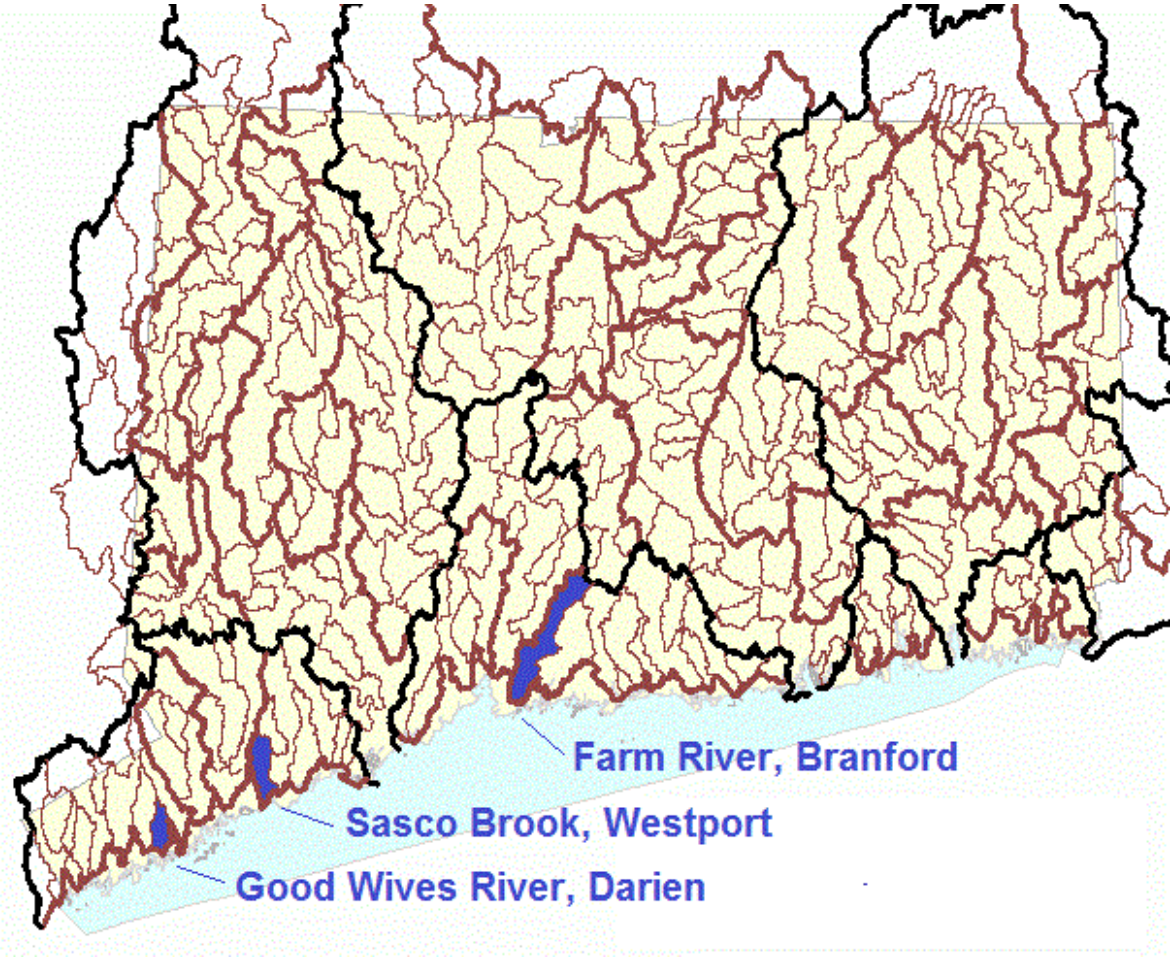
Microbial Source Tracking (MST) Analyses in the Sasco Brook, Lower Farm River and Goodwives River Watersheds.

Project #13-04e Westport Weston Health District Microbial Source Tracking
A project funded by the CT-DEEP through a CWA Section 319 Grant

Project Manager: Mark A.R. Cooper, Director of Health, Westport Weston Health District

Prepared with cooperation from:

Connecticut Agricultural Experiment Station
Yale University



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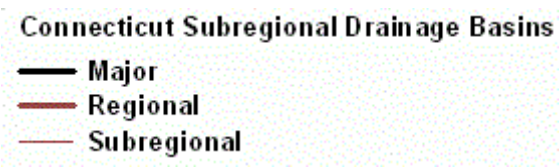

Microbial Source Tracking (MST) Analyses in the Sasco Brook, Lower Farm River and Goodwives River Watersheds.

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Abstract

Goals of this project were the development of a microbial source tracking program protocol that can be utilized to determine the presence or absence of bacteria from various sources within a watershed, monitor the water quality within each selected Connecticut watershed to more accurately identify significant human and nonhuman sources of bacterial contamination, identify the sources of contamination for the development of management strategies for reducing and/or eliminating the identified bacterial sources while raising awareness and support for evidenced based watershed planning.

A growing body of research indicates Microbial Source Tracking (MST) using Real Time Quantitative Polymerase Chain Reaction (qPCR) may be a useful tool for identifying host species contributing bacteria from waters subject to a variety of potential pollution sources. A PCR water sample testing protocol and implementation process was developed and tested for the development of a Connecticut shore line MST program. Three tributaries to Long Island Sound, Sasco Brook in Westport, Goodwives River in Darien, and the Lower Farm River in Branford, were selected as model watersheds to utilize qPCR to detect host specific genetic markers and identify sources contributing to bacteria loading in the watersheds. Although a series of sampling points along each river with a more aggressive sampling schedule that included precipitation events would have been the preferred collection methodology, due to limited project resources, a single sample location was selected for each project watershed with collections conducted in such a way to avoid tidal influence. Water samples were collected monthly over a 12-month period at low tide and analyzed for traditional fecal indicator bacteria (*Escherichia coli*) using culture based methods as well as Bacteroidetes, a largely anaerobic phylum of bacteria commonly used in MST approaches, using culture-independent qPCR. Samples were analyzed for total Bacteroidetes, as well as for host specific detection of multiple sources including human, ruminants, dogs, horses, chickens, seagulls and general avian sources contributing to the fecal bacteria load of the tributaries.

After developing and implementing a microbial source tracking protocol, it is important to note that this study did not detect significant human contributions to the bacteria levels in the three subject watersheds. The result of this study was inconclusive, contributed in part by the project's sample collection methodology. Furthermore, DNA markers associated with feces from poultry, dogs, and cattle were analyzed, but not found in any sample results. Ruminant markers were also used in this study as they encompass agricultural animals, including cattle and sheep, as well as wildlife such as deer. Detection of the ruminant marker was rare at the selected sites with no positive samples in Darien, one in Branford, and two in Westport. Finally, the two assays associated with sea gulls and general avian sources failed to pass the quality screening for successful runs. Steps taken to improve performance of both the GFC (specific for seagulls) and the GFD (associated with birds) assays failed,

resulting in non-specific amplification or no amplification. Due to this inability to pass the quality screenings, these assays are not included in the analyses.

The results indicate that there is not a detected high level of contribution of bacteria from any of the monitored contaminants. While this information does not result in action-based recommendations that can be done to address the elevated *E. coli* levels observed at each of the monitored sites, it still provides a valuable starting point for future work to develop more reliable markers for birds and other potential sources of bacteria, such as rodents.

Introduction

In Connecticut, local health departments are responsible for monitoring water quality at public beaches and closing beaches whenever a risk to public health is present. The Environmental Protection Agency has established water quality criteria as guidance, using the following bacteria as indicator organisms for potential pollution: *Enterococci* for marine waters and *E. coli* for fresh water. Water sampling protocols have been established by the State of Connecticut Department of Public Health, and local health departments are responsible for the water sample collection and submission of them to approved laboratories for analysis. Should samples exceed established limits, the impacted beach is closed until additional sampling and test results indicate bacteria levels are once again within established acceptable water quality criteria. Follow-up action to determine bacteria sources, such as a survey of the watershed (drainage basin), dye testing or more drastic actions, such as closing beaches for longer periods of time, may or may not occur. Water sampling and testing for bacteria is also done to ensure safe water quality in shellfishing areas. For this research paper, the Health Directors in the East Shore District Health Department, the Westport-Weston Health District and the Town of Darien partnered to perform this study as an effort to determine the actual sources of bacteria found in streams flowing into Long Island Sound and also whether that bacteria poses a risk to human health.

Long Island Sound (LIS) has been designated as an Atlantic Ocean tidal estuary of national significance by the United States Congress with a population of over nine (9) million people living within the watershed area. Its water quality is greatly dependent on the quality of water from the many tributaries that flow into it. Variations in water quality of these tributaries within the watershed contribute to the changes in LIS, making the protection and/or the remediation of water quality in the tributaries essential for the continued viability of LIS.

Due to public health concerns, swimming waters and shellfish beds are closed as bacteria levels are elevated, causing residents and commercial users to temporarily, or in some cases permanently, lose access to these resources. Despite actions taken to address point sources of fecal bacteria, elevated bacteria counts in LIS and its tributaries have public health and economic consequences.

As readily identified point sources of pollution have become increasingly regulated, the impact of non-point sources of fecal bacteria has become a greater concern and the need to identify these non-point sources has become increasingly important.

Traditional monitoring for fecal contamination relies on the culturing of fecal indicator bacteria (FIB) such as *Escherichia coli* (*E. coli*) and *Enterococci*. These bacteria, while typically not harmful to humans, are used as proxies for potential pathogens present in fecal contamination. Use of the FIB was adopted due to their ease of quantification and known presence in feces. However, while these methods provide a useful tool to estimate human health risks associated with fecal contamination, numerous shortcomings associated with these methods have since been identified, including weak

correlation with pathogen counts or reported illnesses (Colford et al., 2007; Wade, Pai, Eisenberg, & Colford, 2003) and the fact that samples must be cultured for 24 hours before results are known. This time lapse presents an enforcement dilemma since, in marine waters, the 24 hour time period represents two tide changes likely ensuring completely different water quality between when samples are taken and the results obtained. Additionally, the utility of results from traditional monitoring are limited by an inability to provide information regarding the sources contributing to the elevated bacterial levels.

While the first two limitations directly impact the public health implications of FIB monitoring results, the latter of these limitations is particularly relevant from a bacteria management perspective, as a crucial first step to any mitigation is to identify sources contributing to the elevated bacteria levels. Bacteria found at a recreational beach or shellfish bed may originate upstream elsewhere in the watershed and be transported to the mouth of a water body, making it difficult to pinpoint these non-point sources. Thus, the ability to identify which host sources contribute to elevated FIB counts is particularly relevant at the watershed scale. Thus, water quality managers may have to conduct time consuming and costly surveys which may not disclose the non-point sources affecting water bodies.

As water quality managers have recognized the need for tools that can identify contributions to non-point source pollution, methods that allow for source detection, referred to broadly as Microbial Source Tracking (MST), have received a lot of attention. Recent developments in biotechnology have led to new rapid assessment techniques for monitoring water quality. Many of these methods also have the potential to identify the host species, or source, of the bacteria, a first step to pinpointing the source of contamination. MST has demonstrated potential for widespread employment in the use of genetic markers amplified using polymerase chain reaction (PCR). Using PCR, a genetic marker that is specific to a host of interest can be detected in water samples. With slight modifications to conventional PCR, Real-Time PCR, which allows for not only presence/absence data to be measured, but also can be adapted to provide quantitative measurements (qPCR). These methods have been widely employed in numerous watershed studies (Anderson, Whitlock, & Harwood, 2005; Chase, Hunting, Staley, & Harwood, 2012; Gordon et al., 2013; Harwood et al., 2009; Lee, Weir, Lee, & Trevors, 2010; McQuaig, Griffith, & Harwood, 2012; Rodriguez, 2012) and the development standard methods for implementation of several markers are underway (Orin C. Shanks et al., 2016).

While work has been done to develop and test these genetic markers, little work has been done in LIS to implement these technological developments to evaluate the water quality of streams flowing into the Sound. Studies conducted elsewhere have found these to be useful tools that can provide valuable information about the sources responsible for elevated bacteria levels, and thus are helpful at targeting management strategies to improve water quality (Anderson et al., 2005; Chase et al., 2012; Gordon et al., 2013; Harwood et al., 2009; Lee et al., 2010; McQuaig et al., 2012; Rodriguez, 2012)

Methods

Site selection

As a first step towards developing a future MST protocol for coastal Connecticut, a qPCR based monitoring program at the mouth of three subject watersheds feeding into LIS was implemented. Sites were selected based on historical water quality information including evidence of previously identified and unexplained elevated bacterial counts. The three watersheds totaling 37.4 sq. miles of drainage basin, the Sasco Brook Watershed, the Lower Farm River Watershed, and the Goodwives River Watershed have a history of elevated bacteria counts leading to the closures of beaches for contact recreation (swimming) use and/or shellfishing areas. It should also be noted that kelp farming and

other fisheries are being developed in LIS which could be impacted, resulting in lost economic opportunities for Connecticut, and its shoreline communities. Through the water quality monitoring portion of this project, the watersheds were monitored for a year to collect samples to be used in identifying likely sources of fecal contamination.

Sasco Brook has been identified by the Connecticut Department of Energy and Environmental Protection (CT DEEP) as an impaired water body not meeting state water quality standards due to periodic elevated bacteria levels. High bacteria levels have also resulted in the state-imposed closure of public shellfish beds near the mouth of the brook in Long Island Sound. The Sasco Brook Pollution Abatement Committee (SBPAC), a voluntary alliance consisting of representatives of local, state, and federal agencies, private organizations, and interested citizens, was organized to identify sources of bacterial contamination and pursue initiatives to improve water quality. Several SBPAC initiatives have resulted in the successful reduction of the detected amounts of bacterial contamination in Sasco Brook from several sources. However, unacceptable levels of bacterial contamination continue to occur periodically.

Waste load assumption based methodologies used in other Connecticut studies for estimating the amounts of fecal bacteria generated by potential sources of watershed contamination have been used for the Sasco Brook watershed. Such methodologies include relatively complex, computer driven models that require substantial data sets and a significant number of assumptions and input variables which do not account for bacterial sources from wildlife sources. Application of these methodologies to the Sasco Brook Watershed has indicated that the most significant sources of fecal bacterial contamination may be geese and dogs. However, because of the multitude of calculations and assumptions these methodologies use, they do not provide sufficient confidence for justifying municipal expenses for more aggressive pollution abatement measures. As a result, MST techniques were proposed to test the findings of the computer models, thus providing further evidence as to the sources of bacterial contamination.

Like Sasco Brook, Darien Cove has also been classified as impaired by CT DEEP for not meeting state water quality standards regarding fecal coliform (a class of organisms including *E. coli)* levels. This classification has prevented the harvesting of shellfish for direct consumption in this area. Additionally, water samples collected by the Darien Health Department at various locations along the Goodwives River, the primary source of fresh water to the Darien Cove, have shown consistently elevated levels of bacteria since 2009. According to the assessment conducted by the CT DEEP, likely sources of the bacteria are storm water and non-point sources, including decentralized treatment systems (such as septic systems), vessel discharges, and waterfowl. Detailed sanitary surveys have been conducted throughout the watershed with numerous potential but inconclusive sources of bacteria found.

The area of the Lower Farm River separating the towns of East Haven and Branford is a significant shellfish resource, however it is currently classified as "Prohibited" and "Restricted-Relay", as defined by the United States Food and Drug Administration National Shellfish Sanitation Program Model Ordinance (USFDA NSSP MO), due to the elevated levels of fecal coliform organisms impacting the area. The Lower Farm River watershed, beginning at the discharge of Lake Saltonstall, is a watershed consisting primarily of residential areas, although some agricultural and commercial industries do border the river. In 2012, a study conducted by the East Shore District Health Department and Yale University in the Lower Farm River began to monitor bacteria loading throughout the river (unpublished data). This study used Real Time PCR to distinguish between human and non-point sources of bacteria as well as traditional fecal coliform monitoring to measure bacteria levels.

Sample Collection

Sample locations (as shown on Appendix B maps) were selected near the mouth of each watershed with collections conducted in such a way to avoid tidal influence. One freshwater sample, of greater than 200 ml volume, was collected at each sample location each month during January to December 2016. Collections from each watershed's sample location were typically conducted during the third week of the month in the morning at low tide. The samples were collected in sterile containers from approximately 6-12 inches below the water surface with the mouth of the container pointed away from the sampler. Samples were immediately placed onto ice for transport to the Harbor Watch laboratory in Westport, CT where samples were divided for *E. coli* enumeration and DNA analysis.

Sample Filtration

E. coli enumeration was conducted at the Harbor Watch Water Quality laboratory in Westport, CT, using m-FC media following standard method 922D (Bridewater et al., 2012) and filtered for DNA analysis. Prior to filtration, each water sample was shaken vigorously approximately 25 times.

For qPCR analysis, two independent 100 ml water subsamples were vacuum filtered (polycarbonate filter, 0.2 µm pore size, GE osmotic Inc. 04CP04700) to concentrate bacterial cells. Following the filtration of 100 ml of the sample, the funnel sides were rinsed with 20-30 mL sterile phosphate buffered saline (PBS, Invitrogen 10010-031) and filtration continued until all fluid had passed through the filter media. Following filtration, the filter was removed aseptically. One filter per sample were rolled with sample side facing inward using sterilized tweezers and transferred into cryo-safe tubes containing glass beads (prepared in advance as described below). The other filter was rolled and placed into a sterile polypropylene tube. One water sample filtration blank was processed following the same protocol for each round of samples collected. Filters were transported under ice to the New Haven Agricultural Experiment Station for storage at -80 ^{o}C until DNA extractions were performed.

DNA Extraction and Storage

DNA extractions were conducted on the first set of filters at the Connecticut Agricultural Experiment Station (CAES). Duplicate filters remain frozen at -80C at the CAES. To detect and control for differences in DNA extraction and amplification, a sample processing control (SPC) made from salmon testes DNA (Sigma Aldrich D1626) was prepared and added for quantification in all samples. Fresh Salmon DNA/extraction buffer was prepared by combining stock salmon testes DNA (10 µg/ml) with buffer AE (Qiagen 19077) to make a 0.2 µg/ml dilution prior to extractions.

Once prepared, 590 µL of the Salmon DNA/Extraction buffer was added to each labeled glass bead tube containing the water filters. The tubes were closed and placed in a mini bead beater for 60 seconds at 5000 rpm. Following centrifugation at 12,000 x g for 1 minute to pellet the glass and debris, gloves were changed to prevent contamination. Using a 200 µL pipette, 400 µL of supernatant was transferred to a new labeled 1.7 mL sterile microcentrifuge tube without disturbing the pellet. The collected supernatant was centrifuged for 5 minutes at 12,000 x g and 350 µL of the clarified supernatant was transferred to a new labeled 1.7 mL sterile microcentrifuge tube without disturbing the pellet. Each tube was labeled from before as undiluted or 1x water sample extracts with sample identification. These were the water sample filter extracts. The tubes for method blanks were also labeled and

processed last along with samples. All extracts were immediately refrigerated and then frozen at -80C for long term storage.

Additional 1.7 ml tubes for 5 and 25 fold dilutions were also labeled. Using a micro-pipette, 50 µl of aliquot was added to each 1x water sample extract and each diluted with 200 µL AE buffer to make 5 fold dilutions in appropriately labeled tubes. Method blanks were prepared last in all of the above steps. All extracts were immediately refrigerated and then frozen at -80ºC for long term storage.

qPCR Analysis

Analysis for host associated fecal anaerobic bacteria was conducted at the Yale University DNA Analysis Facility on Science Hill in New Haven, CT using primers that allow the detection of general Bacteroidetes associated markers, host associated markers for common pollution sources, as well as quality assurance assays (Table 1). An ABI 7500 Fast Real-Time PCR analyzer was used with all samples analyzed in triplicate, and the mean count used for quantification of the marker in the reaction.

The General Bacteroidetes (GenBac3) associated marker is used as a measure to assess levels of Bacteroidetes in the water. These organisms, like *E. coli*, are found in the gut of many animals, but are also known to occur in the environment without contributions of fecal matter (Fiksdal, Maki, LaCroix, & Staley, 1985). Host associated markers were selected for use in this study based on the availability of tested methods as well as sources likely to contribute to pollution at the selected sites. Eight markers representing six different host groups were chosen and monitored for all samples. The selected assays consisted of a multiplex assay for the detection of human sources, as well as assays for detection of ruminant sources, cattle, canine, poultry, seagull, and general avian contamination (see Table 1).

Table 1: List of Hosts, primers and probes used in the study.

ASSAY	HOST GROUP	PRIMERS AND PROBES	REFERENCE
TAQMAN ASSAYS			
GENBAC3	General Bacteroidetes	F: GGGGTTCTGAGAGGAAGGT R: CCGTCATCCTTCACGCTACT P: [FAM]CAATATTCCTCACTGCTGCCTCCCGTA[TAMRA]	(Dick & Field, 2004; Siefring, Varma, Atikovic, Wymer, & Haugland, 2008)
HF183	Human	F: ATCATGAGTTCACATGTCCG R: CTTCCTCTCAGAACCCCTATCC P1: [FAM]-CTAATGGAACGCATCCC-[MGB] P2: [VIC]-AACACGCCGTTGCTACA-[MGB]	(Bernhard & Field, 2000; Seurinck, Defoirdt, Verstraete, & Siciliano, 2005)
HUMM2	Human	F: CGTCAGGTTTGTTTCGGTATTG R: TCATCACGTAACTTATTTATATGCATTAGC P1: [FAM]-TATCGAAAATCTCACGGATTAACTCTTG TGTACGC-[TAMRA] P2: [VIC]-CCTGCCGTCTCGTGCTCCTCA-[TAMRA]	(O. C. Shanks, Kelty, Sivaganesan, Varma, & Haugland, 2009)
RUM2BAC	Ruminant	F: ACAGCCCGCGATTGATACTGGTAA R: CAATCGGAGTTCTTCGTGAT P: [FAM]-ATGAGGTGGATGGAATTCGTGGTGT-[BHQ-1]	(Mieszkin, Yala, Joubrel, & Gourmelon, 2010)
COWM2	Cattle	F: CGGCCAAATACTCCTGATCGT R: GCTTGTTGCGTTCCTTGAGATAAT P: [FAM]-AGGCACCTATGTCCTTTACCT CATCAACTACAGACA-[TAMRA]	(O. C. Shanks et al., 2007)

LA35	Poultry	F: ACCGGATACGACCATCTGC R: TCCCCAGTGTCAGTCACAGC P: [FAM] - CAGCAGGGAAGAAGCCTTC GGGTGACGGTA - [BHQ-1]	(Nayak, Weidhaas, & Harwood, 2015)
DOGBACT	Canines	F: CGCTTGTATGTACCGGTACG R: CAATCGGAGTTCTTCGTG P: [6-FAM]- ATTCGTGGTGTAGC GGTGAAATGCTTAG - [BHQ1]	(Schriewer et al., 2015)
SKETA22	Quality Assurance	F: GGTTTCCGCAGCTGGG R: CCGAGCCGTCCTGGTCTA P: [FAM]AGTCGCAGGCGGCCACCGT[TAMRA]	(Haugland, Siefring, Wymer, Brenner, & Dufour, 2005)
SYBR ASSAYS			
GFD*	General Avian	F: TCGGCTGAGCACTCTAGGG R: GCGTCTCTTTGTACATCCCA	(Green, Dick, Gilpin, Samadpour, & Field, 2012)
GFC*	Gull	F: CCCTTGTCGTTAGTTGCCATCATTC R: GCCCTCGCGAGTTCGCTG C	(Green et al., 2012)

* Assays were not successfully implemented

sample processing controls (SPC) were spiked into samples at the time of DNA extraction to allow for the detection of inhibitors that often occur in natural samples. Current SPC methods recommend the addition of a spike consisting of Salmon Testes DNA added to the extraction buffer. Inhibition was measured by comparing the amplification efficiency (cycle threshold) of the blanks compared to the samples and was detected for all undiluted samples, making it necessary to dilute the samples 1:5 with sterile water as recommended (Orin C. Shanks et al., 2016). The SPC assay was repeated for diluted samples and all diluted samples passed this quality assessment. In addition to the SPC, Internal Amplification Controls (UCP1) were added to the GenBac3 assay to detect inhibition of the qPCR assays. Inhibition was tested by comparing the cycle threshold of non-template control wells in each plate to those containing samples or standards. No inhibition was detected in diluted samples. Standard curves were constructed for each plate to allow for absolute quantization using synthetic plasmids consisting of sequences corresponding to the selected markers (Table 2). Standards were diluted from a range of 10^5 to 10^1 and used to construct calibration curves for quantification for each run. Additionally, standards were treated as positive controls to detect successful amplification for each assay as well as to establish the limits of quantification and detection for each assay.

Table 2: Synthetic DNA sequences for standard constructs and internal amplification controls.

ID	SEQUENCE
STANDARD CONSTRUCT 1	ATCGGGGGTTCTGAGAGGAAGGTCCCCCACATTGGAACTGAGACACGGTCCTCGAGCCTACGGGAGGCAGCA GTGAGGAATATTGGTCAATGGGCGCAGGCCTGAACCAGCCAAGTAGCGTGAAGGATGACTGATCGGATCGAC AGCCCGCGATTGATACTGGTAACCTTGAGTGCAGATGAAGTGGATGGAATTCGTGGTGTAGCGGTGAAATGCT TAGATATCACGAAGAACTCCGATTGATCGCGTCAGGTTTGTTTCGGTATTGAGTATCGAAAATCTCACGGATTAA CTCTTGTGTACGCTCTCGAGGACCAGCTAATGCATATAAATAAGTTACGTGATGAGACCGGCGCACGGGTGAGT AACACGTATCCAACCTGCCGTCTACTCTTGGCCAGCCTTCTGAAAGGAAGATTAATCCAGGATGGGATCATGAGT TCACATGTCCGCATGATTAAAGGTATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGCTCGAGATAGTAGGCG GGGTAACGGCCCACCTAGTCAACGATGGATAGGGGTTCTGAGAGGAAGGATCGCGGCCAAATACTCCTGATCGT ACTCGAGATAGGCACCTATGTCCTTTACCTCATCAACTACAGACAAAATTATCTCAAGGAACGCAACAAGC
STANDARD CONSTRUCT 2	CGCTTGTATGTACCGGTACGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCGAG CGTTATCCGGATTTATTGGGTTTAAAGGGAGCGCAGACGGGTTTTTAAGTCAGCTGTGAAAGTTTGGGGCTCAAC CTTAAAATTGCAGTTGATNCTGGAGACCTTGAGTGCAGTTGAGGCAGGCGGAATTCGTGGTGTAGCGGTGAAAT GCTTAGATATCACGAAGAACTCCGATTGATCGCCCTTGTCGTTAGTTGCCATCATTCTCGGCTGAGCACTCTAGGG ATCGATGCTAGCTAGCTAGGCATTACGTACGTAGCGTGTTCCCATGGTTCATTCACCTAAGGCTAAGTCAGGCTC GGTAATGCATGGGATGTACAAAGAGACGCGCAGCGAACTCGCGAGGGCATCGACCGGATACGACCATCTGCCG CATGGCGGGTGGTGGAAAGTTTTTCGATTGGGGATGGGCTCGCGGCCTATCAGTTTGTTGGTGGGGTAATGGCC TACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTC CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCGACGCAGCGTGCGGGATGA CGGCCTTCGGGTTGTAAACCGCTTTCAGCAGGGAAGAAGCCTTCGGGTGACGGTACCTGCAGAAGAAGTACCGG CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTACGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTC GTAGGTGGTTGGTCACGTCTGCTGTGGAAACGCAACGCTTAACGTTGCGCGGGCAGTGGGTACGGGCTGACTAG AGTGCAGTAGGGGAGTCTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCG AAGGCGGGACTCTGGGCTGTGACTGACACTGGGGA
BACR287IAC	ATCATGAGTTCACATGTCCGCATGATTAAAGGTATTTTCCGGTAGACGATGTGTAGCAACGGCGTGTTATAGTA GGCGGGGTAACGGCCCACCTAGTCAACGATGGATAGGGGTTCTGAGAGGAAG
BACP234IAC	AACACGCCGTTGCTACATTGTGCGGCAACGATGTTGTAGCAACGGCGTGTT

For TaqMan Assays, a 20 µl reaction consisted of 10 µl TaqMan Fast Universal Master Mix, 500 nmol l^{-1} of each primer, and 250 nmol l^{-1} of probe. All reactions were performed in triplicate in MicroAmp optical 96-well plates with optical adhesive film. Cycling parameters for all assays included a 2 minute start at 94°C followed by 40 cycles of 15s at 94°C and 32s at 60°C. Cycle threshold for each run was determined by the software provided with the ABI 7500 Fast Real-Time PCR system and used to calculate the concentration using equations fit from the standard curves.

SYBR assays were conducted similarly, with 20 µl reactions consisting of 10 ul Fast SYBR Green Master Mix, and 500 nmol l^{-1} of each primer. Troubleshooting to achieve amplification of the two SYBR green assays was performed in an attempt to achieve specific and reliable amplification. These optimization steps included variations in melting temperature, magnesium chloride, and other PCR additives including Bovine Serum Albumin. However, despite these steps, quality screening from both the GFC and the GFD assay were unsuccessful and thus no results for those assays are included in this analysis

Data analysis and interpretation

Each run was assessed for performance visually using the ABI 7500 installed software. Runs were screened for amplification in negative controls, high standard deviation, and successful amplification in positive controls. Results for each plate that was considered a successful run were exported as Microsoft Excel Spreadsheets using the ABI 7500 software. Analysis of the results and graphics were produced using RStudio (2015).

Results

Quality Assurance steps were implemented at various stages of the project and revealed no violations of the approved plan. Field blanks revealed no evidence of contamination at any stage of the sample handling process. The spiked salmon testes DNA was used as a control and revealed the presence of environmental inhibition in all samples. This was addressed by a dilution factor of five, after which all samples passed this test. Additionally, diluted samples showed no evidence of inhibition in the IAC.

Two of the assays failed to pass the screening for successful runs. Steps taken to optimize both the GFC (specific for seagulls) and the GFD (associated with birds) assays failed to improve performance, resulting in non-specific amplification or no amplification. Due to these failings, these assays are not included in further analyses. This was unfortunate since the up gradient presence of geese and ducks were noted during some of the water sample collections and would most likely be identified in the analysis.

Traditional monitoring for *E. coli* at the three watershed sites revealed the occurrence of elevated bacterial counts at all three sites, but the quantity of *E. coli* and the frequency of exceedance over acceptable standards varied by site and sampling date (Appendix A). The Branford site in the Farm River had lower *E. coli* counts relative to the other locations, with no samples over 1000 CFU/100 ml, but still had moderately high counts greater than 100 CFU/100 ml in 6 of the 12 samples (Figure 1) *E. coli* counts in Darien were significantly higher than those in Branford with 9/12 of the samples over the water quality standards. The samples were significantly higher in summer months, especially in July and August when the samples exceeded 10,000 CFU/100 ml. E. coli levels in Westport were over the regulatory standard in 8/12 of the samples with the August sample having over 10,000 CFU/100 ml. *E. coli* levels were low in the early part of the year when the water was cold, but become elevated in late spring and stayed elevated throughout the fall with higher water temperatures.

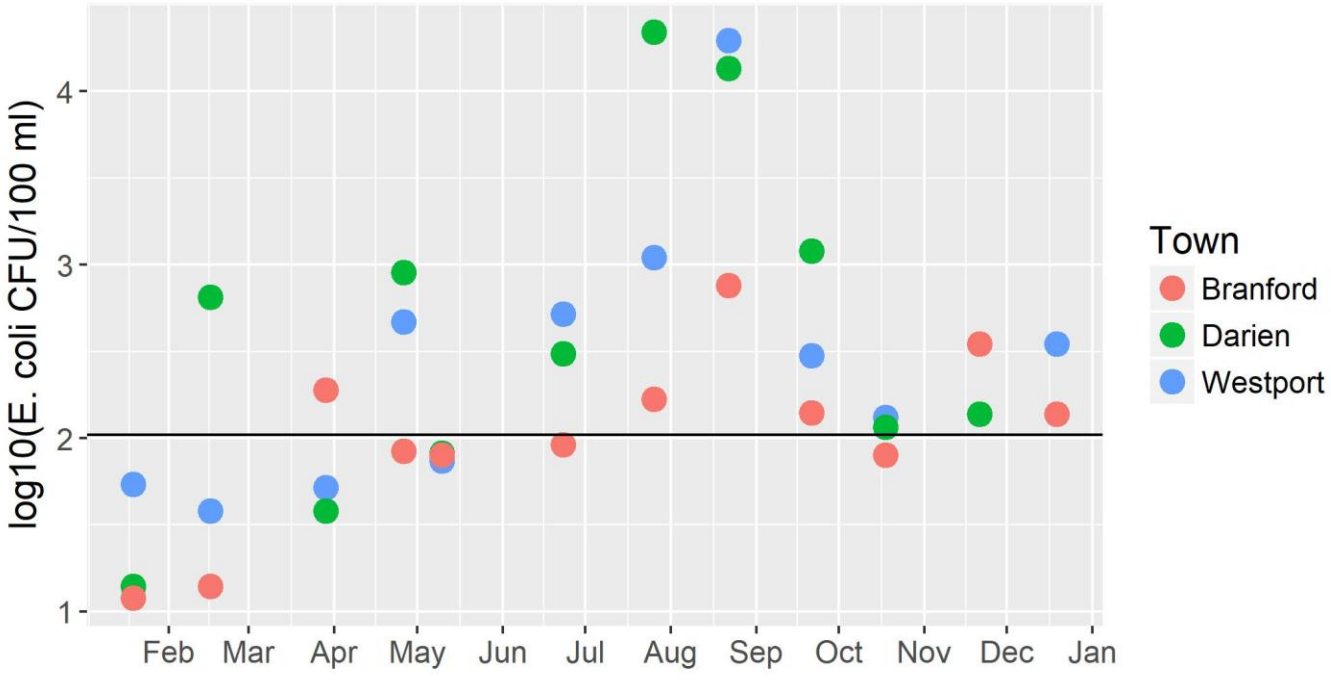


Figure 1: *E. coli* levels at the sites varied with date and location. The black line represents the regulatory cutoff of 104 CFU/100ml. The data are presented on a log scale for ease of visibility.

GenBac3

In addition to *E.coli*, GenBac3, another general indicator of fecal contamination, was quantified using qPCR. This indicator uses an alternative group of fecal bacteria of the phylum *Bacteroidetes*, but unlike the other markers used in this study this indicator is not associated with any particular host.

While the two are not expected to have perfect correlation, as *E.* coli and GenBac3 are general indicators, a relationship between the two was expected. However, the relationship between *E. coli* and GenBac3 was not strong for any of the sites (Figure 2) and no discernible correlation was found for the Branford and Westport sites. There was slightly more correlation found between the levels of *E. coli* and the general marker GenBac3 (R^2 = 0.44) at the Darien site. This correlation is largely influenced by the elevated GenBac3 counts co-occurring with the highly-elevated *E. coli* counts in July and August, while other samples have little to no correlation (R^2 = 0.19 if high counts are removed).

Figure 2a: Little correlation between *E. coli* and GenBac3 markers

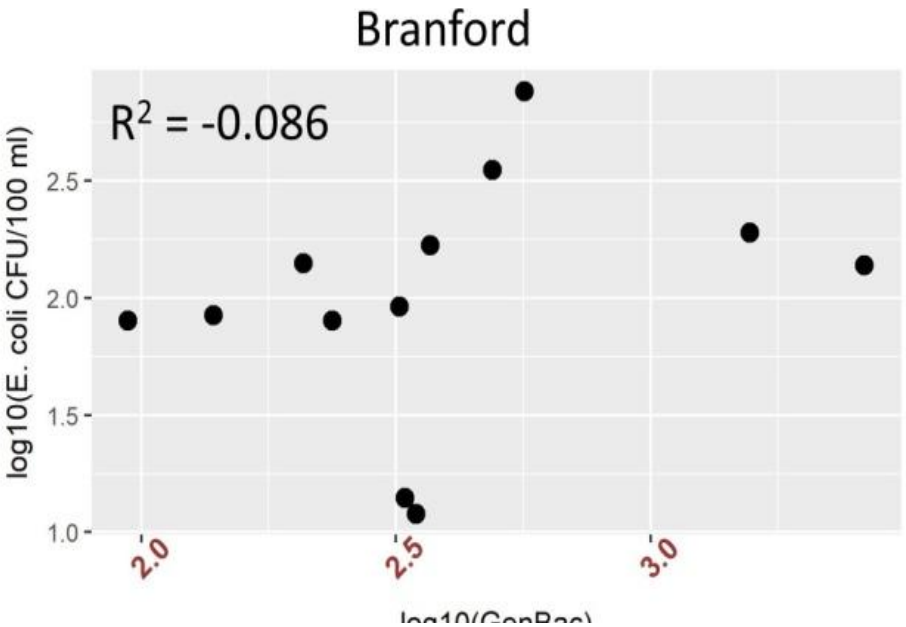


Figure 2b: Little correlation between *E. coli* and GenBac3 markers

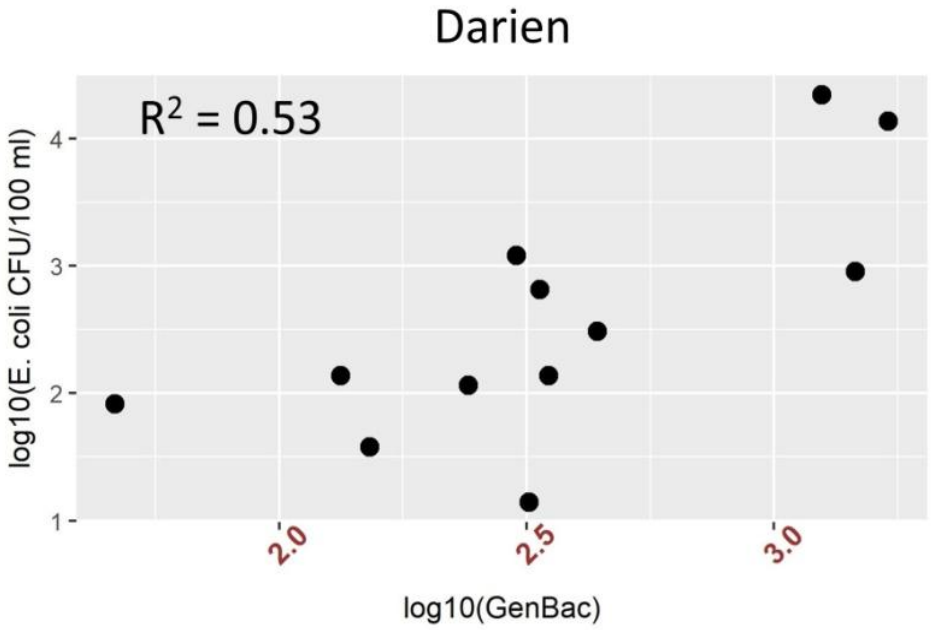
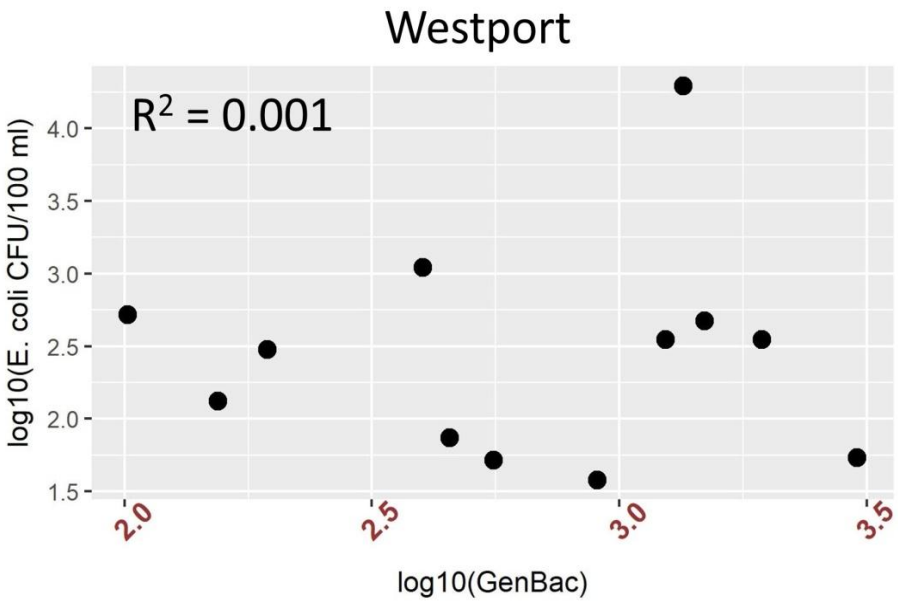


Figure 2c: Little correlation between *E. coli* and GenBac3 markers



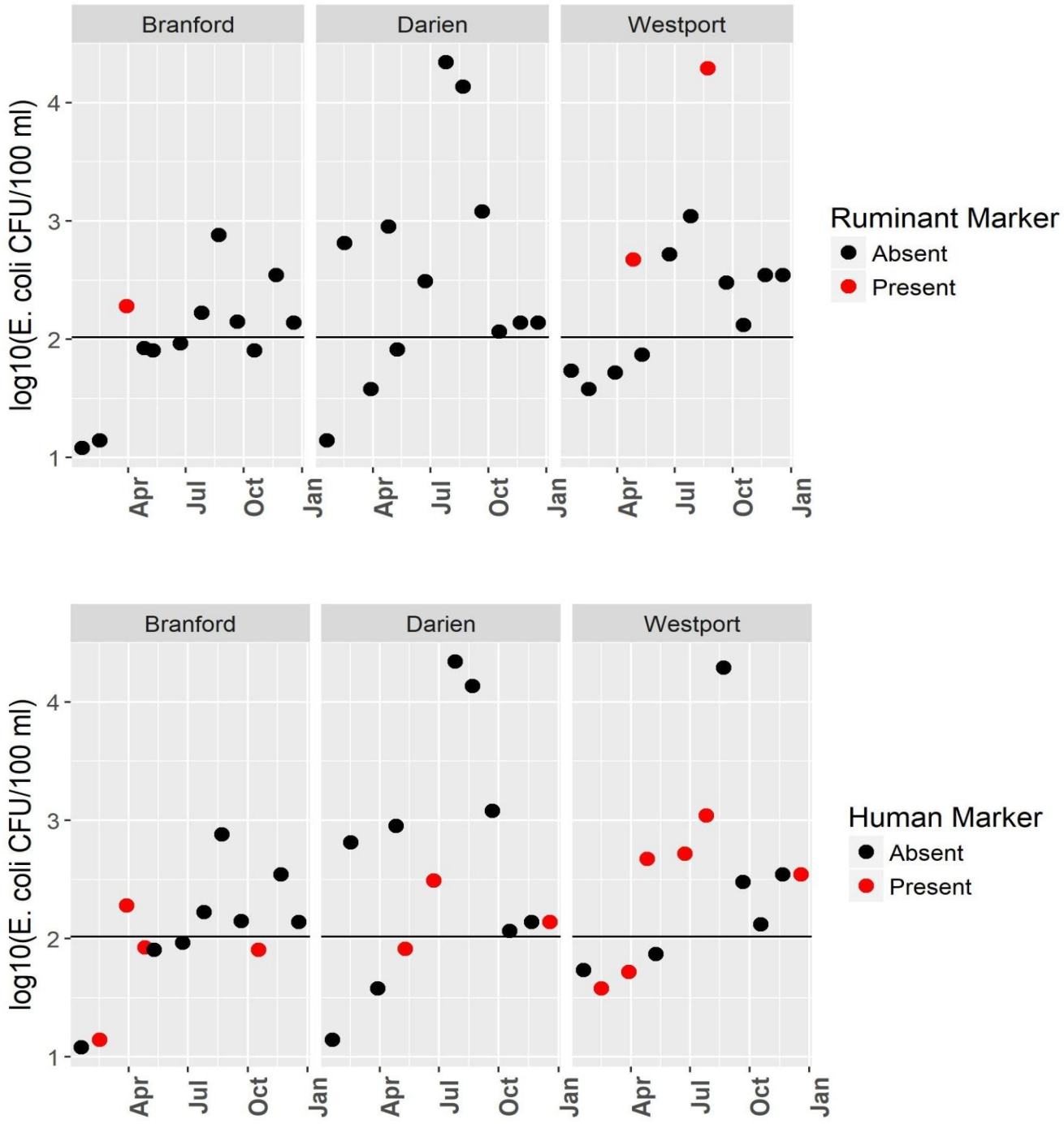
Host specific markers

As human sources are known to present higher health risks than most animal sources of contamination(Soller, Schoen, Bartrand, Ravenscroft, & Ashbolt, 2010), the ability to detect human sources of contamination is of great interest. More research has been done to establish methodologies for the detection of human fecal contamination than for other markers (Orin C. Shanks et al., 2016). Current recommended methods use a multiplex assay for two different human specific markers, HumM2 and HF183.

Monitoring for presence of the human markers indicated that there is little evidence of human sources of contamination at any site. At all sites, the human marker was detected sporadically (4, 3, and 6 of the 12 samples at Branford, Darien, and Westport, respectively) but was only present at levels below the level of quantification but still above the limit of detection. Additionally, when the human markers were detected, there did not appear to be a correlation with elevated *E. coli* concentrations (Figure 3a).

Ruminant markers were also used in this study as they encompass both agricultural animals such as cattle and sheep, as well as wildlife such as deer. There is an over abundance of deer in Connecticut (Connecticut's urban deer plan) with aerial surveys (Bernatas, Susan Aeerial Thermal Infrared Imaging White-tailed Deer Count Westport, Connecticut) indicating up to 32 deer per square mile. However, detection of the ruminant marker was rare at the selected sites with no positive samples in Darien, one in Branford, and two in Westport. Again, however, the detection of the markers at these sites does little to explain the extremely high observations of *E. coli* (Figure 3b).

In addition to these markers, markers associated with poultry, dogs, and cattle feces were analyzed for the samples but were not found in any of the samples.



Discussion

Stream water samples were collected monthly and tested for two general fecal indicators (cultured *E. coli* and a general Bacteroidetes associated marker) and for the presence of host-specific markers that could identify the presence of fecal contamination from human, ruminant, cattle, dog, chicken, seagull, and non-specific birds such as Canadian Geese. Additionally, quality assurance assays including a sample processing control (SPC) and an internal amplification control (IAC) were conducted to assess

possible inhibition or processing errors. Two of the assays (GFC for seagull and GFD for general avian contamination) were not implemented successfully and could not detect positive controls.

The results gathered from the study implemented here do not identify any source as having a consistent impact on water quality at any of the sites. This statement must be made and interpreted with caution, however, as there are a number of limitations both to the design of this study and to the use of host specific markers that must be considered, preventing the conclusion that none of the monitored sources contribute to fecal contamination at these sites.

One of the major limitations to the interpretation of studies comparing different indicators is a known difference in the persistence of indicators in the environment. Even when comparing indicators targeting similar groups of organisms (i.e. Bacteroidetes groups from different hosts), differences in persistence occur (Brooks and Field, 2016). These differences are even more drastic when comparing vastly different indicators, such as *E. coli* and Bacteroidetes markers, as Bacteroidetes are anaerobic and known to decay rapidly outside of the host gut when released into the environment (Kreader, 1995) while *E. coli* have been documented to survive in the environment, including both water and sediment (Wheller Alm, Burke, & Spain, 2003). This differential survival not only makes comparisons between the two markers difficult, but also makes it hard to state conclusively whether the absence of a marker for a particular source means that source did not contribute to *E. coli* levels at a site.

Another limitation to interpretation of source tracking studies is the variability of indicator concentration in host guts. This problem occurs both across species as well as within the same species over time (Dick et al., 2005; Haugland et al. 2010; Layton, Walters, Lam, & Boehm, 2010). One example of this that is particularly relevant for this study is that mammals have higher concentrations of Bacteroidetes in their guts than birds (Kreader, 1995). Additionally, geese have been shown to have lower intestinal *E. coli* counts in the winter season, while the concentration increases in the summer (Alderisio & DeLuca, 1999).

Our data show that there was discrepancy between the *E. coli* and general, non-host associated Bacteroidetes marker (GenBac3). As these are both indicators of the presence of fecal contamination from a range of sources, it is reasonable to expect a relationship between these two indicators, but only weak correlations were observed. One possible explanation for this is that the low concentration of Bacteroidetes markers in geese and variable concentrations of *E. coli* over time could help to explain this pattern. If geese were primarily responsible for elevated *E. coli* levels over the summer, it would be reasonable to expect lower counts in winter as well as a poor relationship between the two general indicators. Unfortunately, due to difficulties in implementing the two selected avian assays, we are currently not able to identify *E. coli* from avian sources.

The data do not suggest high levels of chronic contamination of any sources tested in this study. Additional sources that are likely to be affecting water quality in the area, including horses and rodents, were not tested as there are no existing markers for use that have been tested for in this geographic region. The presence of avian feces was not detected using a marker designed specifically for poultry, but we were unable to test for general avian contamination due to failings of the quality control steps taken for those assays. There have been attempts to replace the avian markers selected for use in this study as implementation has been challenging, but there are currently no tested methods that can be used in their place.

Conclusions and Future Directions

One of the primary goals for this project was to define a microbial source tracking program and test methodologies that could be implemented in future source tracking studies. DNA markers associated with humans, poultry, dogs, and ruminant feces were successfully utilized. The marker for general avian sources of contamination failed due to difficulties resulting from the design of the primers that amplify the markers.

Currently, a number of other markers exist, but have yet to be tested sufficiently for use in studies of this nature at this location. Additionally, other forms of source tracking markers, such as viral markers or chemical tracers, have been identified and could be used to expand and complement results using genetic markers. As developments in biotechnology continue to lead to new and improved rapid assessment techniques, a more consistent and reliable marker for geese is likely to be developed, as well as, additional markers that may enable the detection of other sources of bacterial contamination.

Another goal of this project was to monitor the water quality within each subject watershed to more accurately identify significant human and non-human sources of fecal bacterial contamination to develop management strategies for reducing and/or eliminating identified bacteria sources. Human sources are of more concern than non-human and domestic animals are of more concern than wild animals. Many watershed planning models are based on mathematical assumptions that do not take into account bacterial sources from wildlife sources.

A secondary goal of the project was to validate or refute the findings of the assumption-based method used in the Sasco Brook Watershed Based Plan. The plan assumed that geese and dogs are the most significant sources of bacterial pollution in this watershed and that with human sources were relatively insignificant. This study did not detect evidence of significant human contribution to the bacteria levels at the mouth of the three subject watersheds. DNA markers associated with poultry, dogs, and cattle feces were analyzed for the samples but were also not found in any of the samples. The non-detection of dog DNA markers leads to no conclusion as to whether dogs are a significant source of bacterial pollution in the Sasco Brook watershed. Whether the non-detection of significant human or dog DNA markers was the result of the difference in the persistence of indicators in the environment, variability of indicator concentration in host guts, the limitation of having one water sample collection site in each project watershed is unclear. Increasing the number sampling sites and frequency of collections, along with a sampling schedule to include both precipitation events and fair weather ones within each watershed would provide more data for analysis.

The two assays specific for seagulls and birds that failed to pass the screening for successful runs could not be included in further analyses. Thus, the study could not validate nor refute the significance of geese as a source of pollution, even though they were periodically observed at collection sites. This highlights the need for the development of reliable markers for goose contamination, as well as for other species of birds, especially seagulls.

Although the results of this study did not positively identify a likely source of elevated bacteria levels in these watersheds, they do suggest a possibility that those sources tested for are not contributing to the observed elevated bacteria levels. This information is valuable, especially with respect to the potential threat to public health the discovery of human markers would have represented, but it does not lend itself to the development of action-based recommendations that can be used to address the elevated bacteria levels observed at the monitored sites.

While this study did not identify specific contributions to the bacteria levels in the three subject watersheds for the markers successfully applied, further evaluation is warranted based on the elevated *E. coli* levels observed. The primers used may be subject to geographical variation and need further testing for geographic specificity. The development of reliable markers for other potential sources of bacteria, such as rodents and birds, could be the focus of future study.

Additionally, increasing the number of sampling sites and modifying the sampling schedule to include precipitation events would obtain more data for analysis. A past microbial source tracking study (*Pollution Source Survey and Assessment of the Farm River Watershed in East Haven and Branford*, Connecticut Oct. 2012) in the Farm River found chronic evidence of a human specific marker using a sampling scheme targeting fresh runoff following rain events. This approach could also be used to evaluate whether the discrepancies between *E. coli* and GenBac results are due to the differential survival rates of the two types of indicators.

DNA testing has not identified a source of bacteria in any of the three watersheds subject to this study. While there was no conclusive evidence of human contamination, limitations posed by using indicator organisms make it impossible to state that human contamination does not exist thereby illustrating other methodologies for assessing sources of bacteria need to be developed.

An additional or alternate direction for future studies could be to employ next generation sequencing technologies to assess likely sources of bacteria and to attempt to detect actual pathogens rather than focusing on surrogate indicators such as enterococcus or *E.coli* bacteria.

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Appendix A: Fecal and E.coli results from DNA sample sites.

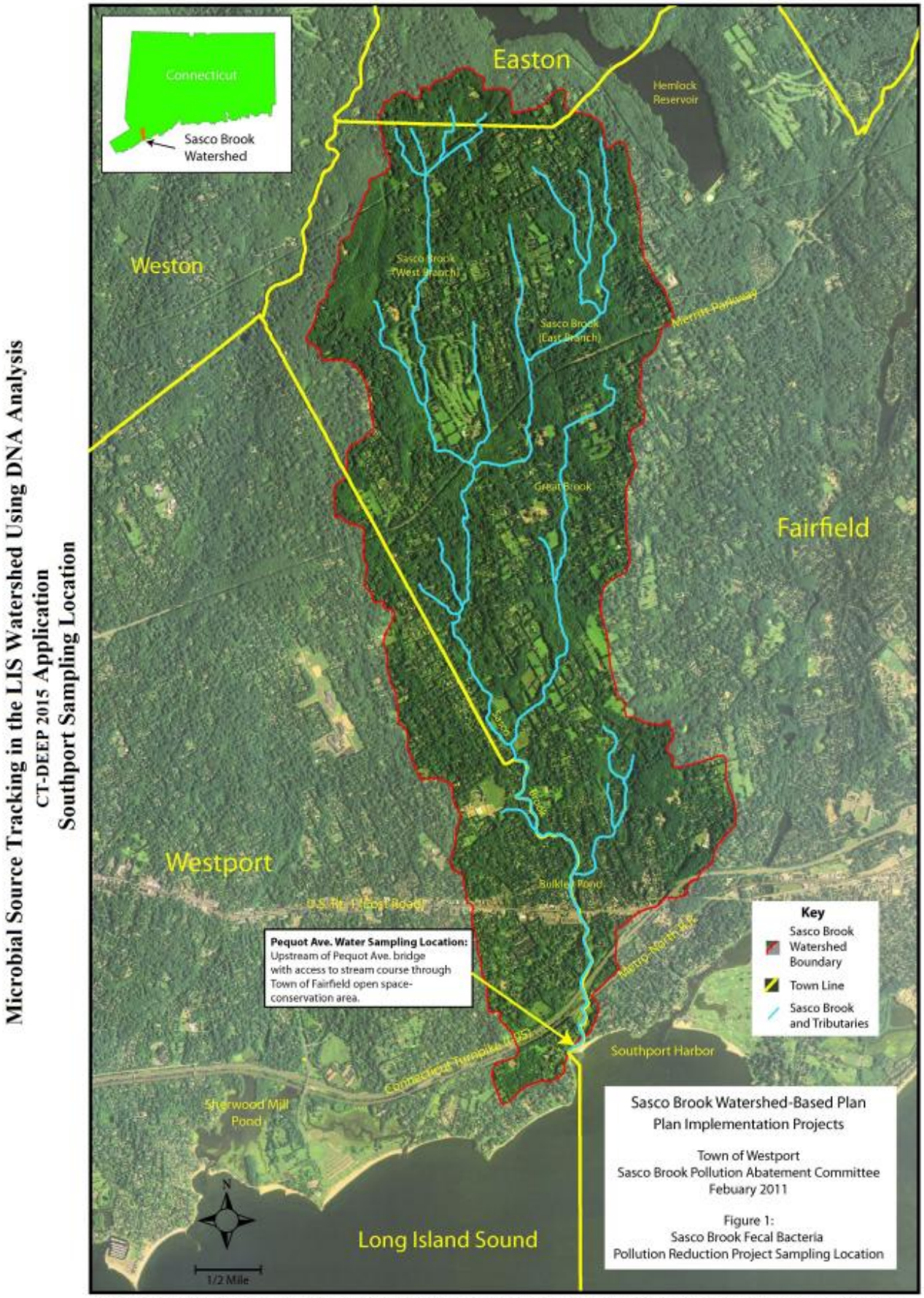
Site	Date	Time	Water Temp.	Fecal Coliform CFUs/100 mL	E. coli CFUs/100 mL	Weather[A]	Air Temp[A]	Discharge Cubic Ft/Sec[B]
Branford	1/19/2016	9:30	34 °F	12	12	Sunny	23°F	
	2/16/2016	10:00	32°F	14	14	Overcast	45°F	
	3/29/2016	10:30	39 °F	290	190	Sunny	47°F	
	4/26/2016	9:05	58°F	112	84	Overcast	50°F	
	5/10/2016	9:19	51 °F	86	80	Sunny	50°F	
	6/23/2016	9:18	71 °F	96	92	P-Sunny	71°F	
	7/26/2016	10:15	76°F	204	168	Sunny	75°F	
	8/22/2016	9:38	76 °F	960	760	Sunny	70°F	
	9/21/2016	9:45	72 °F	150	140	Sunny	70°F	
	10/18/2016	8:49	61°F	80	80	Overcast	65°F	
	11/21/2016	9:00	36 °F	340	350	Overcast	36°F	
	12/19/2016	9:00	36 °F	132	138	Sunny	22°F	
Darien	1/19/2016	10:30	32 °F	18	14	Sunny	23°F	
	2/16/2016	10:15	34 °F	730	650	Overcast	50°F	
	3/29/2016	13:00	49°F	48	38	Sunny	50°F	
	4/26/2016	10:15	54 °F	1300	900	Overcast	50°F	
	5/10/2016	10:00	53°F	96	82	Sunny	56°F	
	6/23/2016	9:25	65 °F	308	308	Sunny	71°F	
	7/26/2016	9:05	72 °F	39000	22000	Sunny	79°F	
	8/22/2016	9:05	72°F	13700	13600	Sunny	74°F	
	9/21/2016	9:40	69 °F	1500	1200	Sunny	78°F	
	10/18/2016	9:40	60 °F	134	116	Overcast	65°F	
	11/21/2016	9:00	34°F	138	138	Overcast	36°F	
	12/19/2016	9:00	34°F	138	138	Sunny	22°F	

Westport	1/19/2016	10:50	31 °F	62	54	Sunny	23°F	10.1
	2/16/2016	10:45	36°F	82	70	Overcast	50°F	23.8
	3/29/2016	10:39	48 °F	60	52	Sunny	50°F	19.3
	4/26/2016	9:22	58°F	560	470	LT Rain	50°F	16.8
	5/10/2016	9:26	58 °F	90	74	Sunny	56°F	10.9
	6/23/2016	8:50	68 °F	580	520	P-Sunny	71°F	0.74
	7/26/2016	10:50	73°F	2100	1100	Sunny	79°F	5.96
	8/22/2016	10:00	72 °F	19600	19600	P-Sunny	74°F	13
	9/21/2016	11:10	73 °F	370	300	Sunny	78°F	0.52
	10/18/2016	9:26	63°F	132	132	Sunny	72°F	0.45
	11/21/2016	11:30	30 °F	340	350	Rain	36°F	4.25
	12/19/2016	9:30	30 °F	340	350	Sunny	22°F	11.2

A - Average weather conditions and temperature obtained from timeanddate.com

B - Data from the U.S.G.S. National Water Information System. River Gauge 01208950 Sasco Brook near Southport is within the same watershed and about 2.7 miles from the Westport water collection site. https://waterdata.usgs.gov/ct/nwis/uv/?site_no=01204000&PARAmeter_cd=00065,00060 Gauge ; There are no U.S.G.S river gauges close enough to the Darien or Branford collection sites to be of use.

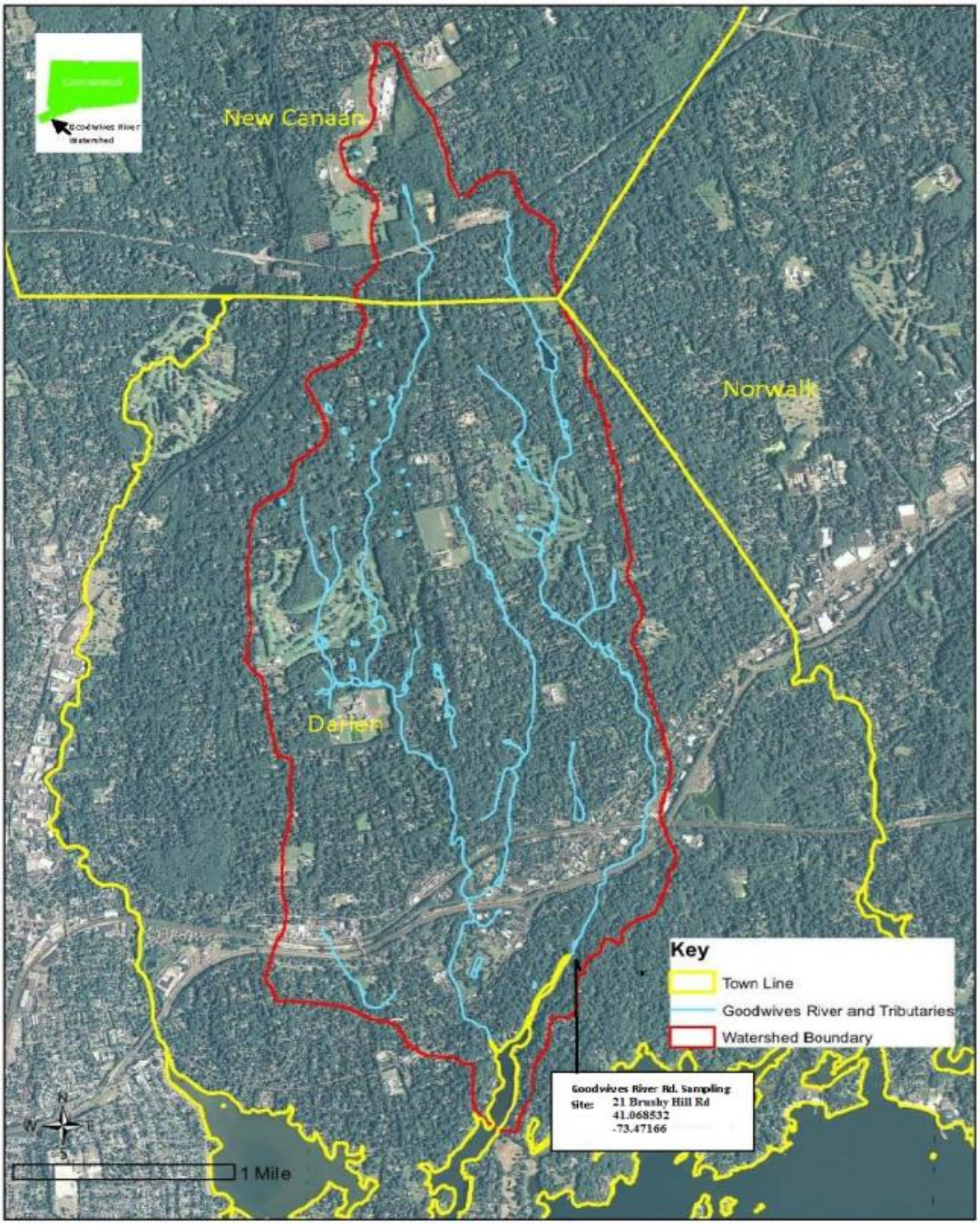
Appendix B - Study Area Watershed Maps



Source: 2006 Connecticut Aerial Photography from University of Connecticut Libraries Map and Geographic Information Center - MAGIC, in collaboration with the Connecticut State Library and the Connecticut Department of Environmental Protection.

Appendix B - Study Area Watershed Maps

Microbial Source Tracking in the LIS Watershed Using DNA Analysis
CT-DEEP 2015 Application
Darien Sampling Location



Appendix B - Study Area Watershed Maps

Microbial Source Tracking in the LIS Watershed Using DNA Analysis
CT-DEEP 2015 Application
Branford Sampling Location