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Controls and Improvements of Molecular Assays for Microbiological Water Testing

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CONTROLS AND IMPROVEMENTS OF MOLECULAR ASSAYS FOR
MICROBIOLOGICAL WATER TESTING

By

David R. Wanless

A THESIS

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
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CONTROLS AND IMPROVEMENTS OF MOLECULAR ASSAYS FOR
MICROBIOLOGICAL WATER TESTING

David R. Wanless

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The monitoring of recreational waters is important to gauge risks to human health. The

methods currently used are time-intensive (18-24 hours to test results) and because of

this, results may be inaccurate. There are now molecular techniques capable of

delivering faster results on multiple microbiological targets, including source tracking

markers. However, these techniques cannot be utilized most effectively until extraction

efficiencies of environmental DNA are known. Earlier studies of bacterial indicators in

recreational beach waters using quantitative polymerase chain reaction (qPCR) showed

variation in the DNA extraction efficiency of filtered marine water samples. Silica-based

binding matrix micro-columns used in DNA extraction kits, have a limited binding

capacity for DNA. The elution of DNA off the column appears to be released in a non-

linear fashion, suggesting that for robust extraction a minimum amount of DNA must be

bound. By using known quantities of *Lactococcus lactis* to mimic the characteristics of

enterococci, along with a minimum amount of background DNA it is possible to

determine DNA recovery for the extraction/purification process. This was accomplished

by adding a known amount of *Lactococcus lactis* cells to samples of recreational water

along with large volumes of water or known amounts of background DNA. After extraction, the *Lactococcus lactis* DNA was quantified using qPCR. The percentage of *Lactococcus lactis* remaining in the elution was then used to quantify the results from the enterococci assay. Source tracking markers for dog associated bacteroidales and gull associated catellococus were adapted into Taqman™ chemistry qPCR assays. These assays were then developed with standards and tested against South Florida water samples. These assays can help provide new information to the contributor of fecal waste to beach and coastal environments. These assays performed well in the field and should be incorporated into the testing of beach water that allows dogs, or has a large gull population.

Dedication page

    I would like to dedicate this thesis first and foremost to my family. I appreciate the patience of my wife Shannon, who's had to put up with more than she should and my son Winston, who every day is an inspiration to me and all those who come in contact with him.

Acknowledgement page

I would like to acknowledge Kelly Goodwin who initially hired me and Jack Fell who initially sponsored me with my thesis effort. Without these two I would not be on the cusp of finishing. I would also like to thank Chris Sinigalliano and Mike Schmale for coming on board and providing me with guidance and assistance.

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1.1 Introduction

The monitoring of near-shore waters is important to gauge health risks to the

public. Waters that are contaminated with fecal material have the capacity to

infect those individuals who come in contact with it. These infections can range

from mild skin irritations and upset stomach to potentially serious infections (Lee

et al. 2002, WHO 2003). Also chronically polluted beaches can have negative

impacts on the businesses that surround the beach in question (Rabinovici et al.

2004). Current beach monitoring techniques for microbial water quality are

antiquated. They rely on classical microbiological techniques that need overnight

incubations before results are known (US EPA method 1600). Over the past 10

years there has been research to shorten the time from sample collection to result.

Many of these techniques require the extraction of DNA from the sample. During

these extractions there is loss of sample. These losses occur due to incomplete

lysis of cells, loss from human error, or to partial elution from binding matrix.

The following is a brief overview of the history of recreational water sampling,

some common bacterial pathogens spread by the fecal-oral route, and some of the

new rapid methods.

Recreational water testing has been a concern since the turn of the 20th

century. It was observed that swimmer health was linked to the amount of

sewage discharged into the body of water (Simons et al. 1922). After some initial

studies (Streeter 1951, and Scott 1951) the first guidelines were published that stated that waters with less than 1000 coliforms per 100mls were safe to swim and bathe in.

Coliform bacteria are rod-shaped gram-negative non-spore forming bacteria that can ferment lactose; some examples of coliform bacteria are *E. coli* and enterococci. Coliforms are abundant in the feces of warm-blooded animals, but can also be found in the aquatic environment, in soil and on vegetation. While coliforms are not normally causes of serious illness, they are easy to culture and their presence is used to indicate that other pathogenic organisms of fecal origin may be present.

In the 1950's and 1960's the public health service studied relationships between water quality and swimmers health (Stevenson 1953). These studies were conducted in Lake Michigan and the Ohio River. From these studies it was determined that a new category of indicator bacteria, fecal coliforms should be used. These fecal coliforms are a sub-set of coliforms; they are rod-shaped, gram-negative, non-sporulating bacteria. Fecal coliforms are capable of growth in the presence of bile salts. The National Technical Advisory Committee (NTAC, 1968) to the U.S. Federal Water Pollution Control Administration developed in 1968 a national fecal coliform guideline of 200 fecal coliforms per 100 ml for fresh and marine waters, which were based on the two fresh water studies of Stevenson (1953).

In 1972, the Environmental Protection Agency (EPA) completed The Clean Water Act (CWA). The report provided guidelines for acceptable levels of biological and chemical contaminates, and suggested the use of total and fecal

coliforms counts as an indicator of fecal pollution (Quality Criteria for Water. EPA-440976023).

The clean water act empowered the United States Environmental Protection Agency with implementing pollution control programs by setting standards for pollutants in surface waters (USEPA, 2002b). The clean water act also made it illegal to discharge pollutants from a point source into navigable waterways without a permit (USEPA, 2002b). The clean water act was successful in reducing water pollution from point sources (USEPA, 1996). Unfortunately, efforts to control non-point sources have not responded to these regulations and still pose a problem in water pollution (USEPA, 1996).

In 1986, The Clean Water Act was modified, due to the studies from Dufour and Cabelli (Dufour 1984, and Cabelli 1983). The resulting report titled Ambient Water Quality Criteria for Bacteria (USEPA, 1986) recommended the replacement of coliforms and fecal coliforms with *Escherichia coli* (*E. coli*) and enterococci, for fresh water quality indicators, and enterococci alone for marine waters. In future studies it was shown that enterococci were better suited for the marine environment than *E. coli* because enterococci rarely replicate in polluted waters and survive longer than *E. coli* (APHA, 2005).

During the latest round of recreational water testing a theory of the ideal fecal indicator was published (Feachem et al. 1983). In the paper it describes what attributes the ideal fecal indicator bacterium should have:
• A normal member of the intestinal flora of healthy people
• Exclusively intestinal in habitat, and hence exclusively fecal in origin when found in the environment

• Absent from nonhuman animals

• Present whenever fecal pathogens are present

• Present in higher numbers than fecal pathogens

• Unable to grow outside the intestine

• Resistant to natural antagonistic factors

• Easy to detect with microbial growth media and isolate

• Nonpathogenic

The decided upon indicator organisms enterococci and *E. coli* have many of these attributes.

In 2000 congress passed the BEACHES act (BEACH Act of 2000) is an amendment to the Federal Water Pollution Control Act (Clean Water Act, 33 U.S.C. 1313). This act gave assistance to states to help them implement switching from coliforms and fecal coliforms to enterococci and *E. coli* (fresh water only). The BEACHES act also required the EPA to address the time lag between sample collection and public notification. The method currently used to enumerate these bacteria is to grow them on selective media with filtered water or water samples have been incubated for 18-24 hours. Today most water managers use either enterococci and/or *E. coli* as their fecal indicating organisms.

There are many types of pathogens that are associated with fecal-oral route transmission. Pathogens can be classified as bacteria, protozoan, virus, and certain types of fungi or parasitic worms (USEPA, 2006). Some of the common bacterial pathogens that can spread from fecal-oral route are: pathogenic *E. coli*, *Shigella* spp., *Vibrio* spp., *Salmonella* spp., and *Camplobacter* spp. (Maier et al. 2000).

Pathogenic *E. coli* is a gram-negative bacillus that can produce symptoms from mild diarrhea to possible kidney failure. In May 2000 an outbreak of pathogenic *E. coli* occurred in Ontario, Canada killing 7 people and sickening more than 2,300. The cause of the outbreak was due to cattle manure that was land applied and entered the groundwater near the town's well (O'Connor, 2002). *Shigella* spp. is a gram-negative bacillus that can also produce diarrhea. This bacterial infection is more prevalent in children. *Vibrio* spp. are gram-negative bacilli which certain species can also cause diarrhea. *Salmonella* ssp. are also gram-negative bacilli that can cause vomiting, diarrhea, and in rare cases typhoid fever. *Campylobacter jejuni* is the leading cause of bacterial diarrheal illness in the United States, more than *Shigella* spp. and *Salmonella* spp. combined (CDC, 2005).

Along with these bacterial pathogens there are also many more viral and protozoan pathogens that can also spread through the fecal-oral route. The protozoan *Cryptosporidium* is transmitted by ingestion of oocysts excreted in human or animal feces (Maier et al. 2000). In 1993 *Cryptosporidium parvum* caused the largest documented waterborne disease outbreak in United States history. Over 400,000 people suffered from gastroenteritis, and more than 100 people died. The outbreak was blamed on a failed filtration process at the Milwaukee Wisconsin treatment plant (Corso et al. 2003). Infectious viruses that have been found in water systems include Enterovirus, Rotavius, Hepatitis A, and Reovirus (Bosch, 1998). These excreted viruses can affect the respiratory, ocular, gastrointestinal, and myocardial processes of the human body (Fleisher et al. 1998, Griffin et al. 2003, and Pina et al. 1998). But with our current system of

indicator organisms (no virus or protist) these pathogens are linked rightly or wrongly to the bacteria.

Due to the need to move swiftly to minimize risk to human health from contaminated water, there is an increased interest in utilizing molecular techniques to decrease testing and response time. Some of the molecular techniques being investigated are Luminex, qPCR, TMA, and immunoassays.

Luminex was originally developed to have multiple assays run for small volumes of sample (blood from test subjects). Luminex fluorescently color-codes tiny beads, called microspheres, into hundreds of distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, such as DNA, allowing the capture and detection of specific analytes from a sample. Inside the Luminex analyzer, a light source excites the internal fluorescent dyes that identify each microsphere particle, and also any reporter dye captured during the assay. Multiple readings are made on each bead set, which further validates the results. Using this process, xMAP Technology (Luminex corp.) allows multiplexing of up to 500 unique bioassays within a single sample, both rapidly and precisely.

TMA stands for transcription mediated amplification. TMA is similar to qPCR in that it amplifies a genetic target in the bacteria and uses a fluorescent probe for detection (Piersimoni et al. 2002), but differs in that it is a single-primer isothermal method that targets RNA rather than DNA. Bacteria are filtered and their RNA is extracted. This releases the target 23s rRNA molecules, which are hybridized with species-specific probes that are then captured by magnetic beads. After capture, a magnet is used to separate the bead-bound RNA from the sample and a small portion of the captured target material is subjected to TMA, which is

an isothermal reaction that incorporates reverse transcriptase and polymerase enzymes to amplify the 23SrRNA gene without any need for thermal cycles.

qPCR stands for quantitative polymerase chain reaction. Probe based qPCR was first developed in 1996 by applied bio-systems. This method uses primers and a probe with a 5' fluorochrome and a 3' quencher that are specific to a piece of target DNA. As both primers and probe anneal to the target DNA the polymerase chain reaction causes the primers to extend and cleave the fluorescent probe. This cleaving removes the fluorochrome from the quencher and creates a fluorescent signal that is read by the machine after each cycle. This method is a great advancement over regular PCR because by comparing the fluorescent signal to that of known concentrations of standard one can quantify the starting amount of target in the sample, and it eliminates the need to run the sample after cycling on a gel. Many assays have been developed to analyze environmental water samples for the fecal indicating bacteria enterococci (Haugland et al. 2005) and other alternative markers (Jiang et al. 2007, Dick and Field, 2004, Kildare et al. 2007, and Shanks et al. 2009). Positive correlations between enterococci sp. classical techniques and qPCR have been observed (Haugland et al. 2005). It has also been shown that a positive correlation exists between enterococci sp. as measured by qPCR and levels of gastro-intestinal illness rates (Wade et al. 2006). To use these assays on a water sample it needs to be filtered and the nucleic acid extracted. Then an aliquot from the elution is tested in the qPCR assay.

Immunoassays use antibody specificity to bind a target. A target specific immunological dipstick (which is manufactured by many life science companies) is combined with liquid growth media and incubated for 4 – 6hours at constant

temperature on an orbital shaker. Once incubation is complete, the dipstick, which contains antibodies specific to *E. coli* is immersed in the growth media. This method produces a binary answer. If the original concentration of *E. coli* was greater than 400 per 100mL of water, then a black bar becomes visible on the dipstick, indicating a positive result.

With each of the above-mentioned technologies and others not mentioned, there is a push for more automation of sample processing. Currently there are a wide variety of robot arms and pipettes that are capable of sample processing. Qiagen has introduced several platforms of automation for use in experimental laboratories and forensic labs. With automation, controls are needed for optimization, and for process efficiencies. However, many of these automated extraction and purification systems cannot deal effectively with the variability and complexity of environmental samples.

For most of these methods, nucleic acids are extracted from a water sample for testing. However, each of these molecular techniques shares some common problems: one must account for the loss of nucleic acid and/or incomplete lysis of the cells between the original sample and elutant, and to insure that the sample is not inhibited by environmental contaminants present in the water sample which may co-purify with the nucleic acids. After accounting for these issues, an accurate test can be obtained and appropriate action taken.

Inhibition has been observed in environmental water samples. Samples routinely contain humics, tannins, and other substances that can inhibit molecular reactions (Wilson 1997). These substances can also impact the sensitivity of qPCR reactions (Audemard et al. 2004, and Loge et al. 2002). Inhibition has been

shown to be more prevalent in non-purified samples that are crudely processed such as bead-beat lysate which is currently favored by the EPA due to its speed (Shanks et al. 2012). Other methods such as DNA purification/extraction have less of an issue with inhibition, since most contaminating substances are removed from the sample during processing.

Extraction controls are incorporated to quantify the loss of DNA from either the incomplete lysis of cells or the loss of DNA during the extraction and purification process. Extraction controls differ from inhibition controls because extraction controls vary from sample to sample due to that loss of DNA, but inhibition controls should not vary from the amount spiked into the reaction. Any large deviation of an inhibition control indicates that the sample is inhibited and further dilution or clean up is needed. Salmon sperm DNA is a readily available commercial product that many studies have used to determine extraction efficiency (Haugland et al. 2005 and Peed et al. 2011). Haugland et al. 2005 used 64 nano-grams of salmon sperm to act as an internal positive control. They assumed that the differences between samples during the extraction process were negligible for crude bead beat lysates, and that the salmon sperm was needed only to show gross inhibition of the sample. This was due to the fact that their samples were crudely processed and had few steps between collection and placement into the qPCR reaction, but an extraction control would be more convincing since it takes into account the lysis step which the salmon sperm DNA does not. An extraction control would also be necessary to compare their results to other studies that used different processing techniques. However the variability from cell lysis was probably minimal as compared to variability from extraction and

purification recovery. Thus these salmon controls that are spiked into the samples before bead beating are likely to predominately reflect inhibition for crude lysates.

In two separate papers, Nobel (Nobel et al. 2006 and Nobel et al. 2010) has described the use of *Lactococcus lactis* as a specimen processing control (SPC). In the 2006 paper they used 100,000 cells as their spike to their sample. But *L. lactis* cells were only used to determine if there was any inhibition and to determine the amplification efficiency of their samples. These papers show that *L. lactis* is a viable candidate not only an inhibition control but an extraction control as well. In the 2006 paper's discussion there is a complaint of underestimation of enterococci qPCR results when compared to the microbiological results. This result may be explained by the use of an extraction control that shows poor extraction efficiency.

In the research paper by Stoeckel (Stoeckel et al. 2009), the aim of that study was the detection of extraction efficiency for microbial source tracking markers. But the two controls that they investigated were a plant pathogen, and a plasmid target inside *E .coli*. These controls were chosen due to the fact that they were structurally similar to the source-tracking targets of bacteroidales (both cell types are gram-negative). They found positive correlation between their spikes and their targets but the overall extraction efficiency was low, averaging 2-5% (Stoeckel et al. 2009). They had difficulty getting accurate estimates of their spikes, relying on plate counts and a rough estimate of plasmid copy number per cell for the plasmid control. There was also the problem of preferential treatment of genomic DNA to plasmid DNA. In 2010 the plant pathogen genomic DNA spike was again used in a study by Dick (Dick et al, 2010). Recoveries were

better than in the previous study and had a range of 2.5-73% recovery. These studies show that extraction methods can show a wide range of recoveries and that it is important to include them with the target results.

In the research paper by Lavender (Lavender et al. 2009) they used *Lactococcus lactis* as a sample process control (SPC) for *E. coli*, and salmon sperm gDNA for the sample process control for enterococci. They used purified target along with the same amount of SPC and processed them in the same manner. By comparing the two results as a relative recovery they were able to fix all SPC's from the samples to the calibrator's value. There are multiple issues with this approach. First *L. lactis* is a poor choice as an extraction control or a sample processing control for *E. coli*. *E. coli* is a gram-negative rod, while *L. lactis* is a gram-positive cocci (sphere).   Also the use of a purified target cell spike along with an SPC is not processed in the same tube as your unknown samples. A comparison and enumeration based on this SPC control could be problematic. Siefring in 2008 used *L. lactis* as a control for the evaluation of 3 different instruments and reagents (Siefring et al. 2008.). They used it as an extraction control and an inhibition control. After overcoming some cross-reactivity problems between their *L. lactis* assay and *E. faecalis* they were able to use the *L. lactis* spike for improved accuracy and precision of their results. When they used *L. lactis* as a control it eliminated significant differences in all 3 systems for *E. faecalis* and eliminated differences for 2 out of the 3 for *B. fragilis*. They spiked with 100,000 *L. lactis* cells and used the crude bead-beat lysate extraction method. This method that they used for the evaluation of different instruments and reagents could also be applied to regular water samples. When

they did apply the extraction control *L. lactis* to the results of *E. faecalis* the differences in the machines and reagents used were diminished.

In the research paper published by Viau and Peccia in 2009, (Viau and Peccia, 2009) they used *L. lactis* as an extraction control for enumerating enterococci from bio-solids. They spiked their samples with 1,000,000 of *L. lactis* and extracted the DNA using a commercially available kit (MoBio PowerSoil DNA kit, Mobio Laboratories, Carlsbad, CA). In their results they stated that they were able to achieve an average of 55% recovery from their samples. But they did not demonstrate how they were able to quantify their cell spikes.

1.2 Objectives

The possible inaccuracy and inefficiency of current testing methods necessitates finding a more precise and practical method for laboratories to determine recreational water quality of public water sources. Current research utilizing the real-time PCR shows promise. Primers and probes have been reported in the literature for testing enterococci, *E. coli* and for other alternative markers. These tests are not standardized and are not currently in routine use, nor have they been evaluated to determine their efficiency in a multiple public health laboratories.

The hypothesis of this study is that a protocol can be developed for the real-time PCR enterococci assay that also includes whole cell extraction controls. This method is anticipated to give a more precise, and accurate estimate of enterococci and help in comparison of results from lab to lab due to the

differences in extraction procedures. This method would also help reduce examiner interpretation with each sample having its own unique extraction efficiency. This may, in turn, aid in the prevention of illness, and help reduce the time that beaches are closed by the use of new faster technology with the re-assurance that whole cell controls give these assays.

This study evaluates the potential use of real-time PCR to determine enterococci extraction efficiency by spiking the extractions with whole cell *Lactococcus lactis*.

The study has four specific aims:

1) To determine the amount of background signal for *Lactococcus lactis* that occurs within normally sampled waters

2) To gauge the extraction efficiency of *E. faecalis* and *L. lactis* with commercially available extraction methods.

3) To determine the sensitivity and statistical relationship between these two cell types as they are simultaneously processed.

4) To deploy these extraction controls to recreational water samples to improve the accuracy and precision of the estimates of enterococci in those samples.

1.3 Materials and Methods

Microbial strains and positive controls

Enterococcus faecalis (ATCC 29212) and *Lactococcus lactis* (ATCC 11955) and *Bacteroidales thetaiotaomicron* (ATCC 29741) were used as stock cultures and as purified enumerated cell stocks supplied in BioBalls™ , or by Cepheid

(Sunnyvale, CA). Cultures of these cells were grown up in LB broth and

extracted using Promega wizard gDNA purification kit. Standard curves were

calculated from these purified gDNA extracts by using a fluorometer (Qubit

dsDNA Quantit kit Invitrogen), and then using the size of each strain's genome

(*L. lactis* 2.37x10^6, *E. faecalis* 3.2 x10^6, and *B. thetaiotaomicron* 6.26 x10^6,). A

standard curve was diluted to genome equivalents for all three stocks. This was

done by converting the genome into weight and then diluting the concentrated

stock to the appropriate dilution ((bp#)(660) = MW, MW/Avogadro = g/DNA).

Quantitative Polymerase Chain Reaction

Quantitative PCR was used to determine the amounts of target in the sample. The

3 assays used were 23s enterococci assay ("Entero1a" Haugland et al. 2005), a

16s *Lactococcus lactis* assay with the probe and reverse primer developed during

these experiments (based on Aymerich et al. 2003), and a 16s "GenBac3" assay

for bacteroidales (Seifring et al. 2008). In the reaction Quanti-TECT ™ Probe

PCR Kit (Qiagen, Cat # 204343) was used as master-mix and final concentrations

for the primers and probes were 900 nano-molar and 300 nano-molar respectively.

Nuclease free water from the kit was used to bring the final volume to 25μl. All

plates were run with a genomic DNA Standard curve and no template controls.

No template controls have all elements of the master mix except for template, this

allows for measuring for master mix contamination. The following qPCR

conditions were used in the experiments: an initial 95°C for 15min, then 45 cycles

of 95°C for 15sec, and 60°C for 60sec. These reactions were performed on

several different qPCR instruments, Bio-rad i-Cycler, MJ-Chromo4, and ABI-Step1+.

Determination of background *Lactococcus lactis* in Florida waters

Water samples were collected from three counties in Florida. These samples were taken from 2007-2010. Between 200~1000mls of water sample were collected and filtered on 0.45um pore-sized Whatman nitrate cellulose filters. These filters were then placed in bead beat tubes (Lysing matrix Z tubes Cat# 116910500) and processed in Fast DNA spin kit according to their protocol (MP Biomedicals Cat # 6540-600). After processing, all samples were eluted into 100ul of buffered water. One µl of each sample was then analyzed using qPCR *L. lactis* 16s assay.

Determination of filters to be used in extraction protocol

$1x10^4$ cells of *E. faecalis* and $3.5 x10^4$ cells of *L. lactis* were loaded onto both Whatman cellulose nitrate filters and Pall Supor-200 polyethersulfone with sterile PBS. These filters were rolled up and placed into bead beat tubes (Lysing matrix Z tubes Cat# 116910500) from the Fast DNA spin kit (MP Biomedicals Cat # 6540-600). 600uls of AE buffer (Qiagen cat # 19077) and .2mg/ml of Salmon sperm DNA (Invitrogen Cat# 15632-011) were added to the tube. Samples were bead beaten in a Fast prep FP120 homogenizer bead mill (Qbiogene/MP Biomedicals) for 2 rounds of 30 seconds each at speed 5.0. This experiment was repeated with 3 treatments of varying amounts of *E. faecalis* and *L. lactis* (High treatment had $5 x10^6$ *E. faecalis* and $1.5 x10^7$ *L. lactis*, Medium treatment had

$1x10^5$ *E. faecalis* and $3x10^5$ *L. lactis*, and Low treatment had $1x10^4$ *E. faecalis* and $3x10^4$ *L. lactis*).

Determination of extraction method

Fifteen extraction tubes were prepared with $5x10^6$ cells of *E. faecalis* and $1.5x10^7$ *L. lactis* cells. Ten filters were 0.45um pore-sized Whatman nitrate cellulose, and 5 filters were .2um pore-sized Pall Supor-200 polyethersulfone. The cells were added to 200mls of 1XPBS and filtered. The Five Pall Supor-200 and five Whatman filters were placed into bead beat tubes (Lysing matrix Z tubes Cat# 116910500) from the Fast DNA spin kit (MP Biomedicals Cat # 6540-600). 600uls of AE buffer (Qiagen cat # 19077) and .2mg/ml of Salmon sperm DNA (Invitrogen Cat# 15632-011) were then added to the tube. The five remaining Whatman filters were placed in the lysis matrix A tubes and processed using the Fast DNA spin kit protocol (MP Biomedicals Cat # 6540-600). This experiment was repeated with 3 different cell spike treatments. Treatments consisted of a High ($5x10^6$ of *E. faecalis* and $1.5 x10^7$ *L. lactis*), medium ($5x10^4$ *E. faecalis* and $1.5 x10^5$ *L. lactis*), and low ($5 x10^3$ *E. faecalis* and $1.5 x10^4$ *L. lactis*) cell spikes with 2 un-spiked replicates and 2 blank filters with 260ml of PBS.

Background gDNA experiment

After determining the amount of *L. lactis* in Florida water samples the next experiment was to determine the minimum amount of nucleic acid required for maximum elution recovery from the binding resin of the Fast DNA spin kit. The experiment consisted of 7 amounts of background DNA. The treatments

consisted of a steady amount of target gDNA from *L. lactis* 100,000 genome

equivalents (.26ng) along with varying amounts of salmon sperm gDNA

(Invitrogen Cat# 15632-011) from .5μg to 32μg (0μg, .5μg, 1μg, 2μg, 4μg, 8μg,

16μg and 32μg). Each treatment used 4 replicates, except the 0 treatment which

had 3 replicates. Samples were added straight to the Fast DNA spin kits bead beat

tubes and processed using the Fast DNA spin kit protocol (MP Biomedicals Cat #

6540-600). After processing, all samples were eluted into 100ul of buffered

water. One μl of each sample was then analyzed using qPCR. The qPCR

reaction was run with a standard curve of *L. lactis* genome equivalents. This was

calculated using the amount of purified gDNA and its genome size as previously

stated. The results were then multiplied by the amount of elution, in this case 100

and then divided by the amount of genome equivalents spiked into the tubes, in

this case 100,000.

Determination of *L. lactis* and *E. faecalis* extraction efficiency

Fifty four samples of varying amounts (From 1×10^7 cells to 1×10^2 cells) of *E.*

faecalis and *L. lactis* were processed using Whatman filters and the Fast DNA

spin kit protocol (MP Biomedicals Cat # 6540-600). Cell concentrations were

first determined by direct counting using a hemeocytometer, then by using a

fluorometer. After processing, all samples were eluted into 100ul of buffered

water. One μl of each sample was then analyzed using qPCR.

Comparison of genomic DNA spikes to Cellular spikes

Three treatments compared the difference between cellular spikes and genomic DNA spikes. Treatment one consisted of 4 replicates of 20,000 cells of *L. lactis* and *E. faecalis* (52pg, 70pg respectively) along with 8μg of salmon sperm DNA. Treatment 2 consisted of 4 replicates of 20,000 cells of *E. faecalis* and 100,000 genome equivalents of *L. lactis* DNA along with 8μg of salmon sperm DNA. Genome equivalents were determined by converting a genome into weight and then diluting the concentrated stock to the appropriate dilution. Treatment 3 consisted of 4 replicates of 1×10^5 genome equivalents of both *E. faecalis* and *L. lactis* along with 8μg of salmon sperm DNA. One tube was run with just the salmon sperm DNA and another tube was run with just reagents. These samples were placed into the Fast DNA spin kit and processed according to the Fast DNA spin kit protocol (MP Biomedicals Cat # 6540-600). All samples were eluted into 100ul of buffered water. One ul of each sample was then analyzed using qPCR.

Decrease in Coefficient of Variance by spiking triplicate water samples

Water samples were collected at Hobie beach, which is a bay beach, located on Virginia Key in Miami Florida. These samples were part of an epidemiological study conducted over several months in 2007-2008 (Fleisher et al. 2010, and Sinigalliano et al. 2010). Composite samples consisted of using part of each individual sample collected on that particular day. These 1L samples were filtered on 0.45um pore-sized Whatman nitrate cellulose filters along with cell spikes. These samples were spiked with 8.3×10^5 cells of *L. lactis*. These samples were then placed into the Fast DNA spin kit and processed according to the Fast

DNA spin kit protocol (MP Biomedicals Cat # 6540-600). All samples were eluted into 100ul of buffered water. One ul of each sample was then analyzed using qPCR.

Alternate indicator extractions with Enterococci and *Lactococcus Lactis*

Bio-ball® (BTF Precise Microbiology, Inc. www.bioball.com) pre-calibrated cell spikes were used to determine the efficiency of extraction between the 3 cell types. Bio-ball samples for *B. thetaiotaomicron* and *E. faecalis* were used along with lyophilized *L. lactis* cells from Cephid (www.cepheid.com). These cells were diluted to 100 cells per sample then filtered on 0.45um pore-sized Whatman nitrate cellulose filters along with 20ml of 1X PBS. 5 replicates were filtered along with 1 PBS blank. These filters were rolled up and placed in the Fast DNA spin kits bead beat tubes. 8µg of salmon sperm DNA was also added to the spin kits bead beat tubes. The tubes were then processed using the Fast DNA spin kit protocol (MP Biomedicals Cat # 6540-600). After processing, all samples were eluted into 100ul of buffered water. One µl of each sample was then analyzed using qPCR.

1.4 Results

Abundance of background *L. lactis* signal in South Florida waters.

This was the first step in determining if *Lactococcus lactis* would be an appropriate control for recreational water samples. 55 samples were tested from 3 Florida counties (Lee, Volusia, and Miami-Dade). In these samples the amount of *L. lactis* signal observed ranged from non-detect to 108 cell equivalents per 100mls (table 1.1).

Sample	Volume (ml)	County	*L. lactis* results per 100mls	% of 1E5 lactococci spike	% of 1E3 lactococci spike
Daytona pier water 1	260	Volusia	N/A	0%	0%
Daytona pier water 2	260	Volusia	N/A	0%	0%
Daytona pier water 3	260	Volusia	N/A	0%	0%
Face FCO 116	315	Broward	0.2	0%	0%
FCO 118	360	Broward	0.4	0%	0%
FCO 120	350	Broward	0	0%	0%
FCO 122	455	Broward	0	0%	0%
FCO 124	70	Broward	23.3	0%	2%
FCO 126	145	Broward	0.2	0%	0%
FCO 128	165	Broward	21.1	0%	2%
FCO 130	320	Broward	0	0%	0%
FCO 132	440	Broward	0	0%	0%
BCH 577	1000	Miami-Dade	16.9	0%	2%
BCH 578	1000	Miami-Dade	6	0%	1%
BCH 579	1000	Miami-Dade	3.5	0%	0%
BCH 580	1000	Miami-Dade	0.4	0%	0%
BCH 581	1000	Miami-Dade	0.6	0%	0%
BCH 582	1000	Miami-Dade	0	0%	0%
BCH 583	1000	Miami-Dade	0.6	0%	0%

BCH 584	1000	Miami-Dade	0.1	0%	0%
BCH 585	1000	Miami-Dade	0	0%	0%
BCH 586	1000	Miami-Dade	0.1	0%	0%
BCH 587	1000	Miami-Dade	12.3	0%	1%
BCH 588	1000	Miami-Dade	39.5	0%	4%
BCH 589	1000	Miami-Dade	4.4	0%	0%
BCH 590	1000	Miami-Dade	1.6	0%	0%
BCH 591	1000	Miami-Dade	6.8	0%	1%
Hobie 4	1000	Miami-Dade	0.2	0%	0%
Hobie Blank	1000	Miami-Dade	1.2	0%	0%
DOH 9/21/2010 1	1000	Miami-Dade	0.2	0%	0%
DOH 9/21/2010 2	1000	Miami-Dade	7.4	0%	1%
DOH 9/21/2010 3	1000	Miami-Dade	5.8	0%	1%
DOH 9/21/2010 4	1000	Miami-Dade	0	0%	0%
DOH 9/21/2010 5	1000	Miami-Dade	1.2	0%	0%
DOH 9/21/2010 6	1000	Miami-Dade	24.3	0%	2%
DOH 9/21/2010 7	1000	Miami-Dade	0	0%	0%
DOH 9/21/2010 8	1000	Miami-Dade	108.2	0.1%	11%
DOH 9/21/2010 9	1000	Miami-Dade	1.8	0%	0%
DOH 9/21/2010 10	1000	Miami-Dade	0.4	0%	0%
DOH 9/21/2010 11	1000	Miami-Dade	0.6	0%	0%
DOH 9/21/2010 12	1000	Miami-Dade	0.2	0%	0%
DOH 9/21/2010 13	1000	Miami-Dade	0	0%	0%
DOH 9/21/2010 14	1000	Miami-Dade	0.1	0%	0%
DOH 9/21/2010 15	1000	Miami-Dade	0.6	0%	0%
DOH 9/28/2010 1	1000	Miami-Dade	0.1	0%	0%
DOH 9/28/2010 2	1000	Miami-Dade	1.2	0%	0%
DOH 9/28/2010 3	1000	Miami-Dade	0.3	0%	0%
DOH 9/28/2010 4	1000	Miami-Dade	0	0%	0%
DOH 9/28/2010 5	1000	Miami-Dade	0.5	0%	0%
DOH 9/28/2010 6	1000	Miami-Dade	0.3	0%	0%
DOH 9/28/2010 7	1000	Miami-Dade	0	0%	0%
DOH 9/28/2010 8	1000	Miami-Dade	0.4	0%	0%
DOH 9/28/2010 10	1000	Miami-Dade	0.4	0%	0%

Table 1.1: Determination of *Lactococcus lactis* background signal in recreational waters of Florida. The yellow cells indicate the highest background signals observed.

Determination of filters to be used in extraction protocol

Whatman cellulose nitrate filters and Pall Supor-200 polyethersulfone were compared to determine which filter extracted with the better efficiency. The same amount of *E. faecalis* and *L. lactis* cells were loaded onto the filter with sterile

PBS. The results were that Pall Supor-200 had higher extraction efficiency in bead beating only extractions, averaging 14% recovery, while the Whatman filters averaged 11% (Figure 1.1). After analyzing these results by ANOVA there was no statistical significance between these two types of filters. Along with the testing of the two types of filters the values of the two cell types were compared. *E. faecalis* results from the two filter types were compared to the *L. lactis* results. A 95% correlation was observed between the two cell types (figure 1.2). This experiment was repeated with 3 treatments of varying amounts of *E. faecalis* and *L. lactis* added to the filters and the results were similar to the first experiment. There wasn't a statistical difference between the values of the two types of filters, and again the *L. lactis* and *E. faecalis* values were highly correlated at 99% (figure 1.3).
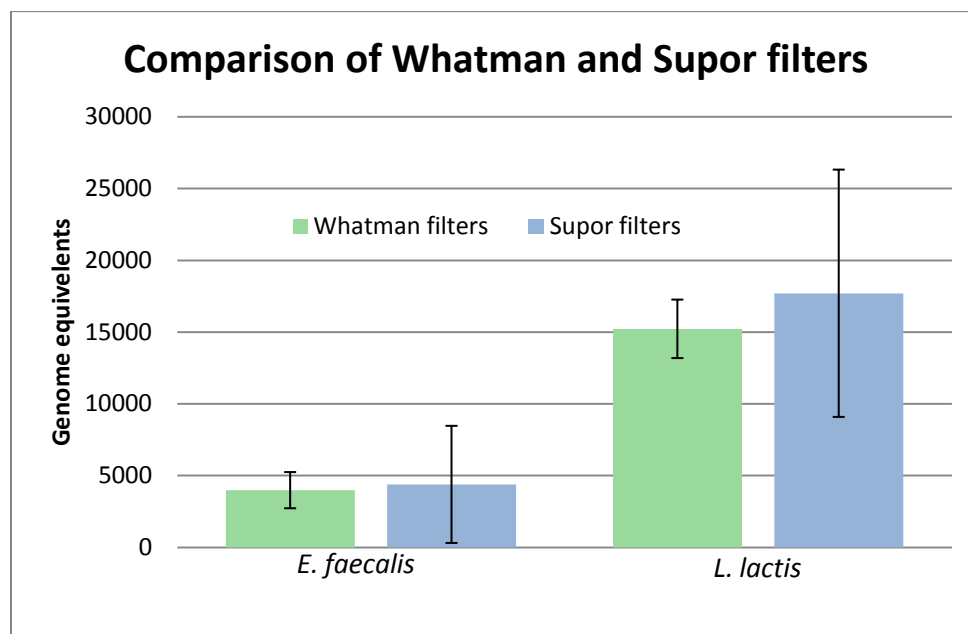
Figure 1.1 Comparison of Whatman and Supor filters with *E. faecalis* and *L. lactis* cell spikes. Samples were processed using crude bead-beat lysate method. ANOVA single factor was conducted on the *E. faecalis* (F=.61) and *L. lactis* (F=1.02) samples (F-Critical 5.31 α=.05)
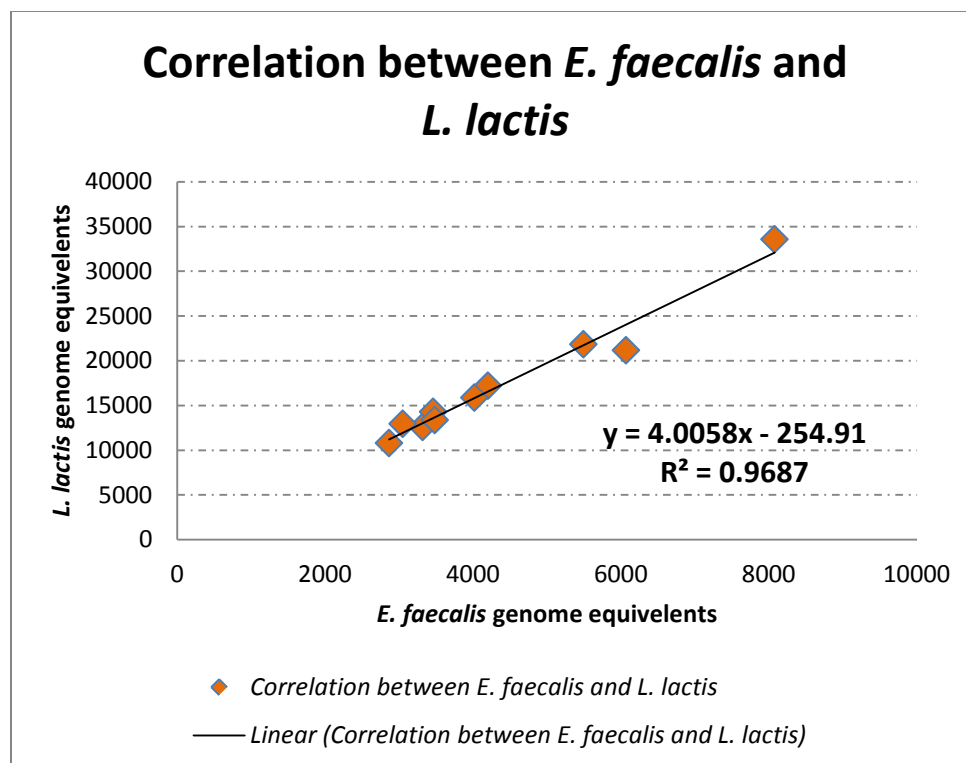
Figure 1.2: Correlation between *E. faecalis* and *L. lactis* using whatman and supor filter types.
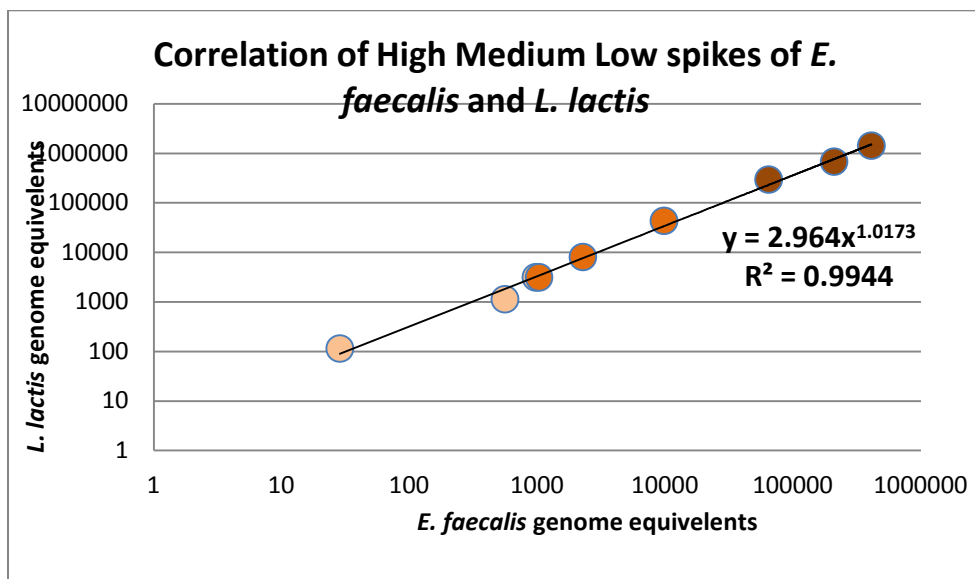
Figure 1.3: Correlation of High, Medium, and Low spikes of *E. faecalis* and *L. lactis* using Whatman and Supor filters with the bead beat crude lysate method. The light orange circles represent the low spike of *E. faecalis* and *L. lactis*. The

orange circles represent the medium spike of *E. faecalis* and *L. lactis*. The dark orange circles represent the high spike of *E. faecalis* and *L. lactis*.

Determination of extraction method

Two methods (bead beat crude lysate and a commercially available DNA extraction kit) were compared to see which provided the highest recovery of the cell spikes. Each sample tube was prepared in the same manner and then selected for either the bead beat method or the DNA extraction kit method. The results (Table 1.2) show that there were small differences between the final outcomes of the methods. The plant DNA extraction kit returned higher values for enterococci than the bead beat lysate method, but after analyzing these results with one-way ANOVA there was no statistical significance between the enterococci results. The *L. lactis* results did show statistical significance between the two methods with higher qPCR results for the plant DNA extraction kit. These methods were also investigated for their overall sensitivity. This was determined my using the raw C(t) (cycle threshold) of the samples. C(t)is a measure of signal strength between the two methods showing that the method that has the lower C(t) value is more sensitive to the signal. In Figure 1.4 the DNA extraction kit method averaged 19.77 C(t)'s for enterococci and 16.75 C(t)'s for *L. lactis*. The Bead beat extraction method averaged 23.1 C(t)'s for enterococci and 21.09 C(t)'s for *L. lactis* (Figure 1.4). These results were analyzed by one-way ANOVA and found these C(t) differences to be statistically significant.

Extraction method	*E. faecalis* genome equivalents	*E. faecalis* standard deviation	*L. lactis* genome equivalents	*L. lactis* standard deviation
Fast DNA spin kit	4.73E+06	2.26E+06	2.85E+07	1.18E+07
Bead beat crude lysate Whatman filters	2.39E+06	7.57E+05	9.13E+06	2.45E+06
Bead beat crude lysate Supor filters	2.89E+06	1.22E+06	1.17E+07	5.17E+06

Table 1.2. Results of the comparison between a crude lysate extraction method (Bead beat methods) and a DNA extraction kit (Fast DNA spin kit). Tubes were spiked with $1x10^6$ -$1x10^7$ cells based on hemeocytometer results.

Average C(t) values for *E. faecalis* and *L. lactis* using 2 different methods of extraction

High spike corresponds to 5×10^6 *E. faecalis* cells and 1.5×10^7 *L. lacits* cells
Medium spike corresponds to 5×10^4 *E. faecalis* cells and 1.5×10^5 *L. lactis* cells
Low spike corresponds to 5×10^3 *E. faecalis* cells and 1.5×10^4 *L. lactis* cells

Fast DNA kit High spike
Fast DNA kit Med spike
Fast DNA kit Low spike

BBLP Whatman High spike
(Bead beat lysate protocol)
BBLP Whatman Med spike
BBLP Whatman Low spike

BBLP Supor High spike
(Bead beat lysate protocol)
BBLP Supor Med spike
BBLP Supor Low spike

Figure 1.4. Average C(t) values for *E. faecalis* and *L. lactis* using 2 different methods of extraction. Average *L. lactis* C(t) values are on the y-axis, while average *E. faecalis* C(t) values are on the x-axis. Error bars represent standard deviations of the samples. Orange symbols correspond to the use of the Fast DNA extraction kit. Green and Blue symbols indicate the use of the bead beat lysate protocol method. High spike corresponds to 5×10^6 *E. faecalis* cells and 1.5×10^7 *L. lactis* cells, medium spike corresponds to 5×10^4 *E. faecalis* cells and 1.5×10^5 *L. lactis* cells, and low spike corresponds to 5×10^3 *E. faecalis* cells and 1.5×10^4 *L. lactis* cells

Determination of the amount of background DNA needed for recovery of cell spikes.

It was observed that experiments performed using low amounts of starting material in a purified matrix such as PBS that the cell spikes were undetectable (data not shown). A theory was proposed that the kit needed a certain amount of background nucleic acid to elute our target cell spikes. The results of Figure 1.5 show that adding varying amounts of background DNA changes the recovery of our *L. lactis* gDNA spike. 8 treatments of 0, .5, 1, 2, 4, 8, 16, and 32 micrograms of salmon sperm DNA was added to the extraction kit along with 1   genome equivalents (.259 nano-grams of gDNA) of *Lactococcus lactis*. The results showed that by adding 0 micrograms returned 0% recovery of our *Lactococcus lactis* spike. But the percent recoveries increased as the amount of background DNA was added. The average percent recoveries (standard deviation) for the background added samples were as follows .5ug 7% (5%), 1ug 11% (3%), 2ug 19% (9%), 4ug 30% (4%), 8ug 27% (3%), 16ug 24% (3%), 32ug 20% (5%) (Figure 1.5). The addition of 4 micrograms of salmon sperm DNA had the highest improvement in our 1E5 cell spike recovery. Even loading massive amounts of salmon sperm DNA (32ug) still allowed for the detection of our signal. This suggests that a minimum of 4ug of total DNA needs to be loaded to the DNA binding resin column of the fast-prep DNA purification kit for most effective recovery of the specific target DNA, especially at low levels of target DNA. Thus, unless there is sufficient total DNA in the environmental sample being processed, recovery of target DNA is likely to be poor from low concentration of total sample DNA.
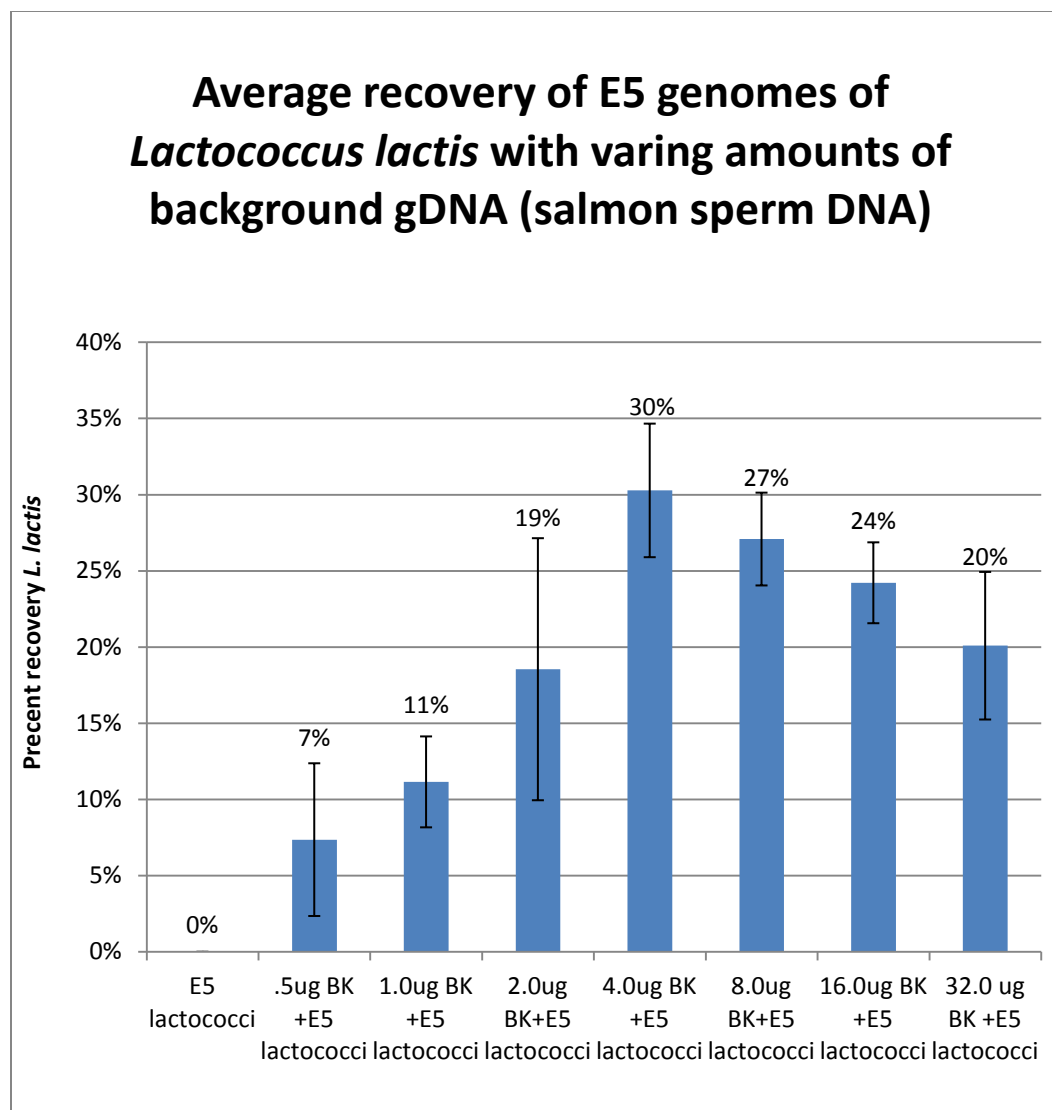
Figure 1.5: Average recovery of *L. lactis* gDNA with varying amounts of background gDNA. Error bars are the samples standard deviation.

Determination of *Lactococcus lactis* and enterococci extraction efficiency

Water filters spiked with varying amounts of *Enterococci faecalis* and

Lactococcus lactis were processed and then compared to determine their

correlation. A total of 54 samples were processed. Using the Pearson Product

Moment Correlation (PPMC) these samples achieved an r-value of .981 with a p

value of 8.8×10^{-42}. These samples are also represented with a regression graph

(figure 1.6).
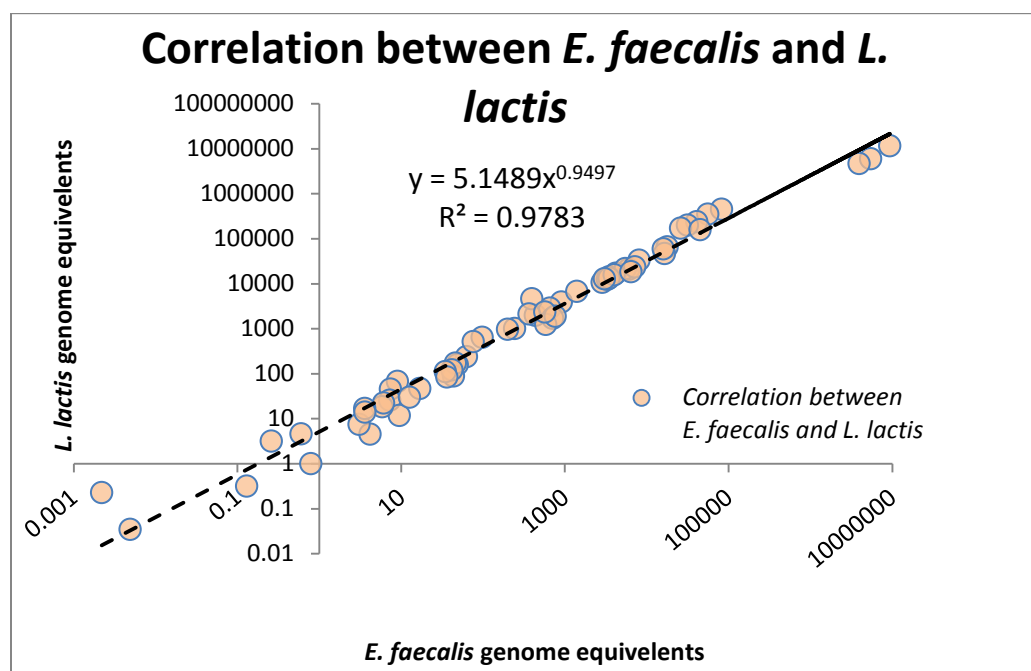
Figure 1.6: Correlation between *E. faecalis* and *L. lactis*. Orange circles represents 54 samples of varying amounts of target.

Comparison of genomic DNA spikes to whole cell spikes.

This experiment was run to determine if whole cell spikes and DNA spikes could

be used interchangeably. Four samples containing equal amounts of cells for *L.*

lactis and *E. faecalis* were processed and run on their respective qPCR assays.

These samples achieved a .87 r-value with a p value .06 (figure 1.7). Another 4

samples were run with equal amounts of genomic DNA from *Lactococcus lactis*

and *E. faecalis*. These samples had an r-value of .92 with a p value of .04. The

third set of 4 sample tubes had an equal amount of *E. faecalis* cells and

Lactococcus lactis genomic DNA. These samples had an r-value of .46 with a p

value of .31.

Comparison of genomic DNA spikes to whole cell spikes

E. faecalis cells and *L. lactis* gDNA
y = 0.3938x + 376.29
R² = 0.466

E. faecalis gDNA and *L. lactis* gDNA
y = 0.5486x + 5.3971
R² = 0.9209

E. faecalis cells and *L. lactis* cells
y = 0.7843x + 3.383
R² = 0.8768

L. lactis genome equivelents

E. faecalis genome equivelents

- *E.faecalis and L.lactis cell spike comparison*
- *E.faecalis cells and L.lactis gDNA spike comparison*
- *E.faecalis gDNA and L.lactis gDNA spike comparison*

Figure 1.7: Comparison of genomic DNA spikes to whole cell spikes. Red symbols represent *E. faecalis* cells extracted with *L. lactis* DNA. Green symbols represent *E. faecalis* DNA extracted with *L. lactis* DNA. Blue symbols represent *E. faecalis* cells and *L. lactis* cells extracted together.

Decrease in coefficient of variation (CV) by spiking triplicate water samples

Triplicate water samples were processed with a whole cell *Lactococcus lactis*

spike. These water samples were taken 7 times over the course of a few months.

The method used to evaluate precision was by looking at the samples' coefficient of variation. Coefficient of variation is a measure of the standard deviation divided by the mean. As precision increases the CV should decrease. CV is expressed as a percentage. For the enterococci samples, (table 1.3) the coefficient of variation decreased when the whole cell spike *L. lactis* extraction efficiency was incorporated in the results. These extraction efficiencies were incorporated by taking the calculated enterococci results and dividing them by the percent efficiency of the *L. lactis* in the tube. The improvement in CV ranged from 1% to 42%, with an average of 19% improvement. These *Lactococcus lactis* extraction efficiencies were also used as extraction controls with a different cell target *Catellicoccus marimammalium*, a gram-positive, catalase-negative bacterium as a marker for gulls. The difference in CV between the samples before applying the *L. lactis* % efficiencies to the samples afterwards ranged from –9% to 73% with an average of 24% improvement in CV, for target recovery assessments, when corrected by recovery of the *L. lactis* controls (Table 1.3).

Sample date	Coefficient of Variation of enterococci	Coefficient of Variation of enterococci adjusted with *L. lactis* recovery	% CV difference
12/15/2007	31%	25%	6%
1/12/2008	18%	8%	11%
2/16/2008	134%	97%	37%
3/8/2008	70%	28%	42%
3/22/2008	14%	13%	1%
3/29/2008	85%	84%	1%
4/12/2008	79%	45%	33%
Average	62%	43%	19%
Sample date	Coefficient of Variation of Gull-2 marker	Coefficient of Variation of Gull-2 adjusted with *L. lactis* recovery	% CV difference
12/15/2007	30%	34%	-4%
1/12/2008	17%	11%	7%
2/16/2008	44%	18%	26%
3/8/2008	81%	44%	37%
3/22/2008	20%	29%	-9%
3/29/2008	109%	36%	73%
4/12/2008	82%	43%	39%
Average	55%	31%	24%

Table 1.3: Coefficient of variation before and after the incorporation of *L. lactis* extraction recovery and the difference between the two calculations. The table highlighted in orange indicates the results for enterococci. The section of the table highlighted in purple indicates the results for the gull-2 marker (*C. marimammalium)*.Extraction efficiency was incorporated into the calculated results of the two markers by taking the results of the qPCR assay and dividing it by the percent recovery of the *L. lactis* that was spiked into the tube.

Alternate indicator extractions with enterococci and *L. lactis*

Along with enterococci, another category of fecal indicating bacteria

bacteroidales has been suggested to augment current water quality standards.

Bacteroidales could provide even more information via source tracking of the

fecal inputs of water samples. In this experiment 5 replicate samples of known

amounts of *E. faecalis*, *L. lactis* and *B. thetaiotaomicron* were processed. These

samples were processed and run for qPCR assays on each type of the bacteria.

The extraction efficiency of the *L. lactis* spike was applied to correct the recovery

estimates of both enterococci and bacteroidales spikes. The CV improvement for

enterococci was from 25% to 3%. The CV improvement for bacteroidales was

from 20% to 12%. The total number of cells in the samples was compared to the

computed values, which take into account the extraction efficiency of *L. lactis*.

For enterococci the average was 107 cells in each tube (Table 1.4). The cell spike

was listed as 100 cells and this overage is in line with the manufactures specs.

However for bacteroidales the average was 155 cells in each tube. This count is

higher than what could be accounted for in manufacturing error.

1.5 Discussion

From the first experiment it appears that there's not an over abundance of *L. lactis*

in the recreational waters of south Florida. One could spike the water samples

with $1x10^5$ of *L. lactis* and be confident that the ambient amount in the water

would have little effect in determining their extraction efficiency.

Sample Cell spikes	E. faecalis cell equivalents	B. theta-iotaomicron Cell equivalents	L. lactis % recovery	E. faecalis cell equivalents adjusted with L. lactis % recovery	B. theta-iotaomicron Cell equivalents adjusted with L. lactis % recovery
100-1	49.4	61.2	47%	104.5	129.4
100-2	30.0	51.2	29%	103.6	176.8
100-3	36.5	55.6	33%	109.4	166.8
100-4	25.5	34.3	24%	105.9	142.0
100-5	36.2	52.9	33%	108.4	158.2
Cells-Blank	2.0	0.0	0%		
10 Cell	2.5	3.2			
Average	35.5	51.0	33%	106.3	154.6
Standard deviation	9.0	10.1	0.1	2.5	19.0

Table 1.4. Results from Cell spike experiment. Samples 100-1 through 100-5 were spiked with the same amount (100) of *E. faecalis*, *L. lactis*, and *B. thetaiotaomicron* cells. Cells blank had no spikes placed in its tube, and 10cells was a spike of 10 cells placed into the qPCR reaction for quality control.

Although it would also be wise to periodically run a sample without spike to make sure that levels of ambient *L. lactis* remain low. The addition of a $1x10^5$ spike into the sample could be troubling for some researchers because of applying its extraction efficiency to target cells, which are usually found in much lower concentrations. The results show that one could use lower spikes but these spikes may run into a sample that does contain enough ambient *L. lactis* to skew the efficiency results. It would also be beneficial to perhaps run samples that compared cell spikes of vastly different concentrations to see if the correlation and percent recoveries were similar to the ones reported here.

The second experiment tried to determine if there was a difference in extraction efficiency between two different types of filters. The two filters chosen Whatman and Supor filters are used frequently in current recreational water testing. The results showed that there was little difference between the two types and pore sizes of filters.

Current methods for testing water quality with molecular assays are divided in two camps; one that favors bead beat crude lysate, and one that prefers using a DNA extraction kit, which yields purified nucleic acid. The bead beat method is fast, going from filtered water sample to qPCR machine in a matter of minutes. The extraction method takes much longer from water sample to qPCR. It can take up to an hour or more depending on type of kit that is used, skill of lab technician, and number of samples (Griffith and Weisberg 2006). While bead beat lysate has little problem with scaling up to more samples, the DNA extraction method lags as the number of samples increases. But there are large advantages in using the DNA extraction method. The first advantage is the

sensitivity of the sample. In the third experiment it was shown that the C(t) values for *Enterococcus faecalis* and *Lactococcus lactis* improved by 4 C(t)'s over the bead beat method. 4 C(t)'s on the qPCR machine represent 10-fold increase in sensitivity. Another advantage of the DNA extraction method are that its samples are much less likely to be inhibited than bead beat lysate method. During the DNA extraction process, substances that can cause inhibition are usually washed away, leaving only the purified nucleic acids (Schriewer et al. 2011). The third advantage of using an extraction kit instead of the bead beat method is the ability of long-term storage of the samples. The purified nucleic acids can be frozen and re-analyzed at a later date with little degradation. Unfortunately for the bead beat lysate method all DNA damaging enzymes and other damaging debris are not removed from the sample and will rapidly degrade the sample after it is processed, thus providing only a short time-frame for analysis of samples and no opportunity to re-analyze at a later date.

During subsequent experiments of spiking relatively low amounts of *L. lactis* and *E. faecalis* in sterile PBS or sterile water (results not shown), we were unable to detect a signal from the elutant using qPCR. But if these same cell spikes were processed with ocean water (these samples of ocean water were also processed un-spiked to make sure that there was little to no ambient quantities of *L. lactis* or enterococci in them) the signal was detected with qPCR. This was only a problem when processing the samples using our fast DNA extraction kit with PBS or Sterile water. When using the crude bead beat lysate method, signal strength was similar regardless of liquid matrix. Because of these results a hypothesis was proposed that the DNA extraction kit needed a certain amount of

total nucleic acid added to it for robust nucleic acid elution. The background experiment provided strong evidence that there indeed is a threshold of DNA/RNA needed for efficient recovery of DNA from the binding resin of the kit. It is important to remember that these kits were originally designed to process high levels of cell growth to generate high quality purified DNA, but recovery is not quantitative. When adding low amounts of cells to the kits the DNA/RNA is either too tightly bound to the binding resin, or too much of the DNA/RNA is washed away in the ethanol clean up steps. Now that this factor is known it was possible to move forward and test other aspects of the cellular relationship between *L. lactis* and enterococci. It is also interesting to note that if using this DNA extraction kit it may be possible to improve extraction efficiency by adding background DNA. The problem here is to be able to estimate the amount of DNA that is present in the filtered water sample. But as the experiment showed that there is little danger in overloading the column. Even when 32 micrograms of salmon sperm DNA was added to a sample it still had an extraction efficiency of over 20% (which is only a 10% decrease from the highest observed extraction efficiency). Although most environmental samples already have sufficient total DNA to avoid this problem and give efficient elution of target DNA, when dealing with low concentration samples, incorporating 1-2 micrograms of salmon sperm DNA to water samples may improve extraction efficiency, which would then increase the overall sensitivity of detection.

After determining the need for background DNA it was possible to determine the relationship of extraction efficiency between *L. lactis* and enterococci. By comparing the results of the Pearson Product Moment

Correlation (PPMC) these samples achieved an r-value of .981 with a p value of

8.8×10^{-42}. The R value is close to a perfect correlation of 1 at .981. This shows

that *Lactococcus lactis* and *Enterococci faecalis* are highly correlated. Also the

very small p value gives a high level of confidence in these results.

The next experiment performed was to determine if salmon sperm DNA

correlated as well as *L. lactis* to *E. faecalis*. Using whole cell spikes have several

drawbacks. The first problem was that whole cell spikes are hard to quantify.

Some methods to quantify cells use a hemocytometer or with flow cytometry.

But some of these methods may be beyond the resources of the average health

department labs and even with such instruments there are still areas of variation.

With using a hemocytometer the condition of the cells, such as clumping, can

make results widely variable (Bailey et al. 2007). Clumping can also be a

problem with flow cytometry. Also there is residual exogenous DNA from cells

that have died during the preparation of the cell spikes. Even by washing the cells

numerous times, this exogenous DNA can still persist in the washed control cell

spike, throwing off calculations. We were only able to achieve consistent, and

accurate cell spikes buy purchasing them from commercially quantified

lyophilized bio-balls for a considerable price. These cell spikes were tested for

accuracy by both plate counts and qPCR. Another problem is limited shelf-life of

washed cell suspensions. New batches of whole cell controls have to be made

frequently, with potential for variation from batch to batch. Maintenance and

preparation of these whole cell controls is time and labor intensive and an

inherent source of variability. Conversely DNA controls are relatively easy to

quantify, with either a spectrometer or a fluorometer. It is also cheap to purchase

and doesn't have the storage limitations, cell spikes should be stored at -80°C, where as DNA can be stored at -20°C. These are tools that readily available to health department labs or are low cost options, with much less time and labor as compared to microscopes and flow cytometry. If spiking with DNA could work as well as cell spikes then it would represent a low cost option for extraction efficiency assessment. Unfortunately, DNA spikes did not prove to be as effective as an extraction control. What was found was that cell spikes to cells spikes correlated well together and DNA spikes correlated well with DNA, but that DNA spike with whole cells didn't correlate. This suggests that the cell-lysis step in the extraction method is where much of the variation may be occurring.

After determining that cell spikes were superior to DNA in correlation with *E. faecalis*, these cell spikes were then applied to real world water samples. By taking triplicate water samples each spiked with known amounts of *L. lactis*, then applying it's extraction efficiencies to the enterococci results, one could hope to achieve a decrease in the coefficient of variation (CV). This decrease of CV in the sample would improve the precision of the sample, and if the cell spike was accurately quantified then it could also improve accuracy. This decrease was observed in all of our samples. This improvement in accuracy was the specific aim of the study. By being able to take multiple samples of the same water, add an extraction control that would take into account differences in extraction and give the user the same results for enterococci abundance in the water sample. So one could take multiple samples of water and have different labs process the samples as long as there was an extraction control each lab could then give consistent results. This experiment also applied the *L. lactis* extraction control to

a different target (*Catellicoccus marimammalium),* which has been shown to be a fecal indicator of gulls and other sea birds (Lu et al. 2008). An average decrease in CV was observed for the gull signal, but in one sample the CV was larger after applying the extraction efficiency of *L. lactis*.

The final experiment was conducted to determine if it was possible to use *L. lactis* as an extraction control for both enterococci and bacteroidales. The EPA has mentioned that the bacterial order bacteroidales could be used as a new fecal indicator. Strains of the genus have been an integral part of microbial source tracking. Microbial source tracking has the promise of revolutionizing the monitoring of recreational water. Current fecal indicating bacteria are associated with a wide variety of warm-blooded animals. With certain strains of bacteroidales it may be possible to determine the host-source of the contamination. In the experiment the samples were spiked with known amounts of *L. lactis E. faecalis* and *B. thetaiotaomicron* cells. These cells were lyophilized beads with accurate cell counts. These cell counts were also independently verified by growing a sub-aliquot of the cell dilution on agar plates and counting the colonies. By applying the extraction efficiency of *L. lactis* the starting amount of *E. faecalis* was calculated. It was calculated to be 107 cells per tube. This result was slightly higher than what was calculated from the manufacturer but it was within their standard error. The total amount of bacteroidales that was calculated from the *L. lactis* extraction efficiency results was 155 cells. This result was higher than what was originally spiked and outside the manufacturers' error. The cellular make-up of bacteroidales is different than that of enterococci and *Lactococcus lactis*. Bacteroidales are gram-negative cells; enterococci and *L.*

lactis are gram-positive. Gram-negative cells are known to be easier to lyse than gram positives (Mahalanabis et al. 2009). This difference in cell wall makeup may explain the difference in extraction efficiency between the two cell types and why the *B. thetaiotaomicron* was estimated to be much higher than what was spiked. Other researchers have observed this phenomenon (Siefring et al. 2008).

1.6 Summary

From this study it has been determined that the amount of background *Lactococcus lactis* signal in south Florida waters to be minimal (41/55 samples had less than 10 cells per 100ml). By incorporating *L. lactis* cell spike of $1x10^5$ these background levels should not affect the extraction efficiency of the spike. It has also been shown that both the bead beat lysate method and DNA extraction kit can produce similar results from water samples. But it was also shown that the DNA extraction kit could be as much as 10 times more sensitive. This sensitivity is achieved by eluting in a smaller volume than the crude bead-beat lysate, and more complete lysis of cells due to the kits mechanical and chemical lysis. The statistical relationship between *Enterococci faecalis* and *Lactococcus lactis* is that of a positive correlation approaching 1 (.981). This relationship shows that *L. lactis* may be used as a surrogate for enterococci efficiency. With more accurate cell counts the true relationship between enterococci and *L. lactis* will be better understood. Whole cell spikes of *L. lactis* applied to recreational water samples were shown to improve the precision of the enterococci results. It still remains to be seen if *Lactococcus lactis* whole cell spikes can be used effectively for other cell targets

Chapter 2 Dog Associated Marker Experiments

2.1 Overview

Most methods used for monitoring recreational waters use fecal indicating bacteria (Myers et al. 2007). While these indicator organisms can gauge the health risks associated with their levels, they cannot inform the water manager as to the source of these indicators (Field and Samadpour 2007). If the identity of the source is known, then there is the possibility of remediation, or at least the understanding of the true contributors of this contamination.

For beaches that allow dogs there is a greater risk for infection to the average swimmer than at a beach that does not allow dogs (Katagiri and Oliveira-Sequeira, 2008). Dog feces can be a reservoir of many different types of human pathogens. *Campylobacter enteritis*, which is an infection of the small intestine, can cause diarrhea and fever. *Campylobacter* spp. has been shown to reside in 58% of healthy dogs' feces (Chaban et al. 2010). *Cryptosporidium* spp. and *Giardia* spp. have also been linked to Dog feces. 5%-10% of healthy dogs carry *Cryptosporidium* spp. and *Giardia* spp. (Rimhanen-Fine et al. 2007, and Shukla et al. 2006). These diseases can be debilitating to healthy adults and can be even more dangerous to children and those individuals with compromised immune systems (Tangermann et al. 1991, Jokipii et al. 1985, and Current et al. 1983). The monitoring for one of the possible sources of theses pathogens should be included in the beaches that allow dogs.

Several strategies have been utilized to try to determine the source of the contaminants. Culture based, library dependent methods have been used to show the sources of bacteria. These methods include ribotyping, PFGE, DGGE, and AFLP.

Ribotyping extracts nucleic acid from an environmental sample and then digests it with restriction enzymes. The digests are loaded on an agrose gel and this separates the bands. The DNA on the gel is then transferred to a membrane and then a southern blot is performed on the 16S rRNA. This method can distinguish between sources of fecal contamination by identifying patterns in the genetic material of bacterial isolates and matching them with libraries from known sources (Parveen et al. 1999, Carson et al. 2001, 2003, and Scott et al. 2003). But this method is time consuming and labor intensive, and has high levels of false positives (Griffith et al. 2003).

Pulse field gel electrophoresis or PFGE (Schwartz and Cantor, 1984) uses the digestion method of ribotyping along with alternating currents pulsed in the agarose gel. These pulses allow for separation of large pieces of DNA. Because of this, all of the DNA extract, not just the 16s portion as in ribotyping is visualized. These gels are then compared to known sources of contamination. This method is a long process and it's difficult to process large numbers of samples simultaneously. This method was also compared in the study by Griffith but was shown to have the same problems as ribotyping (Griffith et al. 2003).

Denaturing gradient gel electrophoresis or DGGE (Fischer and Lerman, 1983) uses PCR amplification of DNA extracted from mixed microbial communities with PCR primers specific for 16S rRNA gene fragments of bacteria. These multiple products cannot be separated from each other by agarose gel electrophoresis because they all are roughly the same size. But due to the differences between the sequences GC content, they can differentiate the products based on denaturing characteristics. DNA samples are loaded onto an agrose gel along with a denaturing agent. A current is run through the gel and discrete bands appear. These gels are then compared to known sources of contamination. This method is time consuming and requires that you have adequate sequence variability for detection.

The technique of amplified fragment length polymorphism or just AFLP (Zabeau and Vos, 1993) uses restriction enzymes and PCR to create a community profile to compare to known sources. First the sample DNA is extracted and then digested. After digestion the fragmented DNA is then ligated with adaptors and then PCR is performed using primers that are specific for the adaptor sequence. Due to the difference in each community's sequence, each community should produce different banding patterns when visualized on a gel. These fragments can also be analyzed by sequencer machines for an automated process. This method is somewhat time consuming but can be scaled up and automated. But this method is not quantitative only giving a yes/no if a certain band is present. Also the source material must be well characterized to place confidence that a certain band relates to a certain type of contamination.

Another strategy used to determine the sources of contamination is by monitoring chemicals associated with certain types of waste. One category of chemicals that has been used for source tracking of fecal material is antibiotic resistance. Antibiotics are used mainly by humans and livestock (Hager 2001). But different patterns of individual antibiotics exist between these two and others found in the wild (Harwood et al. 2000). A study conducted in Virginia by Hagedorn (Hagedorn et al. 1999) was able to show that the major source of pollution of a waterway was the result of cattle. By being able to discriminate between human and livestock they were able to take steps to remediate the problem. Cattle were restricted from the water way and as a result there was a decrease of 94% of fecal coliforms. This is an interesting method to source track specific polluters. But for bodies of water in which livestock is not a likely contributor its usefulness decreases. Also the very nature of antibiotic resistance is transient (Meays et al. 2004). Resistance is often passed from one bacterium to another and can be expelled when not needed (Maiden 1998). Also humans and livestock are not always taking antibiotics and there is movement in the country to try to limit the amount that is used both commercially and medically (Gustafson and Bowen 1997).

Another chemical that can be used as a chemical source associated with human waste is caffeine (Buerge et al. 2003, Buerge et al. 2006). There is a high concentration in impacted surface waters and a clear anthropogenic origin to caffeine (Wu et al. 2008). Caffeine has also been shown to have a high correlation with fecal coliforms (Wu et al. 2008). Caffeine can be detected much faster than

current microbial indicators. But there are some problems with using caffeine. The chemicals persistence and distribution in the environment may not correlate with a health risk (Standley et al. 2000). Most sewage treatment plants are not designed to remove all chemicals. So correctly treated sewage may have a high caffeine signal, but does not correlate with human health. These same arguments can be made with other chemicals such as pharmaceuticals and whitening agents.

Microbiological agents have been sought after to be used as source tracking markers. Researchers have investigated protozoa, bacteria, and virus that are specific to its host and unlikely to be found elsewhere. These markers could then be tested for in water samples to determine the origins of the contaminants.

Host specific viruses have also been utilized as a target for microbial source tracking. Viruses are more resistant to environmental stress and sewage treatment processes than bacteria (De Leon and Jaykus, 1997). Pathogenic viruses are also responsible for many of the gastro-intestinal illnesses reported from recreational bathers (Bofill-Mas et al. 2011).These viruses can be found directly in water without re-growth and offer not only the indication of viral pathogens but also the host organism. One such virus that has been studied is the F+ RNA coliphage. This virus infects *E. coli* which is already used as a fecal indicating organism. This virus was also chosen because it resembles other pathogenic viruses such as hepatitis A and E, and many enteroviruses in size and shape (Havelaar et al 1993, Hsu et al. 1995, and Sobsey et al. 1995). Studies have shown that the presence of this virus in water correlates to the presence of fecal waste (Contreras-Coll et al. 2002), and with the presence of pathogenic organisms

(Wade et al. 2003, and Colford et al. 2006). Enteroviruses and adenoviruses have also been shown to have human specific variants (Fong et al. 2005, and Noble and Fuhrman, 2001). These viruses have been used to monitor waters in many studies and have done well in detecting human sewage (Noble et al. 2003). But due to the scarcity and relative low presence in populations (Noble et al. 2003) the filtering of larger volumes of water may be necessary.

Another biological organism considered for use as a source tracking marker has been the protozoan cryptosporidium. Studies have shown that there are specific genotypes of cryptosporidium for many targets of fecal contamination (Xiao et al. 2000). This method has also been used to determine the major sources of contamination in real world settings (Xiao et al. 2001). In the study conducted by Xiao in 2001 they were able to determine that humans and cattle were the major contributors of fecal contamination in their body of water. The drawbacks to using this method as a source tracking marker is the relatively low levels of *Cryptosporidium* ssp. in water and the need for water concentration. The amount of water required to be able to detect the *Cryptosporidium* ssp. oocysts is around 40-100 liters. With the massive amount of water needed to be filtered along with the time constraints of the filtering, and the co-concentration of other interfering particles and substances this method is not a viable option for large scale fecal source tracking.

In feces, the most abundant types of bacteria are anaerobic (Savage, 2001). Bacteroidales, which is a subset of the anaerobic bacteria are present in a quantity, of up to 10% of fecal mass (Layton et al. 2006) which is greater than the indicator

organisms currently used in recreational water testing (Eckburg et al. 2005). These bacteria were largely ignored during the time of classical indicator development because they were difficult to culture. This perceived impediment is now looked on as an advantage. Current fecal indicators have been shown to re-grow in the environment (Fiksdal et al. 1985, Kreader, 1995). Bacteroidales do not grow aerobically so re-growth is not a problem and may better correlate with new sources of contamination (Haugland et al. 2010). With the advent of PCR and qPCR, it is possible to skip the re-growth step and directly sample these bacteria from the water. These bacteria have also been shown to contain host specific strains (Dick et al. 2004, Layton et al. 2006, and Okabe et al. 2007).

Dog specific primers were first developed in Kate Fields' lab in 2004 (Dick 2004). These primers were the basis for the Taqman qPCR assay developed by Kildare in 2007 (Kildare et al. 2007). The BacCan-UCD assay was originally reported to be positive for 62.5% of their dog population (5/8), and there was some cross reactivity with human stool 22% (4/18), cat stool 14.3% (1/7).

2.2 Objectives

From this initial report the aim of this study was to convert the original assay to Taqman chemistry. Confirm the presence of the dog associated bacteroidales marker in the south Florida dog population, and then use the assay on beach water that permits dogs and monitor that beach over time. Finally, this study also gauges

beach-goers ability to cross-react with the assay, by testing water samples from a bather shedding study.

2.3 Materials and Methods

Conversion of Dog qPCR assay to Taqman chemistry

From the 2007 paper by Kildare (Kildare et al. 2007) the original probe (BacUni-656p 6-FAM-TGGTGTAGCGGTGAAA-TAMRA-MGB) was a minor grove binding probe and 16bp. That probe was extended to 28bp and converted to Taqman chemistry (Dog specific Probe FAM-ATTCGTGGTGTAGCGGTGAAATGCTTAG-BHQ) (Sinigalliano et al. 2010). The original probe is highlighted in blue. The forward and reverse primers for these experiments were the same as the original Kildare paper.

Positive controls and Standards

A plasmid standard was constructed by cloning a PCR product of the Dog specific primer DF475F and its universal reverse with extracted dog feces as its template. The product was run on a 1% agrose gel and the 251bp product was excised and purified (Qiagen gel extraction kit Cat# 28604). The purified product was then cloned into a zero-blunt topo plasmid (Invitrogen Zero-Blunt cloning kit Ca# K2800-20). The plasmid was then transferred into chemi-competent cells and grown on LB+Kanamycin agar plates. Colonies were selected and placed into cell-pop qPCR and also into 5ml of LB+Kanamycin broth. The colonies that were positive for the product were spun down and had their plasmids extracted.

These extractions were performed with Promega's Wizard plus SV mini prep kit(Cat# A1330). The purified plasmid was digested with the enzyme ECOR1 to insure that it contained insert. Sample plasmid concentrations were quantified by fluorometer and copy number of the plasmid was assigned by using the plasmid plus insert size (3770 base pairs).

Dog stool sample preparations

Dog stool samples were prepared by Jody Harwood's lab at University of South Florida. Between .25-.3 grams of stool was placed in a bead beat tube and processed using the power soil DNA kit (MO BIO laboratories, INC., Carlsbad, CA) one μl of elution(total elution 100ul)was used in the qPCR assay.

Sample Collection

The BEACHES study was an epidemiological study of a heavily used Biscayne Bay (Miami, FL) beach that allows dogs (Fleisher et al 2010, Sinigalliano et al. 2010, and Shibata et al. 2010). Individuals who participated in the study went in the water and collected their own water samples. Samples were collected over a 7-month period (December 2007 - June 2008) resulting in over 600 individual water samples. To calculate the values of figure 2.2, all of the markers were multiplied to achieve 100mls of original sample. Enterococci and human associated UCD marker were calculated in genomes per 100mls, while dog associated bacteroidales marker and gull-2 marker were calculated in copies per 100mls of sample.

The bather study water samples were collected from pools that had been used by a number of bathers at Hobe Beach in Miami, FL (Elmir et al. 2009). Ten bathers were placed in a large pool, with four fifteen-minute bathing cycles. Sand was added to the pool during cycles 3, and 4. A small pool study was also conducted with toddlers in diapers. Water samples were collected from each pool and 1L samples were processed in the same manner as the beaches sampling.

Water sample processing

After the water had been collected,1L of water was filtered on Whatman .45micron 47mm filters no later than 6 hours after is was collected. These filters were rolled up and placed in the Fast DNA spin kits bead beat tubes. The tubes were processed using the Fast DNA spin kit protocol (MP Biomedicals Cat # 6540-600). At the start of the extraction process a known amount (1×10^5 genome equivalents) of *Lactococcus lactis* gDNA was added to each tube that was extracted. This DNA was used as an extraction/inhibition control for the samples. After processing, all samples were eluted into 100ul of buffered water. One µl of each sample was analyzed using qPCR. This 1ul of elutant is equivalent to 10mls of original sample.

QPCR set up

The primer and probe sequences used in this study are as follows: Forward-DF475F-CGCTTGTATGTACCGGTACG, Uni-reverse-CAATCGGAGTTCTTCGTG, and the Dog-bacteroidales probe FAM-

ATTCGTGGTGTAGCGGTGAAATGCTTAGT-BQ.   The probe has been modified from the original minor grove-binding probe (MGB) designed by Kildare (Kildare et al. 2007). The reaction set up was in 25µl final volume. The final primer concentration was 900nm, and the final probe concentration was 300nm. In the reaction Quanti-TECT ™ Probe PCR Kit (Qiagen, Cat # 204343) was used as master-mix. Nuclease free water from the kit was used to bring the final volume to 25µl. The samples were run on an MJ Chromo4 QPCR thermal-cycler and analyzed with opticon monitor-3 software. All plates were run with a standard curve and no template controls (NTC).   All values for dog specific bacteroidales are reported in copy number per µl of elutant.

2.4 Results

Abundance of dog-specific bacteroidales in South Florida dog population.
 From the 29 test subjects 26 were positive for the marker (90%), (Figure 2.1). The lowest sample that was positive was at 9 copies or at 9 times above the no template controls. Only 3 samples were called negative, samples 1, 7, and 24. Twenty out of the twenty-nine samples (69%) were above 10 copies. The samples that exceeded $1x10^{4}$ copies had a copy number range of $2x10^{4}$ to $1.74x10^{8}$ copies per ul of elutant
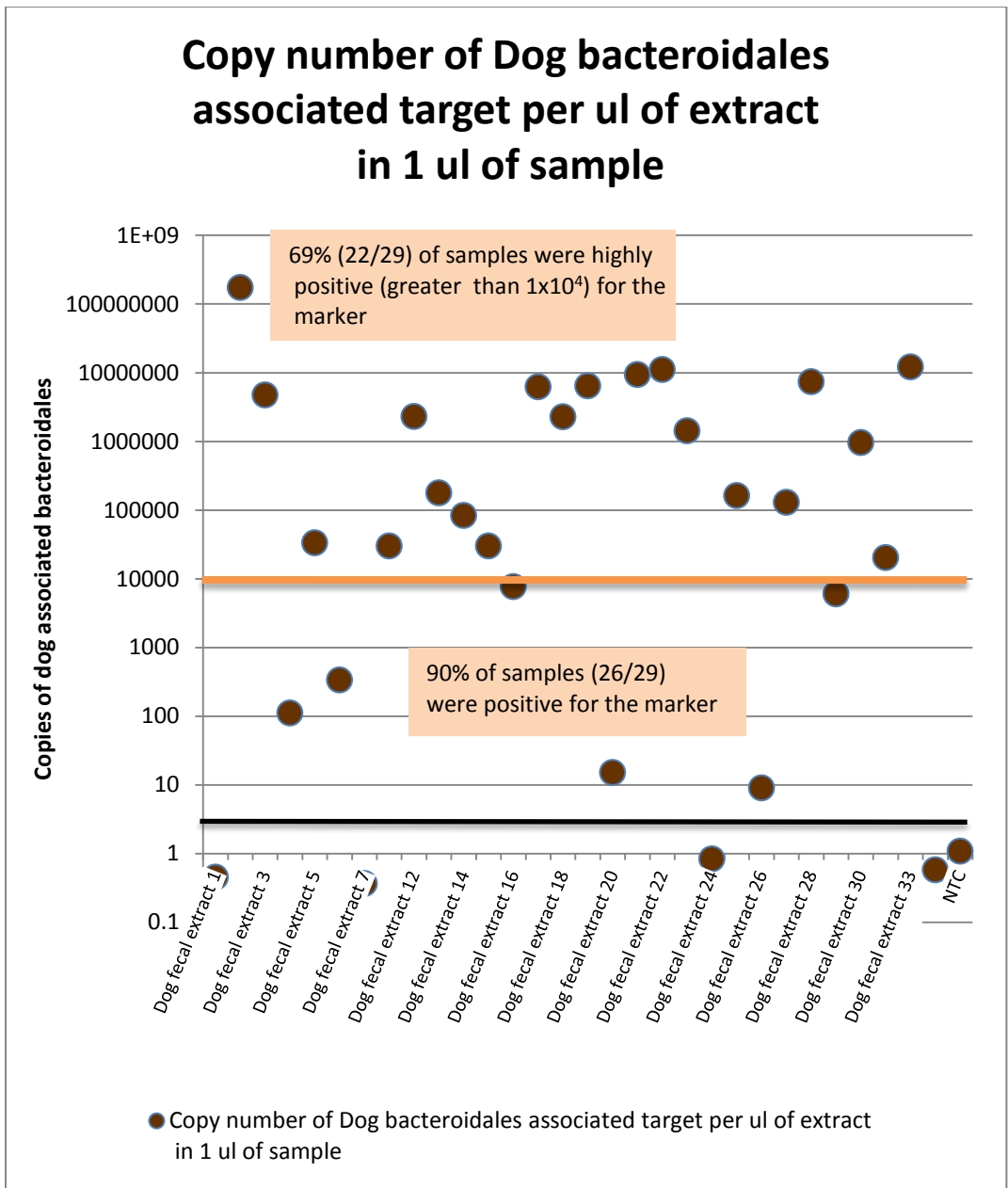
53

Figure 2.1: Copy number of dog associated bacteroidales marker in the South Florida dog population. The orange line highlights the 10,000 copy level, while the black line depicts the threshold for samples to be called positive.

BEACHES sample results

Samples were collected over a 7-month period beginning in December 2007 to June 2008. An average of 42 samples was collected on each of the 15 sampling events. The average copy number of dog-specific bacteroidales taken from each date is detailed in Figure 2.2. The range detected during these 15 sampling events was from .1 copies (4/12/2008) to 161.2 copies (12/17/2007) per 1μl of extraction.   These averages were also compared to the incidence of illness reported during the study. The Pearson's correlation between the average dog signal and illnesses reported (as reported in Fleisher et al 2010) was r =.121. These numbers were also compared with other source tracking, and fecal indicating markers (Figure 2.3). The dog specific bacteroidales marker fluctuated independently from the other source tracking markers during the study.

Bather shedding study

From the 90 samples taken, the highest amount of dog specific bacteroidales detected was .3 copies. This signal was before any bathers had entered the pool.   Other source tracking markers for human bacteroidales, BacHum-UCD, and HF-183 (Kildare et al., 2007, Bernhard and Field, 2000) were tested on these pool samples and became increasingly positive as the bathers continued to bathe. Human bacteroidales, and levels of enterococci were both elevated after humans had used the pool, while dog bacteroidales levels were either undetectable or below .3 copies (Table 2.1).
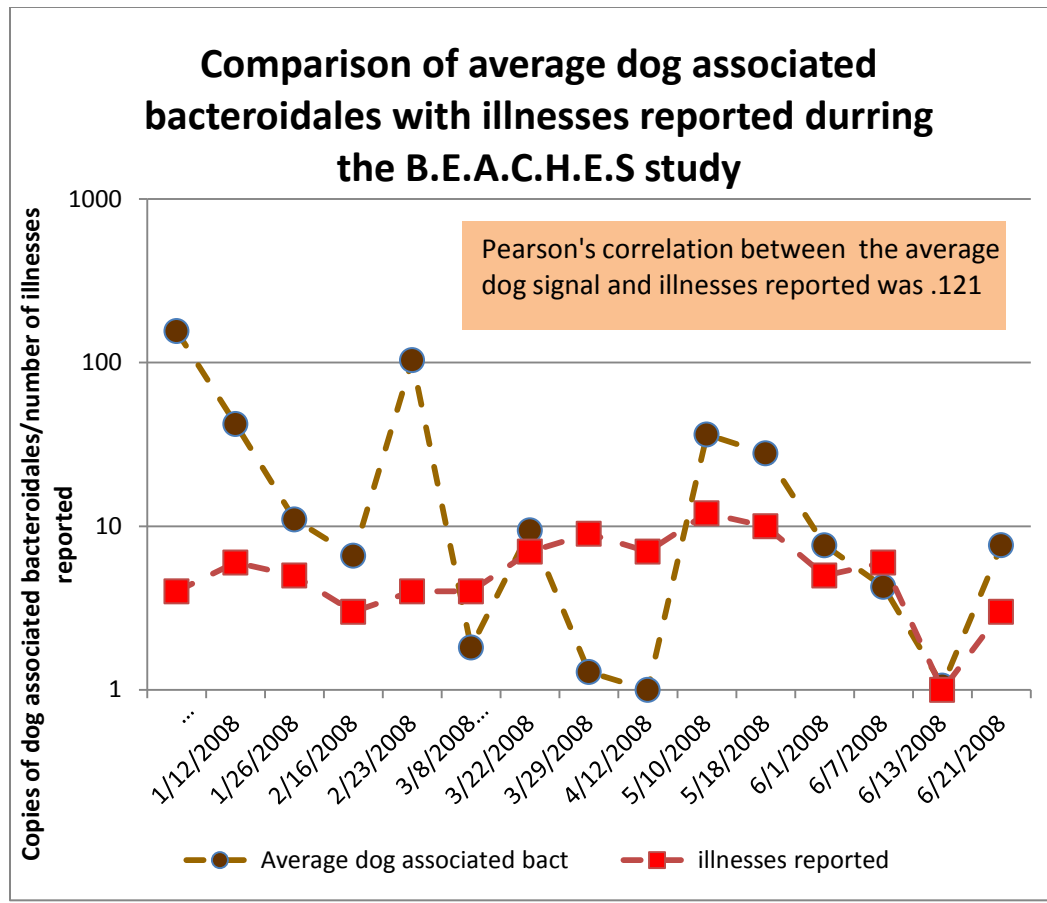
Figure 2.2: Correlation between the average dog associated bacteroidales and illness reported in the B.E.A.C.H.E.S. study. The average dog associated bacteroidales signals are depicted with the brown dots, while the numbers of illnesses reported by swimmers are depicted with the red boxes. Pearson's correlation between the dog associated bacteroidales and reported illness was r=.121 (p>.05)

Figure 2.3: Average copy number/genome equivalent of molecular markers for the B.E.A.C.H.E.S. epidemiological study. These numbers were calculated to 100mls of original sample. This chart shows the fluctuations of different fecal markers over a 7 month period

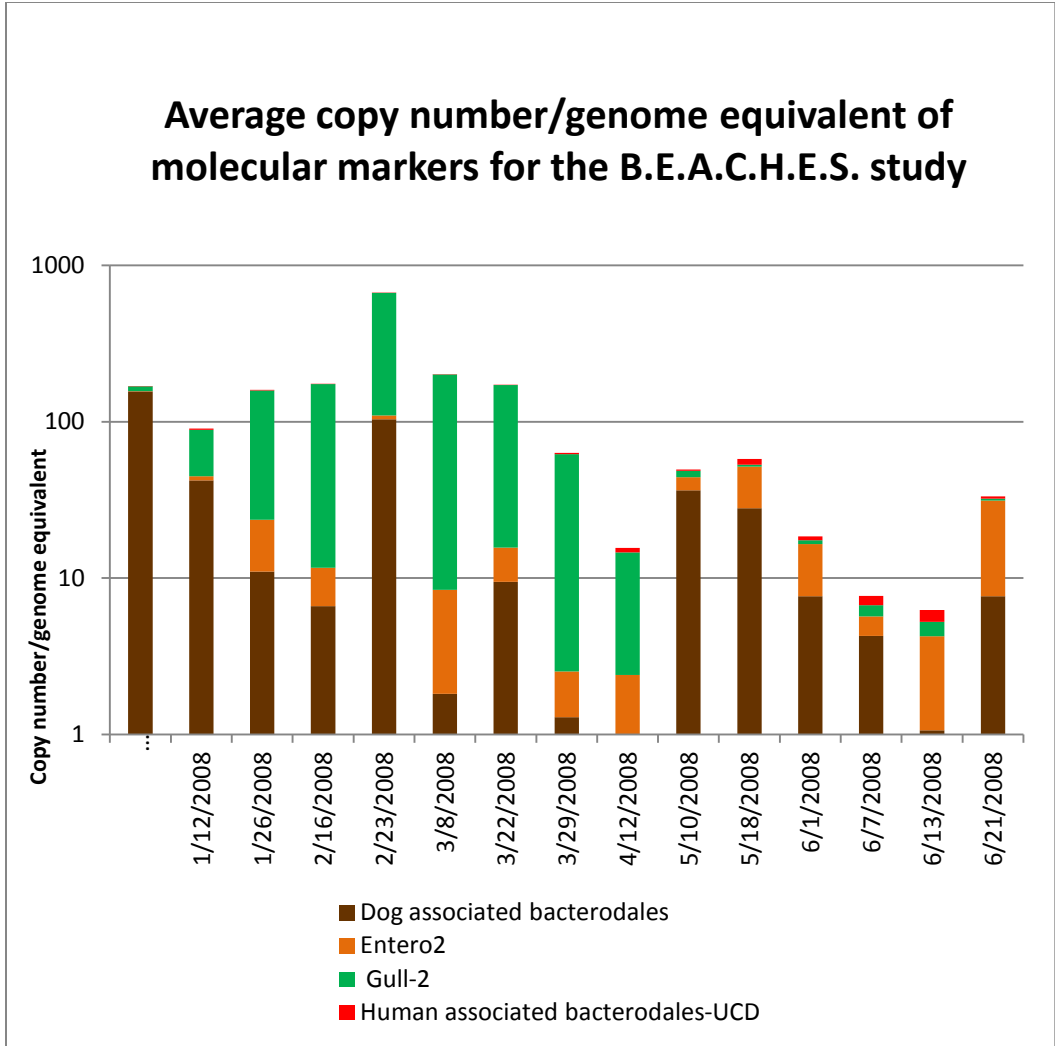| Samples	Enterococci 23s (genome equivalents)	Human bacteroidales marker- UCD(genome equivalents)	Human bacteroidales marker HF-8 (genome equivalents)	Dog specific bacteroidales marker(Copies)
Average from source water Hobie beach	**0.5**	**0.2**	**0**	**0.3**
Bathers group 1 Initial	0.4	0.2	0.5	0
Bathers group 1 Final	**4.2**	**13.4**	0	0.1
Bathers group 2 Initial	0.4	0.2	0.1	0
Bathers group 2 Final	**8**	**2.9**	**1**	0
Bathers group 3 Initial	0.4	0.2	0	0
Bathers group 3 Final	**6.8**	**7.1**	**2.5**	0
Bathers group 4 Initial	0.4	0.2	0	0
Bathers group 4 Final	**9.1**	**3.9**	0.9	0
Bathers group 5 Initial	0.3	0.3	0	0
Bathers group 5 Final	**73.4**	0.9	0	0
Bathers group 6 initial	16	0.5	0	0
Bathers group 6 Final	**22**	0.4	0	0.1
Bathers group 7 Initial	0.9	0	0	0
Bathers group 7 Final	**22.7**	0.8	0	0

Table 2.1: The results from the bather shedding study. Values highlighted in red indicate increased detection of marker after the bathing of the participants.

2.5 Discussion

The prevalence of the dog specific bacteroidales marker in South Florida dog population was high (26/29 samples were positive, with 20/29 samples above 10,000 copies per ul). This marker was present in a majority of dogs in the experiment. This is important because the original assay was developed in

California. By testing the assay on samples from a vastly different geographic location demonstrates that this assay can be used all over the United States and that the target was relatively universal in dogs. While the amount seems to fluctuate between samples, this could be due to sample processing or the differences in diet and heath of the individual animal. Also quantifying the amount of DNA in each extraction and then adding the same concentration to each reaction could be beneficial as well. In this study an aliquot of 1µl from each elutant of 100ul was used from .25-.3 grams of feces. In the Kildare paper (Kildare et al. 2007) they reported 62.5% of their dog samples positive. From the data collected in this study it was found that 90% of the samples were positive for the marker. But four of the positives were low (under 500 copies) and there seemed to be a natural threshold of approximately 1×10^4 copies between those 4 low samples and the rest. By placing the threshold at 1×10^4 copies, 69% of our samples were positive. This seems inline in what was previously reported

The results from the BEACHES study show that the dog specific bacteroidales marker can be used on recreational waters. Changing concentrations of dog specific bacteroidales over the course of the BEACHES study suggests that the marker is detectable and able to fluctuate over time due to the impact from dogs. The levels of some of these samples approach and exceed some of the extracted fecal samples used in the prevalence study. By comparing these results to the reported illnesses there was no correlation between the two, nor was any dose response seen for dog marker and reported illness (Sinigalliano et al. 2010). This may suggest that having dog signal at the beach may not be a

major factor in predicting human health risk from recreational water exposure. It is a widely accepted and held belief that human fecal contamination poses the highest risk to recreational water users. But it has also been shown that there are other pathogens that reside in the domestic animals (Beutin et al. 1999) and wildlife (Atwill et al. 2001, and Graczyk et al. 1998) that frequent these waters. With the implementation of many source-tracking markers, perhaps in the future, when fecal indicating bacteria are elevated, the level of concern could be heightened or alleviated by assessing the secondary levels of source tracking markers. With a certain percentage of beaches allowing dogs, this marker should prove valuable to gauge the amount of fecal contamination that is occurring in the waters from these animals.

In the bather shedding study, no cross reactivity was observed. In previous studies the dog specific bacteroidales marker was tested against extracted human feces (Kildare et al. 2007). It is important to know if the body of water being monitored is impacted by untreated human waste, or other point sources. For most recreational waterways, these point sources have mostly been remediated. Testing cross reactivity by using situations that more accurately depict sources of human contamination in these waters provides a more realistic scenario to test the dog specific bacteroidales. Our findings show more confidence in the specificity of our dog specific bacteroidales marker along with the specificity of other source tracking markers used in the study. It is also interesting to note that human associated bacteroidales markers (BacHum-UCD,HF-183) increased in the study while the dog specific bacteroidales marker

stayed undetectable or low. This study shows that these markers function independently from each other and correlate with the amount and time of humans shedding their microbial flora in the pool.

2.6 Summary

Source tracking markers can be a useful tool to distinguish the different sources of contamination affecting the water being tested. Once an assay has been shown to be prevalent in the type of feces it was designed for, and specific for that target, it can be used to understand the inputs or possibly mitigate the source. Some of the ways this tool can be applied is by providing a spatial component to a testing site, and/or risk assessment. If the site has multiple inputs from streams or currents, these markers may be able to show not only the type but also the flow of these fecal markers (Noble et al. 2006). These source tracking markers can also be used along with traditional markers. If a body of water has high levels of indicator organisms, source tracking markers can be used to augment the estimate of risk involved with swimming in the water. If there were high levels of human markers this could potentially represent a high risk of infection. But if there were low or no detection of human markers and high levels of dog markers then that risk could be viewed as lower risk and may not warrant the closure of the beach. In the BEACHES study, a flow of fecal indicating bacteria was observed after a rain event. This flow was then tested with source tracking markers showing that the Dog specific bacteroidales was not coming

from the run-off. This information could be critical for addressing the problem of

high indicator bacteria at this and other beaches (Shibata et al 2010).

Chapter 3 Gull Associated Marker Experiments

3.1 Overview

Gulls, pigeons, and other seabirds can be a major source of non-point source pollution in beaches and recreational waters. Gulls and other waterfowl's feces can carry fecal indicating bacteria that can cause elevated levels in beach water (McLellan and Salmore 2003, Genthner et al. 2005). It has also been shown that the populations of gulls have been increasing. From 1976 to 1990 ringed-bill gulls increased in population 6-fold to 283,000 breeding pairs along the Canadian portion of the lower Great Lakes. Herring gulls also increased from 440 to 1,300 breeding pairs (Blokpoel and Tessier 1991). These increases have been due to several factors such as the loss of predators, and the over-abundance of food. With more gulls frequenting the beaches, higher levels of closures influenced by non-human fecal sources may lead to negative economic and health impacts in the surrounding area.

Because of this direct link established between gulls/water fowl and elevated levels of indicator organism water managers have been developing methods to minimize their impact. In Chicago they have had beaches that are chronically high in fecal indicator organisms. These beaches also were observed to have large gull populations as well. So they used trained border collies to chase the birds on two of the problem beaches (Chicago park district 2008). What they found was a dramatic decrease in closure days for those beaches. In 2007 without the dogs on one of the beaches they had a 50%

exceeds rate for the beach season. In 2008 with the use of the dogs that exceedance rate dropped to 6%. They then removed the dogs in the 2009 beach season and saw a 66% exceedance rate for the beach. Then they brought back the dogs in 2010 and saw an exceedance rate drop to 22%. This study demonstrates that the removal of large bird populations can have an impact on the rate of exceedances. But this option is a expensive with the need for trained dogs and handlers to be on-site though out beach season. Even with those drawbacks other cites and counties are now looking to try this method. Some of the other deterrent methods used are the removal or inactivation of eggs. With the application of oil over the egg, it can no longer breathe and will die. The oil is non-toxic and a single application of oil prevents over 95% of eggs from hatching, applying two applications is more than 99.6% effective (Blokpoel and Tessier 1992). This method is also expensive with the labor costs and the lag time between eggs and adult birds.

Gulls have been targeted as a possible source for water fowl pollution. Some of the methods in the past have tried to gauge the amount of impact gulls and other water fowl have had on recreational waters. The patterns of antibiotic resistance in *E. coli* have been used to determine whether the source was human/cattle or wild birds (Edge and Hill 2005). The Edge study showed that bird feces had less antibiotic resistance then fecal contamination from other sources. It also showed that at times birds could be the major contributors of fecal indicating bacteria. But this study would be hard to apply if one could not obtain fecal samples of all possible contributors. Also this

study does not allow for a background population of fecal indicating bacteria in the waters which could be a major limitation of that study.

Another method used to source track bird contaminants was DNA fingerprinting (ribotyping). In the Samadpour study (Samadpour et al. 2005) they DNA fingerprinted 40 different groups of *E. coli* from many different sources and then compared these patterns to those of environmental samples. This method requires in-depth local knowledge of the water shed and human interpretation of the banding patterns. This study was carried out in central California and may not be applicable to other areas of the country.

Other methods used to determine the impacts of birds in recreational waters have been direct counting. Klienheinz in 2006 (Klienheinz et al. 2006) used counts of birds on the beaches to see if they correlated with the amount of fecal indicator bacteria. They also tried counting the fecal pellets of the birds that were on the beach. They were unable to correlate either the number of fecal pellets or the total number of birds on the beach with the fecal indicating bacteria. There have been explanations of how the sand interacts with the waste possibly harboring and providing nutrients to the bacteria, only to be released at a later date(Whitman and Nevers 2003).

Recently molecular methods have tried to identify and quantify the impact of gulls/birds on beach waters. In 2008 Lu made a 16s rRNA library from the feces of a gull (Lu et al. 2008). These clones were sequenced and it was shown that 26% of the clones were closely related to *Catellicoccus marimammalium*. Lu then developed a PCR and Sybrgreen qPCR assay to

detect this gene. The assay was both specific for gull and that the target was distributed geographically in the upper great lakes region. Other studies have also developed molecular assays for gull and water fowl. In the study performed by Green in 2011 (Green et al. 2012) they used subtractive hybridization from gull feces to other source tracking targets such as human, dog, cat, cow, and pig. What they found was a Catellicoccus marker similar to what Lu had discovered along with a marker that was associated with Helicobacter. They made a PCR and a Sybrgreen qPCR assay for both targets. They tested the markers against a wide array of other animal feces. What they found for the marker associated with helicobacter was that it was a more general bird marker compared to its catellicoccus target. The more general bird marker called GFD was specific for gull, goose, duck, chicken, and pelican. By having a wider range of targets this marker becomes more of a waterfowl marker and could be more useful to a larger portion of waters and their managers. Recently the specificity of the catellicoccus gull marker found it also detects at least pigeon and pelican feces, thus this may also be considered a "sea bird" marker rather than a "gull" marker. In this context pigeons at beach settings are considered as seabirds as they have similar scavenging behavior and carry many of the same fecal pathogens as gulls (Ryu et al. 2012).

3.2 Objectives

The purpose of this experiment was to develop a probe for the Lu based gull-2 marker. By making the assay a Taqman assay it will increase the sensitivity,

specificity, and standardize it to existing source tracking markers. The next step would be to develop a plasmid standard to use for the quantification of samples. After those goals are complete, testing the assay against some different types of fecal samples that were collected from around south Florida. Once those steps have been completed the marker would be used on water samples that were collected for an epidemiological study and to determine if there are any correlations between high levels of fecal indicating bacteria or with any human health outcomes.

3.3 Materials and Methods

Positive controls and Standards

A plasmid standard was constructed by cloning a PCR product of the Gull-2 specific primers described in the Lu 2008 paper, with extracted *Catellicoccus marimammalium* (DSMZ M35/04/3T, obtained from the Culture Collection of the University of Göteborg, Göteborg, Sweden) as its template. The product was run on a 1% agrose gel and the 412bp product was excised and purified (Qiagen gel extraction kit Cat# 28604). The purified product was then cloned into a zero-blunt topo plasmid (Invitrogen Zero-Blunt cloning kit Cat# K2800-20).
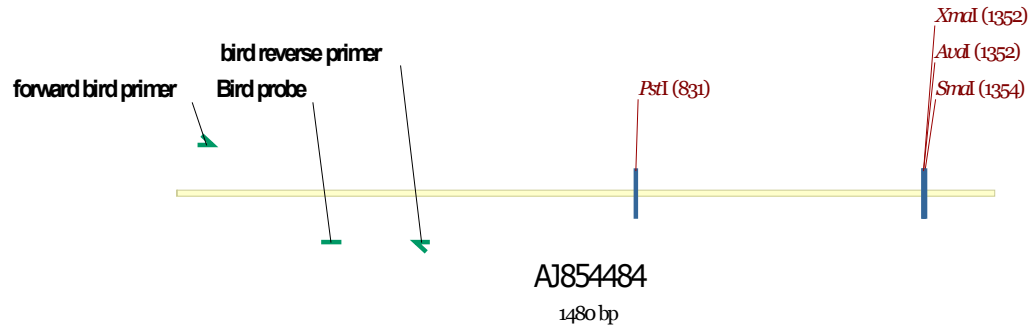
>AJ854484 (41bp - 452bp, direct) 412bp

TGCATCGACCTAAAGTTTTGAGTGGCGGACGGGTGAGTAACACGTG

GGTAACCTGCCCATCAGAGGGGGACAACACTTGGAAACAGGTGCTA

ATACCGCATAATACAGAGAACCGCATGGTTCTTTGTTGAAAGGCGCT

TCTGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGACGGTG

AGGTAACGGCTCACCGTGGCAATGATGCATAGCCGACCTGAGAGGG

TGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGA

GGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGC

AACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGT

TAGAGAAGAACAGGAGCGATAGTAACTGCTCGCTCTTTGAC
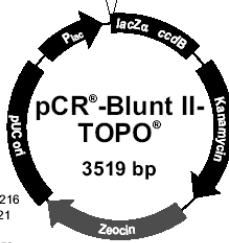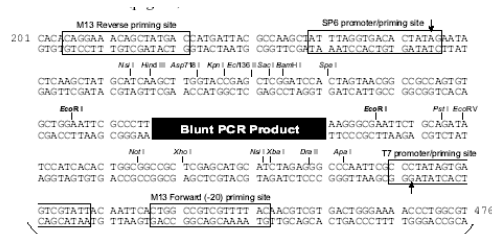
Comments for pCR®-Blunt II-TOPO®
3519 nucleotides

lac promoter/operator region: bases 95-216
M13 Reverse priming site: bases 205-221
LacZ-alpha ORF: bases 217-576
SP6 promoter priming site: bases 239-256
Multiple Cloning Site: bases 269-399
TOPO®-Cloning site: bases 336-337

The plasmid was then transferred into chemi-competent cells and grown on LB+Kanamycin agar plates. Colonies were selected and placed into cell-pop qPCR and also into 5ml of LB+Kanamycin broth. The colonies that were positive for the product were spun down and had their plasmids extracted. These extractions were performed with Promega's Wizard plus SV mini prep kit (Cat# A1330). The purified plasmid was digested with the enzyme ECOR1 to insure that it contained insert. Sample plasmid concentrations were quantified by fluorometer and copy number of the plasmid was assigned by using the plasmid and insert size (3931 base pairs).

Gull sample preparations

Gull stool samples were collected at several different sites in the Miami-Dade metro area. Site one was a boat dock on the 79th street causeway in Miami, site 2 was at Crandon marina located on Key Biscayne, and site 3 was at hobie beach which is located on the Rickenbacker causeway. Between .25-.3 grams of stool was placed in a bead beat tube and processed using the power soil DNA kit (MO BIO laboratories, INC., Carlsbad, CA) one μl of elution (total elution 100uls) was used in the qPCR assay. Other fecal samples were collected in south Florida with a close proximity to AOML/NOAA laboratories. These fecal samples were processed in the same way as the gull fecal extracts.

Water Sample Collection

The BEACHES study was an epidemiological study of a heavily used

Biscayne Bay (Miami, FL) beach that allows dogs, and has impacts from birds

as well (Fleisher et al 2010, Sinigalliano et al. 2010, and Shibata et al. 2010).

Individuals who participated in the study went in the water and collected their

own water samples. Samples were collected over a 7-month period (December

2007 - June 2008) resulting in over 600 individual water samples.

Water sample processing

After the water had been collected, 1L of water was filtered on Whatman

0.45micron 47mm filters no later than 6 hours after is was collected. These

filters were rolled up and placed in the Fast DNA spin kits matrix A bead beat

tubes. The tubes were then processed using the Fast DNA spin kit protocol (MP

Biomedicals Cat # 6540-600). At the start of the extraction process a known

amount of *Lactococcus lactis* gDNA was added to each tube that was extracted.

This DNA was used as an extraction/inhibition control for the samples. After

processing, all samples were eluted into 100ul of buffered water. One µl of

each sample was then analyzed using qPCR.

qPCR set up

The primer and probe sequences used in this study are as follows:

Gull-2 Forward-TGCATCGACCTAAAGTTTTGAG,

Gull-2 Reverse-GTCAAAGAGCGAGCAGTTACTA, and the

 Gull-2 probe FAM-CTGAGAGGGTGATCGGCCACATTGGGACT-BQ. The probe was designed at AOML to pair with Lu's Primers. The reaction set up was in 25µl final volume. The final primer concentration was 900nm, and the final probe concentration was 300nm. In the reaction Quanti-TECT ™ Probe PCR Kit (Qiagen, Cat # 204343) was used as master-mix. Nuclease free water from the kit was used to bring the final volume to 25µl. The samples were run on an MJ Chromo4 QPCR thermal-cycler and analyzed with opticon monitor-3 software or on a Step 1 + from ABI. All plates were run with a standard curve and no template controls (NTC). All values for the Gull-2 assay are reported in Copy number per µl of elutant.

3.4 Results

Preparation of Gull standard curve and annealing temperature

The production of gull-2 plasmid followed a standard approach. The source material was ordered, diluted, and PCR was performed. The correct size band was excised and incorporated into plasmid. After trying multiple annealing temperatures it was decided that 62°C produced the best results with consistent standard curves. The slope for the standards was consistently in the range of -3.3 to -3.5, with efficiency ranges in the high 90%'s. From the plasmid dilution we were able to consistently detect 10 copies of gull-2 plasmid with the assay. With that level of detection it is possible to see a wide range of fecal impacts from gulls on recreational waters.

The abundance of Gull-2 marker in South Florida Gull population.

All of our gull fecal samples came up positive with the gull-2 assay. The

samples ranged from ~6,000 copies per ul of elutant to ~120,000 copies per ul

of elutant (Table 3.1). These copy numbers corresponded to cycle threshold

(C(t)) values of 22.5 to 27. This marker was also tested against a wide variety

of other animals' extracted feces. These included crane (N=2), dog (N=6), duck

(N=5), harbor seal (N=1), Human (N=4), Ibis (N=2), pelican (N=1), snow goose

(N=1), and trumpet swan (N=1). Only the pelican sample came up positive for

the gull-2 assay and at a much lower level than the other gull samples (C(t) 33.7

compared to C(t) of 22-27). One recreational water sample was included to see

if the marker was present in high enough concentration to be detected. This

sample was positive for the marker at 824 copies per ul of elutant.

Presence of Gull-2 marker from the BEACHES epidemiological study.

From the individual samples collected during the BEACHES study one ul from

the elutant was run with the gull-2 assay. Sample dates were averaged and then

compared (Figure 3.1). What was found was a possible seasonal trend of

marker in the samples. The trend started on sampling date 1/12/2008 with and

average of 44 copies per elutant and peaked at sampling date 2/23/2008 with an

average of 557 copies per ul of elutant. The trend ended on 3/29/2008 with an

Description	C(t)	Copies per ul
213 (Rec water sample)	**30.0**	824.0
Crane 4	N/A	
Crane 5	N/A	
Dog 54	N/A	
Dog 55	N/A	
Dog 56	N/A	
Dog 57	N/A	
Dog 58	N/A	
Dog 59	N/A	
Duck 1	N/A	
Duck 2	N/A	
Duck 3	N/A	
Duck 7	N/A	
Gull pigeon 1	26.9	6444.9
Gull/P 3	26.5	8423.2
Gull/P 5	22.5	119671.2
Human 1-1	N/A	
Human 2-1	N/A	
Human 3-1	N/A	
Human 4-1	N/A	
Ibis 1	N/A	
Ibis 2	N/A	
Pelican	33.7	73.9
Snow goose	N/A	
Gull/dunlin	34.36	46.1
Trumpet swan	N/A	
Gull	26.3	9815.5
Harbor Seal	N/A	
Mallard	N/A	
NTC-gull	N/A	

Table 3.1: Gull-2 Taqman qPCR assay tested against a wide range of fecal extracts. Highlighted rows indicate positive results from the assay. Samples that have N/A had no amplification.

average of 12 copies per elutant. This trend did not correlate to the sampling

days that had the most adverse health impacts (these days are in red on the

chart). Also there was no does response relationship observed between

abundance of gull-2 marker and reported illness (Sinigalliano et al. 2010)
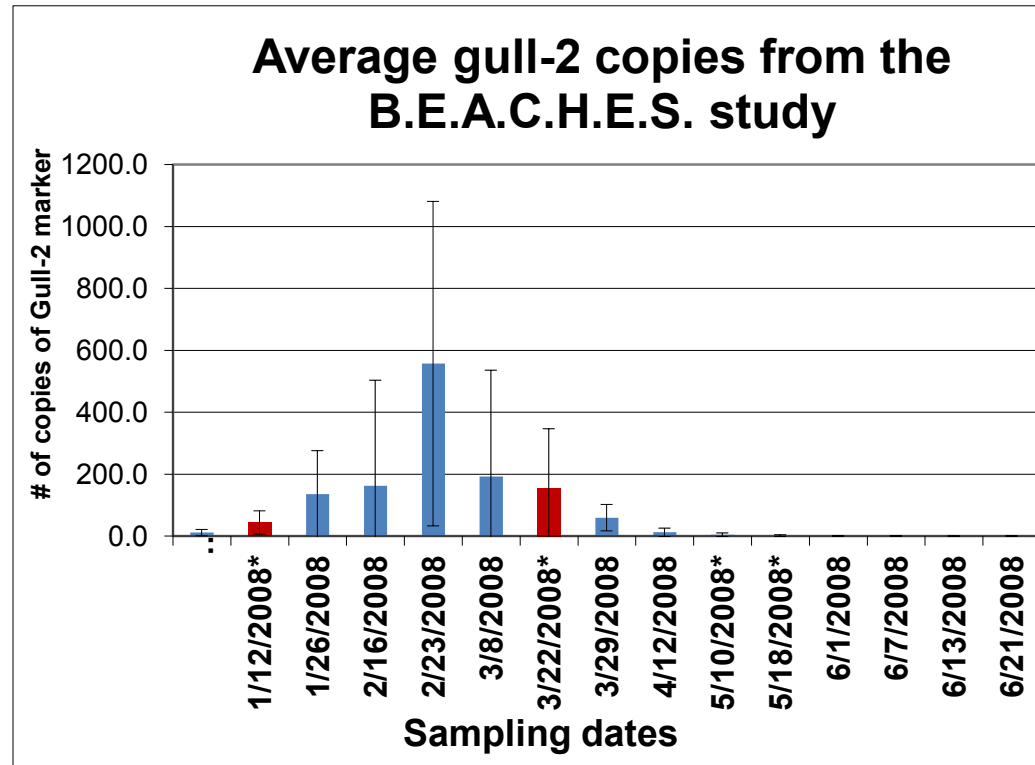

Figure 3.1: Average gull-2 copies from the B.E.A.C.H.E.S. epidemiological study. Samples in red correspond to sampling days with greater reports of bather illness. Error bars represent the standard deviation.

When the amount of gull-2 signal was compared to fecal indicating bacteria

(enterococci for marine waters) there was no correlation between the two

markers with r = -.1 (p≥.05) (Figure 3.2). There was also no correlation

observed between the gull-2 marker and reported human illness with r = -.2

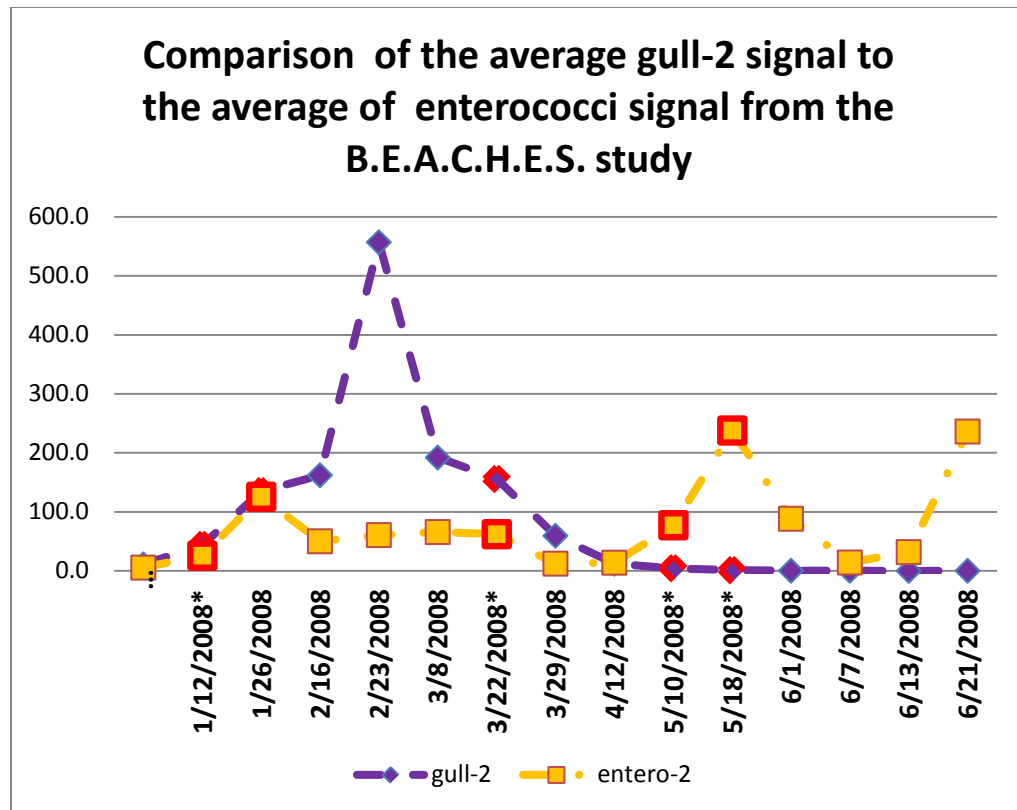(p≥.05). The sample dates with elevated adverse health outcomes are stared, and

filled in red.

Figure 3.2: Comparison between the average gull-2 signal to the average of enterococi signal from the B.E.A.C.H.E.S. epidemiological study. Samples outlined in red correspond to sampling days with greater reports of bather illness.

3.5 Discussion

Preparation of the standard was an easy straight forward process. It would be easy for any lab interested in running this assay to reproduce it. Source material is available through the mail and all the reagents that are needed are available in commercial kits. The slope and efficiency rates for the assay are similar to that of other source tracking markers. It is also interesting to note that the high sensitivity of the assay.

The gull-2 marker continues to be cosmopolitan in the gull samples tested in this study and along with the samples were tested in the original Lu

study (Lu et al. 2008). Geographically speaking this marker has been present in gull feces from the great lakes to the southern Atlantic coast. Further testing is required to see if the marker is present in other locations but the results of the study are promising. It was also shown that there was little cross reactivity between the gull-2 marker and that of other fecal samples tested. Granted that the sample sizes for each of the animals tested was small, it did show that there was no gross cross reactivity with the marker. The one sample that did come up positive, the pelican should be looked on as another shore bird equally likely to be contributing to the wildlife proportion of fecal impacts of coastal waters. It has been shown that pelicans also are carriers of *Clostridium perfringens* (Ankerberg et al. 1984). It has also been shown that the gull-2 assay also cross-reacts with pigeon feces (Ryu et al. 2012). Pigeon and gulls have the same type of food scavenger behavior, and can co-occur in large numbers with gulls. Pigeons have also been shown to carry some of the same fecal pathogens and represent similar public health issues as do gulls (Halde and Fraher 1966).

This experiment also showed that the addition of a Taqman probe didn't adversely change the sensitivity or the specificity of the assay. The conversion of the assay to Taqman also allows for shorter run times (no need for melt curve analysis, as there would be for sybr-green qPCR) and is more consistent with the other source tracking assays that are probe based. What was also important was that the marker was present at a high enough concentration that it could be detected in beach water samples. While some markers are specific for their

target organism if they are not present in great enough quantities to be detected then their usefulness greatly diminishes.

Since it was shown that the marker was present in beach water, the next step was to use it on the samples collected during an epidemiological study that was conducted on a beach that has the presence of gulls (Fleisher et al 2010, and Sinigalliano et al. 2010). Hundreds of samples were collected on 15 sampling days and were all analyzed for the gull-2 marker. What was immediately clear from the results is that on this beach, gulls were possibly seasonally impacting the water with their droppings. During the winter months, From January through March, there was a large increase in gull-2 signal. This signal peaked during the sample date of February 23, 2008. But with these peaks of signal no correlation to adverse human health events were recorded. It was also interesting to see that the gull-2 signal didn't correlate with the fecal indicating bacterial marker of enterococci. At this particular beach, gulls may play a small role in the loading of water with these bacteria. But that's not to say that at more remote sites where there are no known or obvious contributors, that the use of this marker could shed light on one possible source.

3.6 Conclusion

This assay and its standard material are easy to set up and offer a highly sensitive marker for gull feces. There was a slight amount of cross reactivity reported in this study from pelican, but the inclusion of pelican fecal detection may be seen as a positive overall for the marker. This assay is also sensitive

enough to be used in recreational water samples. When included in an epidemiological study this assay didn't correlate with any human health impacts or with the presence of fecal indicating organisms. This could be looked upon as proof that gulls are not a major source of either disease or fecal pollution at this particular site. It's also interesting to point out that the current fecal indicating marker enterococci didn't correlate with human health either. There could be many reasons such as the difference in reporting enterococci between the classical and qPCR techniques. Also the problem exists with the resolution of the study itself, particularly with the participants self reporting illness in a survey.

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