Validation of genetic markers and application to detect fecal contamination at beaches in Coastal Connecticut

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Abstract

Due to shortcomings in traditional methods for detecting and quantifying the presence of fecal waste in waters new methodologies are being explored, with special attention being given to the ability to identify sources of the contamination. In this study, an alternative methodology, which uses Real-Time Polymerase Chain Reaction (RT-PCR) to detect host specific genetic markers to distinguish between human and non-human sources of contamination, was applied to beaches in Connecticut that were experiencing various levels of contamination. Despite some difficulties with this new approach, it was ultimately useful for identifying waste of human origin. Though not quantitative, this study qualitatively provided important baseline information for further studies by indicating beaches to target for more in-depth tracking procedures to detect and correct these human sources impacting the contaminated waters.

Introduction

In the United States, the presence of fecal indicator bacteria is the most frequent cause of waters being classified as impaired. Fecal indicator bacteria (FIB) are used as a proxy to measure the presence of harmful diseases that stem from the presence of untreated waste. Untreated waste is a type of nonpoint source pollution with many possible origins including combined sewer overflow, leaking septic tanks, agricultural runoff, pets, and wildlife. While fecal bacteria are often detected in the environment, there is currently no standard method to easily identify the source of the bacteria, making regulation and enforcement of any limits, such as the established Total Maximum Daily Loads (TMDLs), unfeasible (USEPA 2005). Additionally, without knowing what sources to target, best management practices are either not employed or may be put into effect for sources which are not significantly contributing to the problem.

Presence of untreated waste is a problem from both a public health and an economic standpoint. Consumption of contaminated shellfish or exposure to fecal bacteria in contaminated waters can cause a variety of health problems ranging from skin irritation to serious gastrointestinal illness, making it necessary for officials to prohibit certain activities that would put users at risk whenever bacterial counts exceed the accepted limit (USEPA 1983). Closures of recreational waters can greatly impact the economy of these sites, as many local businesses rely on tourism and visitors (US EPA 1983). Additionally, impairment can cause closures of sites used for shellfish harvesting, which also causes lost revenues to both fisherman as well as businesses relying on the products (Rabinovici). Without being able to identify sources and enforce water quality regulations, the negative impacts on both health and economy cannot be prevented. Thus, there is a great need for a method which can identify the sources of bacterial contamination.

While identification of the source to the individual site responsible for contamination is the ultimate goal in source tracking, an important first step is to distinguish the host species of the detected bacteria. Identification of bacteria from human hosts is especially key as human waste is both more dangerous and potentially easier to remedy. Human fecal matter is potentially more harmful to other humans as many viruses and pathogens tend to be host specific, making the classification of human contamination crucial (Field and Samadpour 2007). Additionally, from a regulatory standpoint, identifying and correcting a human source is often more feasible than regulating input from other sources. Therefore, while identification of all sources is important, finding a way to accurately identify human waste is a priority.

The current standard methods to detect untreated waste use fecal indicator bacteria to assess the possibility of pathogens in the water (Field and Samadpour 2007). These fecal indicator bacteria are most commonly *Enterococci* or Fecal Coliform/*E. coli*. These bacteria are the recommended indicator bacteria by the EPA, and have been well studied to determine the correlation with diseases as well as the levels that indicate a water body is unsafe for certain uses. *Enterococci* are used at marine sites, as they are primarily only capable of surviving for short periods outside of the gut but can survive in marine water and are well correlated with

pathogen survival. Fecal coliform, however, are typically used to detect waste for freshwater sites, and although they can be used in marine sites are typically better indicators for freshwater. These FIB have been used for years, and are recommended by the EPA based on their correlation with pathogens and the simplicity of culturing the bacteria to measure and quantify the living cells.

Despite the benefits of the traditional FIB, there are several drawbacks to the standard methods of detecting waste. First, while they are correlated, traditional FIB are not always directly related to the presence of pathogens. In some cases, the indicator bacteria have been shown to grow outside of the gut, and even in sand, meaning contamination may appear to be occurring even when it is not (Yamahara et al. 2009). Additionally, long incubation periods mean that contamination may not be detected before users of the water are exposed, leading to possible illness while waiting for results. However, the most significant limitation in the standard methods is that they fail to provide information regarding the host species of the bacteria, and thus do not assist in identifying and regulating the input of the bacteria, greatly limiting their usefulness in correcting water contamination.

In recent years, many researchers have proposed new methods for source identification, most of which rely on either a chemical or biological source indicator (Stoeckel and Harwood 2007). An ideal source indicator would be host specific, widely distributed in the host population, stable in the host across time and geographic range, quantitative, relative to regulatory tools, and relevant to health risk (US EPA 2005). While no new method has been able to meet all of these criteria, research into possible indicators has provided a number of candidates, many of which have simply not been tested well enough to determine their potential for future use.

Microbial source tracking uses microbes to not only detect the presence of waste but also uses certain features of the microbes to identify the source. Within this broad category of source tracking are a number of approaches that use polymerase chain reaction (PCR) to amplify certain host specific DNA sequences in the bacteria. The sources of the bacteria can then be determined based on the presence or absence of the markers that identify potential hosts. These approaches have shown potential to either replace or supplement the traditional testing, as they are relatively fast, simple, and inexpensive (US EPA 2005). However, the bacterial genome is prone to fast changes and variations, meaning the markers must be validated in time and location. Validation of the markers consists of a step testing waste from a variety of known sources for the markers, therefore conforming the sensitivity (found in bacteria from the expected hosts) and specificity (not found in bacteria from other host species) of the markers.

In the past, many types of bacteria, including those from the genus *Bacteroides*, were discounted as potential FIB due to difficult cultivation procedures, an obstacle that no longer limits researchers when using PCR. *Bacteroides* were selected as a potential FIB based on their high concentration in fecal matter and their lack of survival under aerobic conditions, characteristics which still make them appealing today (Kreader 1995). Specific assays for use in PCR allow the detection of a variety of host specific genetic markers from *Bacteroides*. Additionally, recent technological developments have improved upon these methods, and Real-Time

Polymerase Chain Reaction (RT-PCR) can be used to both identify and quantify these bacterial DNA sequences (Seurinck et al. 2005).

Among the many DNA sequences that have been identified are general *Bacteroides* markers and human specific markers. The Bac32F/Bac708 primer pair amplifies a 16S rRNA fragment that was developed for use as a genetic marker by Bernhard and Field (2000a) in one of the first attempts to use PCR to indicate the presence of *Bacteroides* in contaminated waters. The marker has since been used for a number of studies in a variety of locations indicating the presence of the marker in *Bacteroides* from all hosts tested (Seurinck et al 2005, Gawler et al. 2007**,** Shanks et al 2009). The human specific genetic marker, amplified using the HF183F/Bac708R primer pair, was also developed by Bernhard and Field (2000b) and is found nearly exclusively in bacteria from human hosts.

While it has not been contested that the universal marker is found in all *Bacteroides*, researchers have not yet definitively concluded that HF183 is a universal human specific marker. However, use of RT-PCR to detect the human specific marker has been shown in several studies to have significant potential. Seurinck et al. (2005) first modified the human specific marker developed by Bernhard and Field (2000a) by shortening the sequence to make it more compatible with RT-PCR. Since then, a number of studies have sought to determine the potential of this marker to accurately detect human fecal contamination. Studies conducted in Belgium (Seurinck et al. 2006), California (Kildare et al. 2007), France (Gourmelon et al 2007), Australia (Ahmed et al 2008), and Kenya (Jenkins et al. 2009) have all shown promising signs that this marker may be used to identify human fecal contamination. Each of these studies concluded that the HF183 marker was usable in the region of the study after showing that it was sensitive and specific to human feces. While this method shows promise, the presence of host specific genetic markers to be used must be validated through demonstration that the markers are both specific and sensitive to the host at the time and location of sampling.

The use of PCR and RT-PCR to detect bacterial contamination has several advantages over the standard method. Aside from the ability to identify the source, this approach to FIB detection has potential to be more reliable and reproducible than standard methodology as this approach does not depend on the ability of cells to grow, and also detects individual markers rather than colony forming units. Another significant improvement over the standard methodology is the lack of a delay between sampling and analysis, with a potential turn around of less than three hours. This improvement in turnaround time is significant as it can expedite detection and response to beach contamination. However, as this methodology has not been thoroughly tested there is no available data on the relationship between pathogens and this marker.

In this study, this method was tested and applied at bathing waters and potential recreational shellfish harvesting sites in East Haven and Branford, Connecticut. Sites were selected based on historical levels of bacterial contamination and sites and locations were added or changed in response to observed patterns in contamination. These sites are each unique in their inputs to the system as well as the potential sources for contamination. By working with the

East Shore District of Health Department, I was able to collect water samples for both standard method analysis as well as samples for analysis using the new methodology simultaneously, to provide samples for comparison of the two methods.

It was hypothesized that the standard methods and the universal marker detected by the new method would be correlated, and a relationship could be determined that would allow the new marker to be used as a proxy for the standard method. Additionally, it was expected that as standard method testing showed contamination, the human marker would be found, meaning that contamination was believed to be from a human source.

Materials and Methods

Sample Collection

Fecal samples were collected prior to the summer sampling period, from June to August, to verify the specificity of the markers. Non-human samples were collected from throughout the area surrounding the town of Branford from farm animals and domestic pets, and human samples were collected from the Branford Waste Water Treatment Plant. Animal samples were collected using sterile 2 ml tubes to collect small amounts of bacteria. Once collected, samples were cored using a 1 ml syringe to subsample approximately 0.2 ml of the original sample. Samples were either continued in the DNA extraction process immediately or were stored in RNAlater (Qiagen), a preservation agent that allows the sample to be stored at room temperature for up to 5 days without DNA degradation (Nechvatal et al. 2007). Waste water plant samples were collected in 0.5 L sterile containers and brought back to the lab immediately, where approximately 10 ml of the sample was collected using a 47 mm diameter 0.22 µl pore size filter. The DNA was then extracted directly from the filter as described below.

Water samples were collected from the towns of East Haven, Branford, and North Branford during the summer ranging from June to August. Four sites were selected for regular monitoring, with additional sites included when bacteria were believed to be present and as detection patterns emerged throughout the summer. Sites included both bathing water sites as well as potential shellfish harvesting sites. The bathing water sites are public access beaches in the towns, and were monitored at least weekly by the East Shore District Health Department (ESDHD), with additional samples taken when problems were reported. Sampling strategies for the shellfish harvesting sites include the sampling of the locations at ebb or low tide, with emphasis on samples taking place within 3 days of rain events, although dry weather samples were also collected.

Traditional water sampling techniques used by the ESDHD were used in conjunction with samples collected for the analysis of the genetic markers. Traditional sampling protocol consists of collecting approximately 100 ml of water in 125 ml sterile bottles. Once collected, samples were stored on ice until being analyzed by the appropriate lab (The State Department of Health Lab in Hartford for Bathing water samples or the Bureau of Aquaculture Lab in Milford for Shellfishing samples).

For genetic analysis, 1 L of water was collected and stored on ice for no longer than 4 hours until brought back to the lab in the Environmental Science Center at Yale University. At the lab, samples were filtered using the filtering protocol described above for the wastewater samples with the exception that the volume varied, ranging between 100 and 550 ml, depending on the turbidity of the sample and the volume that would pass through the filter before becoming saturated. Once collected on the filter, DNA from filters were either isolated immediately or filters were frozen at -80°C until extraction and analysis could be done.

DNA Extraction

DNA from fecal samples was isolated using the Qiagen Qiamp Stool Mini Kit. Following extraction, samples were diluted to make the DNA concentration easier to use in downstream applications. Diluted DNA was aliquoted and frozen at -20°C until further analysis. DNA from water samples was isolated using the MoBio Power Water Kit, allowing for extraction directly from the filters. DNA from these samples was not diluted, and was aliquoted and frozen until further use.

Conventional PCR

Conventional PCR was conducted on some samples in order to detect the presence or absence of the two sequences. PCR reactions were contained 5 µl 5x buffer, 1.5 µl $MgCl_2$, 2 µl dNTPs, 0.5µl each primer (see Table 1), 0.5 µl BSA, 0.2 µl Go Taq Polymerase (Promega), and 2 µl DNA, diluted with RNAse free water to 25 µl. The temperature program was as follows: 35 cycles of 94°C for 1.5 minutes, 60°C for 1.5 minutes, and 72°C for 2 minutes with a final elongation time of 10 minutes at 72°C and products were visualized on a 1% agarose gel. The marker was considered present when a band of the appropriate size was visible.

RT-PCR

Both the human specific and universal markers were quantified using RT-PCR with the following reaction parameters: 10 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.5 µl each 10 µM primer, 6.8 µl RNase Free Water, 0.2 µl Bovine Serum Albumin (BSA), and 2 µl template DNA. The reagents, excluding the DNA, were combined and mixed before being dispensed. Reagents and template DNA were loaded into MicroAmp Optical 96-well reaction plates with optical sheets using the Eppendorf epMotion 5070 automated pipetting workstation to ensure accurate and consistent pipetting between samples and replicates. The 20 µl reactions were conducted in triplicate for each template DNA with the following temperature parameters using an Applied Biosystems 7500 Fast Real Time PCR System: 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Melting curves followed each reaction to detect specific product with human specific products melting around 74°C and universal products melting at approximately 80°C.

Table 1: Primers used for analysis of genetic markers

Primer Pairs	Use	Sequence (5' to 3')
Bac303F/Bac708R	Universal, RT-PCR	GAAGGTCCCCCACATTG / CAATCGGAGTTCTTCGTG
Bac32F/Bac708R	Universal, Conventional PCR	AACGCTAGCTACAGGCTT / CAATCGGAGTTCTTCGTG
HF183F/265R	Human, RT-PCR	ARCATGAGTTCACATGTCCG / TACCCCGCCTAC
HF183F/Bac708R	Human, Conventional	ARCATGAGTTCACATGTCCG / CAATCGGAGTTCTTCGTG

Standard curves were constructed from dilutions made using plasmids into which the markers had been cloned, which were donated by Katharine Fields, Oregon State University. Clones had to be used to construct the standard curve, as the bacteria containing the human specific marker are not culturable. Frozen clones were grown to create a stock solution, from which DNA was extracted using a Qiagen Plasmid Midi Kit. The total plasmid count was then quantified using a Nano-Drop, and dilutions were made to create standards of the appropriate values.

State Lab Analysis

Water samples were sent to the Department of Health State Lab or to the Bureau of Aquaculture Lab and were analyzed based on standard protocol. Bathing water samples were analyzed for the presence of *Enterococci* based on Colony Forming Units (CFU) analyzed following EPA Protocol 1600 or 1106.1 or using the Entero-alert method. Water samples for the shellfish harvesting sites were analyzed by the Bureau of Aquaculture for the presence of Total Coliform and E. coli also based on the number of CFU following the standard methods.

Results and Discussions

Limitations

During the course of this study, several obstacles were encountered which interfered with the intended objectives. Due to difficulties optimizing the protocols described above for the samples, in some cases all DNA extracted was used in the trial and error phase, and analyses could not be conducted. As a result, some samples did not undergo all types of analysis. Duplicate filters were collected and DNA was extracted again where feasible, however, due to the original selection of a DNA extraction kit that proved to be unsuccessful, some of the spare filters had already been used. Nevertheless, as these difficulties resulted in random analysis differences they are not expected to bias the results.

In addition to the optimization difficulties, once the protocols were optimized they still failed to provide reliable results, with high amounts of variation being present between different runs containing the same DNA as well as between field duplicates. Field duplicates and blanks were used as a form of quality control; however, instead of containing similar values the duplicates consistently had a high range of variability. Despite these differences, there was a consistent finding when viewed simply as a detection of the marker, with all blanks indicating no presence of the marker and all duplicates consistently showing either the presence or absence of the mentioned marker. In depth statistical analysis will be conducted in the future to compare the amount of variation following this methodology to that of the standard methods. Therefore, due to the high variation of values determined even after optimization, data presented in this paper will be analyzed based on a presence/absence basis, rather than a quantitative basis.

Specificity of the Markers

As anticipated, the universal marker was found in high amounts in all fecal samples tested. The human marker was sensitive and detected in the sewage samples even when diluted by a factor of ten with sterile water. The marker was specific to the sewage samples with the exception that the marker was found in low concentration in one sample collected from a dog when analyzed using RT-PCR, suggesting a possible issue with the specificity. However, as the level was low, and found in only one of the eight dog samples, it is still usable as a human indicator.

Conventional PCR

Conventional PCR analysis was conducted for samples collected during the latter portion of the summer. Prior to this date, it was not recognized that higher volumes of water were necessary to detect the markers, and thus only when volumes greater than 100 ml were filtered was a conventional analysis conducted. It was originally intended to analyze the earlier samples using conventional PCR, however, all DNA from the samples was necessary for analysis in RT-PCR and was not available for analysis. As a result, there are fewer data points in this section, and they are all from Late July or the month of August, which by the traditional methodology showed lower frequencies of contamination. Despite the low contamination levels indicated by the standard testing, every sample analyzed with conventional PCR showed the presence of the universal marker, while the human marker was absent from every sample, with the exception of the Lanphier's Cove Beach Sample from July 26.

RT-PCR

While RT-PCR can be used to quantify the markers present, it can also be used to simply detect the presence or absence of the marker. In this context, the RT-PCR analysis was successful, with blanks and duplicates being consistently present or absent for the samples analyzed. However, when quantitative analysis was conducted, the values had high amounts of variation between field duplicates, suggesting the values found in this study may not be reliable beyond a semi-

quantitative sense. With this in mind, the data presented below are to be used only in a qualitative or semi-quantitative sense, as hard numbers appear to be unreliable.

The universal marker was detected in all but one sample analyzed (BPB 8/16), and was consistent with the above-mentioned results from the conventional PCR with the exception of this one site. The presence of the universal marker at all sites in high numbers suggests that the marker is there at all times, not just in the case of contamination events. There did not appear to be any correlation (R^2 = 0.014) between the standard values and the marker used in this study. This is believed to be due in part to the high amount of variation in this study, as well as the high amount of variation in findings of the standard method, making any correlation between two somewhat unreliable datasets difficult. Additionally, as there were no steps taken in this project to distinguish between live and dead cells, the use of the universal marker may not be helpful in identifying contamination as these markers have been shown to survive for several weeks in the environment. In contrast, the standard methods rely on live bacteria for counts of colony forming units, which must be alive at the time of sampling and analysis, making direct comparisons between the two markers difficult.

Based on this finding, it is unlikely that the universal marker can be used as a substitute for traditional methods. As the traditional marker is correlated with pathogens, and the universal marker tested was not correlated with the standard marker, it seems unlikely that the universal marker would be correlated with the pathogens known to affect contaminated waters. Therefore, until a method to distinguish the live cells is developed, it is unclear whether the marker is faulty or whether the live cell distinction is the cause of the lack of correlation. Current research has tested methods for the distinction of live cells using propidium monoazide (Bae and Wuertz 2009) but this technique was not implemented in this study.

The human specific marker, which was only detected in one of the sites using conventional PCR, was detected in much higher frequency using RT-PCR. RT-PCR is a more sensitive technique, and was likely simply able to detect the marker at lower concentrations. The quantities reported by the instrument, while not used for determination absolute values, are useful in showing that most of the samples had very low numbers of the markers present relative to the universal marker, suggesting that while the human marker was present it may not have been the sole source of bacteria. However, this may also be due in part to the fact that the universal marker may indicate closely related bacteria, not limited to those of the *Bacteroides* genus. This may also help to in part explain why there was no correlation between the standard marker and the universal marker. Thus, it can be concluded that this universal marker may not be a suitable choice for replacement of the standard methodology.

Human Specific Marker By Site

Originally, this project was intended to include samples taken at four bathing water sites (codes BPB, CAB, LCB, and SRC). However, it was expanded to contain samples from several other beaches. While the numbers may not be reliable for absolute quantitation, the presence of the human marker was far more prevalent at

some sites than others (see Table 2 for details), suggesting potential at these sites for future studies to further testing and implementation of source specific management strategies. Sites of special interest for future studies are those that experience frequent high levels of bacteria as indicated by the standard markers, and are also shown in this study to have a consistent presence of the human specific marker. The shellfish harvesting sites (2.1, 2.4, and 2.6) are from the Town of East Haven, which is considering opening the shellfish harvesting beds near the town beach. However, due to potential bacterial contamination, an in-depth sampling regime has been implemented to provide regular monitoring so that it can be determined if the waters are safe for harvesting. The remaining sites are bathing water sites, which are public beaches where residents of the area are allowed to swim. These sites were monitored at least once a week by the ESDHD, with samples being taken again when the bacterial levels exceeded the limit. Only when a beach was in violation of the limit for two samples was the beach closed, and no longer open to the public, following standard procedures by the Connecticut Department of Public Health.

Figure 1: Map of East Haven sites, consisting of both shellfish harvesting sites and bathing water sites

Figure 2: Branford sampling sites, showing all sites East of the Farm River

Site 2.1

Site 2.1, a potential shellfish site in East Haven, was located directly below a storm drain. As a result, the site is highly influenced by rainfall, with the quantity and quality of outflow from the drain being highly variable depending on recent weather. The human marker was not present any of the three times the site was sampled for conventional PCR analysis. Although conventional PCR indicated no presence of the human specific marker, with RT-PCR the human specific marker was detected 4 of the 7 times sampled. This site experienced elevated bacteria levels (>35 total coliform CFU) during 40% of samples (4/10 samples as determined by the Bureau of Aquaculture lab), but these elevated bacterial events were not well correlated with the presence of the human marker, suggesting that there may be contamination issues from a different source. Another likely source of bacteria is from urban runoff entering the storm drain during rain events, which can contain unattended pet waste or fertilizer that washes into the storm sewers and through the drain.

Site 2.4

Site 2.4 is another potential shellfish site in East Haven, although this location contains water from the drainage of a tidal marsh rather than the Long Island Sound. This site consistently experienced elevated bacteria levels as measured by the standard method, with very few samples experiencing bacterial counts lower than 100 CFU/100 ml. One possible explanation for this is the storm drain located adjacent to the sample site; however, as the samples were collected upstream of the drain and during low tide, when the river was flowing out, this drain should not have drastically impacted the site. Additionally, the drain was quite often at very low flow, especially during dry weather when the site was still experiencing elevated bacteria, suggesting the bacteria were present in the stream, not simply entering from the drain.

This site also experienced no presence of the human marker as shown by the conventional PCR, but did show one occurrence of the human marker as detected with RT-PCR. This site consistently experienced high amounts of bacteria as detected using the standard methods. While only one sample showed the presence of the human marker this site was not sampled regularly until well into the project, when contamination problems were observed using standard markers. Further studies would be necessary to determine whether the human marker is in fact present on a consistent basis.

Site 2.6

Site 2.6, another potential shellfish harvesting site, is located at the mixing of the tidal marsh waters with the waters of the Long Island Sound and experiences impacts of the marsh as well as the waters of the Sound. This site, like the other two shellfish harvesting sites, experienced no detection of the human specific marker when measured using conventional PCR, but did show a 40% detection rate with RT-PCR, higher than that detected at Site 2.4. Like Site 2.4, this site was not consistently monitored with the new methodology; however, the standard methodology indicated bacterial contamination for some samples, but far fewer

than that of Site 2.4, suggesting the mixing with the Sound waters was diluting the contamination. Additionally, there does not appear to be any correlation between elevated bacterial levels from the marsh and those of Site 2.6, suggesting that perhaps elevated bacterial levels in Site 2.6 are coming from another source entirely. This hypothesis is supported by the fact that the human marker was detected in the 2.6 site, even when not detected in Site 2.4. Once again however, this presence of the marker did not seem to be correlated with the detection of bacteria by the standard methods.

Branford Point Beach

The Branford Point Beach (BPB) is a bathing water site that has a history of contamination in recent years, a trend which was continued this summer with the total number of *Enterococci* exceeding the acceptable limit 7 times this summer, leading to closure of the beach once due to continued elevated bacterial counts. This site is a popular public beach, adjacent to a small park and a fishing dock, and attracts relatively large crowds from local residents. While the beach is not located in a highly developed site, it is located in a small cove near the output of the Branford River, and is potentially impacted by any bacteria carried by the river. Additionally, as the beach is located in a cove, the water may be more stagnant, causing the contamination to continue rather than disperse into the larger body of water.

This beach was one selected originally for monitoring, and while the quantities of the markers do not correlate with the counts from the standard markers, the human marker was detected by RT-PCR in 100% of the samples, suggesting at least a portion of the bacteria impacting the beach are from a human source and that these humans sources are consistently contributing to the bacteria levels. As this beach has consistently high bacterial counts, as well as a consistent presence of the human marker, this site is a good candidate for continued study to determine with more certainty the problems associated with human sewage entering the location with special focus being placed on the impacts of the Branford River on the water quality of the beach.

Clark Avenue Beach

Clark Avenue Beach (CAB) is another site with a history of elevated bacterial levels. Like BPB, samples at CAB exceeded established limits many times, resulting in one beach closing. This beach is a fairly small public beach located on a minor road and is mainly used by residents of the Short Beach Community. The area is primarily residential, with the exception of the Yale Yacht Club being located immediately adjacent to the public beach. This site is located in a cove just east of the outflow of the Farm River, and likely receives some water from the flow from the river. The Farm River, which separates the towns of Branford and East Haven, flows from Lake Saltonstall, a drinking water supply reservoir operated by the Regional Water Authority, suggesting the river should be free of bacteria from above this point. However, it is not clear whether these flows are contributing to the bacterial counts in the site.

The human marker was detected at this site for 50% of the samples. Additionally, at this site, there did appear to be a correlation between the exceedences and the presence of the human marker, with bacterial counts being somewhat higher when the marker was detected than when it was not. This site was the only one that showed this kind of relationship, which suggests that perhaps the problems experienced during exceedence of limit were caused by human sources. However, once again, this merely suggests that human contamination may be a problem, and as it was not consistent, much more extensive sampling is recommended to identify and correct contaminant sources.

East Haven Town Beaches

The East Haven Town Beach Sites (EHE and EHW) were also bathing water sites, although they did not have a history of a problem with contamination and were thus not included in the original study. However, on one occasion this summer the beaches were closed due to excess bacterial counts, and as a result were sampled a few times. No conventional PCR was done on these sites, but the RT-PCR did suggest the presence of the human marker on one of the four occasions it was sampled. However, the presence of the human marker was not detected at the time of the closing, suggesting human impact may not be the main cause. This site is located very near the sample sites for the shellfishing, and as a result is of high importance for continuing studies to determine sources so that shellfishing beds can be opened in the future.

Lanphier's Cove Beach

Lanphier's Cove Beach is another bathing water site, and although historically this site does not have a history of contamination, this site consistently had elevated bacterial counts during the summer. While this beach was only closed once, bacterial counts were elevated throughout the summer, though not always above the limit at which a beach is deemed to be unsafe. Like Branford Point, this beach is also located very near the outflow from the Branford River, and is likely experiencing the same inputs. This relationship is indicated by the correlation of bacterial counts by the standard methods between the BPB samples and the LCB samples (R^2=0.78)

In addition to elevated counts, this site tested positive for the human specific marker each time sampled with RT-PCR, and was also the only site to test positive using conventional PCR. The one positive test had a very high count as indicated by quantification of the marker, supporting the belief that the conventional PCR may not have been sensitive enough to detect the marker on all occasions, but is effective when the marker present in high concentrations. The consistent presence of the human marker as well as the consistent elevated bacterial counts suggests this site is ideal for continued studies to determine the sources of contamination. Once again, elevated bacterial counts and the close proximity to the Branford River suggest further investigation of contamination in this river is necessary to detect the sources impacting the beaches.

Stony Creek Beach

Stony Creek Beach is overall a clean beach, with only one exceedence this summer and no closings. This beach is located in a small community full of residences and a few private businesses. Despite the lack of elevated bacterial levels, this beach was sampled on three occasions for the human marker, and all three samples indicated the human bacterial marker was present in low concentrations. Although this site does not currently experience exceedences, humans may be having an impact, and if problems should develop this site could benefit from additional studies.

Sunrise Cove Beach

Sunrise Cove Beach, another bathing water site, was also selected in the original sampling scheme due to a history of contamination. This beach is located near the LCB site, in a region known as Double Beach. While the total count only exceeded the limit twice this summer, and re-sampling allowed the beach to remain open, SRC did consistently show fairly high counts, even if they did not exceed the limit. This is surprising, as one would expect the beach to be experiencing similar flows from the Branford River as BPB and LCB, but this beach seemed to have lower counts. This beach may be experiencing only a portion of the flows, and the hydrology of the region should be better defined before impacts of the river on any of the sites can be confirmed.

Despite having lower counts than other sites, the human marker was detected with RT-PCR each time sampled, suggesting this beach is consistently experiencing the impacts of bacteria from human sources. As this beach is known to experience high contamination, and in this study was shown to consistently have the human specific marker, this beach should be investigated in more depth to determine the sources of these bacteria.

Table 2: Frequency of marker detection for the various markers shown in both total counts and as percentages of the samples analyzed.

Sample Code	Human Marker, Conventional PCR	Human Marker using RT-PCR	Universal Marker, Conventional PCR	Universal Marker, RT-PCR
2.1	0/3 (0%)	4/7 (57%)	3/3 (100%)	6/6 (100%)
2.4	0/2 (0%)	1/3 (33%)	2/2 (100%)	1/1 (100%)
2.6	0/2 (0%)	2/5 (40%)	2/2 (100%)	4/5 (80%)
BPB	0/5 (0%)	10/10 (100%)	5/5 (100%)	8/9 (88%)
CAB	0/5 (0%)	6/12 (50%)	5/5 (100%)	10/10 (100%)
EHE	NA	1/3 (33%)	NA	2/2 (100%)
EHW	NA	0/1 (0%)	NA	1/1 (100%)
LCB	1/5 (20%)	9/9 (100%)	5/5 (100%)	8/8 (100%)
SCB	NA	3/3 (100%)	NA	2/2 (100%)
SRC	0/5 (0%)	10/10 (100%)	5/5 (100%)	9/9 (100%)

Conclusions

Although the results of this study were not consistent with those originally hypothesized, this study still showed several significant findings. First, by showing no correlation between the universal marker and the standard markers, this suggests that this marker is not a good candidate to replace the traditional markers. However, this marker does still have potential if a way to distinguish the live and dead cells is implemented, which may increase the correlation between the traditional and new markers. Future studies should further investigate this correlation.

While the universal marker did not seem to be a good candidate for replacing the traditional method, when used as a supplement the human marker does show potential for the detection of human sources. Rather than replacing traditional methodology and quantifying human impacts, this marker can be used as a tool to determine areas of special interest for detailed studies and regulatory actions. It was originally thought that a stronger correlation would be present between the markers and the traditional FIB, but despite the lack of a relationship this data is still useful as a pilot project for future studies in the area to focus on the sites experiencing human contamination as it suggested sites which may be experiencing bacteria from human sources.

Based on the data for the individual sites, it has been shown that areas of interest to continue monitoring and expand the project are the BPB, CAB, LCB, and SRC sites. As they are likely a major conduit for pollution, it is recommended that special attention be paid to the rivers that flow into these sites as they are likely a major cause of impairment. Additionally, the SCB site should be examined more closely in the future if problems with bacterial counts emerge.

Future work to continue investigation of the use of this methodology should include a more regular monitoring strategy with a larger sample database. However, future work in the area will be simplified by the trial and error done in this study to optimize the protocols used in this study thus providing future researchers with the knowledge and notes to accomplish analysis without such steps. Overall, this method is still believed to show potential for source identification, even if quantification of the impacts seems unlikely at this time.

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