundamental level. There were points in my instructional labs in which I would be far ahead of my peers due to my time spent conducting research, such as DNA extractions. At the same time, there would be points where I would learn the theory in class, and it would reinforce the importance of using the correct technique in research. This was imperative with qPCR, and the significance of minimizing deviation from the proportions of DNA extract to reagents because of dilutions effects.

This experience did not go without frustrations. There were points in time when I believed I was not the right person for research because of ongoing failures. In my year and a half there, I was unable to master the technique required to have consistently successful qPCR experiments. It felt like no matter what I tried, focusing extremely hard on my aseptic technique, micropipette skills, or serial dilution steps, the qPCR would fail to reach a high standard of quality. Either my standard curve was far from the preferred slope, or a negative control would amplify, rendering the whole experiment a failure. It began a point of dread when a qPCR had to be done, and there were always slight nerves when it was time to look at the results. There is no feeling quite as disheartening, in a research lab, like seeing hours of hard work evaporate into thin air when that negative control amplifies.

All those frustrations, however, were overshadowed by the ability to live out my childhood dream of being a scientist. My goal as a kid has always been to be in the science field and don a researcher's coat, and I can finally say I achieved it. I was also surrounded by enthusiastic peer researchers that made the experience more than enjoyable and provided positive reinforcement at each step of the way. It was always bittersweet to see a fellow student that I had connected with graduate and thus depart the lab to pursue their career.

This opportunity to be a part of Dr. Gedalanga's lab would not have been possible without Dr. Gedalanga welcoming me in, and personally teaching me many of the laboratory techniques. He was a great mentor and encouraged me to keep trying my best when I was failing to get an experiment right. The ability to work on an impactful research project funded by Santa Ana Watershed Project Authority's (SAWPA) internship program also made the experience all that much more rewarding. The program connected me with other researchers in the program that are conduction related work in their field. Being able to network with them made me feel like part of a bigger organization that was making an impact in the community. SAWPA was instrumental in ensuring that my internship was going well, checking in on the progress, and offering guidance at all times. When the coronavirus pandemic required a shutdown for the ongoing laboratory work, they offered possibilities to work remotely and continue with the efforts of researching with other projects.

Overall, this experience was a positive impact on my undergraduate career that has left a significant impression on my life. Conducting research is much more than just the hands-on laboratory activities; there were countless hours spent digging into literature as well as learning to assemble a poster presentation and a formal research paper. I learned how to use EndNote, a reference manager that I will undoubtedly use for graduate school. Although I will not be

pursuing research as a career, since my passion lies in the construction field, I will jump at the opportunity to research my graduate studies if the possibility arises. Ultimately, I learned that you have to be passionate about the projects you undertake, so being in the right laboratory that aligns with your interest is crucial, and that is what Dr. Gedalanga's laboratory provided for me.

Appendix A

Table 1. GPS Coordinates of sampling sites that this study used.

Site Number	Location	Latitude	Longitude
1	Veolia Water Treatment Plant	34°02.887’	117°21.392’
2	McLean Park	33°58.099’	117°26.104’
3	Van Buren Bridge	33°57.797’	117°27.905’
4	Paradise Knolls GC	33°58.204’	117°29.296’

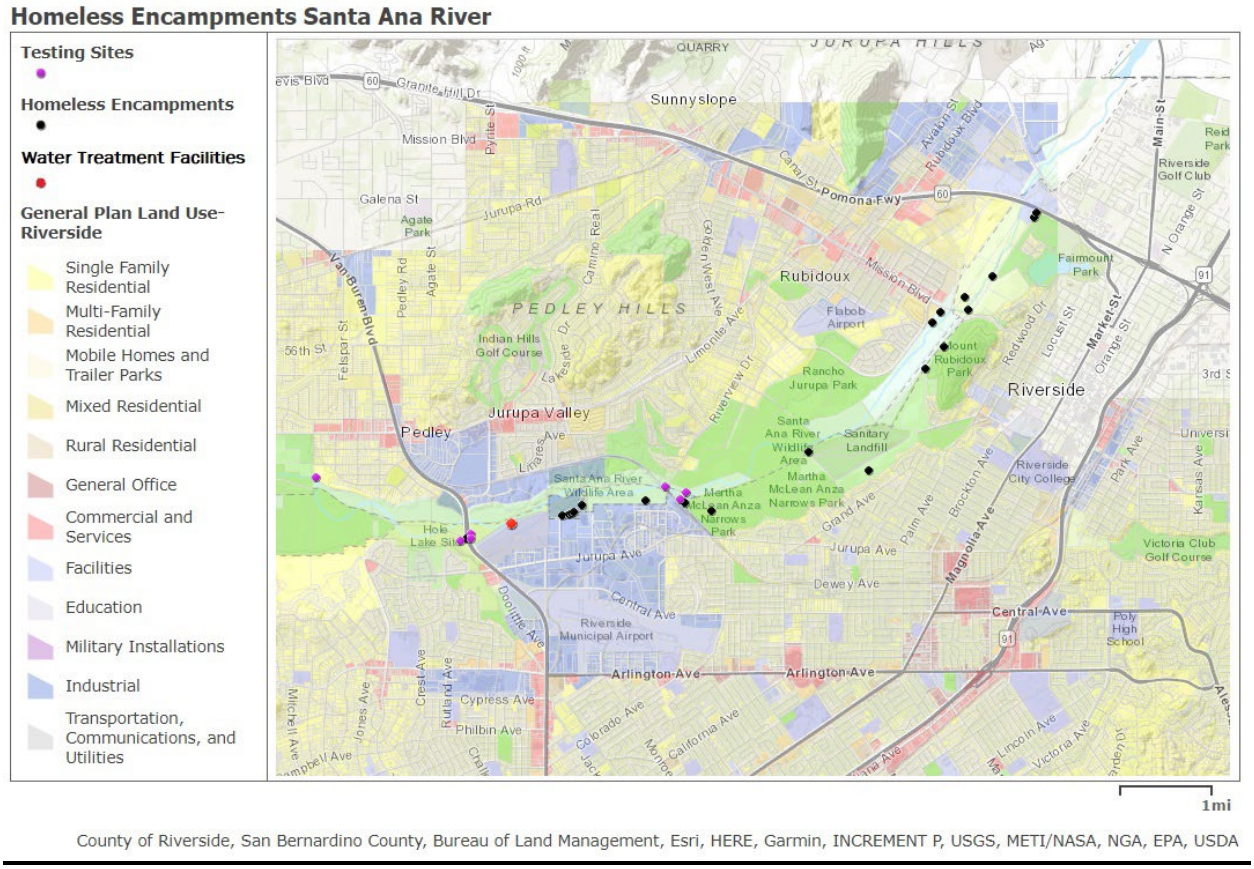

Figure 1. Map indicating previous testing sites, water treatment plant as well as homeless encampment sites.

Table 2. Sampling dates converted to week number to compared date temporally.

Sampling Date	Week
10/26/2018	1*
11/30/2018	6*
12/7/2018	7*
1/11/2019	12*
2/1/2019	15*
3/8/2019	20*
3/22/2019	22*
7/16/2019	39**
8/1/2019	41+
8/15/2019	43+
10/25/2019	53
2/7/2020	68
3/6/2020	71

*Sampling and laboratory work conducted previously. (Puga and Nguyen, 2018)
**Sampling and laboratory work conducted previously. (Lynam and Yacoub, 2019)
+Sampling conducted previously. (Lynam and Yacoub, 2019) Laboratory work was a part of this study.

Table 3. Primer sets which are specific for either the total microbial community or human-associated fecal bacteria.

Assay Name	**Target Microbial group**	**Frag; Anneal Temperature**	**Target/ Gene**	**Primer Sequence (5'-3')**	**Primer Name**	**Citation**
UniBac	Total Bacteria/ Archaea	180bp; 61.5℃	16S rRNA	AAACTCAAAKGAATTGACGG	926F	(De Gregoris et al, 2011)
				CTCACRRCACGAGCTGAC	1062R	
HF183/	Human	155bp; 59.0℃	16S rRNA	ATCATGAGTTCACATGTCCG	HF183	(Green et al, 2014(

BacR 287				CTTCCTCTCAGAACCCCTATCC	BacR28	

Appendix B

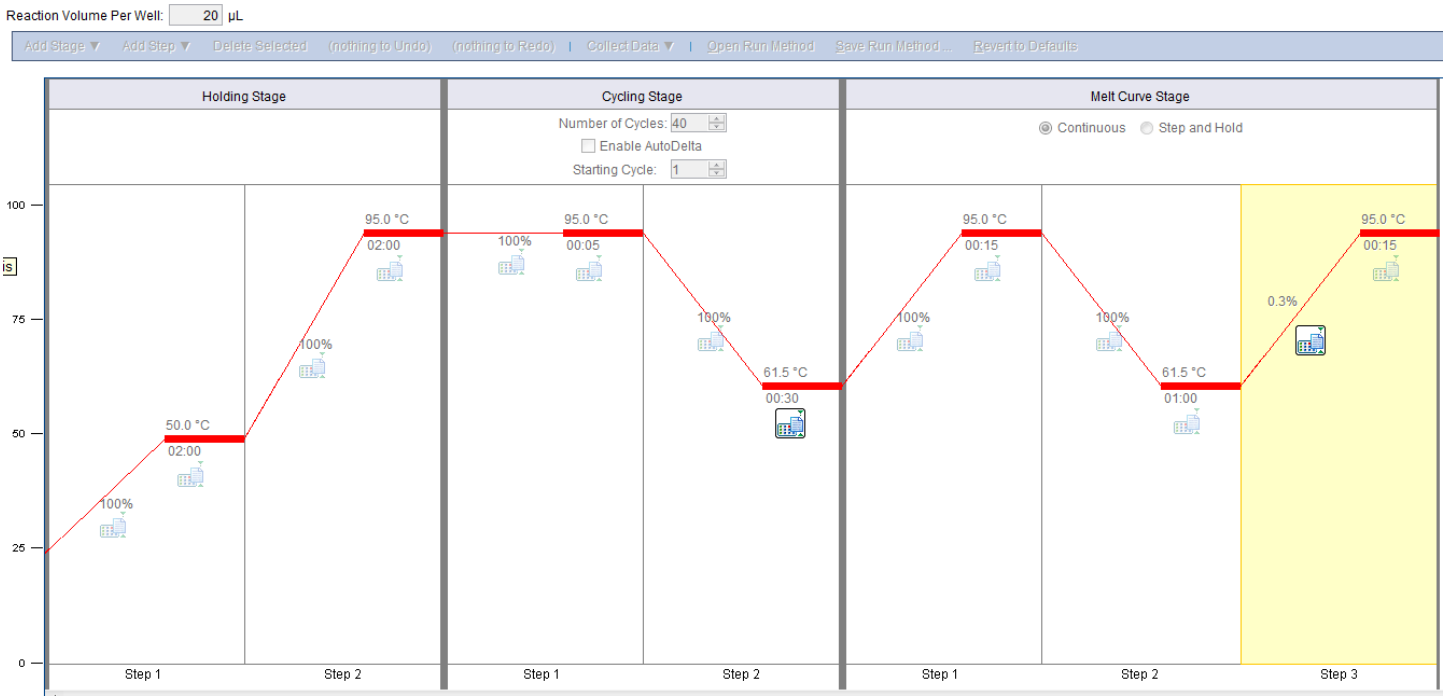

Figure 2. The run method employed for Total Bacteria amplification. Displayed is total volume per well at 20uL and the 40 cycles used for amplification

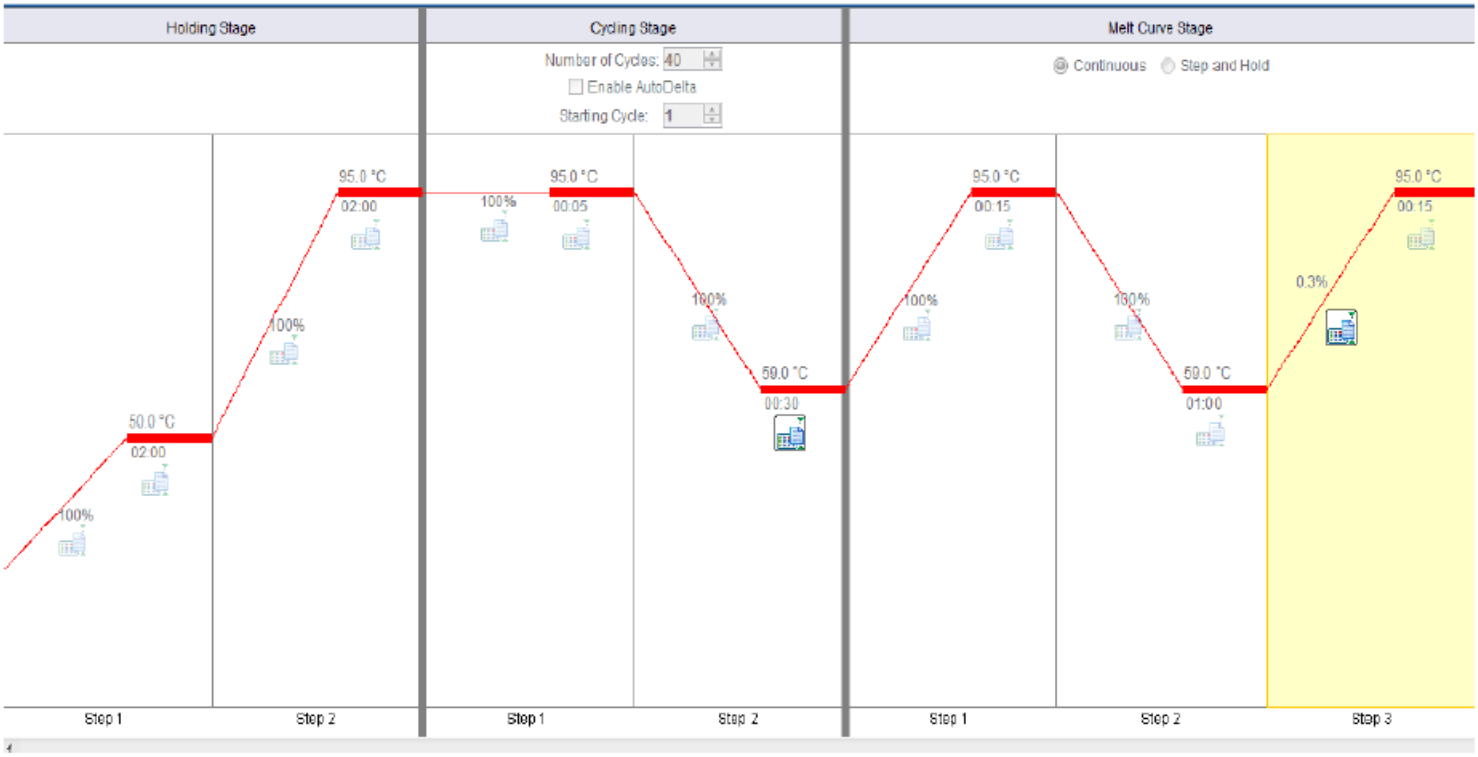

Figure 3. The run method employed for Total Bacteria amplification. Displayed is total volume per well at 20uL and the 40 cycles used for amplification

Table 4. Environmental parameters collected throughout the history of this ongoing study. Data for weeks 68, 71, and portions of 53 are unavailable due to the campus lockdown.

Week	Site	pH	Water Temperature (C)	DO	ORP	Conductivity	Discharge
1	1	8.2	26.4	9	N/A	5.80	42.7
	2	8.4	20.45	7		8.68	
	3	8.45	21.3	7		9.24	
	4	8.25	24.1	5		9.38	
6	1	8.65	14.85	7	N/A	163	437
	2	7.85	14.75	3.75		1043	
	3	8.15	15.65	7		497.5	
	4	8	16.95	5		535	
7	1	8.6	13.85	7	N/A	142	567
	2	8.15	13.75	5.5		909	
	3	9.05	15.45	7		1206	
	4	6.25	16.00	6.5		355	
12	1	N/A	21.2	8	N/A	N/A	57.9
	2	N/A	17.8	6			
	3	N/A	17.8	7			
	4	N/A	19.5	7			
15	1	N/A	N/A	N/A	N/A	N/A	148
	2	N/A	15.8	7			
	3	N/A	16.7	7			
	4	N/A	17.3	7			
20	1	7.77	19.45	9.80	-41.85	N/A	153
	2	7.93	15.8	8.91	-49		
	3	8	16.7	9.08	-53.15		
	4	7.95	17.8	8.85	-50.15		
22	1	N/A	21.25	10.36	-52.75	N/A	81.2
	2	7.94	17.7	8.49	-49		
	3	8.12	19.45	9.13	-59.95		

	4	8.06	19.65	8.74	-57.05		
39	1	7.95	28.8	8.52	-49.8	N/A	37.5
	2	8.05	25.2	7.82	-56		
	3	8.10	28.7	7.81	-60.2		
	4	8.12	29.1	7.3	-59.4		
41	1	N/A	28.15	8.33	-44	N/A	34.6
	2	8.05	22.05	8.39	-53.7		
	3	8.27	24.35	8.15	-67		
	4	8.11	26.05	7.61	-59.2		
43	1	7.89	27.75	8.62	-48.1	N/A	40
	2	8.02	20.9	8.99	-51.7		
	3	8.28	25.25	8.18	-67.7		
	4	8.1	26.85	7.6	-59.2		
53	1	7.79	23.9	8.28	-37	N/A	33.4
	2	7.95	17.6	8.4	-48.7		
	3	8.27	18.15	9.17	-66.1		
	4						
68	Data unattainable due to COVID lockdown						
71							

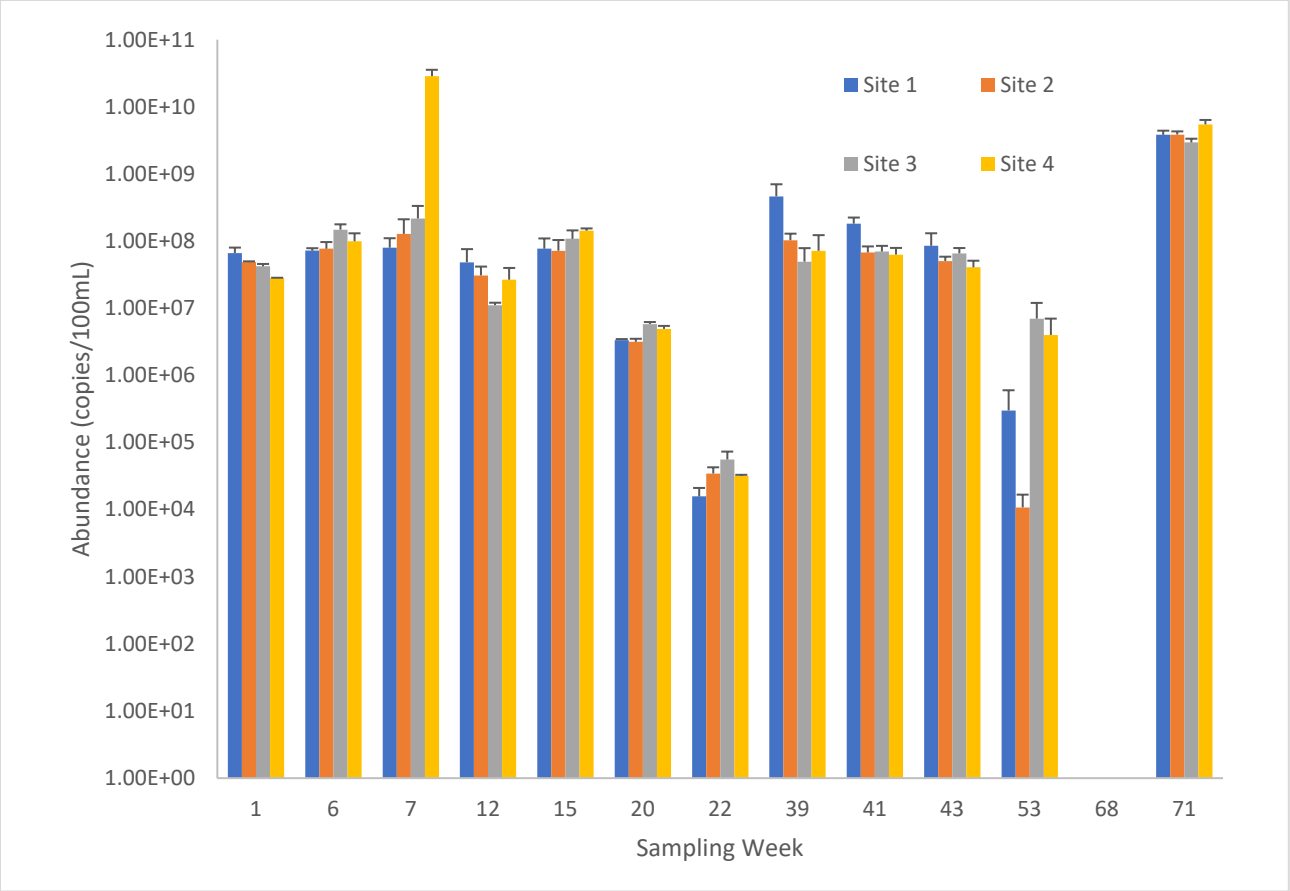

Figure 4. Total Bacteria concentrations in a logarithmic scale from the qPCR assay throughout the history of this SAWPA project. Note the absence of data from week 68 from the lost sampling due to material failure.

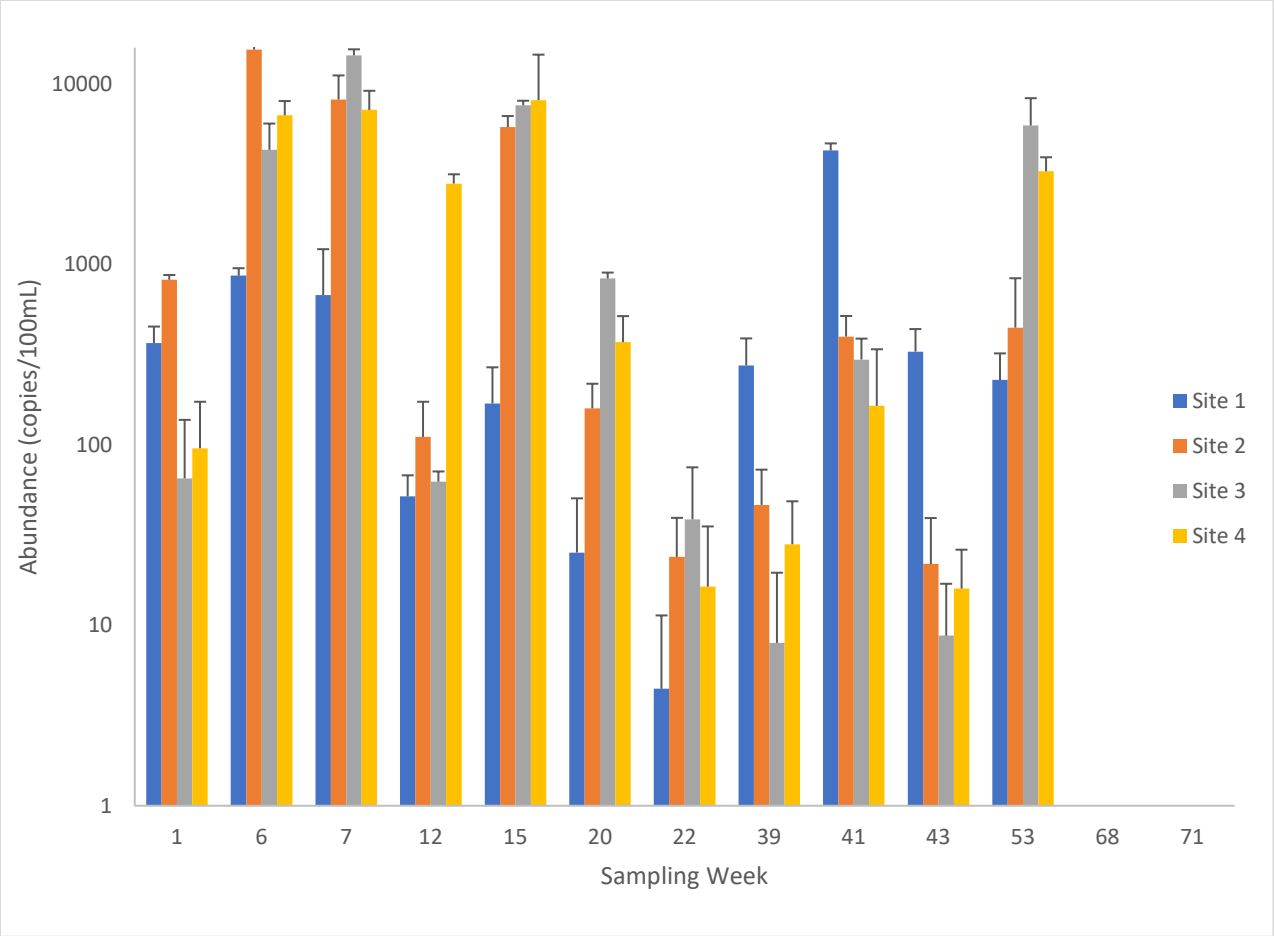

Figure 5. Total Bacteria concentrations in a logarithmic scale from the qPCR assay throughout the history of this SAWPA project. Note the absence of data from week 68 from the lost sampling due to material failure. Week 71 assay could not be reconducted before COVID lockdown.

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