

Composition of Generic and Host-Specific Bacteria in Feces of Specific Hosts using Culture and QPCR

Ali Kattan

Abstract This study investigates the ratios of specific bacteria in fecal samples of several different hosts. These hosts are common sources of fecal pollution in watersheds and include horse, cow, dog, goose and human. To address the concentrations and variability of host-specific and generic bacteria of the host individuals the following measurements were made from the fecal samples: dry weight, QPCR of host-specific and generic bacteria, and culture-test for presence of *E. coli* and *Enterococcus*. The variability of these bacteria ratios was calculated using QPCR to give an estimate of the accuracy and sensitivity of the QPCR method for a contaminated watershed. Percent compositions were determined from each host using cycle threshold (Ct) values and supplemental dry-weight and culture-test data. Culture data results of the ratio of *E. coli* to *Enterococcus* of human and non-human sources confirm previous findings of ratios of Fecal Coliform to Fecal Streptococcus for human and non-human sources except for horse ratio. Culture data showed cows, humans and dogs to contain the highest *E. coli* concentration in their feces. Preliminary QPCR data of initial cell concentrations for total *E. coli* per gram dry weight for goose and dog do not correlate with initial cell concentrations for total *E. coli* from culture data. Future QPCR data for all bacteria is to be collected to give more accurate data.

Introduction

Gastrointestinal disease or gastro enteritis is the most common disease among recreational swimmers in fresh and salt-water (Pruss 1998, Wade et al. 2003). Extensive research has shown that recreational swimming at fecal contaminated beaches, fresh- or salt-water, is strongly correlated with gastrointestinal disease among those swimmers (Cabelli et al. 1982, Seyfried 1985, Pruss 1998). These findings were made around the globe in Spain, Canada, Hong Kong and several areas in the United States (Seyfried 1985, Van Asperen 1995, Thompson 1999, Wade et al. 2006).

Fecal concentrations in coastal and inland water bodies can originate from these point sources; raw sewage discharges, storm water, human sewage lines, effluents from wastewater treatment plants and industrial sources. Non-point sources include agricultural, wildlife and urban runoff and affect water quality to the same degree. Runoff of fecal concentrations into bodies of water contains high bacterial counts of *Escherichia coli* and genus *Enterococcus* (APHA 1998, Prieto 2001). Previous research has shown that bacteria indicators, *Enterococcus*, *E. coli*, and total bacteria, are the most efficient in determining fecal concentrations in fresh and salt-water samples (Seyfried 1985, US EPA 2000, Prieto 2001). These bacteria are native to the intestinal tract of mammals, reptiles, and birds, which depicts the presence of animal fecal concentrations in bacterial contaminated waters. It is important to detect the concentrations of these bacterial organisms in water in order to prevent humans from being infected. Beach closures for more than twenty-four hours prevent humans from becoming infected and are not reopened until bacteria concentrations go below levels set by the state of California and US EPA (US EPA 2000). The ability to detect high levels of bacteria quickly is what concerns watershed and beach managements and is the subject of a great amount of research today. Traditional methods of measuring concentrations of fecal contamination are culture methods of Total Coliform and Fecal Coliform bacteria. Though effective, culture-based methods for the detection of these indicator bacteria take more than 24 hours and do not distinguish between different hosts (Emori and Gaynes 1993).

Recent methods of measuring fecal contamination in water reservoirs involves microbiological methods of measuring generic indicators using library dependent methods such as DNA fingerprinting of *E. coli* and ribotyping of other generic bacteria (Wiggins 1996). Microbial source tracking methods of *E. coli* and *Enterococcus* bacteria are rapid yet present

some problems. These problems are, genomic library-dependent methods are needed to accurately quantify their differential distributions, high variability in *E. coli* populations within host species has been documented (Scott et al. 2003) and bacterial indicators are aerobic and can grow outside of the intestinal tract leading to false-positives in measuring their concentrations (Wiggins et al 1999). To quicken the process the Polymerase Chain Reaction (PCR) is an inexpensive procedure, which has been improved to detect host-specific sequences of bacteria organisms in water samples (Bernard and Field 2000). Despite recent improvements upon this PCR approach, much work is needed in order to apply the technique to any watershed (Simpson et al. 2002). By developing an inexpensive and rapid method of detecting fecal concentrations by specific hosts, disease prevention and watershed remediation can be most effective in watershed environments. A fully developed PCR method could take no more than four hours and would prevent disease contamination at a watershed.

Detection and quantification is only the first step to disease prevention as remediation of the watershed is crucial in order to prevent further contamination. This can be done for non-point source pollutants by identifying the host species of fecal concentrations. An anaerobic bacterium species, *Bacteroides*, has been discovered to be host-specific in fecal samples (Xu et al. 2003). Their host-specificity has been narrowed down to different nucleic-acid sequences in warm-blooded hosts. These DNA and RNA sequences can be amplified through the Polymerase Chain Reaction for their detection in fecal samples. PCR is a microbiological procedure used to amplify strands of DNA in extracts in a relatively short amount of time. Bernard and Field (2000), using Restriction Length Polymorphism, determined host-specific markers and sequences of *Bacteroides* for humans and cows. Quantitative-PCR (QPCR), or Real-Time PCR, assays have since been developed to directly measure the concentrations of total *Bacteroides* (Dick and Field 2004), host-specific *Bacteroides* (Weurtz 2006), *E. coli* (Nelson 2006), and *Enterococcus* (He and Jiang 2005). QPCR uses fluorescent signals, or probes, to mark each sequence and can be used to measure the number of cells in the sample. This project uses these QPCR assays to improve techniques in quantifying host-specific bacteria in environmental samples.

Bacteria compositions in the gastrointestinal tract differ among different hosts with variation in the abundance of *E. coli* and *Enterococcus* and genetic differences in *Bacteroides* for human, cow, horse and dog, and *E. coli* for bird (Gordon 2001). It is unknown how these compositions vary between individuals within a host group (e.g. difference between one horse and another

horse). Using advances in QPCR technology, this study seeks to determine the compositions of host-specific and generic bacteria of the following hosts: humans, horses, cows, geese and dogs. These hosts are most frequently responsible for fecal contamination in watersheds (APHA 1998). A table portraying the differences in host-specific and generic bacteria compositions could help develop a QPCR method in detecting and quantifying fecal contamination in environmental water samples.

The objective of this study is to determine if the composition of host-specific *Bacteroides*, *Enterococcus*, *E. coli*, total bacteria and host-specific *E. coli* are stable among different hosts of feces samples. Stable, as defined by this research project, characterizes the distribution of the measured bacterium that falls within a narrow range of the mean value for the measured values for that specific bacterium for a specific host. In terms of percent, if the mean percentage is 50% host-specific *Bacteroides* of total *Bacteroides*, a range of 20-70% would be sufficient for this table. However if the mean is 1% a range of .01-10% would be too large to make a conclusive range. The main hypothesis of this study is that the mean concentrations of host-specific and generic bacteria in each host will remain stable. If the hypothesis holds true, this study will provide a significant step to the future goal of using QPCR as a microbial source tracking method for detecting hosts and concentrations of fecal contamination in a watershed. In order to fully apply this QPCR approach to detect the concentrations of bacteria in a water sample, further research needs to look into the relative die off rates of each bacterium. Using the specific composition of bacteria in each host and the relative die-off rates of each bacterium, QPCR could be used to specify the animal host of the fecal contamination and would allow watershed and beach managements to remediate the non-point source pollutant. For instance, if human fecal concentrations are detected in a water sample a broken human sewage line could be remedied in order to reduce fecal contamination.

Another aim of this study is to determine how many environmental samples of water would need to be collected in order to be confident in the dominant source of feces in the watershed. This will be done by looking at the stability of the composition at different sample intervals of each host. The null hypothesis is if ratios vary drastically it will show that this QPCR approach may not be an efficient Fecal Source Tracking method. However, regular QPCR can still be applied to watersheds to determine host-specific *Bacteroides* and other bacteria but the detection and quantifying of each bacterium is a single PCR approach. Confirmation of the null hypothesis

will open up further research of the factors influencing the variation of bacterial compositions in different hosts.

Methods

Sampling Locations The locations for fecal sample collection for the different hosts were the following: Dog fecal samples were collected from Ohlone Dog Park in Berkeley, California; horse samples were collected from Skyline Ranch in Oakland, California; cow samples were collected from Briones Regional Park in Lafayette, California; bird, specifically goose, fecal samples were collected from Lake Merritt in Oakland, California; human samples were collected from human volunteers in a housing setting. By taking an online course through Collaborative IRB Training Initiative, the UC Committee for Protection of Human Subjects gave consent to sample feces from human volunteers.

Sample Collection Fecal samples were collected upon thirty minutes of excretion from the specific host. This was done to ensure that bacteria were still alive to be able to process culture tests and QPCR on the extracted DNA. Samples were handled with sterile gloves and placed in sterile plastic tubes. Approximately five grams of fecal matter were needed from each individual sample for data collection. Each sampling trip consisted of sampling four-five individual hosts. Fecal samples placed in tubes were immediately placed on ice ($\sim 3^{\circ}\text{C}$) in a cooler. This was done to ensure the samples remained fresh and that bacteria were alive. Once samples were collected they were transported within an hour back to the laboratory at UC Berkeley.

Dry Weight Upon arrival to the laboratory the first measurements made were dry weight of each fecal sample. Replicates were done for each sample and approximately 2.5 g were weighed out for each in sterile pans. Wet weight was measured and then replicates were placed in the drying oven at approximately 40°C for 24 hours and then measured again for dry weight. Samples were then placed back in the oven for one hour and measured again to see if dry weight decreased more than 1 mg. If so, samples were placed in the drying oven for another 24 hours and the process was repeated.

Culture Measurements Culture measurements were collected to compare concentrations of total *E. coli* and total *Enterococcus* with QPCR results for each host. Initial *E. coli* and *Enterococcus* concentrations were measured to make sure that there were sufficient bacteria to do quantitative measurements (QPCR) on the fecal samples. To accomplish this I made dilutions of different magnitudes of the fecal samples and used Colilert and Enterolert procedures as

culture tests. These data provide a quantitative measurement of the amount of *E. coli* and *Enterococcus* in each of the fecal samples. If sufficient bacteria were present in each of the dilutions, the samples were then prepared for DNA extraction and ultimately QPCR procedure.

Dilutions of fecal concentrations were made using deionized water and NaCl to create a concentration of NaCl similar to that of a bacterial cell at 6.5% (Scott et al., 2002) in 100 ml Colilert bottles. 1:10 dilutions were made from 0.250 g of fecal matter in 100 ml of deionized water. Using the IDEXX Colilert and Enterolert Water Quality measurement kit the dilutions were then placed in quanti-trays and a reagent was added to fluoresce the presence of *E. coli* and *Enterococcus* bacteria. These trays were then placed into an incubation chamber at 34°C and 41°C for *E. coli* and *Enterococcus* respectively, for 24 hours. After the incubation time, trays were analyzed for fluorescence by counting wells that fluoresced under Ultra-Violet light using a UV lamp (IDEXX Laboratory Inc., 2006). These values were then plotted onto a standard table to be converted into Most Probable Number (MPN) of bacterial cells of *E. coli* and *Enterococcus* per gram dry weight of feces. To convert the MPN value into a concentration per gram of dry feces the ratio of the average wet weight to dry weight of the host was used for conversion and dilutions were accounted for. The IDEXX Colilert method has been approved by the US-EPA as an efficient method of measuring concentrations of *E. coli* and *Enterococcus* in a water sample. I recorded these values to be later compared with concentrations of *E. coli* and *Enterococcus* using QPCR.

DNA Extraction Sterile procedures were needed for this set of methods in order to avoid obtaining false-positives for bacteria concentrations. This included using sterile gloves at all times and wiping down all lab equipment with ethanol. A Mo-Bio Fecal DNA Extraction kit was used for extraction of DNA from fecal samples. The purpose of the extractions was to isolate PCR compatible genomic bacterial DNA from the fecal samples. The protocol used for this procedure was set by the Mo-Bio kit, which also included reagents and tubes for the procedure (Mo-Bio Laboratories Inc., 2006). Replicates of 500 mg of each fecal sample were placed in bead beating lysis tubes. To lyse the cells, lysis, Inhibitor Removal and bead solutions were added to the tubes. To further lyse the cells the tubes were mechanically vortexed for 3-5 minutes at a time. The DNA would then be washed through a silica spin filter and vortexed again to separate the DNA from solution. The extracted DNA samples at approximately 0.05 ml were labeled and placed in a freezer, ~3°C, until QPCR was done on the samples. The Mo-Bio

Extraction kit has been used by numerous law enforcement agencies and commonly by zoologists.

QPCR This portion of the study was done by the Graduate Student, Sarah Silkie. Professor Kara Nelson in the Department of Civil and Environmental Engineering reported a PCR assay for the detection of *E. coli*, which was used for the QPCR of *E. coli* in this study (Nelson et al., 2006). Primer sequences were used from two previous studies for the host-specific *Bacteroides* bacteria for the different hosts (Bernhard and Field 2000; Seurinck et al. 2005). QPCR were run for each host-specific *Bacteroides* and for bird-specific *E. coli* for geese. QPCR was also done for *Enterococcus*, *E. coli*, Total *Bacteroides* and Total Bacteria for each fecal sample. Controls of known bacteria, *Pseudomonas syringae*, were done with each QPCR cycle to eliminate the possibility of false-positives in the data. QPCR data presented in this paper are initial cell concentrations of total *E. coli* per gram dry weight for goose and dog.

Analysis QPCR data gave me quantitative values of bacteria in each of the samples. QPCR data were compiled for each host and analyzed to determine a stable composition of general indicator bacteria and host-specific bacteria for 16 DNA samples for each host. To determine stability the best statistical approach for these data was to calculate the means for each host and calculate the standard deviations with 95% confidence. An ANOVA (Analysis of Variance) test was performed using separate variables as the different bacteria to determine if the percent compositions vary significantly from sample to sample in each host.

Results

Culture Measurements Ratio of *Enterococcus* to *E. coli* The ratio of *E. coli* to *Enterococcus* concentrations (EC:ENT) was used to differentiate human from non-human sources of fecal contamination. *E. coli* bacteria species, which constitutes most of Fecal Coliform, and *Enterococcus* bacteria, which constitute most of Fecal Streptococcus, were quantified using Colilert and Enterolert data and an MPN Calculator.

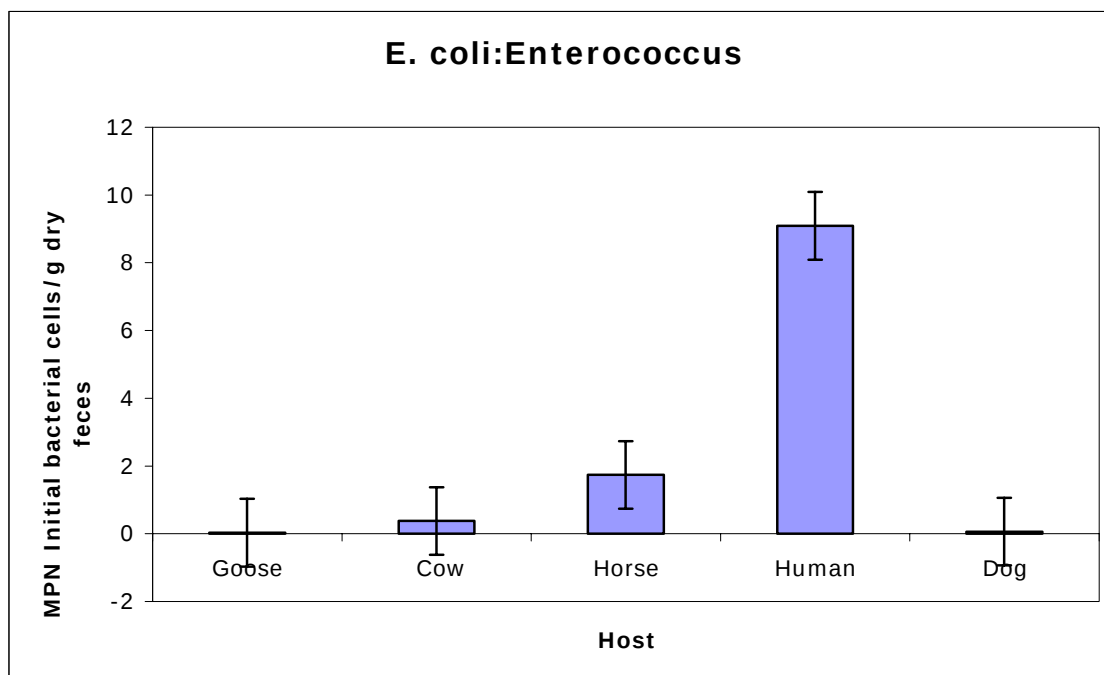


Figure 1. Graph of mean values of ratios of *E. coli* to *Enterococcus* in measured hosts. Values are Most Probable Number of cells per gram dry weight of feces (MPN/g dry feces).

Table 1. Average presence of *E. Coli* and *Enterococcus* and EC:ENT Ratio. Values are Most Probable Number of cells per gram dry weight of feces (MPN/g dry feces).

Host	Goose	Cow	Horse	Human	Dog
MPN <i>E. coli</i> /g dry feces	8.18E+01	7.31E+04	5.88E+03	2.36E+04	3.24E+04
Std. Dev.	1.53E+01	1.04E+05	8.25E+03	2.39E+06	3.94E+04
MPN Ent/g dry feces	4.12E+03	6.51E+05	4.56E+03	3.26E+05	1.05E+06
Std. Dev.	2.57E+03	1.03E+06	3.93E+03	3.19E+05	1.80E+06
EC:ENT Mean	2.77E-02	3.75E-01	1.73E+00	9.09E+00	6.22E-02
Std. Dev.	1.87E-02	5.83E-01	1.41E+00	6.07E+00	9.87E-02

Human ratio of *E. coli* to *Enterococcus* (EC:ENT) showed a mean value of 9.09 Most Probable Number (MPN) of initial bacterial cells per gram of dry feces with a standard deviation of 6.07 (Table 1). Non-human mean ratios of EC:ENT were under 1 except for the value for horse with a mean ratio of 1.73 and standard deviation of 1.41 (Table 1). Relative ratios of EC:ENT show human to be vastly greater than the other non-human sources (Figure 1). Average values of MPN of initial *E. coli* cells per gram of dry feces revealed that cows have the highest concentration (7.31E+04), dogs to have the second highest concentration (3.24E+04) and humans to have the third highest (2.36E+04) (Table 1). Average values of

MPN of initial *Enterococcus* cells per gram of dry feces showed dogs to have the highest concentration ($1.05\text{E}+06$), cows to have the second highest concentration ($6.51\text{E}+05$) and humans to have the third highest ($3.26\text{E}+05$) (Table 1).

QPCR A standardized scatter plot was used to determine initial cell concentrations of each of the measured bacteria for each host. This scatter plot uses results of $\log_{10}$ cell equivalents of Total Bacteria, *E. coli*, *Enterococcus*, Total *Bacteroides*, host-specific *Bacteroides* and bird-specific *E. coli* on QPCR-measured cycle threshold values (Ct) for DNA extracts. The linear model for *E. coli* was developed by Sarah Silk (2006) for the purpose of this study. A linear regression model was used as a standardized plot to determine an estimated mean detection limit of cells for *E. coli* (Figure 2). The development of the linear regression used two different treatments to eliminate false-positives that are easily produced when amplifying the *uidA* gene for *E. coli*. These two treatments were ultrafiltration and the use of Dnase, which are plotted on the same graph as the no treatment (Figure 2). The use of ultrafiltration proved to be the most reliable in eliminating false-positives (Silk 2006).

Preliminary QPCR data collected for this study is total *E. coli* for the hosts: dog and goose. QPCR data was compared with culture data for initial cell concentration of total *E. coli* (Figure 3). Between QPCR and Colilert measured techniques, goose *E. coli* concentration differed by $1.26\text{E}+5$ cells and dog *E. coli* concentration differed by $1.54\text{E}+04$ cells. The number of samples for preliminary QPCR data for each host was 4. More QPCR data with a larger sample size will be collected for total *E. coli* to give a more accurate description of the concentration in these two hosts.

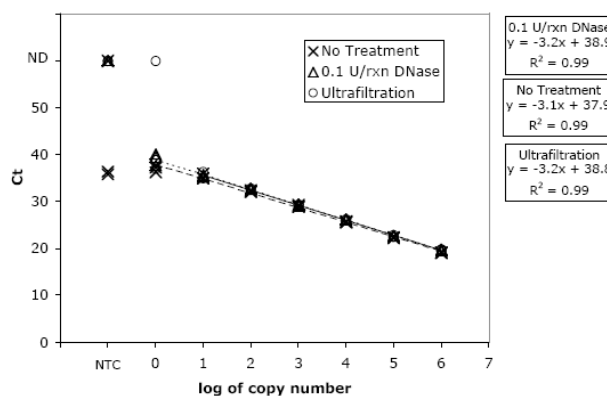


Figure 2. Effect of Decontamination Procedures on *E. coli* QPCR Method.

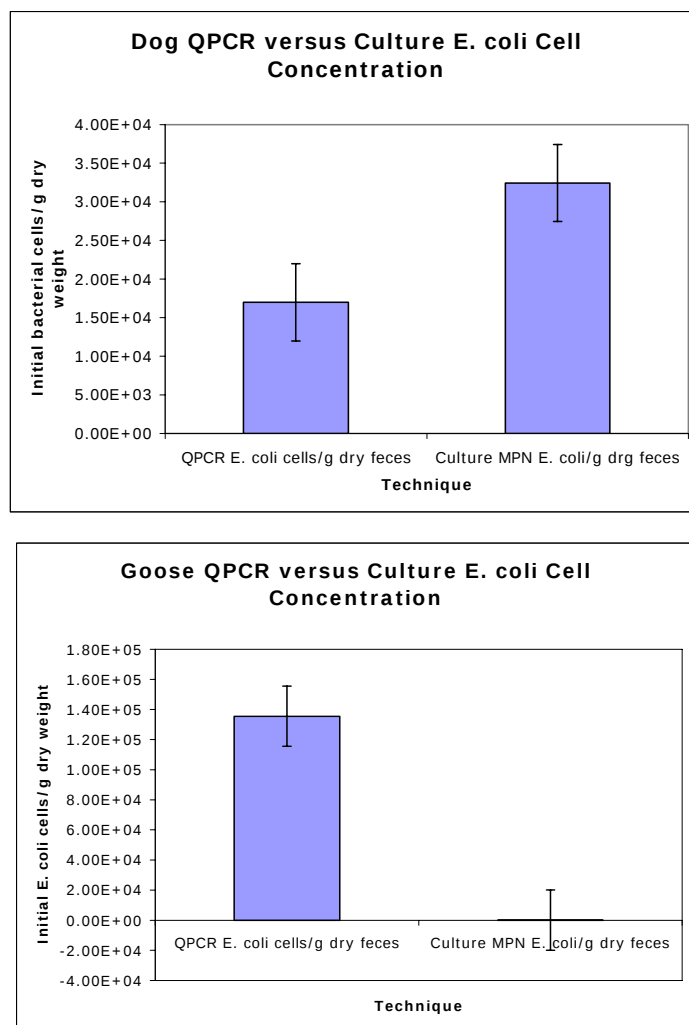


Figure 3. Comparison of quantification of cell concentration using QPCR and Culture (Colilert) for total *E. coli* for dog and goose.

Initial cell concentrations of DNA strands specific to each of the bacteria categories are averages of the 16 samples collected for each host (Table 2). This is the expected table this study seeks to accomplish once all QPCR data is collected. Non-shaded values are true values collected for total *E. coli* for dog and goose. Standard deviations will be calculated using 95% confidence distribution. If the mean of a specific bacterium composition is within a narrow distribution of the total data collected, this number will be used as a library for future source tracking in the environment. Percentages of host-specific bacteria will be determined by the fraction of, for example, human-specific *Bacteroides* to total *Bacteroides*.

With stable values of host-specific and generic compositions these numbers can be used as a library to help determine the dominant fecal source of an environmental sample. Also, analysis

of the future data can show how many samples would need to be collected in order to get an accurate description of the dominant source-pollutants.

Table 2. Expected average of initial bacterial cell concentrations per gram of dry weight using QPCR.

Host	Total Bacteria	<i>E. Coli</i>	<i>Enterococcus</i>	Total <i>Bacteroides</i>	Dog-specific <i>Bacteroides</i>	Human-specific <i>Bacteroides</i>	Cow-specific <i>Bacteroides</i>	Bird-specific <i>E. Coli</i>	Horse Specific <i>Bacteroides</i>
Dog	10 ⁵	3.52E+03	10 ⁵	10 ⁸	10 ⁹	10 ²	0	0	0
Human	10 ⁷	10 ⁹	10 ⁷	10 ⁹	10 ¹	10 ⁹	0	10 ¹	0
Cow	10 ⁸	10 ²	10 ⁶	10 ⁹	0	0	10 ¹⁰	10 ²	10 ¹
Horse	10 ⁶	10 ³	10 ¹⁰	10 ¹⁰	0	0	10 ¹	0	10 ⁹
Goose	10 ⁴	8.18E+01	10 ⁹	10 ⁷	10 ¹	10 ³	0	10 ⁹	0

Discussion

The QPCR data for this study will hopefully provide a reliable genomic library of bacterial concentrations of the different hosts: dog, horse, cow, human and goose. The goal of this study is to provide a significant step toward evaluating the pollutants to a watershed. By determining the average values of bacteria from feces for each host measured this library can prospectively be used to evaluate the pollutants of an affected watershed.

The culture results for *Enterococcus* and *E. coli* provide a framework for the QPCR data. Previous studies have found stable Fecal Streptococci to Fecal Coliform ratios (FC:FS) for human and non-human sources as a tool for microbial source tracking (Sargeant et al. 1999, Scott et al. 2002). The Fecal Coliform, which is mostly *E. coli*, to Fecal Streptococcus, which is mostly *Enterococcus*, ratios (FC:FS) discovered by these studies were less than .7 for non-human sources and greater than 4 for human-sources. The ratios calculated in this study correspond to these ratios with human EC:ENT > 4 (human: 9.09), and non-human EC:ENT, < .7, (cow: .375, dog: .0622, goose: .0277), except for horse with a value of 1.73. Thus, the culture tests mostly match up to earlier studies and show that the ratios of *E. coli* to *Enterococcus* differ between human and non-human sources, and differ among the non-human sources as well. However this approach is misleading, unless the FC and FS die off at identical rates, the ratio will not mirror the initial cell FC:FS providing inaccurate results. It is difficult to determine the age of pollution of fecal sources due to the problem of differential die-off. The FC:FS and EC:ENT ratios provide a framework for this study and for future research, however it is an unreliable microbial source tracking method (Scott et al.

2002). This problem also arises with QPCR microbial source tracking, due to the generic and host-specific bacteria dying off at different rates. The ratios determined in this study reflect bacteria that are still alive due to fresh fecal collection and the placement of samples on ice.

Further analysis of the culture data shows cow fecal sources to have the highest concentration of *E. coli* cells per gram of dry feces (mean = $7.31\text{E}+04$) and dog to have the highest concentration of *Enterococcus* cells per gram of dry feces (mean = $1.05\text{E}+06$). Human and dog hosts also have high concentrations of *E. coli* with values of the same magnitude as cow *E. coli* concentration (human: mean = $2.36\text{E}+04$, dog: mean = $3.24\text{E}+04$). In terms of *E. coli* concentration, these host fecal sources are the most harmful when runoff into water. These three hosts, cows, humans and dogs, should be monitored more carefully when assessing bacterial contamination in an environmental water body and for remediation.

Preliminary dog QPCR data of initial *E. coli* cells per gram of dry feces slightly corresponds with the culture concentration of *E. coli* for dog (QPCR mean = $1.70\text{E}+04$, std. dev. = $5.13\text{E}+04$; Culture mean = $3.24\text{E}+04$, std. dev. = $3.94\text{E}+04$). With such a large standard deviation for QPCR, more samples need to be quantified to determine a more accurate value (QPCR sample size = 4). QPCR data for concentration of *E. coli* for goose does not correspond with culture results (QPCR mean = $1.36\text{E}+05$, std. dev. = $1.51\text{E}+05$; Culture mean = $8.18\text{E}+01$, std. dev. = $1.53\text{E}+01$). More samples also need to be quantified for goose in order to get a more accurate value for *E. coli* concentration (QPCR sample size = 4).

One of the questions of this study is to determine the number of samples that would need to be collected from a specific host in order to have values of initial cell concentrations with standard deviations that are not too large. By taking means of the initial cell concentrations at sample intervals of 4, 8, 12 and 16 we will be able to determine the approximate amount of samples needed from each host. With high variability in cell concentrations more samples would need to be collected for the host and analyzed for specific bacteria. If the number of samples that provide stable values is 8 one would need to collect at least 8 samples of water to evaluate the presence of the host fecal source-pollutant. Initial QPCR data for total *E. coli* concentration for dog and goose show a sample size of 4 to be inaccurate with a low correlation with culture data. The proposed library of bacteria composition for the common hosts and the average number of samples needed to collect to determine these values are not

the final steps toward evaluating the fecal contamination of an environmental water body. Further research needs to look at the relative die-off rates of each bacterium in order to make an accurate assessment of the initial bacterial cell concentrations in water.

The greatest challenge in this study is obtaining QPCR data that does not vary drastically and provides substantial quantitative values that would enhance the reliability of the developed library. In order to obtain average values of bacterial concentrations with little standard error, a greater number of samples should be collected. Future research can focus on this issue if this library does not hold for an environmental watershed.

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