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Factors Influencing Persistence of Fecal *Bacteroides* in Stream Water

Alyssa K. Bell
University of Tennessee - Knoxville

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To the Graduate Council:

I am submitting herewith a thesis written by Alyssa K. Bell entitled "Factors Influencing Persistence of Fecal *Bacteroides* in Stream Water." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Geology.

Larry McKay, Major Professor

We have read this thesis and recommend its acceptance:

Alice Layton, Ed Perfect, John McCarthy

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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**Factors Influencing Persistence of Fecal
Bacteroides in Stream Water**

A Thesis Presented for
the Master of Science
Degree
The University of Tennessee, Knoxville

Alyssa K. Bell
August 2007

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ABSTRACT

Fecal contamination leads to increased risk of exposure to enteric pathogens in aquatic environments used for drinking water, recreation, and commercial shellfishing. Current indicators of fecal contamination recommended by the EPA such as *E. coli* and enterococcus can fall short of meeting ideal indicator criteria by having widely-varying persistence in the environment, reproducing in the environment, occurring in the gut in low and variable concentrations, and requiring time-consuming assays. Furthermore, both these indicators lack the degree of host specificity needed for use in identifying sources of fecal contamination, which is an important tool for identifying and reducing fecal inputs to the aquatic environment. Preliminary studies have suggested bacteria belonging to the genus *Bacteroides* can be an alternative to current fecal indicators. This thesis assessed the utility of *Bacteroides* as a fecal indicator first through a review of the literature investigating the use of *Bacteroides* and highlighting current research needs. Next, a series of microcosm experiments investigating the effects of varying environmental parameters on the persistence of the *Bacteroides* 16S rRNA marker in stream water were performed. These studies used real-time PCR detection to develop decay curves for *Bacteroides* concentrations under different conditions of water filtration, initial fecal aggregate size, initial fecal concentration, temperature, and fecal source organism. Statistical models were used to determine the significance of the decay curves and identify those variables having the greatest significance to the rate of decline of

Bacteroides. The results of this study indicated that filtration, temperature, and the initial fecal aggregate size had significant effects on the rate of removal of the *Bacteroides* genetic marker from stream water. The decline was significantly less in filtered (0.25 log removal/day) versus unfiltered stream water (0.67 log removal/day), 5 °C (0.32 log removal/day) versus 25 °C (1.62 log removal/day), and coarse (0.62 log removal/day) versus fine aggregate size (1.41 log removal/day). The initial concentration of fecal matter and the host organism did not have significant effects on removal. This suggests that the primary mechanism behind decline of the *Bacteroides* marker in stream water is biologic removal, such as grazing by protozoa or infection by bacteriophage. Finally, this thesis presents the development of the EqBac assay, a new quantitative PCR assay using the *Bacteroides* 16S rRNA gene from horses to detect equine fecal contamination.

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CHAPTER I

INTRODUCTION TO FECAL CONTAMINATION IN THE ENVIRONMENT

Fecal contamination is a common problem in surface water systems. Waters with fecal contamination pose health risks through exposure to enteric pathogens and may also cause economic damage through the inability to use contaminated waters as recreational areas or sources of shellfish (EPA, 2005). Fecal contamination can originate from natural causes, such as storm water run-off containing waste from wild animals, or from human-influenced sources, such as agricultural runoff, leaky sewage systems, or septic tanks (EPA, 2005).

Some examples of enteric pathogens that can be introduced to water through fecal pollution include strains of the bacteria *E. coli*, *Shigella*, *Campylobacter* and *Salmonella*, the viral pathogens in the rotavirus and calicivirus families, and the parasites *Cryptosporidium* and *Giardia*. Exposure to these pathogens can result in gastroenteritis, meningitis and liver failure. In extreme cases, exposure may be fatal. Fecal contamination of municipal drinking water systems may result in disease epidemics that can affect hundreds to thousands of people. One such incident occurred in Walkerton, Ontario in May 2000. Walkerton is a town of 4800 residents located northwest of Toronto, Ontario. Storm-enhanced runoff from a cattle farm infiltrated a shallow drinking water supply well, contaminating the city's water with *E. coli* O157:H7 and *Campylobacter jejuni* (Hrudey et al., 2002).

As a result of this contamination, 2300 people reported experiencing symptoms of gastroenteritis, for which 65 people were hospitalized, 27 developed serious kidney complications, and 7 died. This episode represents one of the worst public health disasters in Canada (Ali, 2004). Other serious epidemics include the outbreaks in 1993 of cryptosporidiosis in Milwaukee, Wisconsin, and North Battleford, Saskatchewan (Hrudey et al., 2002).

The risks of fecal contamination are not limited to drinking water. Swimming or bathing in polluted waters can also cause pathogen exposure. Currently, gastroenteritis caused by consumption of waterborne enteric pathogens is the leading cause of death in children of third-world countries (UN Atlas of the Oceans, 2007). Every year an estimated 250 million cases of gastroenteritis globally can be attributed to contact with waterborne pathogens in the ocean (Shuval, 2001). This translates into \$1.6 billion/year spent globally in health expenses and lost wages (GESAMP, 2001). Also, shellfish can concentrate pathogens in muscle tissue, leading to exposure for those who consume undercooked or raw shellfish (Shuval, 2001). Exposures such as these constitute significant costs in terms of economic loss from health expenses and shellfishery closures. The economic loss to the global shellfish industry for the closure or restriction of fishing areas ranges from \$10 – 20 billion/year, while the consumption of contaminated shellfish costs approximately \$7.2 billion/year in health expenses (GESAMP, 2001). These economic losses and health risks point to fecal contamination of surface

waters as a water quality issue equal in importance to the supply of pathogen-free drinking water.

The U.S. Clean Water Act of 1977 was established to protect surface waters and ground water aquifers. While specific regulatory standards are established on a state-by-state basis, the EPA is charged with making quality assessment recommendations and overseeing water quality evaluations for the entire U.S. Current water quality evaluations indicate that within the United States of the 33% of assessed surface waters, 40% of river miles and 45-50% of lake and estuary square miles fail recreational or fishing quality standards, with fecal contamination constituting a primary cause of impairment. Also, an estimated 14% of U.S. ocean coasts are impaired by fecal pathogens, however only 6% of coast lines in the U.S. were assessed (EPA, 2002b).

In the state of Tennessee, 50% of stream miles and 99% of lake acres are assessed every five years for water quality (Denton et al., 2006). The most recently published report on Tennessee water quality labeled 46% of stream miles and 29% of lake acres as impaired or threatened for recreational quality standards (Denton et al., 2006). In Tennessee, geologic factors such as karst topography and anthropogenic factors such as agriculture and industrial pollution contribute to the susceptibility of groundwater to contamination from surface water (TDEC, 2003). This provides an important link between surface and ground water quality. The Tennessee Department of Environment and Conservation (TDEC, 2003)

reported 39% of groundwater supplies have a high risk of surface contamination and 59% have a moderate risk of surface contamination. These high values indicate that improved surface water quality is an important factor in controlling and protecting sources of drinking water in Tennessee.

Monitoring Fecal Contamination in Surface Water

The wide variety of pathogen types and testing methods required for direct pathogen monitoring make this approach to water quality assessment a difficult, expensive, and time-consuming process. As a result, pathogen monitoring is rarely carried out and instead the occurrence and concentration of “fecal indicators” are monitored. These indicators, usually bacteria such as *E. coli*, fecal coliform, or enterococcus, are much easier to detect and less expensive to monitor than pathogens. Ideally, a fecal indicator organism would meet a number of criteria. Indicator organisms should exclusively be from the intestines of warm-blooded animals and not found naturally in water, nor should they be able to grow in water (Maier et al., 2000). Thus, the use of organisms found only in fecal matter ensures that their presence in the environment is due to fecal contamination and not some other environmental source. Second, the density or concentration of indicators in fecal matter should reflect the degree of fecal pollution (Fiksdal et al., 1985). Indicators that meet this criterion can be used to establish quantitative guidelines for regulatory purposes. Also, indicator organisms should be present whenever

fecal pathogens are present, and should persist slightly longer than pathogens in the environment (Maier et al., 2000). The correlation of pathogens to indicators should ensure that samples testing negative for fecal indicators will not contain pathogens. Finally, testing for the indicator should be simple and inexpensive (Field et al., 2003b). Simple and inexpensive testing allows all source waters or water systems to be monitored, even water systems with relatively small budgets.

Current Fecal Indicators

A number of different fecal indicators are currently used. *Escherichia coli*, *Streptococcus. faecalis*, total coliform, fecal coliform and fecal streptococci are all used as indicator organisms (Maier et al., 2000). Of these, the EPA recommends the tests for *E. coli* and enterococcus for recreational and drinking water and the fecal coliform assay for shellfishing waters (EPA, 2002). The importance of indicator organisms in ensuring safe drinking and recreational waters justifies an evaluation of current indicator organisms in order to better understand research needs in water quality analysis. A comparison of current indicator organisms to the ideal indicator criteria discussed above can be found in Table 1.1.

The first requirement of a fecal indicator is that it only occur in fecal matter, however none of the current indicators meet this criterion. *E. coli* and enterococcus have both been found in algal mats in Lake Michigan (Whitman et al., 2003) and in a variety of refuse flies (Szalanski et al., 2004). While

Table 1.1. Comparison of recommended fecal indicators to the ideal indicator criteria collected from Maier et al., 2000; Field et al., 2003a; and Scott et al., 2002.

Current Indicator Organisms

		<i>E. coli</i>	Enterococcus	Fecal Coliform
		Ideal Indicator Criteria	1 strictly enteric life-history	NO - found in chlorophytes and flies
2 No growth outside host	NO - regrowth in wide variety of environments	MIXED - regrowth observed in beaches, not in freshwater streams	NO - regrowth in wide variety of environments	
3 constant density	NO - low, variable density	NO - low, density	NO - low, variable density	
4 correlate to pathogen presence	MIXED - correlated to disease outbreaks, not to pathogens	MIXED - correlated to disease outbreaks, not to pathogens	NO - not correlated to disease outbreaks or pathogens	
5 slightly out-persist pathogens	NO - longer persistence than pathogens	NO - longer persistence than pathogens	NO - longer persistence than pathogens	
6 fast, inexpensive testing	Cheap - YES Fast - NO	Cheap - YES Fast - NO	Cheap - YES Fast - NO	

flies are unlikely to contribute significantly to environmental levels of indicator organisms, algal mats have been shown to pose a potentially significant environmental source. Lake Michigan traditionally has high levels of fecal contamination, as measured by levels of *E. coli* and enterococcus.

While the level of fecal pollution in Lake Michigan might be legitimately high, this cannot be determined using organisms that naturally occur in the prevalent algal mats present in the lake and on the beach (Whitman et al., 2003). The fecal coliform assay, the standard for shellfishing waters, tests for a number of enteric bacteria including *E. coli*. In addition to being subject to the limitations imposed by *E. coli*, other bacteria associated with this assay, such as *Klebsiella* and *Enterobacter*, are found naturally in sediments and groundwater (EPA, 2002), as well as plant material and paper mill effluent (Doyle and Erickson, 2006).

It is important that within fecal matter the indicator is found in a consistent density capable of predicting the density of environmental contamination. Indicators must also be present in large enough numbers to be easily detected after dilution in the environment. The density of pathogens in one individual host must be similar to the density in another individual host. Again, none of the present indicators meet this requirement. *E. coli* occur in low densities that vary widely with the organism's health. A study of mice found approximately 10^3 *E. coli* cells/gram of tissue sampled in all healthy adult mice, however this number increased dramatically in sick mice (Schaedler et al., 1965). As *E. coli* is one of the bacteria included in the

fecal coliform assay, the density of fecal coliform bacteria also varies with an individual's health. Furthermore, the density of *E. coli* recovered from an environmental sample does not reflect the level of contamination due to its ability to grow in the environment (Shanks et al., 2006). While enterococcus is present in larger quantities averaging 10^6 cells/gram fecal matter, this number varies over 5 orders of magnitude in human subjects (Donskey et al., 2000). These studies show that current indicators do not exhibit a strong density relationship with fecal matter.

An important criterion with implications for both persistence and environmental occurrence is the inability of the indicator organism to reproduce or survive outside of the host. If capable of reproducing in the environment, the organism is no longer an accurate indicator of fecal pollution and cannot be correlated with the presence of pathogens. Within the current literature about the survival of *E. coli* and enterococcus there exists some debate as to the environmental conditions under which the organisms may or may not grow. A number of field and lab studies of temperate streams and rivers and subtropical estuaries and fresh waters all indicate that while *E. coli*, enterococcus, and other fecal coliform bacteria may persist for days to weeks in the environment, they do not grow or reproduce (Davenport et al., 1976; McFeters and Stuart, 1972; Anderson et al., 2005). However, many more studies have documented the growth of *E. coli* or enterococcus in a variety of environments. Subtropical and temperate estuaries (Rhodes and Kator, 1988; Desmarais et al., 2002), tropical rain

forests (Carrillo et al., 1985), Antarctic streams (Smith et al., 1994), groundwater systems (Banning et al., 2002), and drinking water treatment facilities (LeChevallier et al., 1996) are examples of some of the diverse environments in which currently recommended indicators have been documented to grow.

The disparity of these results (i.e., growth versus non-growth in the environment) can be attributed to sampling methods employed in the studies. With one exception, all studies that did not document growth tested water samples that were in some way kept restricted from sediment. The exception to this evaluated persistence using the number of culturable cells retrieved from daily and weekly samples (Anderson et al., 2005). This method would fail to account for viable but non-culturable cells, and so the potential for growth cannot be ruled out. All studies that documented the growth of *E. coli*, enterococcus, or fecal coliforms sampled sediment or soil associated with water. A study by Desmarais et al. (2002) demonstrated that while *E. coli* would not grow in water samples alone, the addition of sterile sediment and the simulation of tidal wetting and drying stimulated significant growth of *E. coli* and other fecal coliforms, as measured by culture assays. Despite earlier research that indicated a lack of growth in the environment, recent research has shown that, at the very least, soil or sediment increases the persistence of current indicators, and that in many cases growth is observed. Evidence indicating the ability of currently recommended fecal indicators to grow in the environment implies that occurrence of these indicators is not

restricted to fecal contamination. Additionally, even a very small amount of growth will result in a very different persistence pattern of indicators as compared to pathogens.

For a fecal microorganism to be an effective indicator of pathogens it is necessary that occurrence of the indicator correlate with occurrence of pathogens. Hence, direct assessment of the correlation between current indicators and disease is also desirable. However, given the wide variety of pathogens, often with large differences in density and persistence, it is highly unlikely that a single indicator organism will correlate to all pathogens. A number of epidemiologic studies have correlated sickened beach users with *E. coli* and enterococci counts (Pruss, 1998; EPA 2002). However, more direct studies focusing on actual pathogens rather than disease have found *E. coli* is not correlated with the presence of *Cryptosporidium* oocysts (Bonadonna et al., 2002), *Campylobacter* spp., *Giardia* spp., noroviruses (Horman et al., 2004), and *Yersinia enterocolitica* (Lund, 1996) in the environment. While fewer studies have been published on enterococcus, it has been shown not to correlate with the presence of enteroviruses (Noble and Fuhrman, 2001), but it does have a better correlation with a variety of other pathogens than that demonstrated by *E. coli* (Cabelli et al., 1982; Pruss, 1998). Regardless of the indicator's ability to grow or reproduce in the environment, the overall persistence of the bacteria must correlate with that of pathogens. Several studies have found that both *E. coli* and enterococcus can persist in a viable, non-culturable state much longer than most

pathogens (Lund, 1996; Hartke et al., 1998; Desmarais et al., 2002), but they do not persist as long as *Salmonella* (Rhodes and Kator, 1988). These findings imply that a positive assay of current indicator bacteria does not necessarily indicate the presence of some pathogens, while a negative assay does not rule out the possibility of *Salmonella* contamination.

A final aspect of indicator criteria is the cost and time involved in detecting the organism. Assays that are expensive or require highly trained technicians are not practical options for many water treatment and monitoring facilities. All current indicators were chosen because they meet the low cost criterion. The *E. coli*, enterococcus, and fecal coliform assays are inexpensive culture-based methods that require very little training. However, the culture requires 24 to 48 hours to grow, meaning that contaminated water would not be recognized as such for up to 48 hours after sampling. All of the criteria discussed above are necessary for the accurate assessment of surface water quality. The failure of current indicators to meet these criteria demonstrates a need for more appropriate indicators.

Microbial Source Tracking

Identification of fecal contamination sources (i.e., human, cattle, etc.) can assist environmental regulators and watershed management groups in reducing contamination by focusing cleanup efforts on major fecal sources. The use of host-specific fecal indicators to identify sources of pollution in a watershed has been termed microbial source tracking (MST). MST is based

on the assumption that appropriate indicators may be found that allow fecal pollution to be characterized in terms of the host of origin (Simpson et al., 2002). While a wide variety of chemical and microbial indicators have been proposed for MST (Scott et al., 2002), this review will focus on bacterial source tracking with current fecal indicator bacteria.

A number of different methods of source tracking exist that can generally be described as using either genotypic or phenotypic characteristics to identify the indicator organism. Genotypic methods, such as the polymerase chain reaction (PCR), rely on the identification of some portion of the organism's genome, while phenotypic methods, such as antibiotic resistance analysis, rely on the identification of the products of a biologic process that differ according to the source of the organism. Methods that fall into either of these categories can be further described as being culture and library dependent or independent. In general, most authors agree that indicators using genotypic, culture independent assays, which do not depend on a local fecal library, are the most useful for microbial source tracking (Scott et al., 2002; Field et al., 2003a; Griffith et al., 2003). Methods such as these avoid the time-consuming process of culturing samples and do not require the collection of an extensive local reference library, which can be expensive, time-consuming, and result in sampling biases (Simpson et al., 2002). Specific methods that have been recommended for MST include a number of PCR methods such as host-specific analysis of the 16S rRNA

gene and terminal restriction fragment length polymorphism (T-RFLP) (Field et al., 2003a).

The primary assumption of microbial source tracking is that enteric microbes exist that reflect host fecal origins (Simpson et al., 2002). The degree of host specificity can be as general as human versus non-human, or as specific as differentiation to the species level. Because of its importance to microbial source tracking, a more detailed look at the viability of this assumption is warranted. In warm-blooded animals, 500 - 1000 different species of bacteria (Xu and Gordon, 2003) typically make up 25 - 50% of the total content of the intestinal tract (McBee, 1971). The bacteria in the intestine are under intense selective pressure to evolve symbiotically with the host in order to survive (Xu and Gordon, 2003). Evidence of this symbiosis can be found in the bacterial-influenced development of the host's gastrointestinal system after birth (Hooper and Gordon, 2001) and the constant fluctuations in the distributions of bacteria throughout the intestinal tract in response to changes in the host environment (Xu and Gordon, 2003). The genetic incorporation of this host-specific symbiosis in the bacterial genome has until recently been overlooked because it contradicts the clonal paradigm of bacterial inheritance (Gordon and Lee, 1999).

The clonal paradigm emphasizes the importance of asexual reproduction to bacterial evolution. Asexual reproduction causes vertical transmission of genetic information from a common ancestor without a significant role for recombination events in bacterial evolution (Orskov and

Orskov, 1983). This view does not allow for genetic diversity within a bacterial species as a result of niche specialization, such as host-specificity. The main support for the clonal paradigm comes from multilocus enzyme electrophoresis (MLEE) studies that find high levels of linkage disequilibrium between allozymes in natural bacterial populations (Guttman, 1997). Linkage disequilibrium, the nonrandom distribution of certain combinations of alleles in a population, is significant because it is a common result of strictly asexual reproduction. Recombination is the genetic counterbalance to linkage disequilibrium, a process by which alleles in a genome are randomly rearranged. This has the effect of reducing divergence and linkage disequilibrium across a species (Guttman, 1997). In eukaryotes, recombination usually occurs during meiosis between genetic material obtained from each parent. Bacteria are haploids and so cannot undergo this type of recombination; however they can acquire additional genetic information from the environment (transformation), directly from another bacterial cell (conjugation), or from a bacteriophage (transduction). The clonal paradigm recognizes that these processes occur in bacteria; however their importance is seen as minimal because of the observed high levels of linkage disequilibrium (Orskov and Orskov, 1983). The clonal paradigm indicates that the development of a high degree of host-specificity is not possible under predominantly asexual, vertical transmission of genetic material.

The availability of genome sequence data over the last ten years has drastically changed our understanding of genetic processes within bacteria. Sequence studies have found evidence for extensive recombination events within a variety of bacterial species, a finding at odds with MLEE data of linkage disequilibrium and the entire clonal paradigm (Bisercic et al., 1991; Dykhuizen and Green, 1991; Guttman, 1997). To address these contradictory data, Maynard Smith (1991) proposed a new model of bacterial population genetics that highlights the importance of processes that allow for linkage disequilibrium and recombination. Of primary importance to this model is the existence of ecologic population structure that is genetically transcribed in bacteria. Population structure is capable of producing linkage disequilibria through recurrent selective sweeps, or periodic evolutionary events (Guttman, 1997; Gordon and Lee, 1999). Selective sweeps are the rapid and complete spread of an advantageous allele throughout an ecologically distinct population, resulting in the loss of genetic variability at selected and linked alleles (Guttman, 1997). An example of this process comes from the spread of antibiotic resistance in bacteria such as *Streptococcus* and *Penicillium* (Maynard Smith, 1991).

Under the new model of bacterial population genetics, ancestral genetic sweeps within a restricted ecologic population, such as a single type of host organism, would result in the development of bacteria specialized to specific niches, or to a single host group. The new traits would be non-advantageous outside of the restricted population and thus result in the

establishment of genetically unique populations (Guttmann, 1997). In these settings linkage disequilibrium would be a more recent development of bacteria descended from a common ancestor unique to each population (Gordon and Lee, 1999). This model reconciles seemingly contradictory data regarding the prevalence of both high levels of linkage disequilibrium and recombination in bacteria.

A primary test of this model that is of direct importance to microbial source tracking is the identification of ecotypic population structure within bacterial groups, such as host-specificity. This will validate the primary assumption of microbial source tracking as well as the current model of bacterial population genetics. Studies of *Rhizobium*, a bacterium symbiotically associated with host plants for the fixation of nitrogen, are specific to the host species level (Wernegreen et al., 1997). A broader study directly applicable to microbial source tracking examined the genetic structure of five enteric bacteria from a variety of Australian mammals to determine the extent of variability that could be attributed to host specificity (Gordon and Lee, 1999). This study found that a range of 0.4% to 19.8% of genetic variability was associated with the host family. Genetic variability was the most strongly associated with host family in *Klebsiella pneumoniae* and least associated in *Klebsiella oxytoca* (Gordon and Lee, 1999). While the bacteria considered in this study all have a similar life history, large variations in ecologic niche specialization were found to exist. While some bacteria were very specialized, others were not. The current model of

bacterial population genetics can explain this discrepancy as resulting from the occurrence of selective sweeps in some bacteria but not others, perhaps due to host-bacterial interactions or host-host interactions (Gordon and Lee, 1999). The implications of this for microbial source tracking are profound, and indicate that the selection of microbes for targeted tracking must take into account the population structure of the organism and the relationship it has with its host. Genetic methods of microbial source tracking identify the expression of this population-level genetic host-specificity.

The current model of bacterial population genetics mandates that in addition to the indicator criteria discussed above, microbes for MST must also exist in genetically distinct populations associated with their host organism. Current fecal indicator bacteria such as *E. coli*, enterococcus, and other coliform bacteria can be evaluated with this new criterion. A review of current MST methods for *E. coli* indicate that using pulse-field gel electrophoresis (PFGE), the hosts of 100% of samples could be correctly identified to the species level, while the accuracy of the other six methods tested ranged from 13% to 62% (Stoeckel et al., 2004). However, PFGE has been criticized as a time-consuming and labor-intensive library-dependent assay that is disadvantageous for most MST studies (Field et al., 2003a; Scott et al., 2002; Simpson et al., 2002; Griffith et al., 2003). Using DNA fingerprinting of *E. coli* 65% of isolates could be arranged into host specific groups (McLellan et al., 2003), however fingerprinting is another library-dependent method that is not ideal for MST (Scott et al., 2002; Simpson et

al., 2002). PCR methods on the toxin gene in *E. coli* found that 70% of samples were correctly identified, but 50% of positive samples were false-positives (Field et al., 2003a). A more direct evaluation of host-specificity in *E. coli* as an MST indicator would be a genetic analysis of genome-wide variation. Gordon and Lee (1999) found that only 6% of the observed genetic variation within the *E. coli* genome could be explained by host family variability, while up to 50% was explained by temporal variability (Gordon, 2001). The low level of genetic host specificity demonstrated by *E. coli* shows that this organism is not ideal for microbial source tracking. Enterococcus exhibits some degree of host specificity (Willem et al., 2000; Bruinsma et al., 2002), however studies indicate a large overlap between strains from humans with those from pigs and some overlap between human strains and cattle and poultry strains (Heuer et al., 2006). Additionally, a study of different gene clusters found a uniform distribution of genetic material, and an accompanying lack of host-specificity, across human, animal, and sewage sources (Werner et al., 1997).

Summary

The contamination of surface water systems by fecal matter poses a health risk and causes global economic loss every year through health expenses and the closure of shellfishing waters and public recreational areas. In order to assess the dangers posed by water systems and to remediate existing contamination problems, fecal indicators that provide accurate estimates of

fecal pollution and allow the identification of potential sources of contamination are needed. Currently recommended indicators, such as *E. coli* and enterococcus, have been shown to be unreliable due to reproduction in the environment and a lack of host specificity, suggesting a need for more reliable indicators that provide a more accurate assessment of fecal contamination in water. This study will address this need through a review of research describing one such indicator, *Bacteroides*, as well as investigate the survival of this indicator in stream water.

CHAPTER II

REVIEW OF THE BACTERIAL GENUS *BACTEROIDES* AS FECAL INDICATOR ORGANISMS

Abstract

Current fecal indicators *E. coli* and *Streptococcus* are recommended by the EPA for the assessment of surface water quality; however these indicators fail a number of indicator criteria including reproduction in the environment and presence in low and highly variable concentrations within the host organism. These shortcomings indicate a need for a better fecal indicator that will more reliably and accurately predict the quantity of fecal contamination without significant false positive or negative results. One such indicator, *Bacteroides spp.*, has appeared in the literature repeatedly in recent years. In order to fully evaluate the utility of this genus as a fecal indicator and assess areas of weakness upon which future research should focus, this chapter will provide a review of research into the use of *Bacteroides* as a fecal indicator for water quality analysis and microbial source tracking.

***Bacteroides* as a Fecal Indicator**

Comparison of the life history and ecology of *Bacteroides* to fecal indicator criteria as described by Maier et al. (2000) and Fiksdal et al. (1985) can provide an assessment of the utility of *Bacteroides* as a fecal indicator. The first and most obvious criterion is the occurrence of the bacteria in the

intestines of warm-blooded animals. *Bacteroides* are commensulate bacteria present throughout the intestines of all mammals and birds from a very young age through death and are not endemic to other environments (Schaedler et al., 1965; Holdeman et al., 1976). Second, the organism should not be able to reproduce in aerobic environments, such as surface waters. *Bacteroides* are non-spore-forming obligate anaerobes, indicating that they should not be able to metabolize or reproduce outside of the intestine (Holdeman et al., 1976; Kreader, 1998). However, recent research has indicated that some *Bacteroides* species do have a limited ability to grow in anoxic microenvironments within aerobic environments. *Bacteroides* cells have been found in the interiors of aerobically-grown microbial granules (Tay et al., 2002). The cells were thought to originate from activated sewage sludge used to inoculate the granules. As soon as granules became large enough to contain an anoxic interior region (>250 μm diameter), bacteria transported from the sewage sludge were able to grow (Tay et al., 2002). In addition, a direct evaluation of the potential for *Bacteroides* to grow in aerobic microcosm environments found growth of *Bacteroides* in the lower, anaerobic portion of aerobically incubated sewage sludge (Walter and Field, 2006). These combined studies imply *Bacteroides* are potentially able to survive transport through aerobic environments in a vegetative state.

There are two important implications for these studies to the use of *Bacteroides* as a fecal indicator. First, *Bacteroides* may grow in anoxic microenvironments, such as biofilms or fecal aggregates, within surface

water systems. Second, *Bacteroides* may grow in stagnant pockets within estuaries, bays, or lakes (Walter and Field, 2006). Both of these situations could result in assays that indicated water contamination in either inflated levels or where no contamination was present. Considering the likelihood of either of these situations arising and further investigating these situations experimentally is critical to assessing *Bacteroides* utility as a fecal indicator.

In the environment, biofilms are the most likely microenvironment for *Bacteroides* to survive and grow in. Evidence of increased *Bacteroides* concentrations, as detected by real-time PCR, in biofilms has been attributed to cell survival in anaerobic microenvironments within the biofilms (Savichtcheva et al., *in press*). A study of *Bacteroides* survivability in the water column of surface water would be of direct importance in evaluating the importance of anaerobic microenvironments to inflating *Bacteroides* concentrations in water samples. To date, such a study has not been carried out using molecular methods, however a molecular study of *Bacteroides* survival in tap water has been conducted (Savichtcheva et al., 2005). While tap water is very different chemically and ecologically from stream water, it is still an oxygenated aquatic system, and so has some relevance to environmental applications. This study used a combination of live/dead staining and fluorescence in situ hybridization with *Bacteroides*-specific probes to assess the survival of *Bacteroides* in stagnant tap water (Savichtcheva et al., 2005). Live *Bacteroides* cells from fecal matter were detected after four hours in tap water, but none survived longer

(Savichtcheva et al., 2005). This indicates inflated *Bacteroides* concentrations resulting from growth in anoxic microenvironments may be restricted to locations with extensive biofilm growth.

The third criterion for an ideal indicator bacteria is its occurrence at a constant density that, once introduced to the environment, reflects the level of fecal contamination. *Bacteroides* occur in a predictable concentration constituting approximately 33% of fecal matter weight (Madigan et al., 2003). The arithmetic mean of coliform bacteria per gram of human feces is 1.9×10^7 , while the concentration of the *Bacteroides fragilis* group alone is 1000 times greater than that of coliforms (Fiksdal et al., 1985). The exact density of *Bacteroides* varies two orders of magnitude within a host species and four orders of magnitude across mammalian hosts (Layton et al., 2006). This variance is much more constrained than that observed in *E. coli* (Schaedler et al., 1965) and *Streptococcus* (Donskey et al., 2000), making *Bacteroides* a more useful indicator for quantifying fecal contamination.

Fourth, the presence of the organism should correlate to the presence of pathogens. This is a difficult criterion to assess due to the vast number of possible enteric pathogens. The three main size classes of pathogens, viruses ($<1 \mu\text{m}$), bacteria ($1 - 5 \mu\text{m}$), and protozoa ($5 \mu\text{m} - 1 \text{mm}$), have very different transport behaviors in subsurface and surface waters (Maier et al., 2000). This makes the likelihood of identifying a single organism from any of the above size classes that accurately predicts the occurrence of the others very unlikely (Boehm et al., 2003). Therefore, *Bacteroides* may not be an

applicable indicator for viruses and protozoa (Boehm et al., 2003). However, both EPA-recommended indicators are bacteria and are therefore subject to these same limitations. The presence of *Bacteroides* should also correlate to the presence of similar sized pathogens, such as the bacteria *E. coli* O157:H7 or *Salmonella*.

To date only one study has directly evaluated the correlation of *Bacteroides* to the presence of surface water pathogens. Using real-time PCR assays, Savichtcheva et al. (*in press*) tested approximately 70 environmental and water treatment facility samples for the presence of *Bacteroides* from humans, cows, and pigs, as well as a suite of bacterial pathogens. The study found *Bacteroides* was a good predictor of the presence of *E. coli* O157: H7 and *Salmonella*, but not of *Clostridium perfringens*. Additionally, the pathogens *Shigella* spp., *Staphylococcus aureus*, and *Vibrio cholerae* were not detected in any samples (Savichtcheva et al., *in press*). While this is the first study of its kind, it presents promising evidence that the presence of *Bacteroides* genetic markers correlates to the presence of pathogens.

Additionally, the host specificity of *Bacteroides* has the potential to increase the correlation of *Bacteroides* strains from a host with any pathogenic bacteria for which that host is a reservoir. A recent study examined the correlation of samples that tested positive for cattle-hosted *Bacteroides* and samples that contained *E. coli* OH157:H7, for which cattle are the main reservoir (Walters, 2007). None of the samples negative for

bovine *Bacteroides* were positive for pathogenic *E. coli*, however only in 6% of the samples positive for bovine *Bacteroides* was pathogenic *E. coli* also present, indicating that presence of a positive bovine assay was not sufficient to predict the presence of pathogenic *E. coli*. While this may seem a very low correlation, it is reasonable given the low density of pathogens in fecal matter as compared to *Bacteroides* (Walters, 2007). Also, when considering pathogens it is more important that an indicator return no false-negatives than no-false positives, which is indicated in Walters' study (2007).

The fifth criterion necessitates fast, inexpensive testing methods that result in accurate and precise measurement of fecal contamination. Currently, EPA-recommended indicator assays are culture-based methods (EPA, 2002). As an anaerobe, culturing *Bacteroides* is very difficult, making it impractical as a fecal indicator (Fiksdal et al., 1985). The development of molecular methods for nucleic acid replication and identification has promoted the use of organisms previously impractical as indicators, such as *Bacteroides* (Kreader, 1995). Quantifiable real-time PCR (qPCR) lends a particular utility to this indicator organism for numerous reasons that will be discussed in depth below. In the assessment of *Bacteroides* as a fecal indicator, it is sufficient to recognize that qPCR allows the quantification of *Bacteroides* in an environmental sample in a few hours, making these assays faster with better precision and accuracy than culture-based assays. The final criterion for utility as an ideal indicator organism is an understanding of the persistence of the organism in the environment. To date, for *Bacteroides*

such studies have not been prevalent in the literature. The growth of *Bacteroides* in stream environments as well as the persistence of inactive or dead cells must be thoroughly evaluated. Thus, this study was undertaken with the intent of better characterizing the persistence of *Bacteroides* so that it will have greater utility as a fecal indicator.

Characterization of the Persistence of *Bacteroides*

A primary shortcoming in the use of *Bacteroides* as a fecal indicator is the current absence of data regarding the transport and persistence of *Bacteroides* in stream water. Without some idea of how long the *Bacteroides* genetic marker persists after the initial contamination and the change in marker concentration as time passes, identifying the source of contamination in a watershed can be difficult. In addition to this basic level of understanding, more specific information on the variability of persistence with changing environmental factors is highly desirable. In particular, predicting how marker concentrations change over time and distance in a stream may enable better resolution of source identification in microbial source tracking studies.

Factors Affecting Bacteroides Persistence

Bacteroides may enter a stream from runoff of fecal matter deposited on adjacent land or from direct deposition in surface water. In either scenario, *Bacteroides* are a constituent of particulate fecal matter. As such, the persistence and transport of the bacteria through the water system is affected

by a number of physical and chemical parameters of the stream as well as biotic parameters related to predation within the fluvial ecosystem. These factors may affect the bacteria directly through the destruction or consumption of the bacteria (i.e., by primary factors), or indirectly, through the inhibition of the primary agent.

Many basic properties of stream water might act as primary or secondary factors limiting the persistence of *Bacteroides* genetic markers. Water velocity affects the dispersion and composition of micro-communities and detritus within the channel. An increase in flow velocity can alter the composition of a community by flushing organisms and detritus out of the system (Brookes et al., 2004). Turbulence can act to both break up fecal aggregates and reduce aggregate settling, thus increasing the exposure of *Bacteroides* to grazers in the water column and decreasing marker persistence (Zimmerman-Timm, 2002). Organic carbon is another important factor in a stream related to the amount of dissolved organic matter (DOM) present. Organic matter enters a stream as a result of storm water drainage and seasonal patterns in ecosystem productivity (Allan, 2004). Fecal contamination in particular can be a large source of carbon for a stream and as such is an important source of food and habitat for protozoa and bacterial communities (Sigee, 2005). Fluctuations in temperature are part of seasonal changes that cause large oscillations in stream ecology. Temperature has been shown to be the most important factor in the composition of fresh-water communities (Sigee, 2005; Brookes et al., 2004; Allan, 2004). Grazing

micro-organisms have specific ranges of optimal temperatures for activity. This leads to periods of dormancy in winter and activity in summer that may correlate with high and low marker persistence, respectively (Allan, 2004).

Previous Studies of Persistence

Understanding the persistence of indicator organisms is necessary to correlate occurrence with that of pathogens, detect growth in the environment, and for use as agents of source tracking. Despite this importance, relatively few studies have investigated persistence of the *Bacteroides* genetic marker. The majority of those studies that have been published are rudimentary in the technology used and the types of conclusions drawn, as compared to the technologies available today and the type of information necessary for the use of *Bacteroides* as a fecal indicator.

Fiksdal et al. (1985) carried out the first study to characterize the persistence of *Bacteroides*. This study was undertaken before PCR was widely used, and thus relied upon difficult culture-based methods. Lab microcosm studies and in situ field experiments were carried out in order to both assess the survival of *Bacteroides* in water and compare its survival to that of *E. coli* and *S. faecalis*. *Bacteroides* cells remained viable for a maximum of one day at 12°C in aerobic surface water conditions, an amount of time much shorter than that of either *E. coli* or *S. faecalis* (Fiksdal et al., 1985). This study is supported by the observations of Tay et al. (2002) indicating that *Bacteroides* cells are capable of surviving short transport

distances in aerobic water. Fiksdal et al. (1985) also found non-viable *Bacteroides* cells were detectable by fluorescent antiserum, a non-culture method, for up to 8 days. This value is the most applicable to modern studies, as DNA-based PCR methods do not distinguish between viable and non-viable genetic material. The first PCR-based study to evaluate *Bacteroides* genetic marker persistence was undertaken by Kreader (1998) for both lab microcosms and in situ field experiments. A general *Bacteroides* assay was developed for qualitative PCR in order to evaluate changes in persistence of the *Bacteroides* genetic marker at varying temperatures and with and without predation. Persistence of *Bacteroides* varied from two weeks at 4°C to one day at 30°C, while the absence of eukaryotic predators extended the persistence up to one week (Kreader, 1998).

These initial studies did not use quantitative PCR and did not test a wide range of variables. The use of qPCR would allow precise evaluations of persistence through the development of decay curves. One study that did utilize qPCR found the *Bacteroides* genetic marker was detectable for 24 days at 4°C and 12°C and 8 days at 24°C in lab microcosms spiked with cultured *Bacteroides* cells (Seurnick et al., 2005). These results indicate a higher temperature-dependent variability than that determined by Kreader (1998) using standard PCR. A second study that utilized qPCR methods tested the effect of ambient light on *Bacteroides* genetic marker persistence (Walters, 2007). No difference in marker persistence between microcosms exposed to ambient sunlight and those kept in darkness was observed,

however there was a difference of 6 days in the persistence of *Bacteroides* from human and bovine fecal matter (Walters, 2007). Separating an environment into a number of constituent variables will allow more complex characterization of *Bacteroides* genetic marker persistence than the testing of only one variable. Future research should address this weakness through the investigation of multiple and combinations of variables.

The potential for *Bacteroides* to either remain viable (Fiksdal et al., 1985) or grow in the environment (Tay et al., 2002) has serious ramifications for its use as a fecal indicator. Bacteria that grow in the environment are not absolutely indicative of fecal contamination, and so do not conform to the indicator criteria specified by Maier et al. (2000). These ramifications mandate research into the exact conditions of growth. Walter and Field (2006) observed *Bacteroides* growth restricted to anaerobic micro-environments within stagnant aerobic microcosms. While this removes the concern that *Bacteroides* will grow throughout the environment, it is still possible that *Bacteroides* might persist in anaerobic micro-environments such as stagnant waters of estuaries or lakes (Walters and Field, 2006) or within anaerobic aggregates (Tay et al., 2002). Further research into these types of anaerobic environments and any associated persistence of *Bacteroides* is necessary.

None of the earlier studies directly evaluated any transport effects on the persistence of *Bacteroides*. While laboratory microcosms are very useful for carefully controlled characterizations of variables, the results of such studies are not always directly applicable to a response to the same variables

in a natural setting. Simpson et al. (2004) tracked the dispersion and transport of equine-hosted *Bacteroides* marker downstream from a known point-source. Standard PCR was used to amplify the eubacterial and *Bacteroides*-like assemblage upstream and progressively downstream of the point-source, as well as of the point-source itself. Sequencing of PCR products was phylogenetically analyzed to infer source relationships. The study found that while the bacteria present in the point source were phylogenetically distinct from those in the stream, the samples 5 m downstream of the point-source clustered with those from upstream (Simpson et al., 2004). This appears to imply the point-source was not detectable in the stream; however the PCR methods used in this study were not ideal. General eubacterial PCR primers are not sufficient to overcome dilution effects in a stream and are not applicable to microbial source tracking methods.

The significance of this study comes not from what was determined, but rather the approach that was taken. This is the only in situ transport study of *Bacteroides* published to date. The use of this approach but with host-specific qPCR assays has the potential to provide data on the persistence, behavior, and transport properties of *Bacteroides* that are directly applicable to source tracking studies. Unfortunately, such studies are difficult to undertake because of problems associated with trying to introduce a large point-source of fecal matter to any natural stream. Simpson et al. (2004) were able to get around this problem by taking advantage of an

existing point-source (a collapsed horse manure pile). Nevertheless, this is a potentially important approach that could be better executed in future studies.

***Bacteroides* and Microbial Source Tracking**

The use of host-specific fecal bacteria to identify the sources of pollution in a watershed has been termed microbial source tracking (MST), and is based on the assumption that appropriate indicators may be found that allow fecal pollution to be characterized in terms of origin (Simpson et al., 2002). The host-specificity found in the 16s rRNA gene of *Bacteroides* makes it an ideal candidate for use in microbial source tracking. Additionally, the presence of large quantities of *Bacteroides* in fecal matter ensures its recovery from environmental samples, thus adding to its utility in MST studies (Noble et al., 2006).

To date, several studies have investigated the use of *Bacteroides* as a source-tracking agent. Human and ruminant-specific assays for standard PCR analysis have been successfully used to differentiate between sources of pollution in environmental samples of water, sediment, and fecal matter (Bernhard and Field, 2000b; Bernhard et al., 2003; Bower et al., 2005; Shanks et al., 2006; Lamandella et al., 2007). The detection of host-specific genetic markers has enabled the identification of specific point-sources of contamination such as a sewage treatment facility or housing development (Bernhard et al., 2003; Bernhard and Field, 2000b). Lamandella et al. (2007) were able to map contamination 'hot spots' created by a single type of host

organism. An important aspect of microbial source tracking that has received less attention in the literature is the correlation of indicator organisms to flow patterns or precipitation events. Bower et al. (2005) were able to track changes in human and cow-specific *Bacteroides* into Lake Michigan before and after a rain event in order to better characterize indicator transport. Bernhard and Field (2006) correlated spatial patterns of ruminant-hosted *Bacteroides* to precipitation and run-off patterns. Quantification of fecal contamination has also lead to the correlation of the degree of contamination with tidal patterns (Boehm et al., 2003).

The application of quantitative real-time PCR (qPCR) to source tracking further increases *Bacteroides* utility as a source-tracking agent. Using qPCR the concentrations of different sources have been calculated in several studies, allowing the assessment of the relative contribution of contamination from each source (Boehm et al., 2003; Noble et al., 2006). This is particularly important in the remediation of watersheds with high amounts of fecal contamination. Using only qualitative PCR assays the entire watershed would appear equally contaminated and no clear priorities for remediation would emerge. However, using qPCR *Bacteroides* assays to quantify the level of contamination, researchers have been able to develop prioritized remediation plans for heavily contaminated areas (Noble et al., 2006).

Several drawbacks are highlighted by these studies that illustrate current research needs as well as potential pitfalls in applying molecular

methods. An interesting finding of Lamandella et al. (2007) was a distinct lack of host-specificity of *Bacteroides* from septic, ruminant and wildlife sources in sediment samples as compared to water or fecal samples as determined by phylogenetic analysis. The explanation offered for this trend was the existence of high levels of horizontal gene transfer among closely-associated hosts, however it is questionable how closely wildlife and septic waste interact, and such a trend would not be restricted to sediment samples. Another potential pitfall is developing an assay appropriately specific to all potential sources of fecal contamination in a watershed. The use of a ruminant-specific assay may not be ideal in areas with high populations of multiple types of ruminants. Cross-amplification of cow, deer, and elk strains can result in the inability to differentiate among these sources in a watershed (Bernhard et al., 2003; Lamandella et al., 2007). A solution to this problem is the development of more specific assays for cows (Bower et al., 2005; Layton et al., 2006) or elk and deer (Bernhard and Field, 2000b).

An important point that emerges from a review of studies using *Bacteroides* as a host-specific indicator of contamination is care must be taken in developing and testing PCR assays. Within the 16s rRNA gene certain regions have been identified as being 97-99% similar in human, cat, dog, and gull-hosted *Bacteroides* (Dick et al., 2005a). Such regions must be avoided for the development of host-specific assays. Identifying a region of the gene unique to a specific host is of fundamental importance in using *Bacteroides* as a source-tracking agent (Bower et al., 2005). A classic

example of this is demonstrated by the lower degree of host specificity Lamandella et al. found using assays for human contamination (2007). This was attributed to potentially variable rates of host-specificity in *Bacteroides*, however as other studies have developed successful human assays (Bernhard et al., 2003; Layton et al., 2006), this may not be the case.

Quantitative PCR

The use of real-time PCR (qPCR) assays increases *Bacteroides*' attractiveness as a fecal indicator. Real-time PCR makes the detection of *Bacteroides* fast and inexpensive with accurate and sensitive results. While standard PCR methods provide a presence/absence test for target genes, qPCR enables the quantification of the target gene through the use of fluorescent oligonucleotide probes. The primer/probe assay is designed in the same way as for a standard PCR test, however when synthesized, a TaqMan probe is used that contains a quenched fluorophore reporter (Fig. 2.1). When the probe binds to the target gene during the reaction, enzymes cut the fluorophore reporter from the probe, at which point it emits fluorescence. The qPCR cycler measures the amount of fluorescence from each cycle, which is then calculated into copies of DNA per reaction using a set of known standards included in each reaction. The constant density of *Bacteroides* in fecal matter can then be used to extrapolate the level of fecal contamination in mg/L. This is an important advantage of *Bacteroides* qPCR assays. Not only are results quantifiable, they can be converted into units of

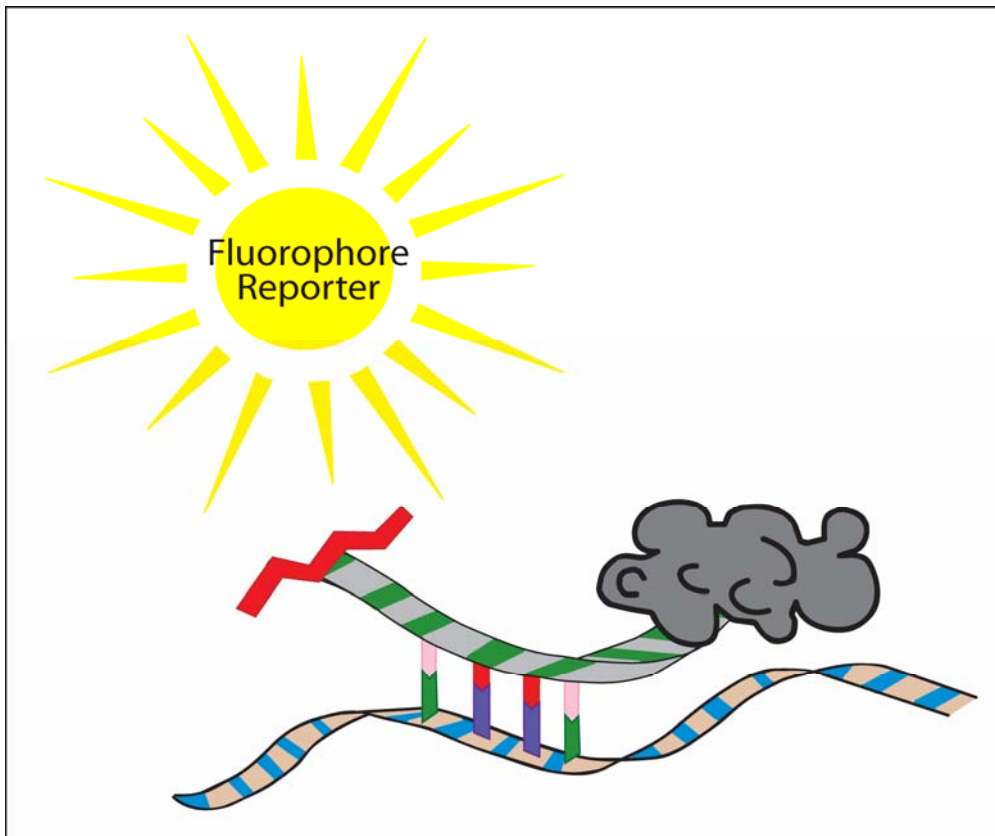
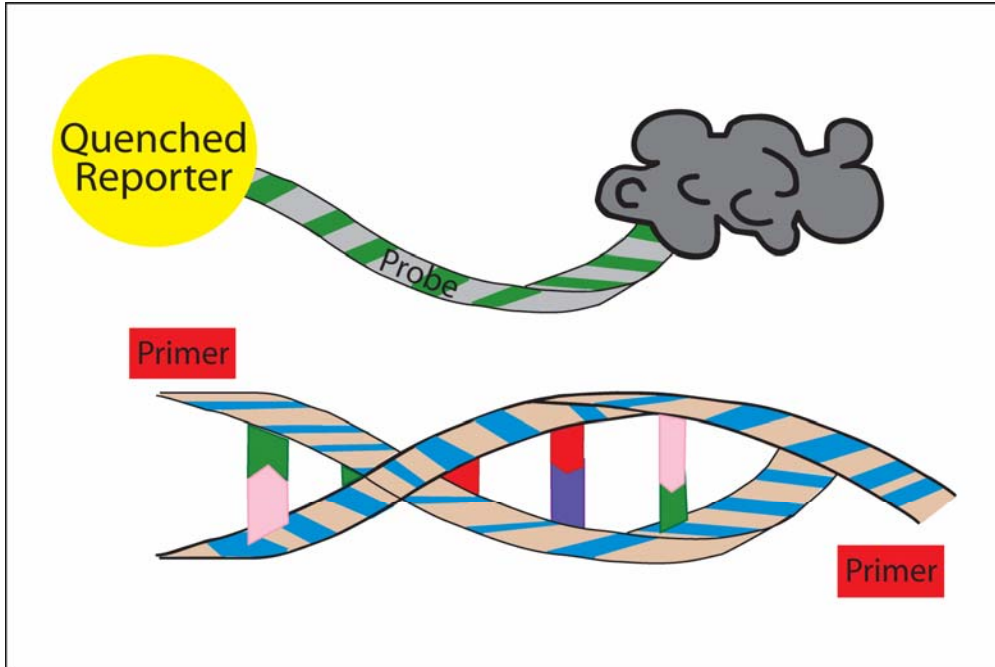


Figure 2.1. Real-time PCR with TaqMan probe uses a fluorophore reporter that is released to emit fluorescence when probe binds to target sequence.

measurement that are easily understood and directly applicable to water quality assessment.

Using molecular methods such as qPCR takes advantage of the host-specificity of *Bacteroides spp.* While most enteric organisms are not genetically unique to a specific host, a number of studies have demonstrated that the *Bacteroides* 16S rRNA gene sequence can be tied to its host using an appropriately sensitive PCR assay (e.g. Dick and Field, 2004; Layton et al., 2006). While a small degree of host specificity has been attributed to indicator organisms such as *E. coli* (Stoeckel et al., 2004), the degree of specificity is very low and highly variable across strains of the bacteria (McLellan et al., 2003; Field et al., 2003b), and even this degree of specificity has been debated (Gordon and Lee, 1999). qPCR assays have been designed by a number of research teams to correctly identify *Bacteroides* specific to cows (Layton et al., 2006; Okabe et al., 2007), humans (Seurnick et al., 2005; Layton et al., 2006; Okabe et al., 2007), pigs (Okabe et al., 2007), and horses (this study). Standard, non-quantitative PCR assays have been developed for a number of other hosts such as horses (Simpson et al., 2004), elk (Bernhard and Field, 2000b) and dogs (Dick et al., 2005b). While the basic indicator criteria discussed above do not require organisms to be host-specific, this trait has a number of important implications for water quality assessment. If fecal contamination can be tied to a specific host, the remediation process becomes much simpler. This is especially important to microbial source tracking, as discussed above.

The third advantage of qPCR is the speed and sensitivity of the process. Nucleic acid extraction, a time-consuming process, is not needed for qPCR analysis of environmental samples (Layton et al., 2006). It is not known whether standard PCR can be used without nucleic acid extraction. Additionally, qPCR does not require the time-consuming process of gel electrophoresis in order to visualize PCR product. Using qPCR, up to 72 environmental samples can be directly analyzed in a single qPCR reaction, which takes from 2.5 – 3 hours to setup and run. This is a significant improvement over traditional culture methods that require incubation periods of up to 48 hours followed by tedious colony counts.

Summary: *Bacteroides* Compared to Current Fecal Indicators

Anaerobic bacteria belonging to the genus *Bacteroides* have been suggested as alternatives to the currently recommended indicators *E. coli* and enterococcus. A direct comparison of these indicators and their adherence to ideal indicator criteria is shown in Table 2.1. *Bacteroides* may be preferable to current indicator bacteria because of their primary occurrence as enteric bacteria, a high degree of host-specificity, and a constant, high density within both fecal matter and the intestine. While qPCR assays are quick and highly accurate, they are also much more expensive and require more technical expertise than culture-based assays. The adherence to criteria regarding environmental growth and pathogen correlation are not fully understood at this time, however it appears from the few studies that have

Table 2.1. Comparison of *Bacteroides* and current EPA recommended fecal indicators *E. coli* and enterococcus to ideal indicator criteria of Maier et al. (2000).

Current Indicator Organisms

		<i>Bacteroides</i>	<i>E. coli</i>	Enterococcus
Ideal Indicator Criteria	1 enteric life-history	YES - strictly enteric	NO - found in chlorophytes and flies	NO - found in chlorophytes
	2 No growth outside host	POTENTIALLY	NO - regrowth in wide variety of environments	MIXED - regrowth observed in beaches, not in freshwater streams
	3 constant density	YES - constant, high density	NO - low, variable density	NO - low, density
	4 correlate to pathogen presence	POTENTIALLY	MIXED - correlated to disease outbreaks, not to pathogens	MIXED - correlated to disease outbreaks, not to pathogens
	5 slightly out-persist pathogens	UNKNOWN	NO - longer persistence than pathogens	NO - longer persistence than pathogens
	6 fast, inexpensive testing	Cheap - MODERATELY Fast - YES	Cheap - YES Fast - NO	Cheap - YES Fast - NO

been published that *Bacteroides* is capable of growth in anaerobic microenvironments (Walters and Field, 2006) and has the potential to better correlate with bacterial pathogens due to high host-specificity (Walters, 2007). While more research needs to be done in these areas, *Bacteroides* has clear advantages as a fecal indicator organism relative to *E. coli* and enterococcus because of the well-documented growth of *E. coli* (e.g. Fiksdal et al., 1985; Rhodes and Kator, 1988; Carillo et al., 1985) and the likely growth of enterococcus (Hartke et al., 1998; Martin and Gruber, 2005) in the environment.

Conclusions: future research directions

From the review presented here a number of conclusions about the use of *Bacteroides* as fecal indicators and future research needs can be made:

- *Bacteroides* meet a majority of the requirements of an ideal fecal indicator organism, many more than are met by currently recommended indicators *E. coli* and enterococcus.
- Assays that detect the *Bacteroides* genetic marker have been successfully employed in a number of lab and field settings.
- The potential for *Bacteroides* to meet all indicator criteria cannot be achieved without detailed characterization of marker persistence in natural environments. In particular, the following lines of research should be explored:

- The effects of multiple or combinations of variable environmental parameters on *Bacteroides* genetic marker persistence.
- The potential for *Bacteroides* to grow or have extended persistence under aerobic conditions, despite the anaerobic metabolism of the bacteria.
- The effects of environmental transport on marker detection.

While all of the future research needs presented above are beyond the scope of this thesis, the effect of environmental parameters on persistence is the first step in meeting all these goals. It is the purpose of this thesis to perform laboratory microcosm studies investigating a number of environmental parameters in order to better constrain the persistence of the *Bacteroides* genetic markers.

CHAPTER III

EVALUATION OF FACTORS INFLUENCING THE PERSISTENCE OF *BACTEROIDES* IN STREAM WATER

Abstract

This chapter presents a series of laboratory microcosm experiments characterizing the effects of environmental parameters on the persistence of the *Bacteroides* 16S rRNA marker in stream water in order to investigate the utility of *Bacteroides* spp. as fecal indicator organisms. Real-time PCR was used to measure marker concentration daily during a series of microcosm experiments carried out with filtered and unfiltered stream water, varying fecal aggregate sizes, fecal concentrations, temperatures, and fecal source animals. Decay curves for the 16S rRNA marker developed from these data indicate that higher temperatures, smaller fecal aggregate size, and water filtration result in lowest marker persistence (2-3 days), while initial fecal concentration does not play a significant role in marker persistence. The longest marker persistence recorded was 14 days when microcosms were held at 5 °C. These data indicate that biotic removal may play an important role in marker decline, with environmental factors that inhibit agents of biotic decline (such as low temperature) resulting in increased marker persistence.

Introduction

Fecal indicator bacteria have a wide range of potential applications in monitoring and controlling fecal contamination in surface waters. Appropriate indicators should be host-specific, exist in a constant proportion in fecal matter, and have a quantitative assay. A number of studies have established that bacteria belonging to the genus *Bacteroides* have a relatively high degree of fecal host-specificity (Bernhard and Field, 2000a; Bernhard et al., 2003), occur in a predictable concentration in fecal matter (Fiksdahl et al., 1985), correlate to the presence of some pathogens (Savichtcheva et al., *in press*), and can be accurately quantified using real-time polymerase chain reaction (qPCR) (Dick and Field, 2004; Layton et al., 2006). A primary drawback in the use of *Bacteroides* as a fecal indicator is the lack of information regarding persistence of its molecular markers in surface water systems. An understanding of *Bacteroides* genetic marker persistence would be useful for microbial source tracking as well as for correlating its presence with that of pathogens and other indicator organisms.

A number of environmental conditions have the potential to remove *Bacteroides* marker from stream water. Biotic decline, such as grazing by protozoa or infection by bacteriophage, may contribute substantially to *Bacteroides* marker decline. Environmental parameters such as temperature may in turn exert some control over the effectiveness of biotic decline, as grazing microorganisms may be less effective at lower temperatures.

Additionally, cell death and chemical break-down of the genetic markers play a role in *Bacteroides* persistence that may be affected by environmental parameters.

Previous research into the persistence of *Bacteroides* in surface waters has focused on lab microcosm and in-situ field studies using culturing methods and PCR detection. The first study of *Bacteroides* survivability found that they remained culturable in aerated freshwater for up to one day, followed by rapid die-off (Fiksdal et al., 1985). Studies using standard presence/absence PCR have also reported persistence times that vary from one day to three weeks at different temperatures (Kreader, 1998; Seurnick et al., 2005). Using river water spiked with fecal matter in both lab microcosms and membrane-filtration chambers within a river, Kreader (1998) found that predation and high temperatures acted to decrease marker persistence to as little as one day at 24°C, while Seurnick et al. (2005) found marker persistence at 28°C was as long as eight days in lab incubations. The differences in persistence observed in these studies may be due to different methodologies or variable environmental parameters, and indicates a need for a better understanding of *Bacteroides* 16S rRNA marker persistence.

Unlike *E. coli* and other fecal coliform bacteria, an important part of *Bacteroides* value as a fecal indicator is that it is unlikely to experience growth in aerobic aquatic environments. However, recent studies have reported that *Bacteroides* can grow in some anaerobic microenvironments within aerobic aquatic systems (Tay et al., 2002; Walters and Field, 2006).

One study has also attributed higher concentrations of *Bacteroides* genetic marker in biofilms to growth in anaerobic conditions (Savichtcheva et al., *in press*). To date, studies of *Bacteroides* survival in stream water have not been performed with molecular methods, however a study of survival in tap water using a combination of live/dead staining and fluorescence in situ hybridization with *Bacteroides*-specific probes found survival to be under five hours (Savichtcheva et al., 2005). Additional testing is still needed to determine whether *Bacteroides* growth is likely to occur in typical stream or lake settings. If growth is possible under conditions similar to those found in the environment, *Bacteroides* utility as a fecal indicator is reduced.

Additionally, focusing on only one or two variables controlling *Bacteroides* persistence (Kreader, 1998; Seurnick et al., 2005; Walters, 2007) may be an oversimplification of the natural environment. Testing of a broader variety of environmental factors will better refine our understanding of *Bacteroides* genetic marker persistence. Finally, only one study has attempted to develop quantitative decay curves for *Bacteroides* markers found in fecal matter (Walters, 2007). This study evaluated the effects of one environmental parameter (ambient light) in a series of microcosm studies, and determined that the amount of ambient light did not have an affect on the persistence of the *Bacteroides* genetic marker (Walters, 2007). More rigorous evaluations quantifying persistence are needed. The high degree of variability in persistence determined by all previous studies indicates that a number of studies developing decay curves are necessary to develop a

practical understanding of *Bacteroides* genetic marker persistence. These previous studies laid the groundwork for an in-depth analysis of the persistence of *Bacteroides* genetic markers using quantifiable real-time PCR (qPCR) assays, which is the subject of this chapter.

The objectives of this study were to evaluate the effects of water filtration, initial fecal aggregate size, initial fecal concentration, temperature, and fecal source organism on the persistence of *Bacteroides* 16S rRNA markers using quantifiable real-time PCR (qPCR). The study presented here builds on previous work (Kreader, 1998; Walter, 2007) in order to test the hypothesis that environmental parameters that are presumed to reduce removal of *Bacteroides* by biotic agents, such as grazing protozoa or bacteriophage, will indirectly act to promote the persistence of the *Bacteroides* genetic marker. Using this guiding assumption, a series of sub hypotheses were formed and tested:

- Water filtration will have an impact on *Bacteroides* genetic marker persistence, with more rapid decline occurring in unfiltered water.
- Large initial fecal aggregate size will increase marker persistence.
- High temperatures will have a strong impact on marker persistence, with more rapid decline occurring at higher temperatures.
- Higher fecal concentration is not expected to influence persistence of the *Bacteroides* genetic markers.

Methods

Sample Collection

Fresh samples of equine and bovine fecal matter were collected from healthy animals on local farms. Samples were collected from a single horse and cow for all experiments. Samples were collected within 12 hours of the beginning of each experiment and stored on ice until the beginning of the experiment. Stream water samples were collected from Second Creek in Knoxville, TN before the beginning of each experiment and transported directly to the lab (located approximately five minutes from sampling site). Sampling for both fecal matter and stream water occurred on 14 August, 13 September, 1 and 24 October, and 10 November of 2006 and 9 January and 22 February of 2007.

Microcosm Persistence Experiments

A series of microcosms subject to varying environmental conditions were used to evaluate *Bacteroides* genetic marker persistence. All microcosms consisted of 100 ml of stream water spiked with fresh fecal slurry contained in a 200 ml Erlenmeyer flask. Microcosms were covered with foil to avoid evaporation of water. The standard microcosm consisted of unfiltered stream water spiked to 100 mg/L of moderately disaggregated fecal slurry, kept for one week in the dark at 25 °C in a PsycroTherm (New Jersey) controlled environmental incubator shaker at a constant 100 rpm. Two 1 ml samples were collected daily from the center of the microcosm using a 1 ml pipette

and frozen in a -80 °C freezer until qPCR analysis (described below). For each experiment one 100 ml microcosm of raw stream water without fecal matter was sampled daily to determine background concentration of *Bacteroides* in the stream water. For each experiment, fecal slurries were made by diluting 10,000 mg/L slurry from fresh fecal matter mixed in filtered stream water into three unfiltered stream water replicates of the desired concentration and one filtered water replicate at the same desired concentration.

The effects of water filtration, initial fecal aggregate size, initial fecal concentration, temperature and fecal source organism on the rates of decline in fecal concentration, as measured by the qPCR analysis of 16s rRNA *Bacteroides* marker, were evaluated independently using a series of laboratory microcosm studies. Experiments were conducted sequentially as described in the flow chart in Figure 3.1. The evaluation of filtered vs. unfiltered stream water was conducted first. This was followed by the initial fecal aggregate size experiment, the initial fecal concentration experiment, the temperature experiment, and finally by the fecal source experiment. The experiments are described below.

Water Filtration

Persistence of the *Bacteroides* genetic marker in microcosms with unfiltered stream water was compared with measurements of persistence in microcosms using stream water filtered through 22 µm filters. The fecal slurry for both tests was made following the general procedures outlined

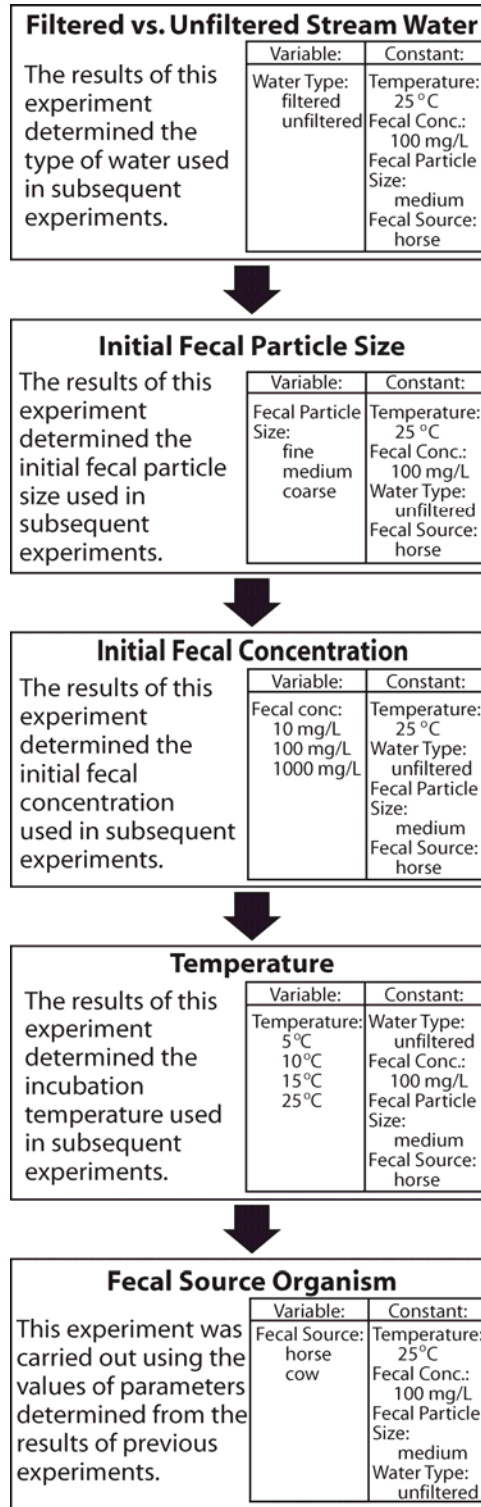


Figure 3.1. Sequential design of microcosm experiments. This chart illustrates the order in which all experiments were carried out.

above with mixing for 10 seconds on a Baxter S/P vortex mixer. Slurry was diluted into triplicate 100 ml samples of both unfiltered and filtered stream water at 100 mg/L concentrations, held concurrently at 25°C for one week, and sampled daily. Additionally, a comparison of the rates of decline between unfiltered and filtered stream water replicates from each of the temperature, concentration, and initial fecal aggregate size experiments (see below) was carried out to observe the effects of biological degradation with varying environmental parameters.

Initial Fecal Aggregate Size

The effects of initial fecal aggregate size on the persistence of the *Bacteroides* genetic marker were evaluated using microcosms prepared with three separate fecal slurries: coarse (hand-separated), medium (vortex for 10 seconds), and fine (blended for 30 seconds on “low blend” in a Hamilton Beach 10 speed blender). Each slurry was diluted into one filtered and three unfiltered stream water samples of 100 ml to a concentration of 100 mg/L, incubated concurrently at 25°C for one week, and sampled daily.

A aggregate size analysis was carried out on three 100 ml samples of each size class in order to ensure consistency in methodology and provide a semi-quantitative measure of the aggregate size distribution within each level. Each slurry was filtered through a column of U.S.A. standard sieves for the following size intervals: #10 (2 mm), #35 (500 µm), #60 (250 µm), #80 (180 µm), #100 (150 µm), and #120 (125 µm), with the water that was filtered classified as containing aggregates < 125 µm. Each sieve was backwashed

with de-ionized water and the resulting liquid vacuum filtered through 22 μm glass fiber filters. Filters were pre-weighed and after filtration were oven-dried at 104°C for six hours and weighed again to determine filtrate weight.

Fecal Concentration

The effects of initial fecal concentration on the persistence of the *Bacteroides* genetic marker were evaluated using microcosms with 10, 100, 1,000, and 10,000 mg/L fecal contamination. Fecal slurry was prepared following the general procedure above with mixing for 10 seconds on a Baxter S/P vortex mixer and diluted to make one filtered and three unfiltered stream water microcosms of 100 ml for each concentration. Replicates and a stream water sample for the background contamination were held concurrently at 25°C for one week and sampled daily.

Temperature

The effects of temperature on the persistence of the *Bacteroides* genetic marker were evaluated using microcosms held at 5, 10, 15, 25, and 35 °C. Fecal slurries were prepared following the general procedure described above with mixing for 10 seconds on a Baxter S/P vortex mixer. The slurry was diluted into 100 ml of filtered or unfiltered stream water at 100 mg/L concentration to make three replicates of stream water and one of filtered water for each temperature to be tested, for a total of 20 slurries.

Temperature variables were held non-concurrently for one week for the 15°C, 25°C, and 35°C tests and two weeks for the 5°C and 10°C tests. For each

test a 100 ml sample of stream water without the fecal slurry was incubated and sampled daily as a monitor of background contamination.

Fecal Sources

Comparison of persistence of the *Bacteroides* genetic marker from different animal feces was carried out using microcosms with cow and horse fecal matter. Fecal slurries were prepared and diluted following the general procedures described above, held concurrently at 25°C for one week, and sampled daily. Each slurry was diluted into one filtered and three unfiltered stream water samples of 100 ml to a concentration of 100 mg/L, incubated concurrently at 25°C for one week, and sampled daily.

Detection and Quantification of Bacteroides

Real-time PCR analysis with a non-host-specific *Bacteroides* assay (AllBac) developed at the University of Tennessee (Layton et al., 2006) was used for detection and quantification of the target gene in water samples from the microcosm experiments. For PCR analysis Quantitect PCR mix (QIAGEN, Valencia, CA) was used with 0.75 µl of the forward primer AllBac 296f and reverse primer AllBac 412r and 0.5 µl of AllBac 375r *Taqman* probe for each 2.5 µl of sample or standard. The PCR protocol consisted of 50°C for 2 minutes, 95°C for ten minutes, and 45 cycles of alternating 95°C for 30 seconds and 60°C for 45 seconds. PCR amplification and fluorescent probe detection was performed with a DNA Engine Opticon continuous fluorescence detection system (MJ Research, Waltham, MA). The threshold

cycle for fluorescence detection was adjusted manually to coincide with the number of cycles to detection for the 10^7 and 10^5 plasmid standards.

For each PCR reaction a set of *Bacteroides* plasmid standards (HU-7 or JC-5) ranging from 2.5×10^7 to 2.5×10^1 copies/PCR, a blank consisting of PCR mix and sterile water, and samples consisting of PCR mix and 2.5 μ l of each sample were all analyzed in triplicate, as well as one additional well of each sample spiked with 2.5 μ l of 10^5 plasmid standard to check for PCR inhibition. In general, PCR inhibition was not a factor in the recovery of a genetic signal, as evidenced by the full recovery of the 10^5 spike. Exceptions at the highest temperature (35°C) and concentration (10,000 mg/L) are discussed more fully in the results. The plasmid standards from each run were used to generate an averaged standard curve from which the concentrations of samples across all runs for a given experiment were calculated (Appendix 1). Published data on the average concentration of *Bacteroides* per gram of fecal matter was used to estimate the concentration in mg/L of fecal matter from the number of copies/PCR reaction (Layton et al., 2006). For all PCR analyses, 10^{-1} mg/L fecal concentration was defined as the limit of reliable detection and any values below this are referred to as below the detection limit. All linear correlations were determined in SigmaPlot 2004, version 9.

Data and Statistical Analyses

The daily measured concentrations of *Bacteroides* marker are presented here as a series of decay curves with accompanying statistical measures. A general linear model using a repeated measures analysis of variance and a Student's *t*-test was used to determine statistical significance of all rates of decline developed for the above experiments using the 'Proc GLM' function of SAS v. 9.1.3 Service Pack 4 (SAS Institutes Inc., Cary, North Carolina). Statistical significance was determined from the *p*-values resulting from a Student's *t* test and a repeated measures analysis of variance. The *p*-values shown represent the probability that a rate of decline can be determined that is as likely or more likely to reject the null hypothesis (e.g. - H₀: the filtered and unfiltered rates of decline are similar) than the rate of decline obtained in the experiment. A *p*-value less than 0.01 for both the Student's *t* test and the repeated measure analysis of variance was taken to indicate statistical significance.

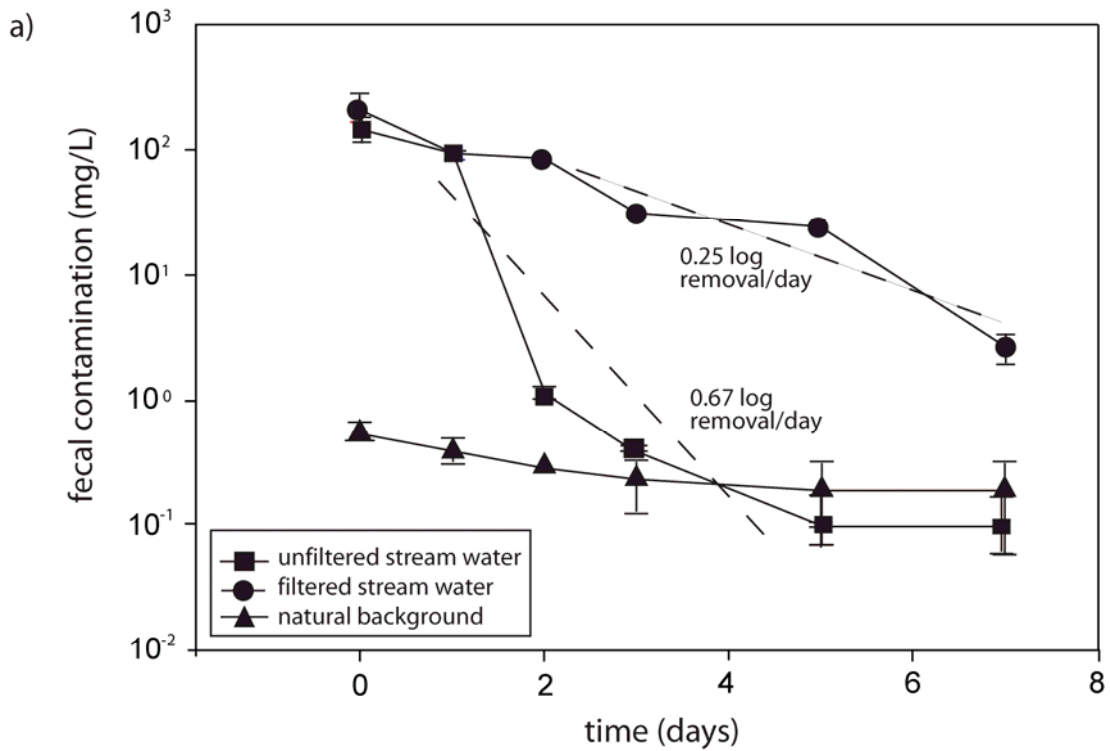
Results

In general, all variables of all experiments exhibited decline in fecal concentration, as measured by the *Bacteroides* 16S rRNA marker with qPCR. For all experiments, the decline in concentration is neither constant nor exponential. Rather, decline is first very slow, followed by a more rapid decline. The length of the initial period of slow decline varies substantially across and within experiments. Because this initial period of slow decline

may be attributed to the adjustment of the natural ecology of the stream water to the microcosm conditions, the decline was described as exponential with a straight line fit to the portion of the semi-log plot such that the initial adjustment time was excluded. Traditionally microbial growth and decay is assumed to be exponential (Bitton et al., 1983; Peiffer et al., 1988; Davies et al., 1991), however this assumption does not account for the lag time that has been observed in this study and several studies of bacterial decay (Gameson, 1984; Barcina et al., 1991; Gonzalez et al., 1992).

Persistence in Filtered vs. Unfiltered Stream Water

The effects of water filtration on persistence of the *Bacteroides* genetic marker was tested in microcosms consisting of 100 mg/L medium aggregate size fecal slurry in 100 ml filtered (presumably with native microorganisms removed) or unfiltered stream water. The decay curves generated from this analysis are found in Figure 3.2. The *Bacteroides* genetic marker in unfiltered water fell below the detection limit by day 5, representing an average decline of 0.67 log removal/day, or 20% of the total concentration lost per day. Samples from the filtered microcosm did not fall below the detection limit during the course of the experiment, and so had a persistence of greater than 7 days with a minimum average rate of decline of 0.25 log removal/day, or 13% of the total concentration lost per day. The differences in decline between the filtered and unfiltered microcosms were statistically significant, with a p -value of <0.0001 for both the F statistic from the repeated



b)

	Time to 10^2 Reduction (days)	Time to Nondetection (days)	Maximum Rate of Decline (log removal/day)
unfiltered	2	4	1.94
filtered	7	>7	0.26

Figure 3.2. Decline of fecal concentration in microcosms as measured by the *Bacteroides* genetic marker in filtered and unfiltered stream water.

measures analysis of variance and the *t* statistic from the Student's *t* test. From these results it was determined that unfiltered stream water would be used for subsequent experiments, as it more closely represented environmental conditions.

Effects of Initial Fecal Aggregate Size

The effect of initial fecal aggregate size on persistence of the *Bacteroides* genetic marker was assessed using microcosms consisting of 100 mg/L fecal slurries mechanically disaggregated to produce fine, medium, or coarse fecal aggregates. An aggregate size analysis was performed in order to determine the representative aggregate size distribution for each mechanical disaggregation treatment (Figs. 3.3 – 3.4). As shown in Figure 3.3, there was a visible difference in the number of aggregates retained on the 2 mm and 120 µm sieves for the three disaggregation treatments. The aggregate size distribution for each treatment (Fig. 3.4) is shown on a semi-log plot similar to that used for soil classification (ASTMD 422-63). Using the ASTM classification, the coarse treatment produced fecal aggregates mainly in the coarse to medium sand size, the medium treatment produced aggregates in the medium to fine sand size, and the fine treatment produced fecal aggregates in the fine sand to silt size range.

The decay curves for the *Bacteroides* genetic marker for the range of size intervals tested is shown in Figure 3.5. The coarse size exhibited a lower rate of decline than the medium and fine sizes. The coarse replicates

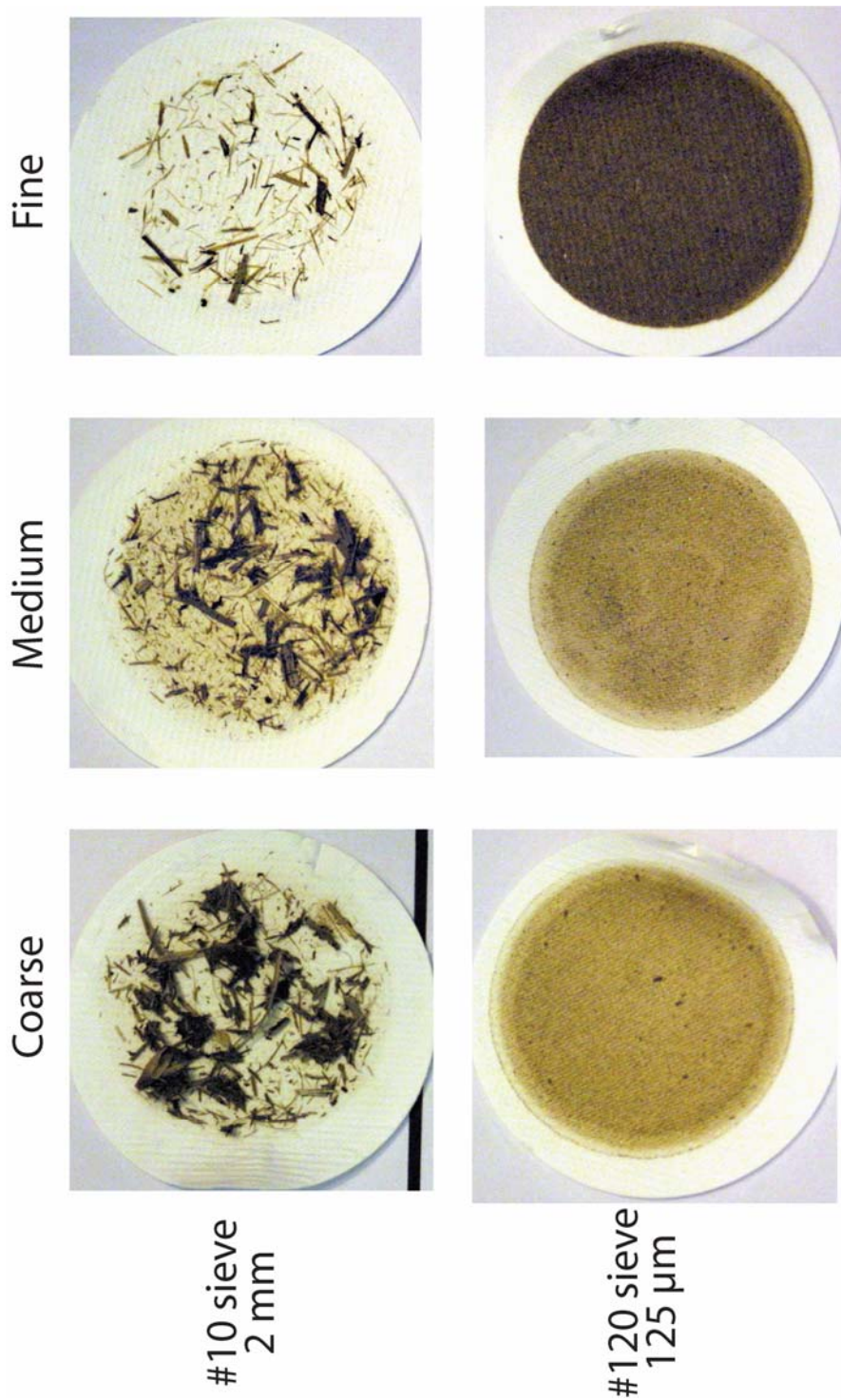


Figure 3.3. Comparison of the finest and coarsest particle size intervals evaluated in the particle size analysis. Photographs were taken of the coarsest and finest filtrates from the particle size analysis of three separate slurries: coarse, medium, and fine.

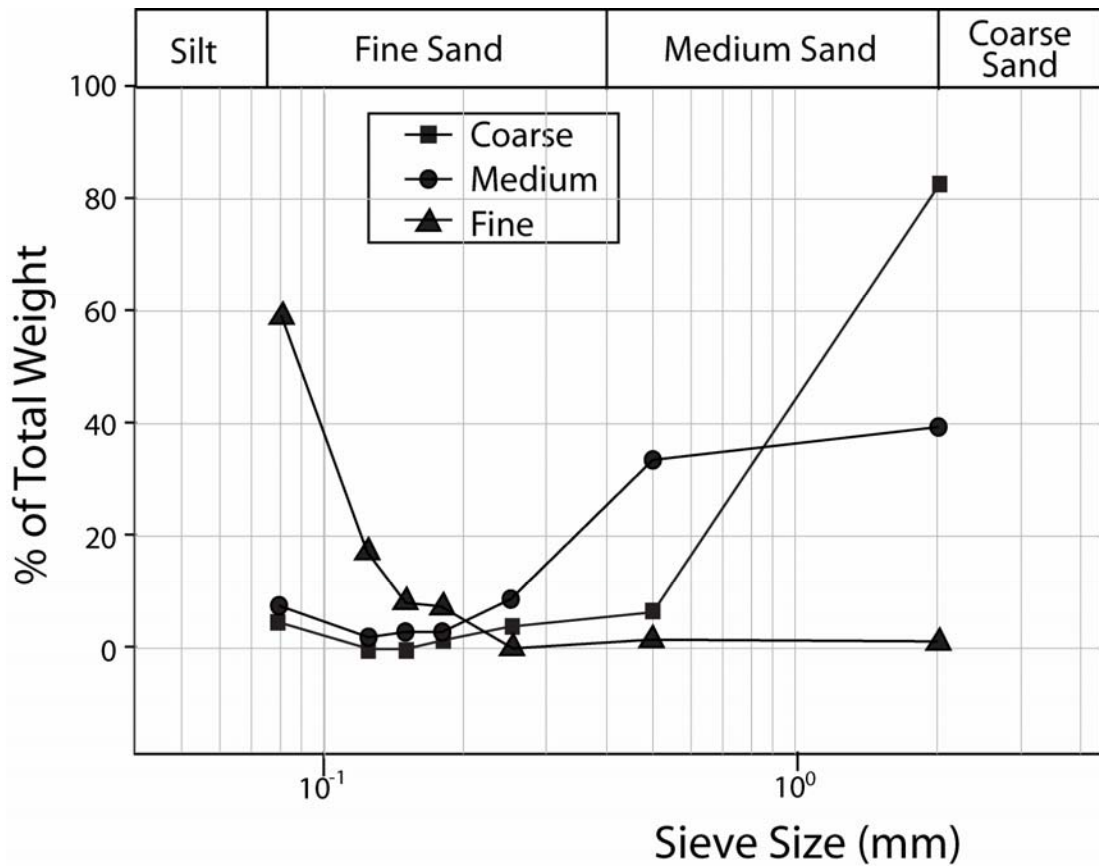
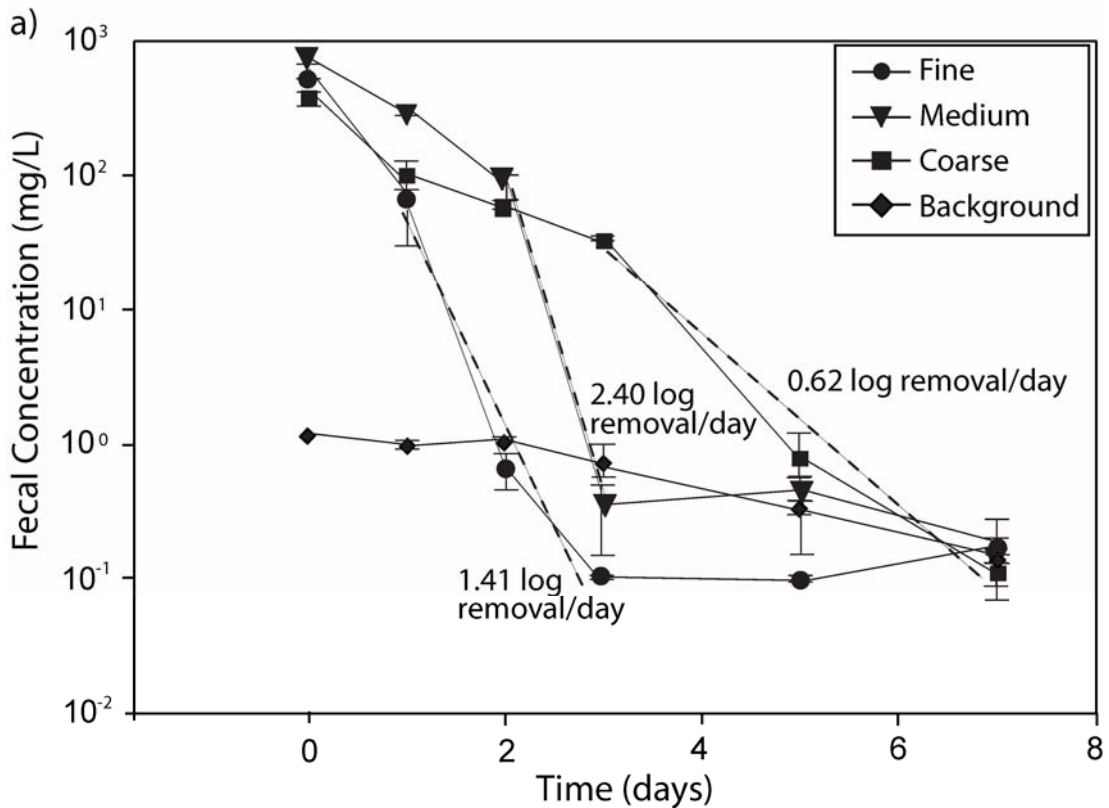


Figure 3.4. Size interval distribution in averages of triplicate slurries for three particle size intervals (coarse, medium, and fine) as reflected in percent of total weight for each filtrate.

had declined to non-detection after 5 days, averaging 0.62 log removal/day, while the medium and fine replicates had declined to non-detection by day 4 with 2.40 log removal/day, and day 3 with 1.41 log removal/day, respectively. Statistical analysis reveals the rates of decline across all three slurries were significantly different, as shown by the 0.0034 *p*-value of the F-statistic from the repeated measures analysis of variance. In addition, a comparison of each curve using the Student's *t* test can be found in Figure 3.5. This analysis shows the rate of decline of the fine slurry was significantly higher than that of either the medium or coarse slurries; however the medium and coarse slurries were not significantly different from each other. Based on these results the medium slurry was chosen as the initial fecal aggregate size for all subsequent microcosm experiments, as it experienced moderate rates of decline.

Effects of Concentration

The effects of initial fecal concentration on persistence of the *Bacteroides* genetic marker were assessed using fecal slurry microcosms of 10, 100, 1,000, and 10,000 mg/L. In real-time PCR analysis samples from the 1,000 mg/L and 10,000 mg/L microcosms had PCR inhibition, and were diluted 1:100 and 1:1000 respectively to eliminate inhibition. This eliminated inhibition for the 1000 mg/L samples, but not for the 10,000 mg/L samples. Thus the 10,000 mg/L samples were removed from consideration in this study.



b)

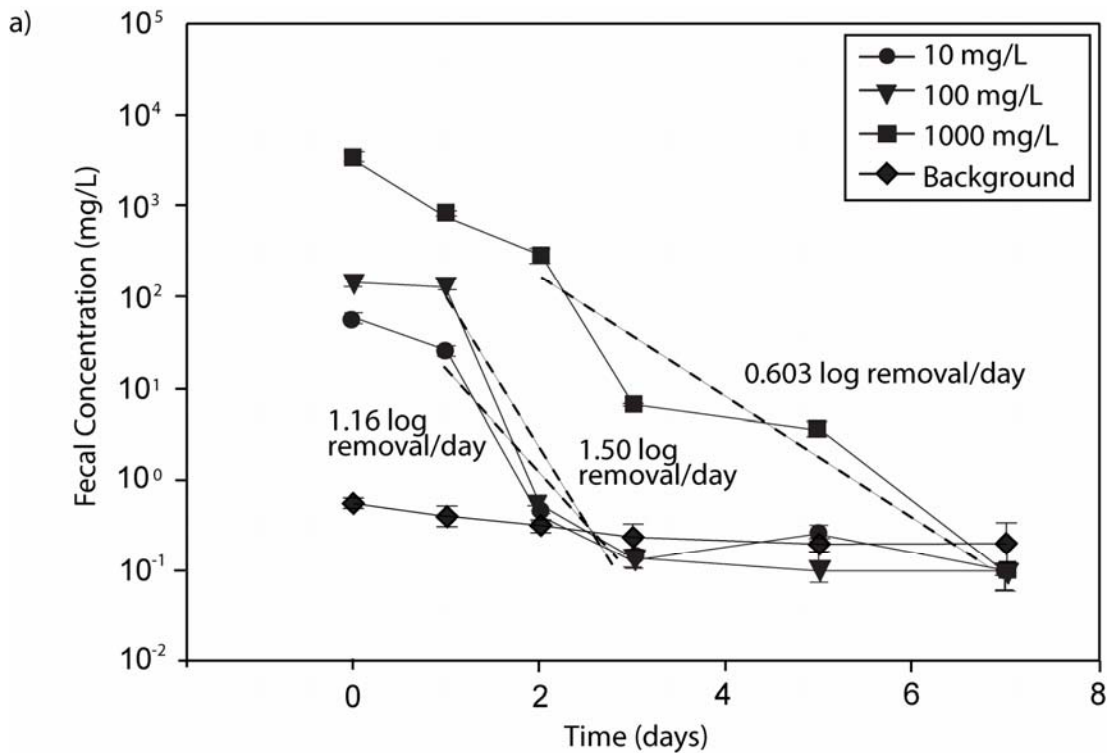
		Time to 10^2 Reduction (days)	Time to Nondetection (days)	Maximum Rate of Decline (log removal/day)
Treatment	Fine	1	2.5	2.01
	Medium	2.5	2.5	2.40
	Coarse	4.25	6	0.81

Figure 3.5. a) Decay curves for *Bacteroides* genetic marker with variable initial fecal particle size. Curves were generated in SigmaPlot using data from qPCR analysis with the AllBac assay. Fecal concentration in mg/L was calculated from an averaged standard curve. b) Statistical analysis of the differences in rates of decline between treatments. *P*-values for the *t* statistic determined from the Student's *t* test using SAS 9.1.3.

The decay curves for concentrations tested are shown in Figure 3.6. The higher concentration, 1,000 mg/L, exhibited a lower rate of decline than the other concentrations. The 1000 mg/L samples in unfiltered stream water declined below the detection limit in 7 days, whereas the 100 mg/L and 10 mg/L samples declined below the detection limit in 3 days. The most concentrated samples had an average rate of decline of 0.60 log removal/day, or 14% of the initial contamination lost per day, while the 100 mg/L averaged 1.50 log removal/day (33% loss/day) and the 10 mg/L averaged 1.16 log removal/day (50% loss/day). While qualitatively there appear to be some differences in persistence across the variables, this observation was not supported statistically. The p -value of the F statistic from the repeated measures analysis of variance was 0.0599, which is not significant. The p -values of the t statistic from the Student's t test comparing each rate of decline are also uniformly non-significant (Fig. 3.6). From these results it was determined that subsequent experiments would use 100 mg/L as the standard concentration of fecal contamination, in order to maintain consistency with previous experiments.

Effects of Temperature

The effects of temperature on the persistence of the *Bacteroides* genetic marker were assessed using fecal slurry microcosms incubated for seven to 15 days at 5, 10, 15, 25, and 35 °C. Samples from the microcosm incubated at 35 °C exhibited PCR inhibition, and so were diluted 1:100 to eliminate



b)

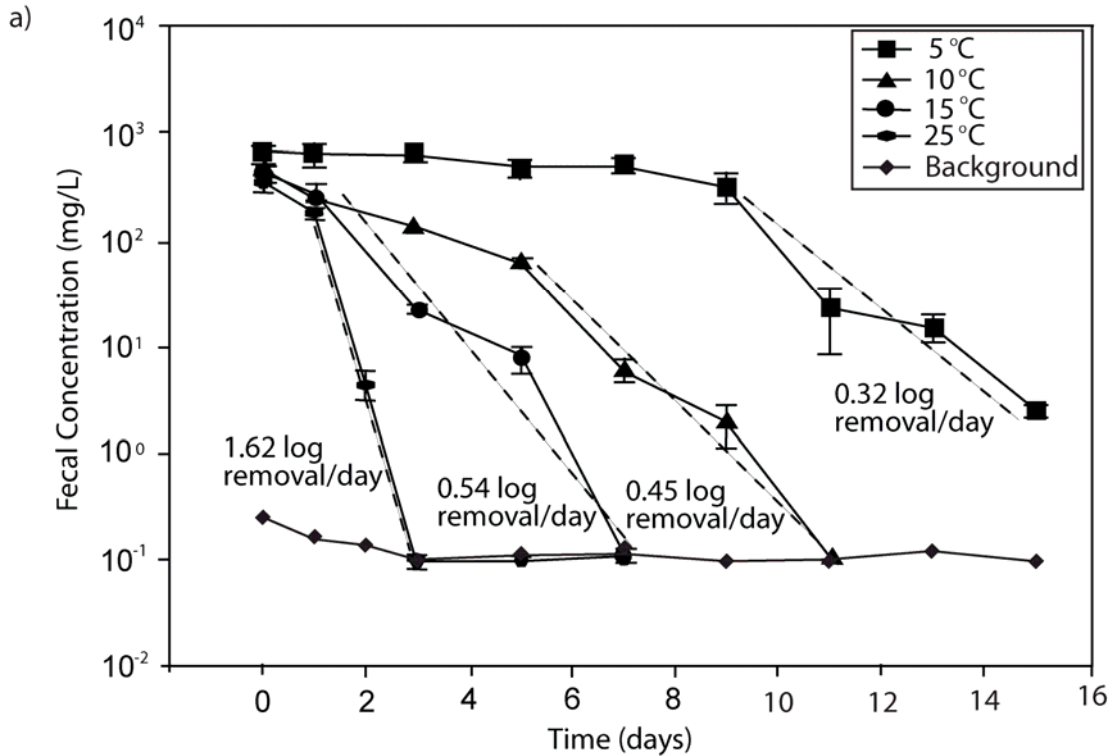
	Time to 10^2 Reduction (days)	Time to Nondetection (days)	Maximum Rate of Decline (log removal/day)
Treatment			
10 mg/L	2	3	1.84
100 mg/L	2	3	2.41
1000 mg/L	2.5	7	1.62

Figure 3.6. a) Decay curves for *Bacteroides* genetic marker with variable fecal concentrations. Curves were generated in SigmaPlot using data from qPCR analysis with the AllBac assay. Fecal concentration in mg/L was calculated from an averaged standard curve. b) Statistical analysis of the differences in rates of decline between variables. *P*-values for the *t* statistic determined from the Student's *t* test using SAS 9.1.3.

inhibition. Despite the dilution inhibition was still observed, and so samples from the 35 °C microcosm were removed from further consideration. The decay curves for each of the temperatures tested are shown in Figure 3.7. Fecal slurries incubated at warmer temperatures exhibited a higher rate of decline than those incubated at colder temperatures. The 5°C replicates did not decline below the detection limit after 15 days, while the 10, 15, and 25°C replicates declined below the detection limit after 11, 7, and 3 days, respectively. Averaged rates of decline were 0.32 log removal/day (6% loss/day) at 5 °C, 0.45 log removal/day (9% loss/day) at 10 °C, 0.54 log removal/day (14% loss/day) at 15 °C, and 1.62 log removal/day (33% loss/day) at 25 °C. Statistical analysis revealed all of these values were highly significant, as shown by the p -value of <0.0001 for the F statistic from the repeated measures analysis of variance test. Individual comparisons using the Student's t test also revealed high levels of significance (Fig. 3.7).

Effects of Fecal Source Organism

The effects of fecal source organism on the persistence of the *Bacteroides* genetic marker were evaluated using microcosms with horse and cow fecal matter. The decay curves for the equine and bovine fecal slurries are shown in Figure 3.8. The horse and cow microcosms both reached the detection limit after 5 days, having very similar average rates of 0.67 and 0.89 log removal/day, constituting 20% initial concentration lost per day for each treatment. The difference between these rates of decline was not statistically



b)

	Time to 10^2 Reduction (days)	Time to Nondetection (days)	Maximum Rate of Decline (log removal/day)
5 °C	2	3	0.58
10 °C	5.5	7	0.65
15 °C	7.5	11	0.95
25 °C	14.5	>15	1.65

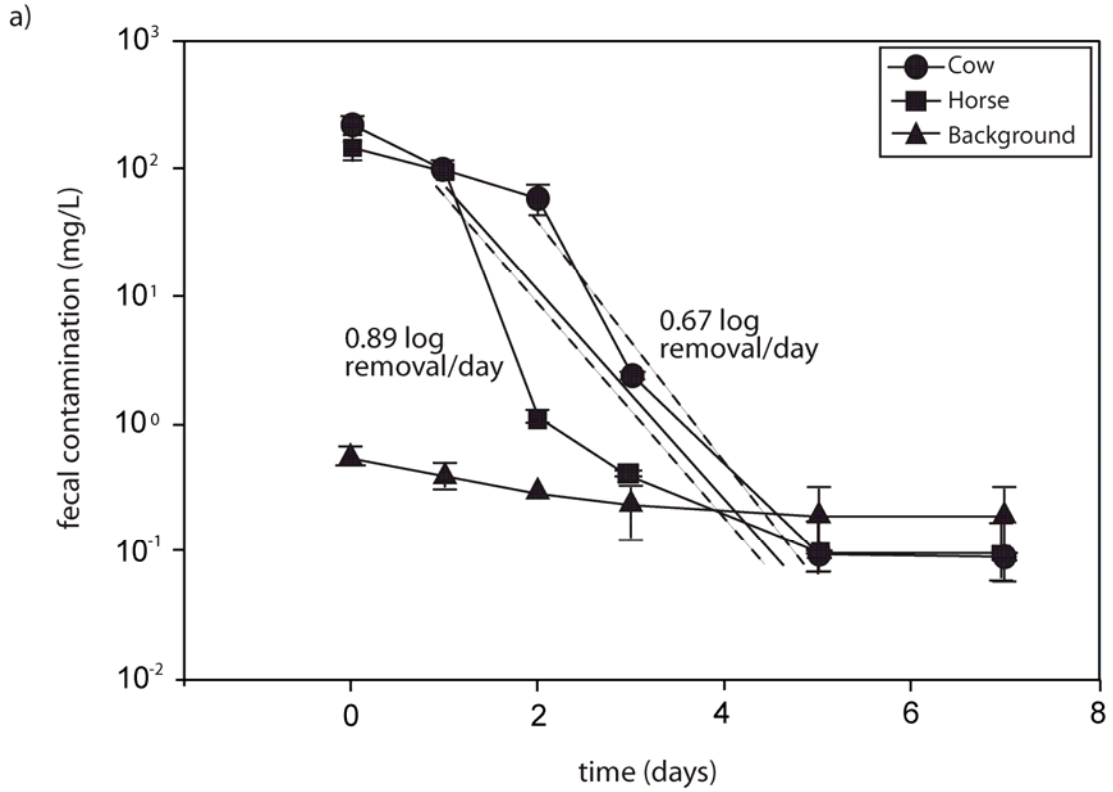
Figure 3.7 . a) Decay curves for *Bacteroides* genetic marker with variable temperature. Curves were generated in SigmaPlot using data from qPCR analysis with the AllBac assay. Fecal concentration in mg/L was calculated from an averaged standard curve. b) Statistical analysis of the differences in rates of decline between variables. *P*-values for the *t* statistic determined from the Student's *t* test using SAS 9.1.3

significant, as determined from the 0.0816 p -values of both the F statistic from the repeated measures analysis of variance and the t statistic from the Student's t test.

Discussion

Persistence of the *Bacteroides* genetic marker was affected by water filtration, temperature, and initial fecal aggregate size. However, the degree of variation ranged between experimental parameters (Figs. 3.9 – 3.10). Of the environmental parameters tested, water filtration and temperature showed the highest range of effects. Fecal aggregate size displayed some variation, but not to the same degree observed for the other parameters. There was no statistically significant change in persistence of the *Bacteroides* genetic marker between horse and cow fecal matter or different initial concentrations of fecal contamination.

It was hypothesized that marker persistence would be negatively correlated with high temperatures, small initial fecal aggregate size, and the use of unfiltered water. This is based upon the assumption that biological agents of degradation are the primary cause of decline in the *Bacteroides* genetic marker. Predatory microorganisms such as protozoa or bacteria can contribute to *Bacteroides* decline by grazing, while bacteriophage can destroy *Bacteroides* cells. As seen from the rates of decline in Figures 3.9 - 3.10, these data support the assumption that biologic degradation controls decline of the *Bacteroides* genetic marker. In particular, temperature was



b)

	Time to 10^2 Reduction (days)	Time to Nondetection (days)	Maximum Rate of Decline (log removal/day)
Horse	3	5	1.94
Cow	2	5	1.42

Figure 3.8. a) Decline in fecal contamination concentration from horse and cow hosts, as measured by the *Bacteroides* genetic marker. b) Decline measurements for microcosms.

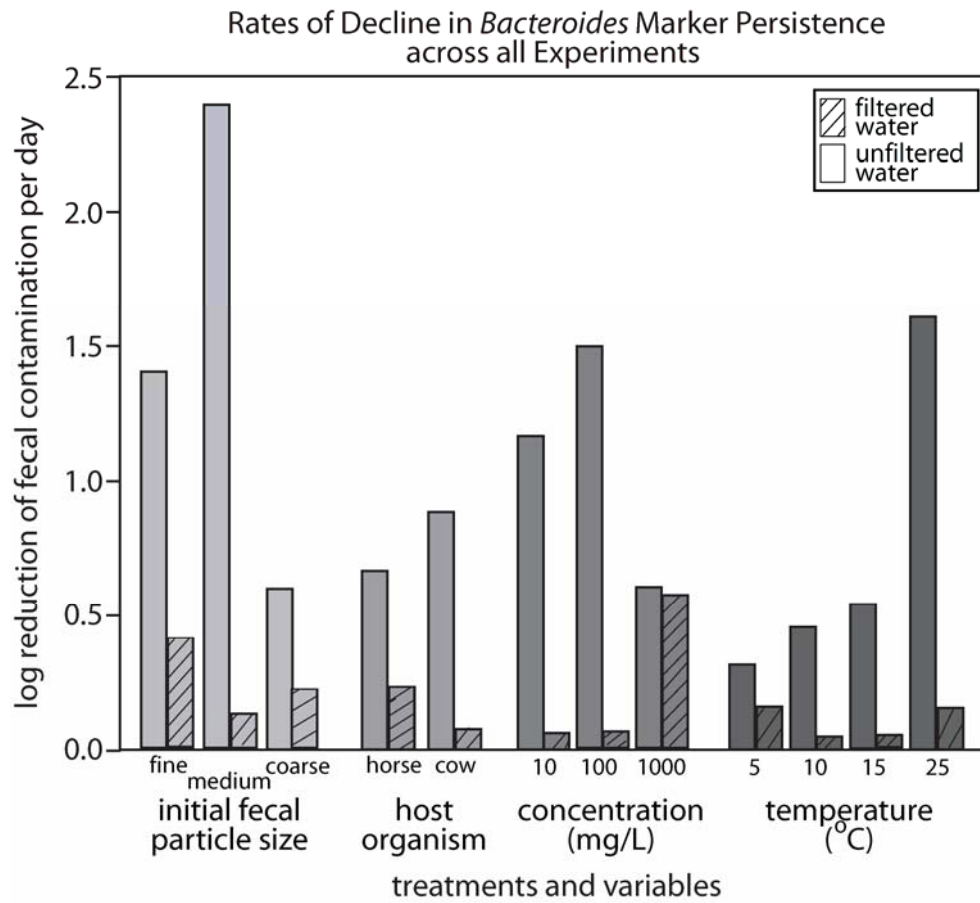


Figure 3.9. Average rate of fecal concentration decline across experiments.

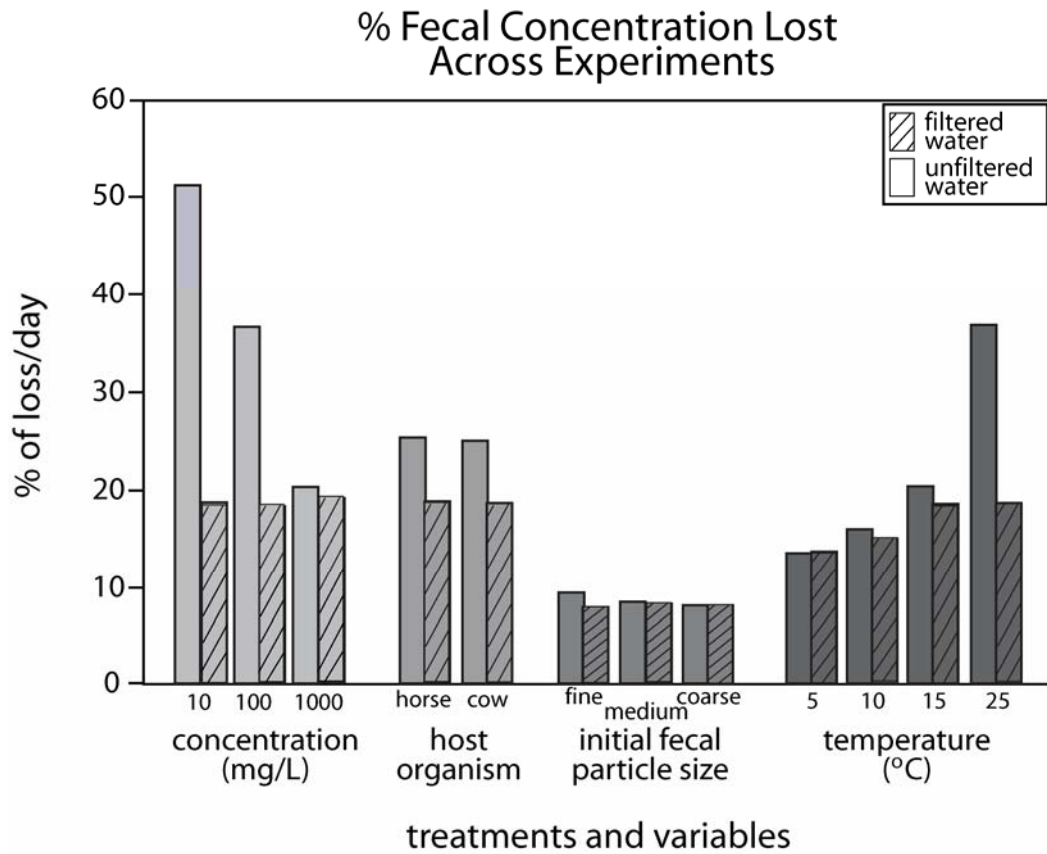


Figure 3.10. Average percent of initial fecal contamination lost per day across experiments.

expected to have the widest range of effect. The rates of decline determined for the temperature experiment support this hypothesis. The temperature data support the global hypothesis, as persistence was longest for treatments incubated at the coldest temperatures. At 5°C and 10°C grazing microbes, such as protozoa, are much less active, so the persistence of *Bacteroides* genetic marker at these temperatures may be attributable to decreased grazing pressure. The aggregate size experiment also showed a significant variation with size, indicating that a measure of protection from grazers may be provided to *Bacteroides* cells within fecal aggregates.

The most direct support for the assumption that biotic degradation controls the decline of the *Bacteroides* genetic marker comes from the water filtration experiment. These data indicate that, with few exceptions, the differences between the filtered and unfiltered water samples tested in all experiments have statistically significant differences in their rates of decline (Fig. 3.11). With very few exceptions, slurry incubated in filtered stream water took a longer time to decline below the detection limit than the same slurry incubated in unfiltered stream water. In the majority of cases, the difference between the persistence of the variables in unfiltered water is much less than the difference in persistence between a single variable in filtered and unfiltered water. This suggests that regardless of the fecal aggregate size, fecal concentration, temperature, or fecal source, the presence of natural particulates (including microorganisms) in the stream water will significantly decrease persistence of the *Bacteroides* genetic

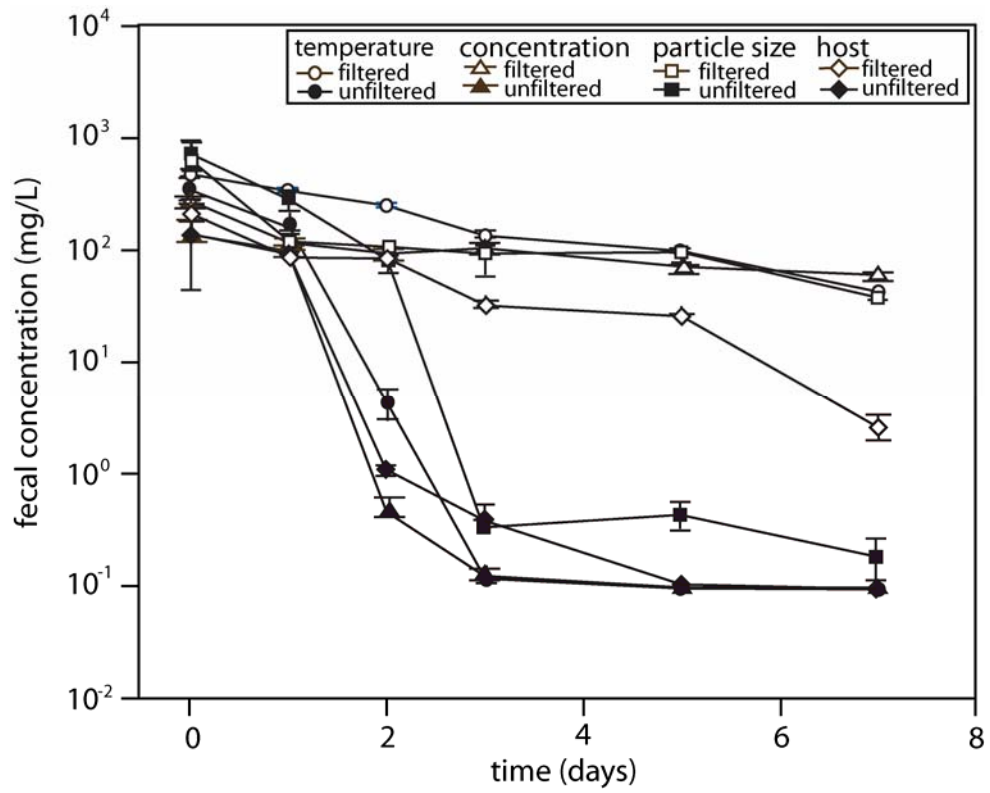


Figure 3.11. Comparison of decay curves generated from all experimental microcosms with 100 mg/L fecal concentration and medium fecal particle size held at 25 °C for one week.

marker. While this is not a direct measurement of the effect of predation, as the decline could be attributed to something else in stream water, biotic degradation is the most plausible explanation.

Comparison to Previous Studies of Persistence

While exact methods and analytical techniques may differ, research conducted in the course of this study overlaps with portions of previous studies. Overlap occurs in the use of microcosms (Fiksdal et al., 1985; Kreader, 1998; Seurnick et al., 2005; Walters and Field, 2006; Walters, 2007) and the evaluation of temperature (Kreader, 1998; Seurnick et al., 2005; Walters, 2007) and predation (Kreader, 1998). The use of microcosms to isolate the effects of a single environmental parameter has been a common theme in studies of *Bacteroides* persistence. While the size and complexity of the microcosms may differ, the concept is similar in all studies. One difference between studies is the type of fecal or bacterial contamination used. This study, as well as that of Kreader (1998) and Walter (2007), used concentrated fecal slurry diluted into the microcosm. An alternative approach is to use pure cultures of *Bacteroides* (Fiksdal et al., 1985; Seurnick et al., 2005) or undiluted sewage (Walters and Field, 2006). These approaches may return results that are less applicable to environmental samples. The lack of fecal matter in pure cultures creates a significant difference between the microcosm and the natural environment, reducing the applicability of the results. The use of undiluted sewage is very similar to situations in sewage

containment or treatment facilities; however, if the sewage is released into the environment dilution and aeration occur that is not accounted for in these studies.

While two previous studies have used qPCR methods (Walters and Field, 2006; Walters, 2007), only one of these studies presents quantitative data on the rates of decline under varying conditions (Walters, 2007). The microcosms in Walters (2007) study were designed to evaluate the effect of ambient daylight on the persistence of the *Bacteroides* genetic marker; however, similarity in fecal concentrations (200 mg/L), temperature (13°C), and dark incubation of some replicates allows us to compare those results to the results of the 10 and 15°C microcosms of the temperature experiment presented in this study (Fig. 3.12).

The results obtained by Walters show a large difference in the concentration and persistence of marker from human and cow fecal matter. This study did not find such a difference in the amount of marker present in horse and cow, however humans were not tested. Previous research, however, has indicated that the initial variation of two orders of magnitude is within the observed limits of variation for these two hosts (Layton et al., 2006). Another difference between Walters (2007) and the present study is the persistence of marker from different source organisms. Walters (2007) observed four days difference in persistence of human and cow marker, while this study did not find any statistically significant difference in the persistence of horse and cow marker. The differences in *Bacteroides* genetic

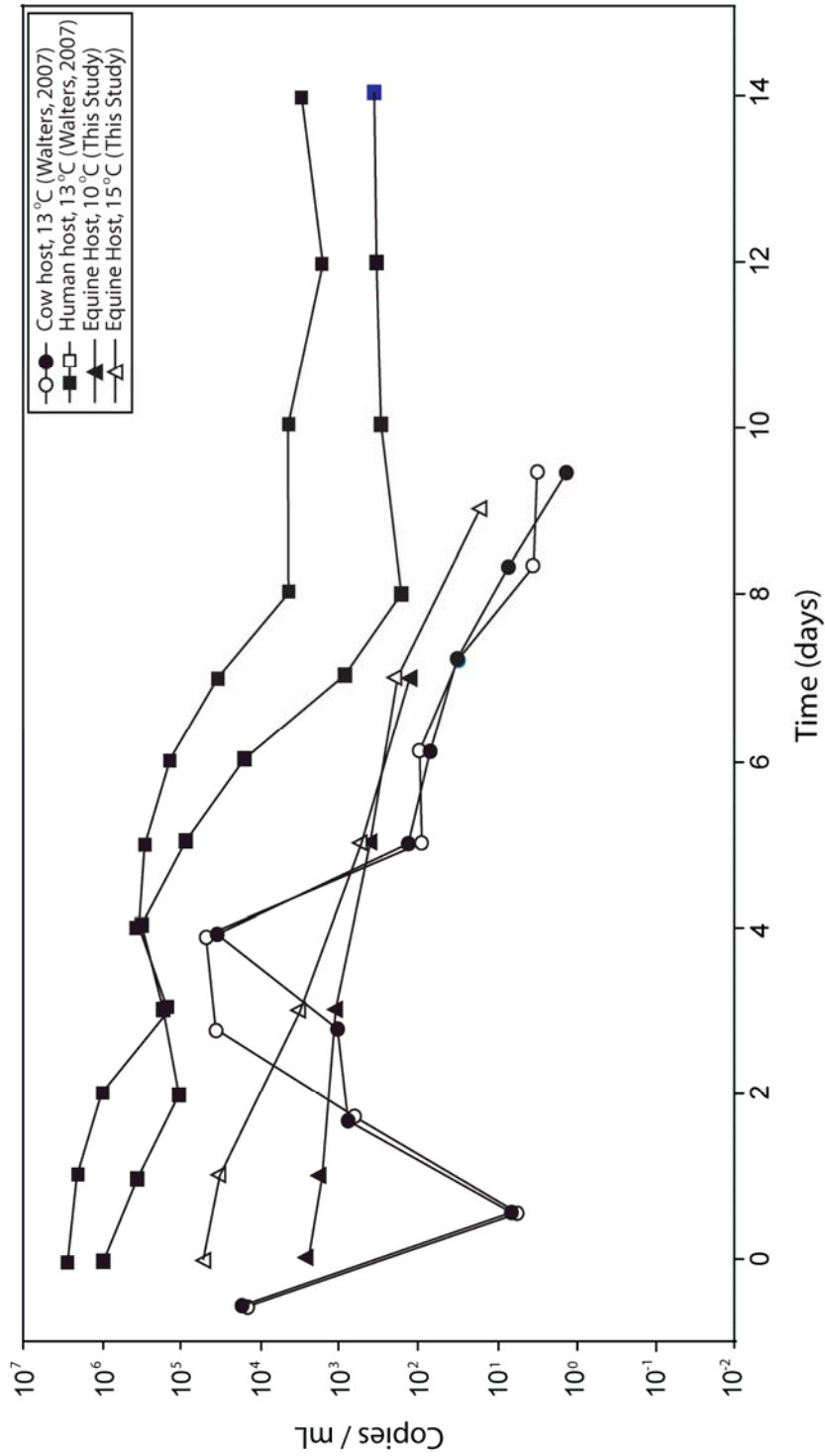


Figure 3.12. Comparison of decay curves generated from two separate studies of environmental parameters affecting persistence of the *Bacteroides* marker.

marker persistence between these two studies might be attributable to the different host animals, qPCR assays, and river water used.

The important similarities from this comparison are the constraints placed on persistence of the *Bacteroides* genetic marker at approximately 10 – 15 °C. The persistence range of 10 – 14 days observed in these experiments falls between previous studies by Kreader (1998), maximum of 8 days, and Seurnick et al. (2005), 24 – 28 days. Because of the general agreement between Walters (2007) and this study, as well as the use of precise qPCR analysis of fecal slurry microcosms, the data presented in Figure 3.12 can perhaps be regarded as a more accurate estimate of *Bacteroides* genetic marker persistence.

The second environmental parameter evaluated by this and a previous study is the effect of biologic degradation on *Bacteroides* marker persistence. Using both filtration and chemical deactivation, Kreader (1998) measured the change in *Bacteroides* persistence using laboratory microcosms with 100 mg/L replicates of human fecal slurry. These methods compare very well to those used in the present study, however the sample analysis method used was non-quantitative PCR. Standard PCR methods do not provide data that can be compared to the decay curves presented here, however the amount of time to nondetection can still be compared. Kreader (1998) found that the removal of aggregates larger than 0.45 µm increased the persistence of the marker 4 – 7 days at 24 °C. The present study found that removal of aggregates larger than 0.22 µm increased the persistence of the marker over

4 days to beyond the temporal range of the experiment at 25°C. This compares favorably with and also builds upon previous research (Kreader, 1998) through the quantification of marker decline at varying temperatures.

Conclusions

This paper presents a quantitative multi-variable analysis of environmental factors that affect the persistence of equine and bovine *Bacteroides* 16s rRNA marker. A number of decay curves have been developed that support the assumption that biologic degradation is the dominant factor controlling marker decline. Specifically, temperature and water filtration have the highest inhibitory effect on decline. Persistence of the marker varied from two days to two weeks, with the longest persistence observed for the 5 °C variable of the temperature experiment and the shortest persistence observed for the fine aggregate size variable of the initial fecal aggregate size experiment. This study also presents data highlighting similarities in marker persistence between horse and cow-hosted *Bacteroides*.

Previous research into the persistence of *Bacteroides* has returned a wide variation of results about the effects of temperature on persistence. The data collected in this study are most similar to those of Walters (2007), falling between the eight day persistence of Kreader (1998) and the 24 day persistence of Seurnick et al. (2005). The decay curves reported here could be used to develop guidelines and constraints for field-scale studies that can

both measure the actual persistence of *Bacteroides* in situ and to investigate the effects of fluvial transport on marker detection.

The data presented here imply that seasonality may have a strong effect on the behavior of the fecal indicator *Bacteroides* in surface water systems. During the summer, the *Bacteroides* marker may only be detectable for 2-3 days after it is introduced to the environment, however in the winter this time may be increased to over two weeks. Additionally, situations that result in the disaggregation of fecal matter, such as fecal matter introduced to streams by run-off, may result in the disappearance of the *Bacteroides* marker after two days, while situations that minimize disaggregation (such as direct deposit of fecal matter in streams) may result in marker persistence of up to one week. These implications are applicable to microbial source tracking as well as developing or implementing remediation plans for watersheds.

CHAPTER IV

DESIGN OF A QPCR ASSAY FOR THE DETECTION AND QUANTIFICATION OF EQUINE *BACTEROIDES*

Abstract

Horses are a significant potential source of fecal contamination in surface waters throughout the country, necessitating the measurement of their contribution to fecal contamination for water quality analysis and microbial source tracking. This chapter introduces the EqBac assay, a qPCR primer and probe set developed to quantify the amount of equine fecal contamination in environmental samples. Potential primer and probe sequences were identified from a phylogenetic tree of 141 16s rRNA sequences from *Bacteroides* present in variety of different animals. The assay was tested and refined using fecal plasmid standards from horse, bird, cow, human, dog, and septic samples until only equine-hosted *Bacteroides* was detected at low numbers of amplification cycles.

Introduction

Fecal contamination of water is a common problem in surface water systems that requires monitoring capabilities for fecal bacteria in drinking, recreational, and shellfishing waters in order to ensure public safety (EPA, 2005). Fecal bacteria are used as indicators of fecal contamination because testing directly for pathogens is made difficult and time-consuming by the variety of organisms and testing methods. A number of different fecal

indicators are currently recognized. *E. coli*, *S. faecalis*, total coliforms, fecal coliforms and fecal streptococci are all used as indicator organisms (Maier et al., 2000). Of these, tests for total coliform and fecal coliform are the most widely used assays, while *E. coli* and enterococcus are the currently recommended indicators (EPA, 2002). Each of these indicators fail to meet indicator requirements due to low fecal concentrations, time-consuming assays, and environmental growth. These drawbacks highlight a need for reliable fecal indicators that accurately assess fecal contamination in water. Additionally, the majority of current fecal indicators are not host-specific, and therefore cannot be used for microbial source tracking. Obligate anaerobes belonging to the genus *Bacteroides* have been suggested as alternative fecal indicators to previously used organisms such as *E. coli* and enterococcus because they are a better fit for indicator criteria and have a high degree of host-specificity (Dick and Field, 2004; Fiksdal et al., 1985; Kreader, 1998; Layton et al., 2006).

Bacteroides have several advantages over other fecal indicators. *Bacteroides* are more abundant in the feces of warm-blooded animals than fecal coliforms and occur in a relatively fixed quantity in fecal matter (Fiksdal et al., 1985; Franks et al., 1998). The anaerobic metabolism of the bacteria prevents growth in the environment (Fiksdal et al., 1985) and limits the occurrence of the group to the interior of warm-blooded animals (Paster et al., 1994). Also, *Bacteroides* are host-specific to the animal species level (Kreader, 1995). To date, the *Bacteroides* 16S rRNA gene from feces of nine

host animals has been sequenced at the University of Tennessee, and most group into genetically distinct clades (Layton et al., 2006).

Previously, several different bovine, human, pig, and equine PCR assays have been developed for standard PCR (Kreader, 1995; Bernhard and Field, 2000; Dick et al., 2005; Fogarty and Voytek, 2005). The development of real-time PCR assays allow the quantification of host-specific fecal contamination, thus providing a more powerful tool for water quality assessment. Currently, general *Bacteroides* assays (Dick and Field, 2004; Layton et al. 2006; Okabe, 2007) as well as human and bovine specific assays (Seurnick et al., 2005; Layton et al., 2006; Okabe et al., 2007) have been developed. This report describes the development of EqBac, a primer and probe set to target equine-hosted *Bacteroides* 16s rRNA. While horses are used agriculturally and recreationally throughout the United States in urban, rural, and national park settings (Fig. 4.1), a method of tracking and quantifying the equine contribution to fecal contamination has not been developed. The EqBac assay described here was tested against cloned *Bacteroides* gene sequences and fecal samples with and without nucleic acid extraction to determine the specificity of the assay as well as establish sensitivity thresholds for quantification.

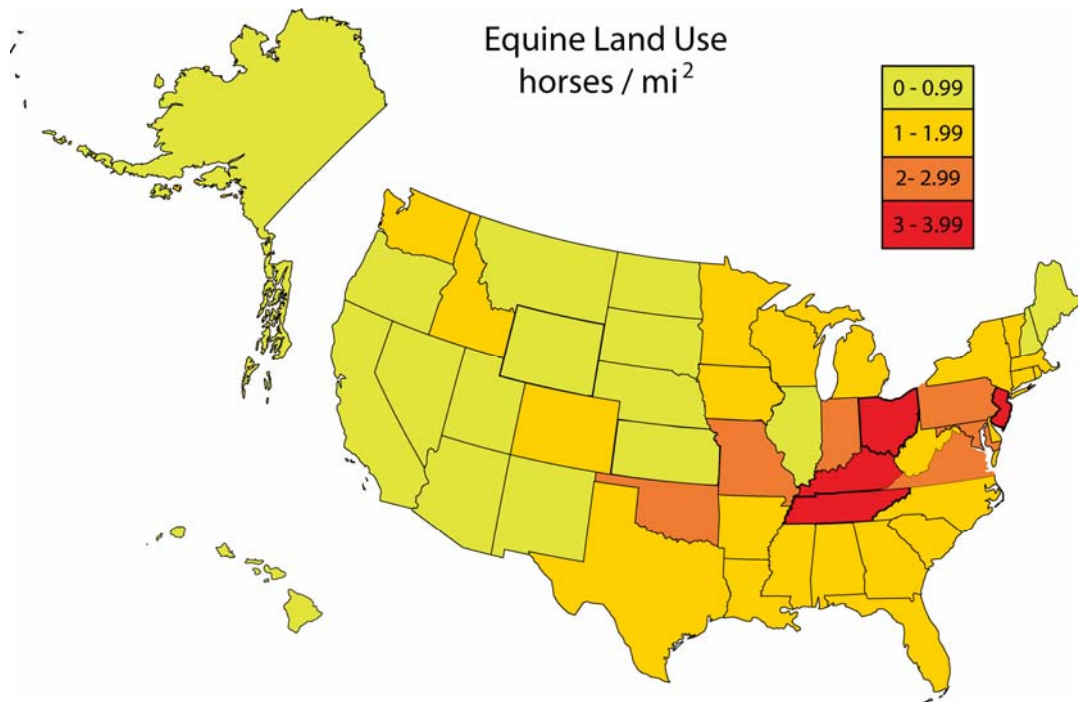


Figure 4.1. Equine land use in the United States. Map shows the number of horses per square mile, as reported by the U.S. Department of Agriculture census, 2002.

Methods

Sample collection and DNA extraction

Individual fecal samples were collected from three healthy pasture and stable fed horses (two horses, TNEQ-8 and TNEQ-9; and one pony, TNEQ-10) at a single farm in Tennessee. Horses were of different breeds, ages, and had different diets. Fecal samples were mixed with a volume of sterile water equal to the wet weight of the feces and frozen at -80°C until extractions were performed. Extractions were made with the FastDNA Spin Kit for soil samples (Q-Biogene, Carlsbad, CA) using 300 µl of fecal slurry in lysis matrix E tubes following the manufacturer's instructions. For each horse 50 µl of prepared DNA was obtained. Concentrations of DNA in samples were measured with a Turner BioSystems fluorometer (Sunnyvale, CA). After concentrations were determined TNEQ-9 was discarded for low DNA concentrations (<25 µg/ml).

Bacteroides 16S rRNA genes from TNEQ-8 and TNEQ-10 sample DNA were amplified using 1 µl each of the primers Bac 32f and Bac 708r (Bernhard and Field, 2000), 2 µl of DNA extract, and 21 µl of sterile water with ready-to-go PCR beads (Amersham Pharmacia, Piscataway, NJ). A touchdown temperature PCR protocol was performed consisting of 5 minutes at 94°C, [15 seconds at 94°C, 45 seconds at 65°C, 60 seconds at 72°C – repeated nine times, decreasing 1°C each cycle], [15 seconds at 94°C, 45

seconds at 55°C, 60 seconds at 72°C – repeated 29 times], 10 minutes at 72°C, and held at 5°C after the end of the program. Following PCR amplification, the product was cloned using the TOPO TA cloning kit (Invitrogen): 1 µl of the 2.1-Topo vector was mixed with 2 µl PCR product, 1 µl salt solution and 1 µl sterile water. The cloning reaction was incubated for 20 minutes at room temperature, after which 2 µl of the reaction was added to chemically competent *E. coli* cells and incubated for 10 minutes on ice, followed by a 30 second heat shock at 42°C. Following the heat shock 250 µl of 50c medium was added and stirred horizontally at 37°C for one hour, after which the medium was cultured on one LB plate each of 10, 25, and 50 µl medium. Colonies from each plate were recultured and plasmids were isolated and screened for gene inserts using the EcoRI restriction digests. Inserts were sequenced at the University of Tennessee Molecular Biology Resource Center in two directions using the M13f and M13r primers.

Phylogenetic analysis

Sequences were compared to the National Center for Biotechnology Information (NCBI) database using a BLAST search (Altschuk et al., 1990) and compiled with other *Bacteroides* and *Prevotella* sequences from seven different host organisms (human, cow, cat, dog, bird, pig and horse), for a total of 141 sequences. ClustalX (version 1.83 for MS Windows XP, Thompson et al., 1997) was used to create a sequence alignment (Appendix

2) and a neighbor-joining phylogenetic tree. The outgroup was defined as the single *Cytophaga* sequence using TreeView (Page, 1996).

Marker identification and PCR assay design

The probe and primer sequences developed for the EqBac assay are shown in Table 4.1. The sequences from the GBac assay, a general *Bacteroides* assay, were developed as part of a general *Bacteroides* assay developed at the University of Tennessee for potential multiplexing of *Bacteroides* assays (Layton et al., 2006). All sequences were conserved across the range of equine sequences targeted for assay development and selected following the guidelines in Table 4.2. Possible probes were checked in the Oligonucleotide Properties Calculator (Cao et al., 1994) for salt-adjusted annealing temperature and any potential secondary structures and then checked for specificity using a BLAST search (NCBI, Altschuk et al., 1990) and Probe Match (Ribosomal Database Project; Cole, et al., 2005). Oligonuclease primers and 6-carboxyfluoroescien (FAM)-BHQ probes were ordered from Biosearch Technologies (Novato, CA).

Assay sensitivity trials

Primer and probe sequences from Table 4.1 were tested by mixing real-time PCR Quantitect PCR mix (QIAGEN, Valencia, CA) with 0.75 µl of the forward and reverse primers and 0.5 µl probe for 2.5 µl of sample or standard. For each PCR analysis a set of plasmid standards ranging from 2.5×10^7 to 2.5×10^1 copies/PCR, a blank consisting of PCR mix and sterile water, and

Table 4.1: Primers and probes tested for the development of the real-time PCR EqBac Assay with sequence information, product length, and annealing temperature. Sequences in bold were selected for the final EqBac assay.

	primer/probe name	sequence (5' - 3')	product length (bp)	annealing temp (°C)
primers	GBac 34f	5' - CGCTAGCTACAGGCTTAACAC-3'	21	61
	EqBac 67f	5' - AGCAATGCCCCGATGGCGAC-3'	20	65
	EqBac 147r	5' - CCTCATTCTAAGGGCTATCC - 3'	21	60
	GBac 313r	5' - GTGGGGGACCTTCCTCTC - 3'	18	61
probes	EqBac 285r	5' - TACCGATCGACGGCATGGTGGGCC - 3'	24	72
	EqBac 90f	5' - CGAATGGGTGAGTAACGCGTATCCAAC - 3'	27	70

Table 4.2. Guidelines for development of real-time PCR primer and probes. Guidelines were developed following the protocols of Applied Biosystems (Foster City, CA).

	Probe	Primer
Requirements	<ol style="list-style-type: none"> 1. 25 - 30 nucleotides long 2. 30 - 89% GC content 3. Avoid nucleotides repeated more than three times, especially Guanine 4. 5' end must not contain Guanine 5. salt-adjusted annealing temperature should be between 68 m- 70 °C 6. Should be located as close as possible (10-20 bases) to one primer 	<ol style="list-style-type: none"> 1. 18 - 24 nucleotides long 2. 40 - 60% GC content 3. Avoid sequences with potential secondary structures 4. 3' end should not contain Thymine 5. Salt-adjusted annealing temperature should be between 58 - 60 °C 6. Both primers should anneal at the same temperature

2.5 µl of each sample were all analyzed in triplicate. The PCR protocol consisted of 50°C for 2 minutes, 95°C for ten minutes, and 45 cycles of alternating 95°C for 30 seconds and 60°C for 45 seconds. PCR amplification and fluorescent probe detection was performed with a DNA Engine Opticon continuous fluorescence detection system (MJ Research, Waltham, MA). The threshold cycle for fluorescence detection was defined manually to correspond with the correct number of copies for the 10⁷ and 10⁵ plasmid standards. The plasmid standards from each run were used to determine the concentrations of the samples within the run. All linear correlations were determined in SigmaPlot 2004, version 9.1.3 (Systat Software, Inc., Cary, NJ).

To test the components of the EqBac assay the 90f and 147r primers were tested separately with GBac 313r and 34f primers respectively for detection of plasmid standards from TN Eq2-2 and TN Eq2-4, the sequences from which they were derived, as well as plasmid standards from *Bacteroides* extracted from other host organisms (avian, canine, human, bovine, and septic samples) and environmental fecal samples. To increase specificity of the EqBac assay a temperature gradient was run from 58°C to 61.4°C during the second half of the cycling portion of the procedure. A final test of the EqBac assay consisted of a number of qPCR tests with a variety of dilutions of DNA and plasmid standards.

Results

Phylogenetic analysis

The neighbor-joining phylogenetic tree created in ClustalX (version 1.83 for MS Windows XP) using 16s rRNA sequences of *Bacteroides* hosted by the following organisms: cows (35 sequences), birds (7 sequences), dogs (9 sequences), pigs (9 sequences), horses (51 sequences), and humans (29 sequences), as well as 1 *Cytophaga* sequence as an outgroup is shown in Figure 4.2. This tree can be divided into major clades that typically correspond to host organism. An exception to this is one clade that is jointly populated with human and pig sequences. This is perhaps a reflection of concerted evolution in bacteria in response to the similar digestive tracts of human and pigs (McBee, 1971; Gashi, 2000; Dick et al., 2005). Equine sequences on this tree were placed in one of two large clades separate from the majority of other sequences (Fig. 4.2).

qPCR specificity

The primers and probes selected for the EqBac assay are shown as bold entries in Table 4.1. Following the initial qPCR analysis of representative plasmids, Eq 67f, GBac 313r, and Eq 285f were discarded because of multiple instances of non-target sequence amplification. The probe Eq90r with primers GBac 34f and EqBac 147r also amplified non-target sequences (avian-hosted *Bacteroides*), a problem that was somewhat resolved by a



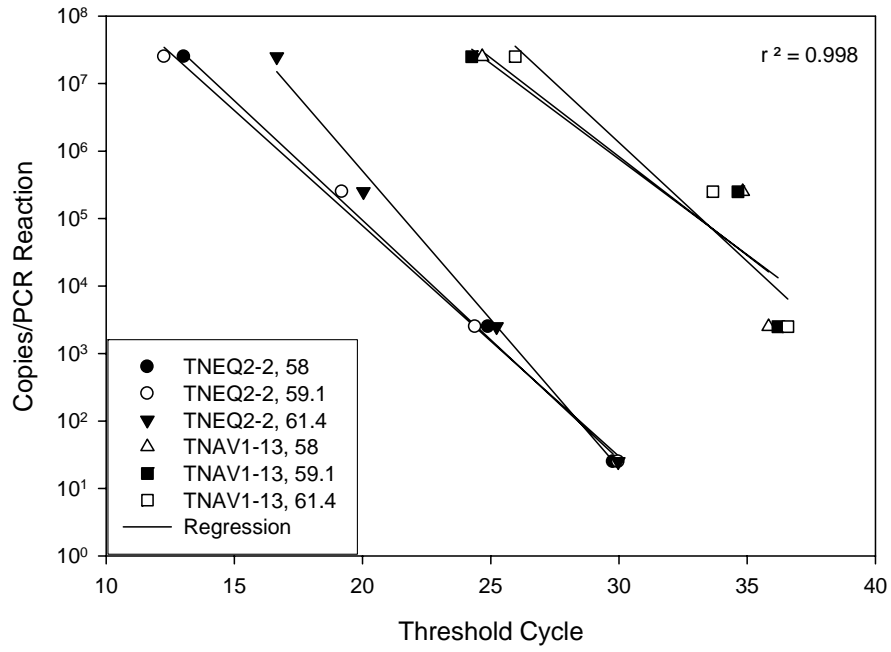
Figure 4.2. Neighbor-joining phylogenetic tree of *Bacteroides* 16S rRNA sequences from 141 host organisms rooted at Cytophaga. Main equine clusters and individual sequences (TN Eq2-2 and TN Eq2-4) targeted for assay development are identified.

temperature-gradient qPCR procedure (Fig. 4.3). The final EqBac assay was selective for equine-hosted *Bacteroides* when 59°C is used as the annealing temperature for the reaction (Fig. 4.4). To evaluate the consistency of EqBac at identifying varying concentrations of fecal contamination a series of six ten-fold dilutions of equine plasmid standards, 2.5×10^7 to 25 copies of target 16S rRNA per PCR, were run following the procedures described above (Fig. 4.5). All curves generated in these procedures are linear, with r^2 values ranging from 0.994 to 0.998. This demonstrates the consistency of the assay.

Conclusions

A number of studies have published real-time PCR assays for the detection of *Bacteroides* 16s rRNA from general, or mixed, sources (Dick and Field, 2004; Layton et al. 2006) as well as from human and bovine fecal contamination (Seurnick et al., 2005; Layton et al., 2006). The advantages of using *Bacteroides* as an indicator of fecal contamination stem from the anaerobic requirements of the bacteria, which limit the capability for environmental growth (Fiksdal, 1985), the predominance of *Bacteroides* in the intestines of warm-blooded animals (Franks et al., 1998), and the host-specific nature of the 16s rRNA gene (Dick et al., 2005). In addition to *Bacteroides* other characteristics, the consistency of *Bacteroides* density in fecal matter allows the quantification of source-identifiable fecal

a)



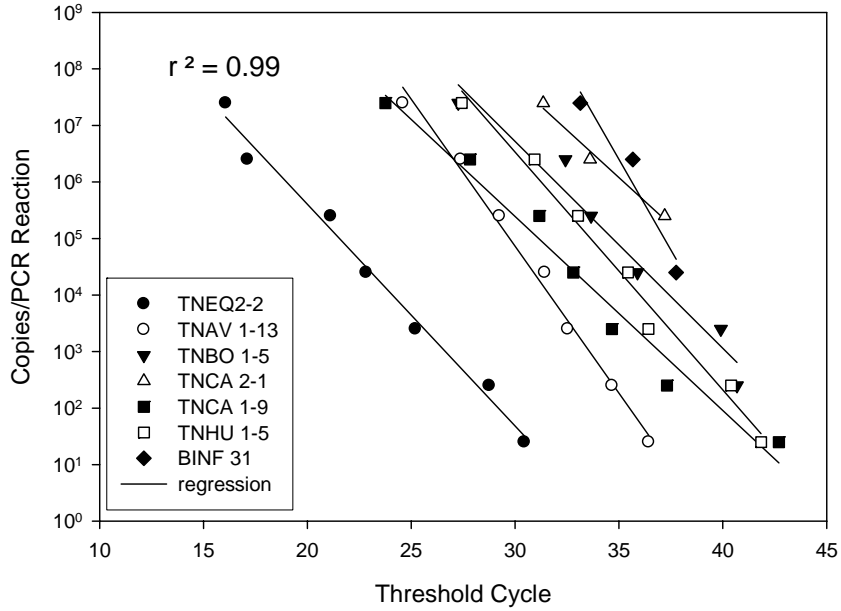
b)

% Cross Hybridization in the EqBac Assay

	TNEQ 2-2 59.1°C	TNEQ 2-2 61.4°C	TNAV 1-13 58°C	TNAV 1-13 59.1°C	TNAV 1-13 61.4°C
10^7	100	10	1×10^{-2}	1×10^{-2}	1×10^{-6}
10^5	100	10	1×10^{-4}	1×10^{-4}	1×10^{-5}
10^3	100	10	1×10^{-3}	1×10^{-3}	
10^1	10	10			

Figure 4.3. a) Variability in threshold cycle for equine-hosted *Bacteroides* 16S rRNA plasmid standards during temperature gradient q-PCR ranging from 58 °C to 61.4 °C. The r^2 value for the 59.1 °C reaction chosen as the final protocol is shown. b) Percent of cross hybridization using the EqBac Assay with target plasmid TNEQ 2-2 and non-target plasmid TNAV 1-13 (bird) at annealing temperatures ranging from 58 °C to 61.4 °C. The standard curve for calculating copies was based on TNEQ 2-2 at 58 °C.

a)



b)

% Cross Hybridization in the EqBac Assay

	TNAV 1-13 bird	TNBO 1-5 cow	TNCA 2-1 dog	TNCA 1-9 dog	TNHU 1-5 human	BINF 31 septic
10^7	3.4×10^{-1}	5.6×10^{-2}	3.7×10^{-3}	5.9×10^{-1}	5.1×10^{-1}	1.1×10^{-3}
10^6	1.1×10^{-1}	3.6×10^{-3}	1.7×10^{-3}	7.8×10^{-2}	9.9×10^{-2}	4.2×10^{-4}
10^5	4.4×10^{-1}	2.3×10^{-2}	2.2×10^{-3}	1.2×10^{-1}	3.5×10^{-3}	1.2×10^{-3}
10^4	3.2×10^{-1}	1.6×10^{-2}		1.3×10^{-1}	2.2×10^{-3}	4.7×10^{-3}
10^3	7.4×10^{-1}	5.4×10^{-3}		1.8×10^{-1}	5.6×10^{-2}	
10^2	1.9×10^0	3.5×10^{-2}		3.3×10^{-1}	4.2×10^{-2}	
10^1	1.8×10^0			2.8×10^{-2}	4.9×10^{-2}	

Figure 4.4. a) Increased specificity of the EqBac assay on target and non-target *Bacteroides* containing plasmids when run at an annealing temperature of 59 °C. R² value for the target gene, TNEQ 2-2, is shown. b) Percent of cross hybridization of non-target *Bacteroides* plasmids in the EqBac Assay. The standard curve for determining the expected copies was generated using the equine target, TNEQ 2-2.

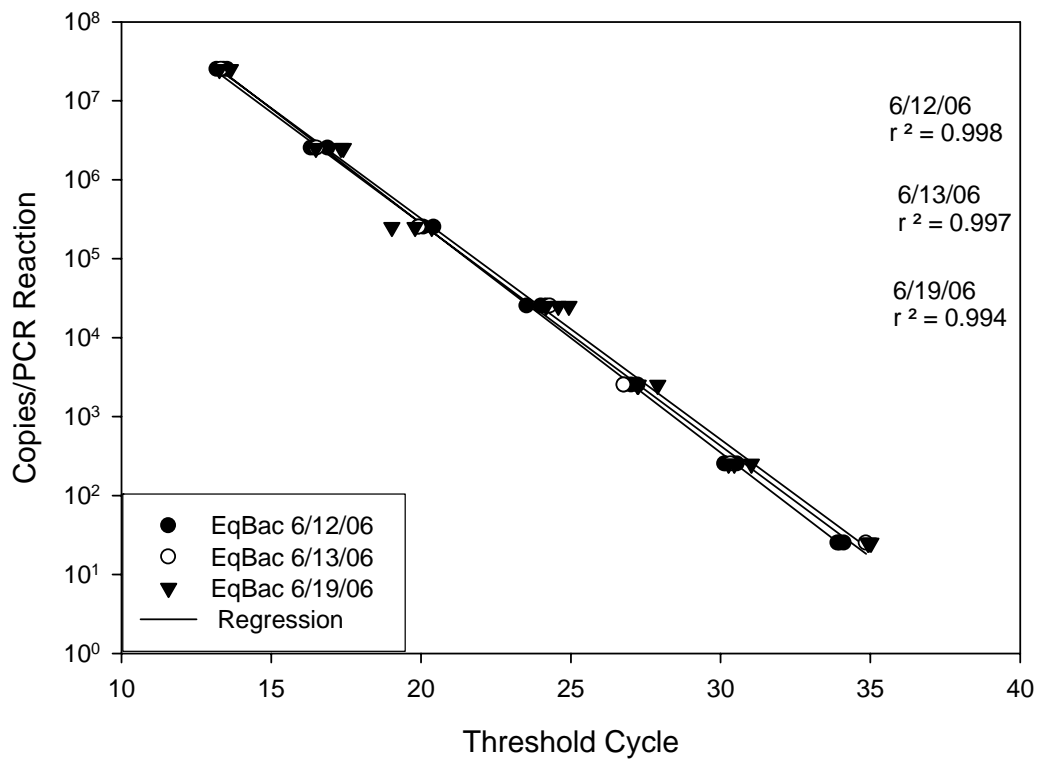


Figure 4.5. Repeated qPCR analysis of the EqBac assay on 16S rRNA equine-hosted *Bacteroides* plasmids to determine assay consistency. R² values are shown for each of three analyses.

contamination. This makes real-time PCR of *Bacteroides* 16s rRNA marker a potentially important tool for water quality analyses.

This paper presents a new real-time PCR assay, EqBac, for the detection of equine fecal contamination in water samples. Horses are a source of agricultural contamination in watersheds throughout the country; however, previously a means of quantifying equine fecal contamination was not available. Previous studies of equine-hosted *Bacteroides* provided assays for non-quantitative PCR (Simpson, et al., 2004); however, such assays are not optimal in analyzing environmental fecal contamination. Future work with this assay should include testing on contaminated water samples from a wide geographic range and inclusion in microbial source tracking projects at the watershed scale.

CHAPTER V CONCLUSIONS AND RECOMMENDATIONS

Fecal contamination of surface water is a serious problem responsible for the majority of infant deaths throughout the world, as well as costing billions of dollars annually in medical expenses, beach closures, and shellfishery closures (Shuvall, 2001). Monitoring fecal contamination of surface waters is a necessity fundamental to evaluating water quality and remediating fecal contamination.

Fecal indicator organisms have the potential to provide an accurate, reliable signal of fecal contamination and the possible presence of enteric pathogens. To do this, indicators should be strictly enteric, with a high correlation to the presence of pathogens as well as provide fast and inexpensive testing methods that provide a quantitative measurement of fecal pollution (Gerba, 2001).

A crucial component to the utility of a fecal indicator is its persistence in the environment. The amount of time that an organism can be detected in the environment is affected by its life history and impacts how well it will correlate to the presence of pathogens. Also, understanding persistence enables indicators to be used for microbial source tracking, which has important applications to watershed-scale remediation of fecal contamination (Savichtcheva and Okabe, 2006). Bacteria belonging to the genus *Bacteroides* have been suggested repeatedly as indicator organisms for both water quality assessment (Fiksdal et al., 1985; Kreader, 1998) and as agents of microbial source tracking (Boehm et al.,

2003; Dick and Field, 2004; Seurnick et al., 2005). While *Bacteroides* have numerous advantages over currently recommended indicators, crucial information about the persistence of the 16s rRNA genetic marker is lacking.

This thesis first presents a literature review of the use of *Bacteroides* as a fecal indicator. This review highlights the need for further research into the persistence of *Bacteroides* in surface water systems. Despite the lack of persistence characterization, a number of studies have successfully developed and applied standard and quantitative PCR assays to the detection of *Bacteroides* genetic markers for water quality analysis (Bernhard and Field, 2000; Dick and Field, 2004; Layton et al., 2006) as well as microbial source tracking (Seurnick et al., 2005; Lamandella et al., 2007; Bernhard et al., 2003). This thesis then presents the first quantitative analysis of multiple environmental factors that affect persistence of the *Bacteroides* 16S rRNA marker, as well as a new qPCR assay for the detection of equine fecal matter in the environment.

Several main conclusions can be drawn from the data presented in this thesis:

- Varying environmental parameters alter the persistence time of the *Bacteroides* genetic marker. The factors that increase persistence are water filtration and low temperature, with coarse aggregate size contributing as well.

- Biologic degradation is proposed as the dominant agent of decline of the *Bacteroides* genetic marker in surface water. Evidence for this conclusion comes from:
 - The increase in marker persistence after water filtration that was observed for the majority of variables tested.
 - The increase in marker persistence that was observed in microcosms with bio-inhibiting conditions such as low temperatures.
- *Bacteroides* marker persistence is thus expected to be longest for fecal matter deposited in water during winter. Lowest persistence times should be observed for fecal matter in water in summer.
- No evidence was found for the growth of *Bacteroides* in microcosm experiments, despite some suggestion from previous research that *Bacteroides* may have the ability to grow in anaerobic microcosms, such as might be found within fecal aggregates.
- The EqBac assay presented here is a potentially important tool for the quantification and detection of equine fecal pollution in water quality assessments and microbial source tracking, however further testing is required.

The data presented in this thesis can be used as a foundation for future studies into applications of the fecal indicator *Bacteroides*. In particular, quantitative molecular methods should be utilized to evaluate persistence under a

broader range of environmental settings than investigated here as well as to develop a better understanding of the effects of environmental transport on *Bacteroides* persistence. Tracking experiments in natural watersheds have the potential to provide direct testing of the expected seasonal patterns of persistence proposed above, as well as further refine the use of *Bacteroides* as an agent of microbial source tracking.

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APPENDICES

Appendix 1: Calculating fecal concentration in environmental samples using qPCR analysis with the AllBac assay.

The use of real-time PCR (qPCR) provides water quality analysts with a powerful tool for both monitoring and identifying sources of fecal contamination. The primary advantage of qPCR is quantitative results that reflect the amount of fecal contamination from a given organism. While qPCR returns the number of copies of target gene per PCR sample, this number can only be translated to a direct evaluation of fecal concentration if the target gene is present in fecal matter in a constant density relationship. A primary advantage of *Bacteroides* as a fecal indicator organism is the presence of the 16S rRNA gene as a constant 30% by mass of fecal matter.¹

In this study, the AllBac qPCR assay (Layton et al., 2006) was used to monitor persistence of the *Bacteroides* genetic marker over time under varying environmental conditions. This appendix describes the steps taken to convert the qPCR output data into mg/L of fecal contamination, as it is presented in this thesis. Figure A.1 provides an example of qPCR output for a number of samples run in triplicate with plasmid standards, blanks, and inhibition spikes. The results for plasmid standards ranging from 2.5×10^7 to 2.5×10^1 are used to create both graphs shown below, from which the copies of target gene in each sample are

¹ See Layton et al., 2006 for exact copies of *Bacteroides* 16S rRNA per mg/L of fecal contamination for a variety of different fecal source organisms.

calculated by the qPCR machine and displayed as an output file, such as that shown in Table A.1.

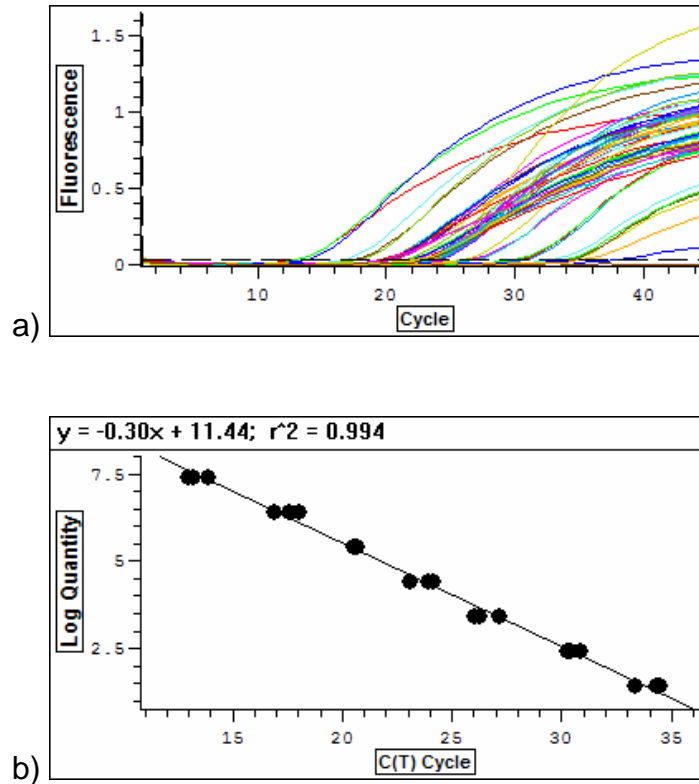


Figure A.1. Sample output files from a qPCR analysis with the AllBac assay. a) Cycles to fluorescence for each plasmid standard. b) Calculation of log quantity of the target gene using the defined quantity in the plasmid standards and the number of cycles to detection.

Well	Dye	Type	Label	C(T)	moles
A1	1:FAM	Standard	Hu 1-4, 2.5e7	13.032	2.50E+07
A2	1:FAM	Standard	Hu 1-4, 2.5e7	13.203	2.50E+07
A3	1:FAM	Standard	Hu 1-4, 2.5e7	13.896	2.50E+07
A4	1:FAM	Sample	Temp5.0-A.Sp	20.078	245537
A5	1:FAM	Sample	Temp5.0-A	22.116	61150.4
A6	1:FAM	Sample	Temp5.0-B	23.325	26812.3
A7	1:FAM	Sample	Temp5.0-C	23.869	18491.2
B1	1:FAM	Standard	Hu 1-4, 2.5e6	17.589	2.50E+06
B2	1:FAM	Standard	Hu 1-4, 2.5e6	16.953	2.50E+06
B3	1:FAM	Standard	Hu 1-4, 2.5e6	18.051	2.50E+06
B4	1:FAM	Sample	Temp5.0-Control.Sp	20.353	203498
B5	1:FAM	Sample	Temp5.0-Control	23.903	18071.3
B6	1:FAM	Sample	Temp5.0-Control	22.984	33816.9
B7	1:FAM	Sample	Temp5.0-Control	23.661	21309.5

Table A.1 Sample output data from qPCR analysis of fecally-contaminated water samples with the AllBac assay.

Because each round of microcosm experiments carried out in this study required multiple qPCR runs, all plasmid standard data were compiled in SigmaPlot to create the averaged standard curves shown in Figure A.2. From these curves the copies of target gene per sample were re-calculated by hand, using Equation A.1,

$$\text{Eq A.1)} \quad \text{copies} = 10^{(mC + b)}$$

where m represents the slope of the averaged standard curve, C represents the number of cycles to detection for the sample, and b represents the y-intercept of the averaged standard curve. This value was then converted into mg/L fecal contamination using the value of 1×10^7 determined for the density of *Bacteroides* target gene in equine fecal matter from Layton et al. (2006) using Equations A.2,

$$\text{Eq A.2)} \quad \text{mg/L} = (\text{copies} / 2 \times 10^7) / 2.5 \times 10^6$$

where the first term (copies) is the number determined from Equation A.1, the second term (2×10^7) is the average number of copies in one PCR sample (from Layton et al., 2006), and the third term (2.5×10^6) is a conversion factor.

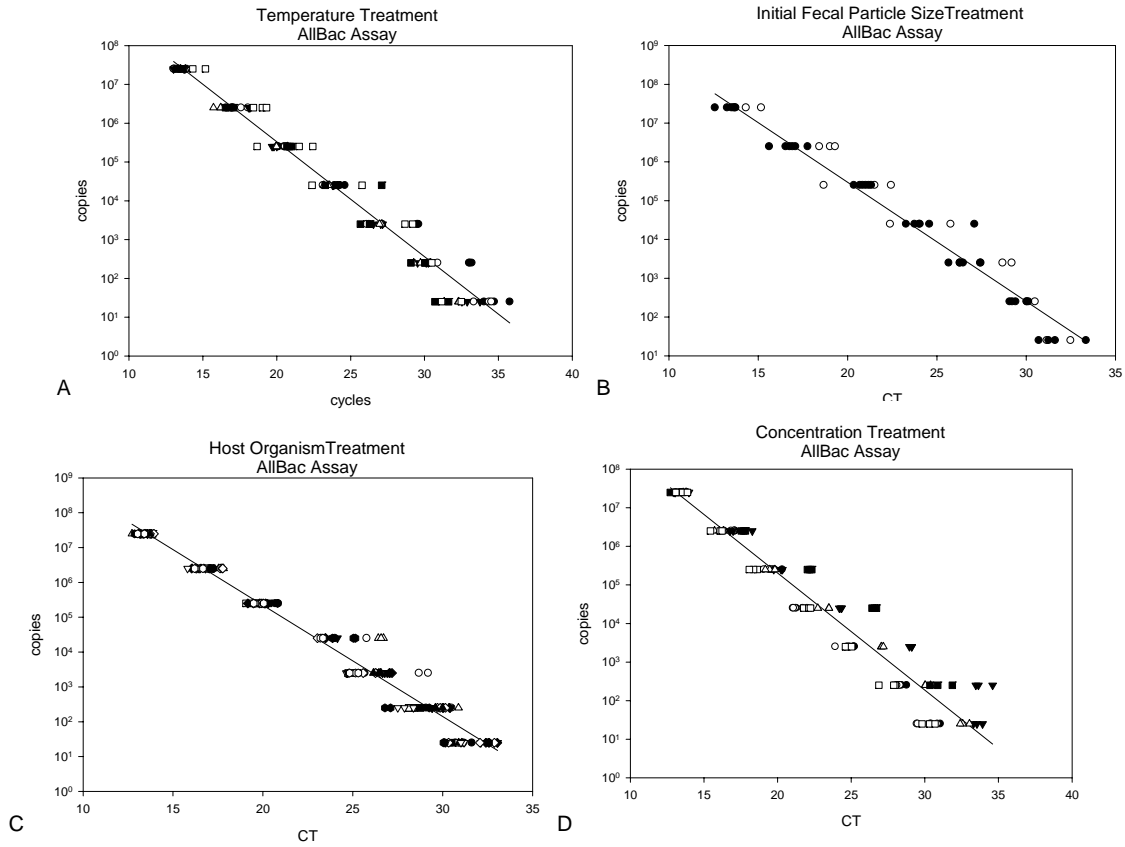


Figure A.2. Averaged qPCR plasmid standard curves for each microcosm experiment. The equation of the regression line was used to hand-calculate the copies of target gene in each sample.

Appendix 2: Multiple sequence alignment of *Bacteroides* 16s rRNA created in ClustalX (Thompson et al., 1997).

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TNBo1-4 -----AACGCTA-GCTACAGGCTTAACACATG
PABo1-8 -----AACGCTA-GCTACAGGCTTAACACATG
TNBo1-9 -----AACGCTA-GCTACAGGCTTAACACATG
TNBo1-5 -----AACGCTA-GCTACAGGCTTAACACATG
TXBo1-8 -----AACGCTA-GCTACAGGCTTAACACATG
PABo1-4 -----AACGCTA-GCTACAGGCTTAACACATG
TNBo2-3 -----AACGCTA-GCTACAGGCTTAACACATG
TNBo2-2 -----AACGCTA-GCTACAGGCTTAACACATG
AF233400_fecal -----AACGCTA-GCTACAGGCTTAACACATG
TXBo1-3 -----AACGCTA-GCTACAGGCTTAACACATG
PABo1-7 -----AACGCTA-GCTACAGGCTTAACACATG
PABo1-1 -----AACGCTA-GCTACAGGCTTAACACATG
TNBo1-6 -----AACGCTA-GCTACAGGCTTAACACATG
TNBo1-2 -----AACGCTA-GCTACAGGCTTAACACATG
TNBo2-9 -----AACGCTA-GCTACAGGCTTAACACATG
TXBo1-6 -----AACGCTA-GCTACAGGCTTAACACATG
TNBo1-3 -----AACGCTA-GCTACAGGCTTAACACATG
TNBo1-8 -----AACGCTA-GCTACAGGCTTAACCCATG
TNBo1-1 -----AACGCTA-GCTACAGGCTTAACACATG
TNBo1-7 -----AACGCTA-GCTACAGGCTTAACACATG
TXBo1-5 -----AACGCTA-GCTACAGGCTTAACACATG
PABo1-3 -----AACGCTA-GCTACAGGCTTAACACATG
TXBo1-10 -----ACGCTA-GCTACAGGCTTAACACATG
PABo1-2 -----AACGCTA-GCTACAGGCTTAACACATG
TXBo1-2 -----AACGCTA-GCTACAGGCTTAACACATG
PABo1-5 -----AACGCTA-GCTACAGGCTTAACACATG
TnPil-7 -----AACGCTA-GCTACAGGCTTAACACATG
TnPil-12 -----AACGCTA-CCTACAGGCTTAACACATG
TnPil-9 -----AACGCTA-GCTACAGGCTTAACACATG
M58762_Bacteroides -----AACGCTA-GCTACAGGCTTAACACATG
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TnPil-8 -----AACGCTN-GCTACAGGCTTAACACATG
TNCA25 -----AACGCTA-GCTACAGGCTTAACACATG
TNCA21 -----AACGCTA-GCTACAGGCTTAACACATG
TNCA22 -----AACGCTA-GCTACAGGCTTAACACATG
TNCA24 -----AACGCTA-GCTACAGGCTTAACACATG
TNBo2-6 -----AACGCTA-GCTACAGGCTTAACACATG
TNHu2-18 -----AACGCTA-GCTACAGGCTTAACACATG
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AF233408_Fecal -----AACGCTA-GCTACAGGCTTAACACATG
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TNHu2-10 -----AACGCTA-GCTACAGGCTTAACACATG
TNHu2-9 -----AACGCTA-GCTACAGGCTTAACACATG
TNHu2-8 -----AACGCTA-GCTACAGGCTTAACACATG
TNHu2-16 -----AACGCTA-GCTACAGGCTTAACACATG
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TNHu1-2 -----AACGCTA-GCTACAGGCTTAACACATG
Bovatus_X839521_ -----AACGCTA-GCTACAGGCTTAACACATG
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TNHu2-2 -----AACGCTA-GCTACAGGCTTAACACATG
TNHu2-6 -----AACGCTA-GCTACAGGCTTAACACATG
TNHu1-1 -----AACGCTA-GCTACAGGCTTAACACATG
TNHu2-7 -----AACGCTA-GCTACAGGCTTAACACATG
Bacteroidesuniformis_AB050110_ -----AACGCTA-G-TACAGGCTTAACACATG
TNHu1-5 -----AACGCTA-GCTACAGGCTTAACACATG
TNHu1-9 -----AACGCTA-GCTACAGGCTTAACACATG
TNHu2-1 -----AACGCTA-GCTACAGGCTTAACACATG
TNHu1-6 -----AACGCTA-GCTACAGGCTTAACACATG
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TNHu1-4 -----AACGCTA-GCTACAGGCTTAACACATG
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TNHu2-19 -----AACGCTA-GCTACAGGCTTAACACATG
TNHu1-10 -----AACGCTA-GCTACAGGCTTAACACATG
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TNCal-7	-----AACGCTA-GCTACAGGCTTAACACATG
TNCal-8	-----AACGCTA-GCTACAGGCTTAACACATG
TNCal-9	-----AACGCTA-GCTACAGGCTTAACACATG
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TNAv1-10	-----AACGCTA-GCTACAGGCTTAACACATG
TNAv1-13	-----AACGCTA-GCTACAGGCTTAACACATG
TnPi1-6	-----AACGCTC-GCTACAGGCTTAACACATG
TnPi2-2	-----AACGCTA-GCTACAGGCTTAACACATG
TNAv1-5	-----AACGCTA-GCTACAGGCTTAACACATG
TNAv1-7	-----AACGCTA-GCTACAGGCTTAACACATG
TNBo2-4	-----AACGCTA-GCTACAGGCTTAACACATG
TNBo2-7	-----AACGCTA-GCTACAGGCTTAACACATG
TNAv1-6	-----AACGCTA-GCTACAGGCTTAACACATG
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PAB01-6	-----AACGCTA-GCTACAGGCTTAACACATG
TNBo1-10	-----AACGCTA-GCTACAGGCTTAACACATG
TXBo1-1	-----AACGCTA-GCTACAGGCTTAACACATG
M58766_Cytophaga	-----CGCTA-GCGACAGGCTTAACACATG
TNEq1-11	-----AACGCTA-GCTACAGGCTTAACACATG
DC113734	-----AACACATG
TNEq-1	-----AACGCTA-GCTACAGGCTTAACACATG
TNEQ8Bac4	-----AACGCTA-GCTACAGGCTTAACACATG
TNEq2-1	-----AACGCTA-GCTACAGGCTTAACACATG
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AY38515	GAGTTTGATYCTGGCTCAGGATGACGGCTA-GCTACAGGCTTAACACATG
TNEq1-8	-----AACGCTA-GCTACAGGCTTAACACATG
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 TXBo1-8 CAAGTCGAGGGGCAGCAC--AGTGTA-GCAAT----ACATGGGTGGCGA
 PABo1-4 CAAGTCGAGGGGCAGCGC--AGTGTA-GTAAT----ACATGGGCGGCGA
 TNBo2-3 CAAGTCGAGGGGCAGCAC--GGTGTA-GCAAT----ACATGGGTGGCGA
 TNBo2-2 CAAGTCGAGGGGCAGCAC--AGTGTA-GCAAT----ACATGGGTGGCGA
 AF233400_fecal CAAGTCGAGGGGCAGCAC--TAGGTA-GCAAT----ACTGAGGTGGCGA
 TXBo1-3 CAAGTCGAGGGGCAGCAT--TAGGTA-GCAAT----ACTGAGATGGCGA
 PABo1-7 CAAGTCGAGGGGCAGCAT--TAGGTA-GCAAT----ACTGAGATGGCGA
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 TNBo2-9 CAAGTCGAGGGGCAGCAC--AAGGCA-GCAAT----GCTGAGGTGGCGA
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 TNBo1-3 CAAGTCGAGGGGCAGCATG-AAAGTA-GCTTGC--TACTTTTGATGGCGA
 TNBo1-8 CAAGTCGAGGGGTAGCACGGAGGA-A-GCTTGC--TTCCTTTGGGTGACGA
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 Bovatus_X839521_ CAAGTCGAGGGGCAGCAT-TTNGTTTCTTGCCTGCAAACTGAA-GATGGCGA
 AB021165Bacteroidesacidofacien CAAGTCGAGGGGCAGCAT-GGGAGTTTCTTGCCTGCAAACTTCC-GATGGCGA
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 TNHu1-1 CAAGTCGAGGGGCATCAG-GAAGAAA-GCTTGC--TTTCTTTGCTGGCGA
 TNHu2-7 CAAGTCGAGGGGCATCAG-GAAGAAA-GCTTGC--TTTCTTTGCTGGCGA
 Bacteroidesuniformis_AB050110_ CAAGTCGAGGGGCAGCAT-GAACTTA-GCTTGC--TAAGTTTGATGGCGA
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 TNHu1-3 CAAGTCGAGGGGCAGCAT-CATCAA-GCTTGC--TTTGTGATGGATGGCGA
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 TNCal-4 CAAGTCGAGGGGCAGCAT-GAAGTTT-GCTTGC--AAACTTTGATGGCGA
 TNCal-7 CAAGTCGAGGGGCAGCAT-GAAGTTT-GCTTGC--AAACTTTGATGGCGA
 TNCal-8 CAAGTCGAGGGGCAGCAT-GAAGTTT-GCTTGC--AAACTTTGATGGCGA
 TNCal-9 CAAGTCGAGGGGCAGCAT-GAAGTTT-GCTTGC--AAACTTTGATGGCGA
 TNAv1-8 CAAGTCGAGGGGCAGCAT-GAACTTA-GCTTGC--TAAGTTTGATGGCGA
 TNAv1-9 CAAGTCGAGGGGCAGCAT-GAGCTTA-GCTTGC--TAAGTTTGATGGCGA
 TNAv1-10 CAAGTCGAGGGGCAGCAT-GAACTTA-GCTTGC--TAAGTTTGATGGCGA

TNAv1-13	CAAGTCGAGGGGCAGCAT-GAACTTA-CCTTGC--TAAGTTTGATGGCGA
TnPil-6	CAAGTCGAGGGGCAGCGG-GNA---T-TGTAGCAATAACAATTGCCGGCGA
TnP12-2	CAAGTCGAGGGGCATCGC-GG-----CGTAGCAATACGCTGGT-GGCGA
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TNAv1-7	CAAGTCGAGGGGCAGCGG-GGGTCTA-GCTTGC--TAGGCTTGCCGGCGA
TNBo2-4	CAAGTCGAGGGGCAGCAT--GAAATCAGCTTGC--TGATTTTGATGGCGA
TNBo2-7	CAAGTCGAGGGGCAGCAT--CATCAAAGCTTGC--TTTGATGGATGGCGA
TNAv1-6	CAAGTCGAGGGGCAGCATAGGGTTCCGGCAACG--GAATTCGGATGGCGA
TXBo1-7	CAAGTCGAGGGGTCTCAAGGG----AGCTTGC--TTC--TGCAGAGCGA
PAB01-6	CAAGTCGAGGGGTCTTTTCT-----AGCTTGC--TA---TAAAGACGA
TNBo1-10	CAAGTCGAGGGGCAGCGGGGTCT--AGCTTGC--TAGATTTGCCGGCGA
TXBo1-1	CAAGTCGAGGGGCAGCGGGAATCT--AGCTTGC--TAGATTTGCCGGCGA
M58766_Cytophaga	CAAGTCGAGGGGTAAACATTGTA-----GCTTGC-----TACAGATGACGA
TNEq1-11	CAAGTCGAGGGGCAGCAT--GATCGAAGCTTGC--TTTGATTTGATGGCGA
DC113734	CAAGTCGAGGGGCAGCGG--GGATGAAGCTTGC--TTCATTTGCCGGCGA
TNEq-1	CAAGTCGAGGGGTAAACGG----GGGCGCTTGC-----GCCGCCGACGA
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TNEq2-8	CAAGTCGAGGGGCAGCGT--GGGAGGTGCTTGC--ACTTCTGACGGCGA
TNEq2-9	CAAGTCGAGGGGCAGCGT--GGGAGGTGCTTGC--ACTTCTGACGGCGA
AY597195	CAAGTCGAGGGGCAGCGT--GGGAGGTGCTTGC--ACTTCTGACGGCGA
TnEq2-2	CAAGTCGAGGGGTAAACAT--GGAGGGAGCTTGC--TTCCTCCGATGACGA
TNEq2-6	CAAGTCGAGGGGTAAACAT--GGAGGGAGCTTGC--TTCCTCCGATGACGA
TNEq2-10	CAAGTCGAGGGGTAAACAT--GGAGGGAGCTTGC--TTCCTCCGATGACGA
TNEQ10Bac2	CAAGTCGAGGGGTAAACATGGNAGGGA-GCTTGC--TTCCTCCGATGACGA
TNEQ10Bac6	CAAGTCGAGGGGTAAACAT--GGAGGGAGCTTGC--TTCCTCCGATGACGA
TNEQ10Bac14	CAAGTCGAGGGGTAAACAT--GGAGGGAGCTTGC--TTCCTCCGATGACGA
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TNEQ10Bac20	CAAGTCGAGGGGTAAACAT--GGAGGGAGCTTGC--TTCCTCCGATGACGA
AY597193	CAAGTCGAGGGGTAAACAT--GGAGGGAGCTTGC--TTCCTCCGATGACGA
AY597189	CAAGTCGAGGGGTAAACAT--GGAGGGAGCTTGC--TTCCTCCGATGACGA
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TNEQ4Bac19M13X2G04	CAAGTCGAGGGGTAAACATGGNAGGGA-GCTTGC--TTCCTCCGATGACGA
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TNEQ10Bac5	CAAGTCGAGGGGCAGCATGGGGCAGCAATGTCC-----CGATGGCGA
TNEQ10Bac3	CAAGTCGAGGGGTAAACAT--GGAGGGAGCTTGC--TTCCTCCGATGACGA
TNEQ10Bac17	CAAGTCGAGGGGTAAACGG----GGGAGCTTGC-----TCCCGCCGACGA
TNEQ4Bac15M13X2C04	CAAGTCGAGGGGTAAACGG----GGGTGCTTGC-----ACCTGCCGACGA
TNEQ8Bac7	CAAGTCGAGGGGTAAACGG----GGGAGCTTGC-----TCCCGCCGACGA
TNEq2-7	CAAGTCGAGGGGTAAACAG--GAGGGAAGCTTGC--TTTCTTGTGACGA
TNEq1-3	CAAGTCGAGGGGCATCGG-----GGGTAGC-AATACCCTGCCGGCGA
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TnEq2-4	CAAGTCGAGGGGCATCGG-----GGGTAGC-AATACCCTGCCGGCGA
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TNEQ10Bac19	CAAGTCGAGGGGCATCGG-----GGGTAGC-AATACCCTGCCGGCGA
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PAB01-4	CCGGCGCACGGGTGCGTAACGCGTATCCAACCTTCCCCTTACTCAGGGAT
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 TXBo1-7
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TNEQ4Bac15M13X2C04	TGAGAGGAAGGTCCCCACATTGGTACTGAGACACGGACCAAACCTCCTAC
TNEQ8Bac7	TGAGAGGAAGGTCCCCACATTGGTACTGAGACACGGACCAAACCTCCTAC
TNeq2-7	TGAGAGGAAGGTCCCCACATTGGAACTGAGACACGGTCCAAACTCCTAC
TNeq1-3	TGAGAGGAAGGTCCCCACACTGGAACCTGAGACACGGTCCAGACTCCTAC
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TNeq1-8	TGAGAGGAAGGTCCCCACATTGGAACCTGAGACACGGTCCAAACTCCTAC
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PABo1-4	GGGAGGCAGCAGTGAGGAATATTGGTCAATGGTCGGAAGACTGAACCAGC
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M58762_Bacteroides	GGGAGGCAGCAGTGAGGAATATTGGTCAATGGCCGGAAGCCTGAACCAGC
TnPil-10	GGGAGGCAGCAGTGAGGAATATTGGTCAATGGCCGGAAGCCTGAACCAGC
TnPil-8	GGGAGGCAGCAGTGAGGAATATTGGTCAATGGCCGGAAGCCTGAACCAGC
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 TnHu1-5 CAAGTAGCGTGAAGGATGACTGCCCTATGGGTTGTAACTTCTTTTATAC
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 TNBo2-7 CAAGTAGCGTGCAGGATGACGGCCCTCCGGGTTGTAACTGCTTTTAGTT
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 TXBo1-7 CAAGTAGCGTGAAGGATGACTGCCCTATGGGTTGTAACTTCTTTTATAC
 PAB01-6 CAAGTAGCGTGAAGGATGACTGCCCTATGGGTTGTAACTTCTTTTATAC
 TNBo1-10 CAAGTAGCGTGAAGGATGACTGCCCTACGGGTTGTAACTTCTTTTATAC
 TXBo1-1 CAAGTAGCGTGAAGGATGACTGCCCTATGGGTTGTAACTTCTTTTGCCG
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 TNEq1-11 CAAGTAGCGTGAAGGATGACTGCCCTATGGGTTGTAACTTCTTTTATAC
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	GGCTTTTTGCATGTACCG-TAAGAAAA
	GGGATAAAAAGCGGGACGTGT-CCC
	* ** * ** *
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TNBo1-9	AGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA-GGATGCGA
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TNBo2-3	GCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGACGGGAGATTAAG
TNBo2-2	GCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGACGGGAGATTAAG
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TnPil-8	GCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGATGGATGTTAAG
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VITA

Alyssa Bell was born August 23, 1982 in Chattanooga, Tennessee. She graduated from high school in Carthage, Missouri in 2000 and went on to earn a Bachelor's of Art in Ecology and Systematics from the Oxbridge Honors Program at William Jewell College in Kansas City, Missouri in 2004. She attended the University of Tennessee from 2005 to 2007 and is expected to graduate in August of 2007 from the Department of Earth and Planetary Sciences with a Master's degree in Geology.