AN ABSTRACT OF THE DISSERTATION OF

Sarah P. Walters for the degree of Doctor of Philosophy in Microbiology presented on December 21, 2006.

Title: Survival and Persistence of *Bacteroidales* Human and Ruminant Specific Fecal Markers and Occurrence with Fecal Pathogens

Abstract approved: _____

Katharine G. Field

Aquatic fecal contamination from non-point sources impairs environmental health and serves as a vehicle for transmission of waterborne disease, resulting in economic losses worldwide. Accurate methods of diagnosing fecal pollution and its source are needed to prevent human exposure, remediate pollution, and reduce economic impacts. In order to obtain this goal, fecal indicator organisms should demonstrate persistence and survival profiles similar to fecal pathogens and be detected when pathogens are present. Molecular markers designed from *Bacteroidales* anaerobic fecal bacteria make good alternative indicator candidates because they identify fecal pollution sources and are not expected to grow in oxygenated environments. To further investigate the utility of these markers as indicators of fecal contamination and predictors of pathogen exposure, we evaluated co-occurrence of pathogenic *E. coli* O157:H7, *Salmonella* spp., and *Campylobacter* spp. with general,

human, ruminant, and porcine-specific *Bacteroidales* molecular markers. We detected host-specific markers in samples where pathogens were present and found a significant correlation between presence of the ruminant markers and *E. coli* O157:H7, and the human markers and pathogenic *Campylobacter* spp. We examined growth and persistence of *Bacteroidales* organisms in aerobically incubated sewage influent using bromodeoxyuridine (BrdU) labeling and immunocapture. We identified growing *Bacteroidales* bacteria in BrdU-labeled DNA fractions after immunocapture with fluorescent fragment detection following a low, quantitative number of PCR cycles. We consistently detected growth of *Bacteroidales* organisms but were unable to detect growth of the human-specific *Bacteroidales* organisms using this method. We constructed microcosms to investigate how ambient light exposure affects survival and persistence of ruminant and human-specific *Bacteroidales* markers, relative to standard indicators. The ruminant-specific *Bacteroidales* markers displayed differential persistence and survival profiles in both light and dark incubations; this was not observed among the human-specific markers. In each microcosm, the standard indicators persisted and survived longer than any of the *Bacteroidales* host-specific markers. Host-specific *Bacteroidales* genetic markers show promise as an accurate, rapid, and reliable tool in health risk based analyses of fecal pollution. The molecular markers are detected when pathogens are present, persist less than 14 days in natural river water at 13°C, and the marker organisms do not proliferate in the environment.

Survival and Persistence of *Bacteroidales* Human and Ruminant Specific Fecal Markers and Occurrence with Fecal Pathogens

by

Sarah P. Walters

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented December 21, 2006
Commencement June 2007

Doctor of Philosophy dissertation of Sarah P. Walters presented on December 21, 2006.

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Sarah P. Walters, Author

ACKNOWLEDGEMENTS

First and foremost I would like to thank Dr. Katherine G. Field for the opportunity to work on the microbial source-tracking project. Dr. Field challenged me to think independently and exposed me to many fascinating aspects of molecular research. I would like to thank Dr. Peter J. Bottomley. Dr. Bottomley was an invaluable source of knowledge, patience, support, and encouragement throughout my tenure as a graduate student. Members of my graduate committee, Drs. Daniel J. Arp, Peter J. Bottomley, Evelyn B. Sherr, and Susan J. Tornquist offered their time and unique perspectives on my project. I could not have completed my education without their assistance and I extend my sincerest gratitude to them.

The Charles E. and Marie Eckelman Graduate Fellowship provided financial support for the majority of my graduate education. The Oregon Sea Grant (04-016) supported my research and absorbed publication costs for the BrdU manuscript. The department of Microbiology allowed me to pursue my academic goals and granted me a teaching assistantship during my first year. The Bill Wick Marine Fisheries Award supplied research funds, making Chapter 5 a reality. Assistance from the Oregon Sports Lottery Scholarship afforded me weekends of snowboarding and thus helped maintain my sanity.

Thank you to the entire staff at the Salmon Disease Laboratory. Your assistance with tank space and engineering for the microcosm experiments was unprecedented and facilitated completion of the experiment without contest. Thank you to all of the departmental office staff. You were extremely helpful no matter what

the request. Sally Tatala is not only an exemplary office assistant she is also a dear friend. Thank you for everything Sally. A special thanks to Paul Grass from Applied Biosystems. If not for Paul's help Chapter 5 could not have materialized. Thank you to Applied Biosystems for providing the demo-QPCR machine.

I want to extend my appreciation to all former members in Dr. Field's laboratory. Linda Dick was steadfast in her friendship and willingness to discuss science with me. Micah Walter and Carolyn Petersen provided humor, friendship, and technical support. Tom Jones, Melissa Younger, and Breanne Borrow listened to me complain and made me laugh regularly. Kevin Vergin helped me get started my first year when I knew nothing and continued to help me throughout the next four years. To all of you, I am sincerely grateful.

I would like to thank all of my friends; your support both professionally and emotionally will never be forgotten. Particular recognition goes to Kevin Hood for helping me in immeasurable ways during the 8 weeks preceding completion of this thesis. Without his willingness to buy groceries, put up with my grumpiness, and take care of my two wonderful Labrador retrievers, August West and Henry, I would not have made it through the final push. Hency Chu and Wei-Wei Chiu are my oldest and dearest friends at Oregon State University. You ladies made life more bearable and I will miss our lunch dates dearly.

Finally, I would like to thank my parents Norman and Tricia Walters for always believing in me and reminding me that I could do anything I set my mind to. A t times you may have questioned my choices but you never told me there was anything I couldn't do. For that I owe you all my love and sincere thanks.

CONTRIBUTION OF AUTHORS

All experiments were conducted in the laboratory of Dr. Katherine Field. Dr. Field was involved in data interpretation as well as preparation and editing of manuscripts. Victor P. J. Gannon provided pathogen data and DNA samples from the Lethbridge Northern Irrigation District watershed, in Alberta Canada and was involved in preparation and editing of the manuscript entitled "Detection of *Bacteroidales* Fecal Indicators and the Zoonotic Pathogens *E. coli* O157:H7, *Salmonella*, and *Campylobacter* in River Water".

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Access to contaminant-free water is essential to all life. Sources of anthropogenic contamination are becoming more prevalent as human population size increases on a global scale. One such type of contamination is fecal pollution. Humans rely on pathogen-free water for drinking, recreation, crop irrigation, and as a source of seafood. As contamination of water with fecal pollution continues to rise, effective means of tracking contamination sources and evaluating human health risks become increasingly important in order to mitigate human exposure. Fecal pollution in aquatic ecosystems not only presents health risks but also impacts the global economy due to loss of revenue resulting from reduced productivity and medical expenses, as well as from closure of watersheds used for recreation and shell fish harvesting. Fecal contamination also adversely affects aquatic ecosystems and is implicated in some diseases of Carribean corals. To preserve ecosystem health across the planet it is imperative that effective, reliable indicators of fecal pollution, that are also capable of identifying pollution sources, are identified, evaluated, and implemented.

Molecular techniques have provided insight into the complexity of communities residing within the intestines of animals. From molecular cloning studies it is evident that the vast majority of animal bacterial fecal flora has not been cultivated. Phylogenetic analyses derived from genetic sequences obtained from cloning studies reveal multiple host-specific groups of anaerobic fecal *Bacteroidales* bacteria. Genetic markers designed from these host-specific sequences have proven

valuable in assessing water quality and identifying host-sources of fecal pollution across the globe.

Environmental growth and persistence, as well as correlation with fecal pathogens are critical elements of an indicator used for health-risk based analyses of fecal pollution. In this thesis, the co-occurrence of select host-specific fecal *Bacteroidales* markers and fecal pathogens is examined and the extra-intestinal survival of human and ruminant-specific *Bacteroidales* bacteria and persistence of the corresponding fecal markers is evaluated. One objective of this work was to determine if *Bacteroidales* host-specific markers are detected when human fecal pathogens are present and if any of these pathogens are associated with the presence of particular host-specific *Bacteroidales* markers. Another objective was to determine how long *Bacteroidales* host-specific marker organisms survive, and the genetic markers persist, in the extra-intestinal environment and how this survival and persistence is influenced by environmental variables.

Chapter 3 describes a study of human, ruminant, and porcine-specific fecal *Bacteroidales* marker co-occurrence with pathogenic *Salmonella* spp., *Campylobacter* spp., and *Escherichia coli* O157:H7 in a rural watershed in Alberta Canada. This study provides insight into the ability of host-specific *Bacteroidales* fecal markers to predict the presence of human fecal pathogens. One striking relationship was observed among the occurrence of ruminant-specific fecal *Bacteroidales* and the presence of *E. coli* O157:H7; the likelihood of isolating *E. coli* O157:H7 was six times greater in samples where ruminant-specific markers were present.

In Chapter 4 results are reported of an experimental study that was carried out to test the application of DNA labeling using bromodeoxyuridine (BrdU), followed by magnetic immunocapture and PCR, as a means of identifying actively growing *Bacteroidales* bacteria. BrdU labeling and immunocapture followed by PCR has been used to identify metabolically active members of bacterial populations. The experiments described in this thesis detail a modified approach to identifying growth of fecal *Bacteroidales* populations using a low, quantitative number of PCR cycles following immunocapture of BrdU-labeled DNA. This study showed that *Bacteroidales* bacteria take up and incorporate BrdU during growth and, using of a low number of PCR cycles, allowed assessment of active populations without false positive results. However, using this modified approach, growing populations comprised of only a small number of target organisms may be missed. We were unable to detect growing human-specific *Bacteroidales* bacteria in aquatic systems using this method.

Chapter 5 describes a study using human and ruminant feces as inocula for survival studies in fresh water microcosms where light penetration of the water column was included as a variable potentially affecting persistence and survival of the *Bacteroidales* genetic markers or marker organisms. In this study the survival of the current standard U.S. Environmental Protection Agency (EPA) indicators for fecal pollution, fecal coliforms (FC), *E. coli* and enterococci, was compared to that of the proposed *Bacteroidales* markers for human and ruminant fecal sources. Current USEPA indicators grow and persist in the environment and as a result do not adequately estimate timing of pollution events or the relative health risks associated

with exposure to fecal pathogens in water. The fact that *Bacteroidales* marker growth and persistence are restricted following extra-intestinal deposition provides evidence that these markers may more adequately estimate water quality and predict human health risk.

CHAPTER 2

LITERATURE REVIEW

Sarah P. Walters

Implications of Aquatic Fecal Pollution

Fecal pollution in water causes economic and environmental loss and human health risks. The presence of human and non-point sewage within aquatic habitats causes eutrophication, predisposing these ecosystems to undergo detrimental shifts in community composition. Sewage outfall and agricultural run-off introduce trace nutrients that facilitate rapid phytoplankton growth, resulting in blooms that reduce the amount of available oxygen in the water column, and can produce harmful toxins resulting in large scale die-off of aquatic organisms and human illness. Aquatic fecal pollution presents a vehicle for the spread of human disease originating from human sources as well as reservoirs of zoonotic pathogens.

Effects of fecal pollution on the environment

Fecal pollution harms the environment and causes habitat destruction. When considered on an annual basis, non-point sources are considered a larger contributor to nutrient loading in watersheds than point sources (National Research Council, 2000), perhaps because they are more difficult to control. Sewage discharges, animal waste, agricultural run-off, and contaminated groundwater contain high concentrations of nitrogen and phosphorus that can fuel marine and freshwater phytoplankton proliferation (Nixon and Pilson, 1983; National Research Council, 1993; Anderson et al., 2002). Phytoplankton blooms are an increasing threat to aquatic life; some species produce chemicals that are toxic to animals and plants. Over the past decade red tides have been linked to the death of hundreds of marine mammals, including endangered

manatees, off the southwest coast of Florida (Bossart et al., 1998; Trainer and Baden, 1999).

Pollution from sewage is also thought to be a contributor to coral disease in the Caribbean (Lipp et al., 2002; Patterson et al., 2002; Whitfield, 2002; Kaczmarsky et al., 2005). White pox, a disease that exclusively affects *Acropora palmata*, the Elkhorn coral, was first identified off the coast of Key West, Florida in 1996 (Holden, 1996; Sutherland and Ritchie, 2004). In 2001 the fecal enteric bacterium, *Serratia marcescens*, was identified as the causative agent of white pox (Patterson et al., 2002); this was the first time a human gut bacterium was shown to cause disease in a marine invertebrate. *Acropora palmata* was once the dominant coral in the Caribbean but has decreased in population size by more than 90% in the past decade and was added to the Endangered Species List in 2006 (Federal Register, 2006).

Human health risks associated with fecal pollution in water

Over the past two decades reports of human gastrointestinal illness associated with waterborne fecal pathogens have increased (U.S. Commission on Ocean Policy, 2004). Traditionally, most epidemiological studies have been conducted in locations where human sewage was the most likely contamination source, focusing on waterborne illnesses resulting from exposure to human effluent (Till et al., 2004). Human and non-human fecal contamination present different threats to human health. Pathogen threats from contact with human feces include adenoviruses and coxsackieviruses, hepatitis C virus, poliovirus, norovirus, rotavirus, *Salmonella*, *Campylobacter*, and *Shigella*, causing clinical diseases such as upper respiratory

infections, conjunctivitis, gastroenteritis, and hepatitis. Zoonotic pathogens are transmissible to humans via a non-human animal host; non-human animal feces can spread many pathogens. Accordingly it is important to consider these pathogen sources when evaluating water quality. *E. coli* O157:H7, for example, is associated with ruminant feces (Kudva et al., 1996; Hancock et al., 2001; Molbak and Scheutz, 2004). Other bacteria including *Salmonella* spp., *Campylobacter jejuni,* and *Leptospira interrogans*, as well as protists such as *Giardia spp.*, *Cryptosporidium parvum*, and *Toxoplasmosis gondii* are associated with wildlife, farm animals and domestic pets (Hanninen et al., 2000; Dubey, 2004; Cox et al., 2005). Identifying the source(s) of fecal pollution can provide insight to which pathogens are potentially present. While swimming-related illnesses are usually not life threatening, some such as those caused by Leptospira, *E. coli* O157, and hepatitis C can be fatal or cause extremely serious infections among immunocompromised individuals, impacting a population's general welfare.

Economic implications of aquatic fecal pollution

Currently over half of the earth's population resides within 100 km of a coastline (Zero population growth, 1991; National Oceanic and Atmospheric Administration (NOAA), 1998 (online); Niemi et al., 2004). Fecal pollution not only damages ecosystems and contributes to human disease, but also presents serious economic implications. Cumulative effects become quite large on a national scale from closures of shellfish farming and beaches, loss of work and productivity due to illness, and medical expenses. In 2000, ocean related recreational activity by itself

contributed more than $200 billion to the U.S. economy (Dorfman, 2006). Approximately 85% of all tourism revenue in the United States is taken in by coastal states. One study estimated economic losses from closing a Lake Michigan beach due to pollution to be as high as $37,000 per day (Rabinovici et al., 2004). Another study conducted in Orange County, California, estimated the economic burden per gastrointestinal illness, acute respiratory illness, ear ailment, and eye ailment as $36.58, $76.76, $37.86, and $27.31 per incident, producing a cumulative financial impact of $3.3 million for the two beaches evaluated (Dwight et al., 2005). These estimates did not take into account personal out of pocket expenses such as prescriptions and over the counter medications. In 2006 there were 24,815 beach closures and advisories reported throughout the country, up 0.5% from 2005 (Dorfman, 2006).

Commercial fishing industries are particularly susceptible to economic hardship brought about by aquatic fecal pollution. The shellfish industry incurs economic losses not only because of eutrophication induced phytoplankton blooms but also due to closures of harvesting activities because of high numbers of fecal indicator bacteria in overlaying waters. Elevated numbers of fecal indicator bacteria are indicative of an increased risk in gastrointestinal illness from consumption of contaminated shellfish.

Indicators of Fecal Pollution in Aquatic Ecosystems

Mandated monitoring of water used for shellfish farming and recreation has greatly reduced the incidence of waterborne disease. Historically, total coliforms (TC) and fecal coliforms (FC) were used as bacterial indicators of fecal pollution. TC and FC can originate from non-fecal sources and, as a result, were replaced by *Escherichia coli* which is more specific to animal feces. Currently, the United States Environmental Protection Agency (U.S. EPA) standard methods for water quality testing rely on growing *E. coli* from freshwater or fecal enterococci from marine water to estimate the human health risk associated with exposure to fecal pollution in waters used for shellfish farming, recreation, or drinking purposes (US EPA, 2001). However, U.S. EPA standard methods do not discriminate among host sources of fecal input. Because effective and efficient mitigation of fecal pollution requires the ability to identify the source(s) of pollution, the standard U.S. EPA methods are undergoing re-evaluation (Fujioka, personal communication) and validation studies using novel source tracking methods are currently under way (Field et al., 2003; Griffin et al., 2003; Harwood et al., 2003).

Standard indicators and source tracking methods

Standard methods for identifying fecal pollution rely on cultivation of *E. coli* and enterococci from environmental sources. Cultivation-based techniques require 18-24 hours before results are available. Often, a health threat from exposure to fecal

pathogens has passed by the time the analysis is completed, and furthermore, individuals are likely to be exposed to pathogens by the time a beach closure decision has been made. *E. coli* and enterococci can persist in a viable but non-culturable (VBNC) state in the environment, a condition that occurs under adverse growth conditions (Wang and Doyle, 1998). VBNC bacteria retain metabolic functions and genes for pathogenicity and can be resuscitated when circumstances conducive to growth are encountered (Roszak and Colwell, 1987; Magarinos et al., 1994; Whitman et al., 2003). Culture-based assays can miss the presence of VBNC fecal indicator bacteria, yielding artificially low quantitative estimates.

Alternative methods using *E. coli* and enterococci to determine the source(s) of fecal pollution are under evaluation. Antibiotic resistance analysis (ARA) (Parveen et al., 1997; Hagedorn et al., 1999; Wiggins et al., 1999) and carbon utilization profiles (CUP) (Hagedorn et al., 2003; Wallis and Taylor, 2003) are phenotypic, library dependent methods that test isolates of *E. coli* and enterococci against a suite of antibiotics or carbon sources to determine sources of fecal input. In a blind study comparing fecal source tracking methods by testing water inoculated with feces, results using the ARA method did not significantly deviate from random guesses (Griffin et al., 2003). Because genes for antibiotic resistance are often conferred by plasmids, they are under strong selective pressure and do not impart geographic stability, requiring large libraries be constructed for each geographic location. In blind studies using CUP, the method produced a higher than expected number of false positives and demonstrated poor host identification (Griffin et al., 2003; Harwood et al., 2003).

Implementation of molecular methods in the field of fecal source tracking revealed differences among the genotypic signatures of *E. coli* and enterococci isolates from different host feces. Ribotyping (Parveen et al., 1999; Carson et al., 2001; Scott et al., 2004), repetitive extragenic palindromic polymerase chain reaction (rep-PCR) (Dombeck et al., 2000; Carson et al., 2003; McLellan et al., 2003), amplified fragment length polymorphism (AFLP) (Guan et al., 2002), and pulse field gel electrophoresis (PFGE) (Myoda et al., 2003) rely on genomic patterns to categorize isolates from various host-animal feces. However, these methods are limited by a cultivation step and due to high levels of genetic diversity, require assembly of libraries from each geographic location (McLellan et al., 2003).

Correlation of standard indicators and pathogens

An indicator should be present at the same time as pathogens and in concentrations proportional to the concentration of pathogens (Tamplin, 2003). The presence of standard indicators, *E. coli* and enterococci, does not correlate well with the presence of many fecal pathogens including *Salmonella* spp., *Campylobacter* spp., *Cryptosporidium* spp., *Giardia*, human adenoviruses, enteroviruses, or hepatitis A (Polo et al., 1998; Jiang et al., 2001; Noble and Fuhrman, 2001; Bonadonna et al., 2002; Lemarchand and Lebaron, 2003; Horman et al., 2004; Jiang and Chu, 2004; Kemp et al., 2005).

Although a significant correlation between the presence of *Cryptosporidium* and thermotolerant fecal coliforms (FC) in sewage effluent, and *Cryptosporidium*, FC, and enterococci (FS) in river water has been reported (Lemarchand and Lebaron,

2003), most studies examining the presence of standard and alternative fecal indicators (*E. coli*, enterococci, fecal coliforms (FC), total coliforms (TC), and f-specific coliphage) indicate there is no correlation with the presence of protozoan parasites *Cryptosporidium* spp. and *Giardia* spp. in sewage effluent or natural surface water (Rose et al., 1988; Bonadonna et al., 2002; Horman et al., 2004). The resistant nature of *Cryptosporidium* oocysts and *Giardia* cysts may contribute to these findings as they can survive for several months in water (Medema et al., 1998). *Clostridium perfringens* spores persist for longer periods in the environment than other fecal bacteria indicator species but this indicator does not appear to correlate with the presence of *Cryptosporidium* spp. or *Giardia* spp. either (Horman et al., 2004).

The relationship between the occurrence of *Salmonella* spp. relative to the fecal indicators *E. coli*, enterococci, TC, and FC indicators is convoluted. A study following extinction rates of *Salmonella* spp., *E. coli*, and enterococci over time found, on average, rates of decay for *Salmonella* spp. were greater than that of the indicator organisms (*Salmonella* spp. > *E. coli* > enterococci), in seawater microcosms (Craig et al., 2003). In a coastal watershed the occurrence of culturable *Salmonella* spp. did not correlate with counts of enterococci or FC in exceedance of established guidelines (Lemarchand and Lebaron, 2003). In river water, when enterococci, TC, and FC counts exceeded established EU guidelines, *Salmonella* spp. were more likely to be recovered. However, *Salmonella* spp. were also recovered 40% of the time when indicator counts were below these limits (Polo et al., 1998).

A small number of studies have reported similarly ambiguous results when evaluating the relationship between presence of indicators and *Campylobacter* spp.

Isolation of *E. coli* or FC does not appear to increase the likelihood of detecting *Campylobacter* spp. in the same sample (Horman et al., 2004; Kemp et al., 2005). *E. coli* can survive up to ten times longer than *Campylobacter jejuni* in autoclaved lake water (Lund, 1996); *E. coli* may be present for extended periods of time after a pollution event.

No clear relationship was found between the occurrence of indicator organisms and adenoviruses, enteroviruses, or hepatitis A in urban rivers (Jiang and Chu, 2004), or in coastal water receiving urban run-off in southern California (Jiang et al., 2001; Noble and Fuhrman, 2001).

Persistence and survival of standard indicators in extra-intestinal ecosystems

To accurately estimate human health risks associated with aquatic fecal pollution, an indicator should not proliferate in the environment but should persist as long as pathogens (Tamplin, 2003). Several studies demonstrate the ubiquity of *E. coli* and enterococci in natural environments. For example, *E. coli* and enterococci naturally occur at high densities in surface and subsurface soils in tropical regions such as Guam and Hawaii, and concentrations of these indicator species are artificially high in tropical streams subsequent to rainfall events (Hardina and Fujioka, 1991; Fujioka et al., 1999). Weekly monitoring of Dunes Creek, Indiana, from 1999 – 2000, identified chronic high levels of *E. coli* throughout the stream. The authors state that direct fecal input did not account for the consistent loading of *E. coli* in this watershed, and survival and multiplication of *E. coli* in the soil and riparian sediments was the most likely source (Byappanahalli et al., 2003). At six freshwater beaches,

concentrations of *E. coli* were 3 – 38 times higher in the upper 20 cm of beach sand than in the water column. Enterococci was most abundant at sand depths of 5 – 10 cm and in greater numbers than *E. coli* (Wheeler Alm et al., 2003). Furthermore, *E. coli* and enterococci persisted and survived for more than six months in dried Cladophora mats stored at 4°C, and grew readily after re-hydration of the mats (Whitman et al., 2003).

More recently, environmental *E. coli* isolates were identified that are phenotypically and genotypically distinct from *E. coli* isolates derived from animal hosts (Power et al., 2005; Byappanahalli et al., 2006), suggesting that *E. coli* can persist as free-living bacteria for extended periods of time, independent of recent fecal input. Thus, the widespread presence of *E. coli* and enterococci in the environment weakens their ability for use in risk assessment and to predict the timing of a pollution event.

Evaluating extra-intestinal growth of enteric bacteria

Typically, studies evaluating survival and growth of allochthonous fecal bacteria utilize cultivation-based techniques. This approach is not useful in studies where organisms of interest are not readily cultured in isolated form or for organisms existing in a VBNC state. Alternative methods have been developed to study metabolically active members of a microbial population. One such method is RNA probing and includes RNA dot-blot hybridization using chemiluminescent or radioactive probes, fluorescent in situ hybridization (FISH), alone or in combination with microautoradiography, and reverse transcriptase PCR (RT-PCR) (Amann et al.,

1995; Lee et al., 1999; Amann and Ludwig, 2000; Ouverney and Fuhrman, 2001; Pernthaler et al., 2002; Rossello-Mora et al., 2003). RNA-based methods rely on the assumption that RNA is rapidly degraded upon cell death or lysis and therefore, RNA is only detected from living cells or from cells that have recently perished.

Alternately, because bacterial growth requires DNA replication, labeling DNA with some traceable compound during growth provides a means of identifying growing members of a population. One method, using the thymidine base analog, bromodeoxyuridine (BrdU) for DNA labeling, has been used to examine metabolically active bacteria within complex communities as well as bacterial response to nutritional supplements (Borneman, 1999; Urbach et al., 1999; Pernthaler et al., 2002; Artursson and Jansson, 2003). When cells are in a state of thymidine auxotrophy they take up exogenously supplied BrdU and incorporate it into nascent DNA. Labeled DNA is separated using magnetic immunocapture and active species are identified by PCR of the labeled DNA fraction. Identification of BrdU labeled DNA indicates growth of bacteria under a prescribed set of parameters.

Bacteroidales Fecal Anaerobes as Host-Specific Indicators of Fecal Pollution

The phrase "the great plate count anomaly" (Staley and Konopka, 1985) was coined to describe the difference between the number of microbial cells observed by direct microscopic evaluation of aquatic samples compared to the number of isolated colonies observed to grow on agar plates from the same samples. Only an estimated 1% of microbial life on planet earth has been successfully cultivated in the laboratory (Amann et al., 1995; Pace, 1997), leaving the remaining 99% undescribed and yet to be discovered. This phenomenon has been demonstrated across a breadth of microbial ecosystems including the gastrointestinal tracts of animals.

Among the majority of uncultivated microbial life are organisms capable of identifying pollution sources. Phylogenetic analyses of 16S rRNA gene libraries have revealed novel lineages among fecal *Bacteroidales* bacteria that are specific to the gastrointestinal tracts of the host species they inhabit (Suau et al., 1999; Bernhard and Field, 2000a; Daly et al., 2001; Simpson et al., 2004; Dick et al., 2005a). By taking advantage of sequence variability within 16S rRNA gene sequences among animal hosts, PCR primers were designed to diagnose and distinguish fecal pollution from human and ruminant sources without cultivation (Bernhard and Field, 2000a). The applicability of this approach was then tested on coastal water samples (Bernhard and Field, 2000b). Successful amplification of human and ruminant-specific *Bacteroidales* 16S rRNA gene sequences from water sources (Bernhard and Field, 2000b; Bernhard et al., 2003; Boehm et al., 2003; Shanks et al., 2006) precipitated development of PCR primers with specificity to elk feces, pig feces, horse feces, and dog feces (Dick et al.,

2005a; Dick et al., 2005b) which have been applied successfully across diverse geographic locations (Boehm et al., 2003; Gilpin et al., 2003; Seurinck et al., 2005; Noble et al., 2006; Walters et al., 2006, in press; Petersen et al., unpublished data).

The *Bacteroidales* group of fecal anaerobes is one of the most abundant groups of bacteria found in mammalian feces, accounting for up to 30% of the total fecal flora (Franks et al., 1998; Sghir et al., 2000). Only a small fraction of fecal *Bacteroidales* diversity is accounted for in culture; the majority of the species within this group remain uncultivated (Suau et al., 1999). Because *Bacteroidales* bacteria are fecal anaerobes and frequently appear to possess host-specificity, it is logical to assume they will not survive long periods of time outside of an animal host. Thus, the presence of fecal *Bacteroidales* in the environment is likely to indicate recent fecal contamination. These attributes make *Bacteroidales* markers attractive indicators for identifying fecal pollution and its source(s).

In order to estimate human health risk from exposure to contaminated water it is critical that an indicator of fecal pollution be detected when pathogens are present in a water source. *E. coli* and enterococci persist and proliferate outside of animal hosts. Therefore, presence of the standard indicators does not necessarily indicate the presence of recent fecal pollution or an imminent health risk. Currently, little is known about the survival of fecal *Bacteroidales* in the environment or how the presence of *Bacteroidales* markers corresponds to the presence of fecal pathogens. Because prolific growth of fecal anaerobes is unlikely in oxygenated environments it is plausible that *Bacteroidales* bacteria will serve as a better predictor of human exposure to fecal pathogens.

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CHAPTER 3

DETECTION OF *BACTEROIDALES* FECAL INDICATORS AND THE ZOONOTIC PATHOGENS *E. COLI* O157:H7, *SALMONELLA*, AND *CAMPYLOBACTER* IN RIVER WATER

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Environmental Science and Technology
American Chemical Society, Washington, D.C.
In press, December 2006

Abstract

Bacteroidales host-specific PCR offers a rapid method of diagnosing fecal pollution in water and identifying sources of input. To assess human health risks from exposure to fecal pathogens, however, *Bacteroidales* markers should be detectable when pathogens are present. To determine if *Bacteroidales* general, human-, ruminant-, and swine-specific markers correlate with certain fecal pathogens, we conducted a retrospective study on water samples for which the presence of *E. coli* O157:H7, *Salmonella* spp., and *Campylobacter* spp. had been determined. We found a positive relationship between detection of the *Bacteroidales* general fecal marker and presence of the pathogens. Detection of ruminant-specific markers predicted *E. coli* O157:H7 occurrence. There was a significant increase in the likelihood of detecting *Salmonella* when a ruminant marker was present, and *Campylobacter* spp. when human markers were present. For pathogens such as *E. coli* O157:H7 that are strongly associated with particular hosts, *Bacteroidales* host-specific markers can estimate the likelihood of pathogen occurrence, enabling more accurate health risk assessments.

Introduction

Human exposure to water contaminated with feces can lead to gastrointestinal, respiratory, and dermatological disease. To reduce diseases associated with fecal pathogens, Canada and United States rely on growth of fecal indicator bacteria (FIB) to estimate the human health risk from exposure to waters used for shellfish farming, recreation, or drinking (U.S. Environmental Protection Agency, 2001). However,

recent studies demonstrate the ubiquitous nature of FIB *E. coli* and enterococci in the environment (Fujioka et al., 1999; Byappanahalli et al., 2003; Wheeler Alm et al., 2003; Whitman et al., 2003; Power et al., 2005). Furthermore, *E. coli* and enterococci can persist in a viable but non-culturable (VBNC) state and resuscitate when conducive growth conditions are encountered (Whitman et al., 2003). Finally, while FIB methods have reduced the incidence of waterborne disease, they fall short in their ability to predict the presence of certain human fecal pathogens (Jiang et al., 2001; Noble and Fuhrman, 2001; Bonadonna et al., 2002; Lemarchand and Lebaron, 2003).

Human and non-human feces pose different threats to human health. Until recently, the focus of concern for water-related illness has been contamination by human effluent (reviewed in (Till et al., 2004)); most epidemiological studies were conducted in locations where human sewage was the predominant contamination source. Non-human feces spread pathogens such as *Salmonella spp.*, *E. coli* O157, *Campylobacter jejuni, Leptospira interrogans, Giardia spp.*, *Cryptosporidium parvum*, and hepatitis E virus (reviewed in (Craun et al., 2004)), and may also serve as a source for emergent zoonotic disease. Outbreaks of *E. coli* O157 have been linked to cattle fecal contamination (Johnson et al., 1999; Licence et al., 2001; Johnson et al., 2003; Craun et al., 2004; Reilly and Browning, 2004), but the source of contamination is unknown for most outbreaks or illnesses caused by fecal pathogens (Craun et al., 2004).

The ability to accurately predict the presence of fecal pathogens is central to assessing health risks associated with exposure to contaminated waters. To estimate human health risk, the presence of a microbial indicator should correlate well with the

presence of fecal pathogens. FIB in water are not well correlated with human enteroviruses (Noble and Fuhrman, 2001; Horman et al., 2004), including adenoviruses (Jiang et al., 2001; Jiang and Chu, 2004), nor with bacterial and eukaryotic pathogens such as *Salmonella* spp. (Lemarchand and Lebaron, 2003), *Campylobacter* spp. (Lund, 1996; Bonadonna et al., 2002; Lemarchand and Lebaron, 2003; Horman et al., 2004), and *Cryptosporidium* spp. The purpose of this research was to establish whether a recently proposed set of fecal indicators, host-specific molecular markers from fecal *Bacteroidales* bacteria, correlate with human pathogens. *Bacteroidales* markers enable certain sources of fecal contamination to be identified (Bernhard and Field, 2000a; Bernhard and Field, 2000b; Dick et al., 2005b), but little is known about their survival and correlation with pathogens (Shanks et al., 2006; Walters et al., 2007, in press).

In a previous study, water samples from our study site, in the Oldman River Basin in southern Alberta, were shown to contain *E. coli* O157 and *Salmonella* spp. (Gannon et al., 2004). In this study we determined whether *Bacteroidales* molecular markers were detected when pathogenic *Salmonella* spp., *E. coli* O157:H7, and *Campylobacter* were present in samples from this site. We also estimated whether we were more likely to encounter one or more of these pathogens when either human-, ruminant-, or swine-specific markers (Bernhard and Field, 2000a; Dick et al., 2005b) were found.

Materials and Methods

Study site

The study site (Figure 3.1) is in southern Alberta, Canada, north of the city of Lethbridge, in a semi-arid climatic zone. The watershed is drained by the Little Bow and Oldman Rivers and their tributaries. This region is home to an intensive cattle industry, other livestock and field crops, and is irrigated by canals and associated holding reservoirs emptying into the Oldman River. Sampling locations were chosen based on surface water flow as opposed to livestock density.

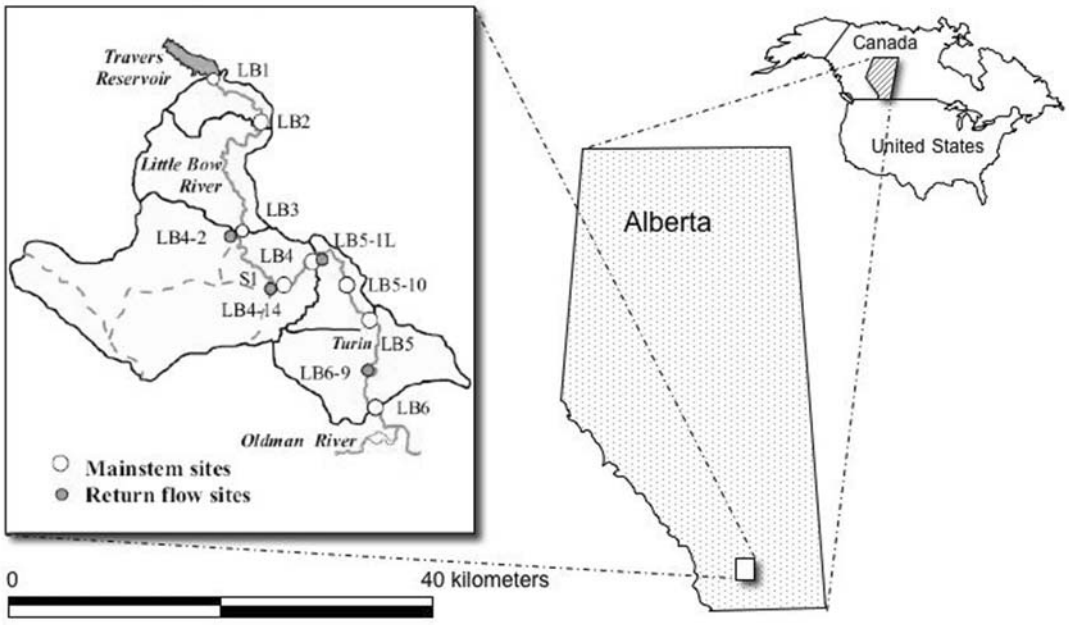


Figure 3.1. Sampling locations within the Lethbridge Northern Irrigation District (LNID).

Sampling

The staff of Alberta Agriculture's Irrigation Branch and Alberta Environment's Environmental Monitoring and Evaluation Branch, Lethbridge conducted water sampling between 2002 - 2004. Sample collection occurred weekly from 23 May through 17 July 2002, every other week, on average, from 7 May through 15 October 2003, and 12 May through 3 November 2004, from approximately 30 cm below the water surface, in sterile 250 ml polypropylene bottles.

Isolation and identification of E. coli O157:H7

The methods of Johnson et al. (Johnson et al., 2003) were employed for isolation of *E. coli* O157:H7. Individual glass culture bottles containing 10 ml of 10X buffered peptone water (BPW) (Oxoid, Nepean, ON, Canada) received 90 ml of each water sample and were incubated at 37ºC for approximately 6 h. *E. coli* O157:H7 was isolated by inoculating 9 ml of modified trypticase soy broth (Oxoid) containing 20 µg/ml novobiocin (Sigma, St. Louis, MO) as per the method of Padhye and Doyle (Padhye and Doyle, 1991). Following incubation, *E. coli* O157:H7 was separated using paramagnetic beads (Dynal, Oslo, Norway) coated with anti-O157:H7 antibody, according to manufacturer's instructions; beads were spread onto sorbitol MacConkey agar (CT-SMAC) (Difco, Detroit, MI) containing 50 µg/ml cefixime (Oxoid) and 2.5 µg/ml tellurite (Oxoid) (Sanderson et al., 1995), and incubated at 42ºC for 24 h. Putative *E. coli* O157:H7 colonies were tested by slide agglutination using the O157:H7 latex agglutination test (Difco). Agglutination positive colonies were assayed using an *E. coli* O157:H7-specific PCR assay (Gannon et al., 1997).

Isolation and identification of Salmonella spp.

Salmonella spp. were isolated using modified methods of D'Aoust and Purvis (D'Aoust and Purvis, 1998) and DeSmedt and Bolderdijk (DeSmedt and Bolderdijk, 1987). BPW cultures (above) were incubated at 37 ºC for approximately 18 h longer. Following incubation, 1 ml and 0.1 ml aliquots of the BPW cultures were transferred to 9 ml of Tetrathionate Brilliant Green Broth (TBGB) (Difco) and 9.9 ml of Rappaport Vassiliadis Broth (RVB) (Oxoid), respectively, and incubated at 42ºC for 24 h. Next, 20 µl of each 24 h RVB culture was inoculated onto Modified Semi-solid Rappaport Vassiliadis agar (MSRV) (Oxoid) (DeSmedt and Bolderdijk, 1987) and a loopful was streaked onto Brilliant Green Sulpha Agar (BGSA) (Becton Dickinson, Mississauga, ON, Canada); cultures were incubated at 42 ºC for 24 h. Putative *Salmonella* spp. colonies were subcultured onto MacConkey agar and incubated at 37 ºC for 24 h. From these cultures colonies were tested by slide agglutination with Salmonella O antiserum Poly A-1 and Vi (Difco) and, if positive, inoculated onto Christensen's Urea Agar, Triple Sugar Iron Agar, Lysine Iron Agar slants, and Motility Indole Ornithine media (Oxoid). PCR assays for detection of the invA gene were performed as described by Chiu and Ou (Chiu and Ou, 1996) on DNA extracted from suspect *Salmonella* colonies. *Salmonella* serotyping was conducted by the Office Internationale des Epizooties Salmonella Reference Laboratory of the Public Health Agency of Canada in Guelph, Ontario.

Isolation and identification of Campylobacter

We analyzed the 2004 samples for *Campylobacter* using the procedures described by Diergaardt et al. (Diergaardt et al., 2004), with several modifications. First, water was filtered through 0.45 µm membrane filters (Pall Gelman Laboratory, Ann Arbor, MI); then the filters were incubated in 30 ml of Bolton's Broth (BB) at 42°C under microaerophilic conditions for 48 h. Next, Campylobacter Blood-Free Selective Agar containing charcoal cefoperazone deoxycholate (CCDA, Oxoid) was inoculated from the BB cultures and incubated at 42°C, under microaerophilic conditions for 48 h. Putative *Campylobacter* colonies were Gram stained, and tested for oxidase and catalase activity. Finally, DNAs extracted from the colonies were tested using a multiplex-PCR assay for 16S rRNA, *MapA*, and *CeuE C. jejuni*- and *C. coli* -specific genes sequences, as described by Denis et al. (Denis et al., 1999).

DNA extraction

DNAs were extracted from samples collected during 2002 using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with the following modifications: water samples were filtered onto 47 mm, 0.45 µm GN-6 membrane filters (Pall Gelman Laboratory). Filters were placed into 15 ml polypropylene tubes containing buffer ASL. After vortexing and heating, filters were discarded and lysates were placed in 2 ml microcentrifuge tubes and centrifuged to pellet cell debris. DNA was eluted in 20 µl of buffer AE, instead of the recommended 200 µl.

DNAs from samples collected during the 2003 and 2004 sampling seasons were extracted using the GenElute Bacterial Genomic DNA Kit (Sigma) according to the manufacturer's directions.

General Bacteroidales and host-specific Bacteroidales marker detection

PCR amplification of general *Bacteroidales* Bac32F (AACGCTAGCTACAGCTT) (Bernhard and Field, 2000b), ruminant-specific CF128 (CCAACYTTCCCGWTACTC) (Bernhard and Field, 2000a) and CF193 (TATGAAAGCTCCGGCG) (Bernhard and Field, 2000a), human-specific HF134 (GCCGTCTACTCTTGGCC) (Bernhard and Field, 2000a) and HF183 (ATCATGAGTTCACATGTCCG) (Bernhard and Field, 2000a), and swine-specific PF163 (GCGGATTAATACCGTATGA) (Dick et al., 2005a) *Bacteroidales* molecular markers, paired with 708R (CAATCGGAGTTCTTCGTG) (Bernhard and Field, 2000b), was used to detect the presence of the markers in the samples evaluated in this study. Amplification reaction conditions were as previously described (Dick et al., 2005a; Shanks et al., 2006). Results were analyzed on 96-well, 2.2% agarose gels, stained with ethidium bromide (Amersham Biosciences, Piscataway, NJ), and visualized and photographed using a UVP gel imager (UVP, Upland, CA).

Statistical design

We evaluated 406 DNA samples using PCR of *Bacteroidales* markers. Of these 406 samples, 285 were negative for pathogens, providing nearly 1.5 times the number of pathogen-negative as pathogen-positive samples (121). To validate the assumption that detection rates did not change between stage one and stage two of the study, 25% of the pathogen-negative samples (9 samples from 2004 and 63 from 2002/03) were randomly selected, using the statistical package S+ (MathSoft,

Cambridge, MA), to be analyzed as part of the stage one analysis; the remaining 75% (25 samples from 2004 and 188 from 2002/03) were set aside for stage two.

Stage 1: Initially we tested pathogen-positive samples using general *Bacteroidales* primers to determine the presence of *Bacteroidales* bacteria in water samples that tested positive for *Salmonella* spp., *E. coli* O157:H7, or *Campylobacter* (2004 only). Next, we evaluated all pathogen-positive samples using *Bacteroidales* ruminant, human, and swine-specific primer sets to ensure that we could detect host-specific markers in pathogen-positive samples. Then, we screened the randomly chosen pathogen-negative samples for the presence of ruminant and human-specific *Bacteroidales* sequences; the swine-specific marker was tested only on samples from 2004 because the swine-specific marker was only detected among the 2004 pathogen-positive samples. We estimated the proportion of samples positive for either *Salmonella* spp., *E. coli* O157:H7, or *Campylobacter* that also tested positive for the presence of general *Bacteroidales*, or *Bacteroidales* source-specific molecular markers, and calculated a 95% confidence interval for each estimate.

Stage 2: This stage of the analysis measured the presence of human, ruminant, and swine-specific *Bacteroidales* molecular markers in samples that were negative for *Salmonella* spp., *E. coli* O157:H7, or *Campylobacter*, and ensured that marker detection rates did not change over time. We analyzed the remaining 213 pathogen-negative samples; human and ruminant-specific primer sets were tested on all pathogen-negative samples and the swine-specific marker was tested on the remaining pathogen-negative samples from 2004.

Statistical analysis

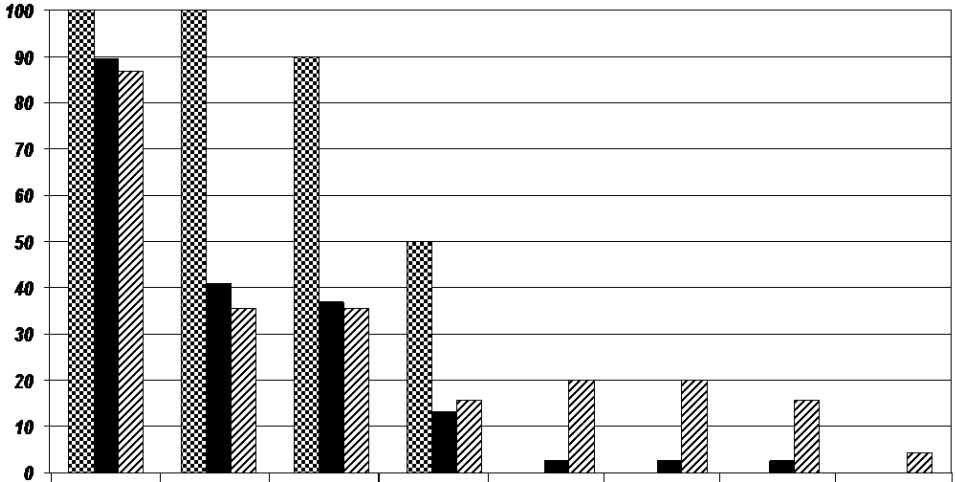
Statistical analyses utilized the S+ statistical package (MathSoft). We analyzed each marker separately; in addition, host-specific genetic markers for ruminants (CF128 and CF193) and humans (HF134 and HF183) were combined by group into two new variables, RUM and HUM, for ruminant and human, respectively. RUM and HUM presence/absence values were scored as 1 if at least one genetic marker for that group was found. We estimated the proportion of pathogen-positive samples that were positive for one or more of the *Bacteroidales* fecal markers and constructed odds ratios, with corresponding confidence intervals, to describe the odds of detecting one of the pathogens when the *Bacteroidales* fecal markers were present. Using logistic regression we determined the likelihood of pathogen detection when *Bacteroidales* source-specific markers were present and accounted for the presence or absence of multiple markers while controlling for the effects of interactions among markers. We sequentially added additional explanatory variables to reduced, single variable, models and compared the models using drop-in-deviance tests; we retained the best models for the regression analyses. We did not account for the sample location or year in our models.

Results

In three years of sampling, *Salmonella* was commonly detected. Among the pathogen-positive samples 76 samples (86.4%) tested positive for *Salmonella*, while over the same three-year period only 12 samples (13.6%) were positive for *E. coli*

O157:H7. During the single year we tested for *Camplylobacter,* it was the most common pathogen detected: 45 of the pathogen-positive samples samples (78.9%) tested positive for *Camplylobacter*, while during the same one-year period, 19 samples (33.3%) were positive for *Salmonella* and 1 sample (1.75%) was positive for *E. coli* O157:H7. Only a few samples were positive for more than one pathogen.

Figure 3.2 shows the percentage of pathogen-positive samples that were positive for each *Bacteroidales* marker, with 95% confidence intervals and standard errors. The highest rates of marker detection in pathogen-positive samples were for the general *Bacteroidales* marker Bac32, and the ruminant markers. Of the total pathogen-positive samples tested in this study, 88.4% were positive for the general *Bacteroidales* marker Bac32F. Bac32F was present in 100% of *E. coli* O157:H7 positive samples, in 89.5% of samples positive for *Salmonella* spp., and in 86.7% of samples positive for *Campylobacter*. In addition, we detected ruminant-specific fecal markers (RUM) in 100% of *E. coli* O157:H7 positive samples. CF128 was detected in 90% and CF193 was detected in 50% of *E. coli* O157:H7 positive samples.



	Bac32	RUM	CF128	CF193	HUM	HF134	HF183	PF163
E. coli	100	100	90	50	0	0	0	0
CI			71.4-108	19.0-81.0				
SE			9.5×10^{-2}	1.6×10^{-1}				
Salmonella	89.5	40.8	36.8	13.2	2.63	2.63	2.63	0
CI	82.6-96.4	29.7-51.8	26.0-47.7	5.56-20.8	-0.97-6.23	-0.97-6.23	-0.97-6.23	
SE	3.5×10^{-2}	5.6×10^{-2}	5.5×10^{-2}	3.9×10^{-2}	1.8×10^{-2}	1.8×10^{-2}	1.8×10^{-2}	
Campylobacter	86.7	35.6	35.6	15.6	20	20	15.6	4.4
CI	76.7-96.6	21.6-49.5	21.6-49.5	4.97-26.1	8.31-31.7	8.31-31.7	4.97-26.1	1.58-10.5
SE	5.1×10^{-2}	7.1×10^{-2}	7.1×10^{-2}	5.4×10^{-2}	5.9×10^{-2}	5.9×10^{-2}	5.4×10^{-2}	3.1×10^{-2}

Figure 3.2. Percentage of samples positive for each pathogen examined in this study that were positive for each *Bacteroidales* marker, followed by 95% confidence intervals (CI) and standard errors (SE).

We calculated odds ratios estimating the odds of one or more of the pathogens occurring when the source-specific markers were present, using data compiled over the three-year sampling period; for *Campylobacter*, we calculated odds ratios for 2004 only. Odds ratios were undefined for *E. coli* O157:H7 relative to general, human-specific, and swine-specific *Bacteroidales* markers, as the general *Bacteroidales* marker was detected in all samples positive for *E. coli* O157:H7 and human and swine-specific markers were detected in none of these samples.

The odds of detecting *E. coli* O157:H7 when CF128 was present were approximately 37 times greater than the odds of finding a positive *E. coli* O157:H7 sample when this marker was absent (95% C.I. from 4.65 – 298, SE = 1.06), and almost 10 times greater when CF193 was present versus when it was absent (95% C.I. from 2.6 – 34, SE = 0.65). When both ruminant-specific markers (RUM) were present, the odds ratio was undefined since all *E. coli* O157:H7 positive samples were positive for at least one of the ruminant-specific *Bacteroidales* markers.

The odds of detecting *Salmonella* in a sample when ruminant-specific markers were present were 2.5 times greater than the odds of detection when ruminant-specific markers were absent (95% C.I. from 1.46 – 4.18, SE = 0.27). When CF193 was detected the odds of isolating *Salmonella* in a sample were 36% greater than if the marker was absent; when CF128 was detected, the odds of isolation were 2.7 times greater than if the marker was absent (95% C.I. from 0.638 – 2.89, SE = 0.386, and 1.58 -4.72, SE = 0.28, respectively). The odds of isolating *Salmonella* when human-specific markers were present were nearly equal to the odds of detection when human-specific markers were absent.

The odds of detecting *Campylobacter* were less than one when ruminant-specific markers were present in a sample versus when they were absent. However, when human-specific markers were present we were more than ten times as likely to observe *Campylobacter* in the same sample (95% C.I. from 1.3 – 88.9, SE = 1.08). We were twice as likely to observe *Campylobacter* in samples that were positive for the presence of the swine-specific marker PF163 (95% C.I. from 0.175 – 22.9, SE = 1.24).

We used logistic regression to account for additive effects among the *Bacteroidales* markers on the odds of pathogen presence, and, taking interactions into

account, to estimate the odds of finding each pathogen when general or source-specific *Bacteroidales* markers were present in a sample. Reduced, one variable models were fit and compared to richer models where additional explanatory variables were added sequentially. Drop-in-deviance tests for the association of the odds of detecting any of the *Bacteroidales* markers when the pathogens were present, after accounting for the occurrence of markers representing each host, suggested that the Salmonella~CF128 and O157:H7~CF128 models were adequate for regression analyses describing the effect presence of ruminant-specific markers had on the presence of *Salmonella* spp. and *E. coli* O157:H7; the Campy~HUM was adequate to describe the ability of human-specific markers to predict the presence of *Campylobacter* in a sample (based on a Chi-Square Distribution; $p>0.25$ comparing all reduced models to full models including additive effects of one or more of the *Bacteroidales* markers).

Regression analysis estimated that we were 6.1 times more likely to detect *E. coli* O157:H7 in a sample if ruminant-specific markers were detected (95% C.I. from 2.2 to 17, SE = 0.52).

The chances of isolating *Salmonella* spp. when CF128 was present were 65% greater than when CF128 was absent (95% C.I. from 1.26 to 2.17, SE = 0.14). Drop-in-deviance tests chose a model accounting for additive effects of the CF128 and HF183 *Bacteroidales* markers to describe the effect the presence HF183 exerted on the odds of *Salmonella* spp. occurrence. However, in a comparison of the full model to a reduced, CF128 only model, the drop-in-deviance test indicated that detection of HF183 had little effect on the presence of *Salmonella* spp. Because the reduced model (*Salmonella*~CF128) best predicted the presence of *Salmonella* spp., we retained this

model to examine the effects of CF128 detection on the odds of *Salmonella* spp. presence.

We were 3.3 times as likely to identify *Campylobacter* in samples testing positive for *Bacteroidales* human-specific markers (95% C.I. from 1.19 to 9.01, SE = 0.52) than in samples where human-specific markers were absent.

Discussion

There was very little co-occurrence of pathogens in samples. The low number of samples that contained more than one pathogen supported our hypothesis that the pathogens are likely to originate from different sources.

Because cattle and other ruminants are known reservoirs for *E. coli* O157:H7 (Kudva et al., 1996; Hancock et al., 2001; Molbak and Scheutz, 2004), it is not surprising that the odds of detecting *E. coli* O157:H7, when ruminant-specific markers were present, were quite high. Several previous studies have connected outbreaks of *E. coli* O157:H7 with cattle feces (Johnson et al., 1999; Licence et al., 2001; Johnson et al., 2003; Reilly and Browning, 2004). Although Bac32F and ruminant-specific markers were detected in 100% of the samples positive for *E. coli* O157:H7, the CF128 and CF193 individual markers were only detected in 90% and 50% of these samples respectively, and *E. coli* O157:H7 was only detected in 7% of the samples positive for ruminant-specific markers.

Low detection of the pathogen compared to ruminant markers could be due to its infrequent occurrence, poor detection rate, or to longer relative survival of

Bacteroidales markers. Recovery of *E. coli* O157:H7 from contaminated water sources during waterborne outbreaks is often unsuccessful, possibly due to the low infectious dose required to cause disease, inadequate isolation methods, or because cells have entered a viable but nonculturable (VBNC) state. Pathogens may be present and infective even when not detected; thus occurrence of *E. coli* O157:H7 and other bacterial pathogens may be systematically under-estimated. Standard indicator methods measuring cleavage of methylumbelliferryl galactocide (MUG), such as Colilert, can miss the presence of *E. coli* O157:H7 because approximately 50% of strains do not cleave MUG (US FDA, 1995; Straub and Chandler, 2003).

Therefore, in the case of a pathogen such as *E. coli* O157:H7, which is strongly associated with a particular source, *Bacteroidales* source specific markers could provide a useful supplement to indicator data, enabling more accurate health risk assessments. Regression analysis estimated that the odds of detecting *E. coli* O157:H7 in a sample was 6.1 times greater if the CF128 was detected. If the average occurrence of *E. coli* O157:H7 in a watershed is known, the risk at particular sampling sites can be estimated based on whether ruminant molecular markers are detected at those sites. Since there are seasonal changes in fecal pathogen deposition and transport due to animal health, reproductive cycles, and rainfall, estimation may be improved by measuring the seasonal occurrence of *E. coli* O157:H7 and correlating it to ruminant *Bacteroidales* markers.

The higher recovery of CF128 than CF193 in *E. coli* O157:H7-positive samples may be due to the over-all higher occurrence of CF128 in cattle in this area. It is known that not all individuals have particular *Bacteroidales* molecular markers in their feces; marker occurrence may vary both spatially and temporally. The frequency

of occurrence of ruminant markers in all water samples in this study was 21.2% for CF128 but only 10.6% for CF193. Thus CF128 may be a better marker than CF193 for both ruminant fecal contamination and *E. coli* O157:H7 in this watershed. This suggests that the occurrence of the various markers within local animals should be estimated before markers are chosen for a particular study. On the other hand, since only 90% of the samples positive for *E. coli* O157:H7 were also positive for CF128, but 100% were positive for RUM (CF128 and/or CF193), adding the second ruminant marker improves ability to predict occurrence of *E. coli* O157:H7.

None of the *Bacteroidales* host-specific fecal markers tested in this study were adequate to predict the presence of *Salmonella* spp. Previous studies (Johnson et al., 2003; Gannon et al., 2004) found that *Salmonella enterica* serovar Rublislaw was the most common enteric *Salmonella* serovar isolated from the surface waters sampled in this study. This serovar has not been found in the feces of cattle in southern Alberta (Van Donkersgoed et al., 1999). Serovar Rublislaw is rarely associated with human or animal disease in Canada, and human infection by this serovar is linked to close contact with captive reptiles (Woodward et al., 1997; BBC, 2000; Ward, 2000). It has been suggested that a great diversity of *Salmonella* serovars occurs in river water as a result of the large number of host species acting as reservoirs (Baudart et al., 2000). Of the other *Salmonella* serovars recovered from these samples, many suggest avian origin (Johnson et al., 2003; Gannon et al., 2004); we did not use an avian-specific assay. The general *Bacteroidales* marker was only found in 89.5% of the samples positive for *Salmonella* spp. *Salmonella* cells may survive longer in water than *Bacteroidales* markers; in addition, our previous research suggests that *Bacteroidales* bacteria may not be as common in birds as in mammals.

The association between human-specific *Bacteroidales* fecal markers and *Campylobacter* is consistent with previous findings that gastroenteritis caused by *Campylobacter* is not zoonotic in origin but is transmitted via human to human contact by fecal waste (Pruss, 1998). Although there is strong support for a ruminant fecal source for *Camplylobacter* infections in New Zealand (Till et al., 2004), ruminants do not appear to be the primary source in this watershed. Odds ratios indicated an over-all increase in the odds of *Campylobacter* detection when ruminant-specific markers were detected. However, after accounting for the presence of ruminant-specific markers in samples negative for pathogens and in samples that were positive for one of the other pathogens tested for in this study, ruminant-specific markers did not predict the presence of *Campylobacter*. Since the likelihood of detecting *Campylobacter* increased more than three-fold when human *Bacteroidales* markers were present, after taking into account the presence and absence of additional host-specific markers, the presence of human markers indicated an increased risk of exposure to *Campylobacter*. This risk could be quantified using seasonal data on *Campylobacter* occurrence. Like *Salmonella*, only 86.7% of *Camplobacter*-positive samples tested positive for the general *Bacteroidales* marker Bac32F, also suggesting birds as one of the sources for the pathogen.

Several pathogen-negative samples tested positive for either ruminant or human-specific fecal markers, or both. This is consistent with a relatively rare distribution of pathogens, whereas *Bacteroidales* are common constituents of feces. It is also possible that the genetic markers for ruminant and human-specific *Bacteroidales* may persist longer than certain pathogens. Little is known as yet about survival of *Bacteroidales* markers in relation to pathogens (Shanks et al., 2006;

Walters and Field, 2006). To be most useful, an indicator should have a survival profile similar to a pathogen.

The *Bacteroidales* PCR data are not quantitative (Suzuki and Giovannoni, 1996; Suzuki et al., 1998) and thus no presumptive statements regarding numbers of *Bacteroidales* markers can be made. Still, pathogen presence/absence compared to sample volume is considered to be semi-quantitative data. We used 90 ml water samples for both *E. coli* O157 and *Salmonella* detection, and 100 ml for both *Campylobacter* detection and DNA extraction; 5% of the extracted DNA was used in each *Bacteroidales* PCR assay. Detection of *E. coli* O157 was specific to a single serotype, whereas detection of *Salmonella* by PCR of the *inv* gene detects up to 1800 serovars (Chiu and Ou, 1996), and the PCR test we used for *Campylobacter* detects two species, *C. jejuni* and *C. coli* (Denis et al., 1999). The PCR assay for general *Bacteroidales* detects a large number of cultivated species of fecal *Bacteroides* and *Prevotella* (Bernhard and Field, 2000b) along with many sequences from uncultivated fecal strains; the host-specific PCR assays for human, ruminant, and swine *Bacteroidales* detect small numbers of sequences from uncultivated fecal strains only (Dick et al., 2005a).

We found that total DNA concentration had little effect on our ability to detect the markers. For example, a DNA sample that was negative for the general *Bacteroidales* marker had a higher DNA concentration than DNA samples that were positive for the marker (data not shown). We also found no evidence of PCR inhibition.

Because our data are binary and represent only a measure of presence or absence of *Bacteroidales* fecal markers, we cannot directly compare our findings to

previous studies using correlation coefficients or regression analysis based on quantitative data. However, Horman and colleagues (Horman et al., 2004) found a significant correlation between *Campylobacter* and *Clostridium* but not between *Campylobacter* and *E. coli*. Kemp and coworkers (Kemp et al., 2005) found that isolation of *E. coli* did not increase the likelihood of isolating *Campylobacter* spp. from the same water sample even though the samples were taken from an area of extensive dairy farming. Here we found roughly 87% of the *Campylobacter* positive samples were positive for the general *Bacteroidales* marker, and 35%, 20%, and 4% were positive for ruminant, human, and swine-specific markers, respectively. We were not able to explain the presence of all *Campylobacter* positive samples using the *Bacteroidales* markers. This is consistent with strong evidence suggesting birds and rabbits, in addition to humans and ruminants, are reservoirs for *Campylobacter* (Hanninen et al., 2000; Horman et al., 2004; Kemp et al., 2005).

This study suggests human fecal pollution is not the predominant source of *Salmonella* spp. in this watershed since human markers were observed in only 2.6% of samples positive for *Salmonella* spp. Agricultural run-off could be a large contributor to *Salmonella* loading in these rivers; ruminant markers were identified in 35.6% of *Salmonella* positive samples. Although many *Salmonella* positive samples were positive for the *Bacteroidales* markers (~50%), we were unable to explain *Salmonella* spp. occurrence in the remaining positive samples using the markers employed in this study. It remains to be determined, through employment of additional host-specific markers, what other fecal sources are responsible for *Salmonella* and *Campylobacter* loading in this watershed. Furthermore, survival of fecal *Bacteroidales* markers

relative to the pathogens assayed in this study, along with other bacterial and viral pathogens, remains to be evaluated.

Acknowledgements

The work on *Bacteroidales* markers was supported, in part, by the Charles E. and Clara Marie Eckelman Graduate Assistantship and Grant NA76RG0476 (project no. R/ECO-04) from the National Oceanic and Atmospheric Administration to the Oregon Sea Grant College Program. We would like to thank Vincente Monleon, Stephen Jensen, and Cody Olsen of the Oregon State University Statistics Student Consulting Service for assisting with the statistical design and interpretation of this study, as well as Linda Dick and Anne Taylor for thoughtful input on the manuscript. We would also like to thank Joanne Little and Andrea Kalischuk, Irrigation Branch, Alberta Agriculture, Ray Walker, Karen Saffran and Wendell Koning, Environmental Monitoring and Evaluation Branch, Alberta Environment, Jim Miller and Bruce Beasley, Lethbridge Research Centre, Research Branch, Agriculture and Agri-food Canada, for collection of the water samples. We would also like to thank Thomas Graham, Susan Ross, Jennifer de Jager, Garrett Kennedy, Cassandra Jokinen, and Laura Milne, and of the Public Health Agency of Canada and Dr. James Thomas and Rommy Rodriguez, University of Lethbridge for their technical assistance. Support for the project was provided by the Public Health Agency of Canada and laboratory facilities were provided by the Canadian Food Inspection Agency, Lethbridge, Alberta.

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CHAPTER 4

PERSISTENCE AND GROWTH OF FECAL *BACTEROIDALES* ASSESSED BY BROMODEOXYURIDINE IMMUNOCAPTURE

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Applied and Environmental Microbiology
American Society for Microbiology, Washington, D.C.
Issue 72, Volume 7

Abstract

Extraintestinal growth of fecal bacteria can impair accurate assessment of watershed health. Anaerobic fecal bacteria within the order *Bacteroidales* are attractive candidates for fecal source tracking because they display host-specific distribution and do not grow well under high oxygen concentrations. Growth of general and human-specific fecal *Bacteroidales* marker organisms in environmental samples (sewage) and persistence of the corresponding genetic markers were investigated using bromodeoxyuridine DNA labeling and immunocapture, followed by PCR detection. Background amplification of unlabeled controls occasionally occurred using a high number of PCR cycles. By incorporating fluorescent detection of PCR products obtained after 15 cycles, determined to be quantitative, we enriched for BrdU-labeled DNA and did not detect unlabeled DNA. Using pure cultures of *Bacteroides vulgatus,* the ability of *Bacteroidales* bacteria to take up and incorporate BrdU into nascent DNA was confirmed. Fecal *Bacteroidales* organisms took up and incorporated BrdU into DNA during growth. In sewage incubated aerobically, at in situ temperature, *Bacteroidales* genetic marker sequences persisted at least 24 hours and *Bacteroidales* fecal bacteria grew for up to 24 hours as well. Detection by PCR at a low, quantitative cycle number diminished sensitivity of the assay such that we were unable to detect fecal *Bacteroidales* human-specific marker sequences in unlabeled or BrdU-labeled fractions, even when using fluorescent detection. Using 30 cycles of PCR on unlabeled fractions, human-specific *Bacteroidales* sequences were detected, and persisted up to 24 hours in sewage. These data support the utility of BrdU-labeling

and immunocapture followed by LH-PCR or fluorescent detection using low cycles of PCR. However, this method may not be sensitive enough to identify cells growing at low densities in aquatic environments.

Introduction

Fecal pollution from failing septic systems, urban and agricultural run-off, and wild animals impacts human and environmental health (Lipp et al., 2002) and causes economic loss (Dorfman et al., 2004) due to closures of shellfish beds and recreational waters. The ability to accurately identify non-point sources of fecal pollution is vital to effective remediation of such pollution. Most U.S. state agencies monitor water quality and estimate human health risk in accordance with the U.S. Environmental Protection Agency (EPA) policies (U.S. Environmental Protection Agency, 2001). These methods rely on cultivation and enumeration of bacterial indicators such as *Escherichia coli* and fecal enterococci. Implementation of these detection methods has substantially reduced the incidence of waterborne disease, but they have failed to discriminate between sources of fecal pollution, and require laborious and time consuming laboratory manipulations. In addition, once in the environment, fecal bacteria may rapidly lose their ability to be cultured using traditional methods, while retaining metabolic functions (Menon et al., 2003) or pathogenicity (Pommepuy et al., 1996; Rahman et al., 1996).

To accurately estimate human health risk associated with exposure to fecal pathogens, an indicator should not proliferate in the environment, should persist as

long as pathogens, and should be present at the same time, in concentrations proportional to the concentration of pathogens (e.g. see (Tamplin, 2003)). *E. coli* and enterococci are not well correlated with pathogenic *Salmonella* spp. (Lemarchand and Lebaron, 2003), *Campylobacter* spp. (Lund, 1996; Bonadonna et al., 2002; Lemarchand and Lebaron, 2003; Horman et al., 2004), *Cryptosporidium* spp. (Lund, 1996; Bonadonna et al., 2002; Lemarchand and Lebaron, 2003; Horman et al., 2004), human enteroviruses (Lemarchand and Lebaron, 2003; Horman et al., 2004), including adenoviruses (Noble and Fuhrman, 2001), or coliphages (Jiang et al., 2001). In addition, *E. coli* and enterococci can survive and persist ubiquitously in natural environments such as fresh water lakes and streams, algal wrack, beach sand, and tropical soils (Fujioka et al., 1999; Byappanahalli et al., 2003; Whitman et al., 2003; Power et al., 2005).

We previously proposed PCR-based detection of 16S ribosomal DNA markers from uncultivated anaerobic fecal bacteria within the order *Bacteroidales* as a rapid method of diagnosing fecal pollution and discriminating its source(s) (Bernhard and Field, 2000b; Field et al., 2003; Dick et al., 2005b; Shanks et al., 2006). Two PCR primers, HF134 and HF183, specifically detect human feces (Bernhard and Field, 2000a). *Bacteroidales* genes can be used as fecal indicators in a real time quantitative PCR assay (Dick and Field, 2004), and recently a human-specific quantitative *Bacteroidales* PCR assay was developed (Seurinck et al., 2005) based on the HF183 primer (Bernhard and Field, 2000a). These assays, combined with quantitative assays for fecal pathogens, offer the potential to determine the associated health risk and make inferences about fecal pollution sources. Several studies suggest *Bacteroidales*

genotypes exhibit geographical stability (Field et al., 2003; Seurinck et al., 2005), as well as host specificity (Bernhard and Field, 2000b; Seurinck et al., 2005; Dick et al., 2005b), and may be appropriate for quantitative source tracking on a global scale.

Application of this method in quantitative source tracking requires an understanding of both the persistence and possible proliferation of these bacteria in the environment. Because *Bacteroidales* bacteria are obligate anaerobes, their survival in the extraintestinal environment is thought to be limited (Kreader, 1995; Kreader, 1998). Present knowledge indicates reservoirs for *Bacteroidales* bacteria are restricted to the body cavities of animals; no strains adapted to aquatic environments are currently known.

We used a cultivation independent bromodeoxyuridine (BrdU) DNA labeling technique to measure the persistence and proliferation of these bacteria. Under thymidine (TdR) auxotrophy, cells pulse-labeled with BrdU, a thymidine base analog, take up and incorporate BrdU into newly synthesized DNA in place of thymidine. Uptake of BrdU and its subsequent incorporation into DNA can therefore be used to identify actively growing bacteria (Steward and Azam, 1999; Pernthaler et al., 2002). Separation of BrdU-labeled DNA using immunocapture, followed by PCR of ribosomal genes in the labeled fraction, allows species level identification of growing bacteria in mixed communities (Borneman, 1999; Urbach et al., 1999; Artursson and Jansson, 2003), bypassing traditional culturing methods. Following immunocapture, successful PCR amplification of unlabeled (supernatant) DNA demonstrates persistence of targeted cells or DNAs, whereas PCR amplification of labeled (immunocaptured) DNA demonstrates their growth. The BrdU-labeling and

immunocapture method has reportedly been successful at identifying metabolically active populations of marine bacteria (Urbach et al., 1999), soil fauna (Borneman, 1999), and arbuscular mycorrhizae (Artursson and Jansson, 2003).

Reported here are the results of a study using the BrdU immunocapture method to follow growth of *Bacteroidales* organisms in sewage. Because strains containing the human-specific *Bacteroidales* markers have not been cultivated, we first tested the ability of *Bacteroides vulgatus*, the closest cultivated phylogenetic relative, to take up and incorporate BrdU into newly synthesized DNA. To circumvent problems arising from background amplification of unlabeled control DNA, we developed a method of fluorescent detection employing quantitative length heterogeneity PCR (LH-PCR) (Suzuki et al., 1998). LH-PCR is a method that chromatographically separates fluorescently-labeled PCR products by length on a DNA automated sequencer such that relative fluorescence of each fragment is proportional to its abundance. Using this technique, Suzuki and colleagues (Suzuki and Giovannoni, 1996; Suzuki et al., 1998) showed that kinetic biases occur as PCR cycle number increases, varying between primer pairs, but that PCR remains quantitative, relative to initial template concentration, at a low cycle number which can be established empirically. We amplified ribosomal gene fragments from strains with different fragment lengths, allowing us to establish quantitative PCR conditions (in which the proportion of fragments in PCR products was equal to their proportion in the template). We then graphically tested the efficiency of the BrdU immunocapture. Finally, we pulse-labeled sewage influent over 24 hours with BrdU to determine if *Bacteroidales* fecal

bacteria grew under environmental conditions, and whether the BrdU method could be used to detect persistence and growth at environmentally relevant concentrations.

Materials and Methods

Bacterial strains

We obtained *Bacteroides vulgatus* ATCC 4245 from the laboratory of Dr. Abigail Salyers, University of Illinois, Champagne-Urbana, IL. The control organism *Fulvimarina pelagi* HTCC 2506 (Cho and Giovannoni, 2003) was obtained from Dr. Jang-Cheon Cho at Oregon State University.

Sewage Influent

Sewage influent was collected from the Corvallis Wastewater Reclamation Plant, Corvallis, OR.

BrdU labeling

Sewage influent and/or pure cultures of *B. vulgatus* and *Escherichia coli* were supplemented with 33 nM TdR (Sigma, St. Louis, Missouri) and 20 µM BrdU (Sigma); unlabeled negative controls received 33 nM TdR in the absence of BrdU. Amended cultures were incubated at 37 ºC until log phase growth (approximately 4 h). Sewage was incubated at in situ temperature (21 ºC) under aerobic conditions for 4, 8, 12, and 24 h. Sewage influent was divided into triplicate tubes representing 4, 8, 12, and 24 h incubations. Supplements were added to sewage samples at 4 h prior to

harvest (i.e. t = 0, 4, 8, and 20 h) such that each sample was only incubated with the supplements for 4 h immediately proceeding DNA extraction.

DNA extraction

Following BrdU labeling, DNA was extracted from cultures and sewage samples.

(i) Bacterial cultures

A log phase culture suspension (4 ml) was pelleted, washed, divided between two 1.7 ml microfuge tubes, and re-pelleted. We extracted DNA from pure bacterial cultures using the DNeasy Tissue Kit (Qiagen, Valencia, Calif.), with the following modifications: two washes with buffer AW2 were used and DNA was eluted twice by adding 50 µl of buffer AE to the silica column, warming to 65 ºC for 10 min, and centrifuging at 10,000 rpm for 1 min.

(ii) Sewage samples

DNA from sewage influent was extracted using the DNeasy tissue extraction kit (Qiagen) with several modifications. First, 7 ml samples were filtered through 0.2 µm Supor membrane filters (Pall Gelman Laboratory, Ann Arbor, MI) under vacuum of at least 15 psi. Filters were rolled and placed in sterile 15 ml polypropylene tubes containing 500 µl guanidine isothiocyanate (GITC) buffer (5M guanidine thiocyanate, 100 mM EDTA, 0.5% N-lauroyl sarcosine), then vortexed for 60 s to completely saturate the filter. Filters were stored at -80 ºC until DNA extraction. After thawing, filters were again vortexed for 60 s. The incubation with buffer ATL and Proteinase K was omitted. Tubes containing filters with GITC received 500 µl buffer AL and were

vortexed for 60 s, then each tube received 500 µl of 100% ethanol and was vortexed for an additional 60 s. Lysates were removed from tubes and loaded onto individual DNeasy spin columns. Filters were discarded. Instead of the recommended single wash step with buffer AW2, three 500 µl washes were used. DNA was eluted twice by adding 50 µl of buffer AE to the silica column, then warming to 65 ºC for 10 min and centrifuging at 10,000 rpm for 1 min.

Immunocapture

To separate BrdU-labeled from unlabeled DNA, we used the immunocapture procedure outlined by Urbach et al. (Urbach et al., 1999). First, 9 µl of herring sperm DNA (1.25 mg/ml; Invitrogen, Carlsbad, CA), per sample, was boiled for 5 min and quick frozen with dry ice and ethanol. Monoclonal mouse-anti-BrdU antibody (0.44 mg/ml IgG; Sigma), 1 µl per sample, was added to the herring sperm DNA, mixed, and incubated in the dark at room temperature for 30 min, with occasional mixing. DNA (1 µg) from pure cultures or DNA recovered from sewage extractions, measured using a PicoGreen assay (Molecular Probes Inc., Eugene, Oregon), was suspended in 10 µl phosphate-buffered saline (PBS), boiled for 5 min and quick frozen on dry ice and ethanol. Each DNA sample received 10 µl of the anti-BrdU/herring sperm DNA mixture. Samples were incubated in the dark at room temperature for 30 min, with occasional mixing. Goat-anti-mouse IgG coated paramagnetic beads (4×10^8 beads/ml; Dynal Biotech, Oslo, Norway) were washed three times with 1 ml PBS/0.1% non-acetylated bovine serum albumin (BSA; Invitrogen) and brought back to the original volume with PBS/0.1% BSA. Twenty five microliters of bead

suspension was added to each sample and was incubated in the dark, at room temperature, with constant mixing for 30 min. Bead supernatants were removed and saved. Remaining antibody-bound DNA was washed 7 times with PBS/0.1% BSA and eluted with 100 µl of 1.7 mM BrdU under constant agitation for 30 min. Recovered DNA was concentrated by ethanol precipitation. Dry pellets were dissolved in 8 µl (immunocaptured) and 20 µl (bead supernatants) molecular grade water with gentle heat and mixing. All washes were performed using a magnetic particle concentrator (MPC-S; Dynal).

PCR detection of immunocaptured and unlabeled fractions

Bead supernatants and immunocaptured fractions were added to PCR mixtures at a volume of 2 µl per reaction. Each 25 µl reaction mixture consisted of 1 X PCR buffer containing 2.0 mM $MgCl_2$ (TaKaRa BIO Inc., Otsu, Shiga, Japan), 0.2 mM dNTPs, 0.2 µM each primer, 0.08% BSA, and 0.025 U Ex Taq DNA Polymerase (TaKaRa BIO Inc.). The amplification conditions included an initial denaturation step of 94 ºC for 3 min; followed by 94 ºC for 1 min, the annealing temperature specific for each primer pair for 45 s, 72 ºC for 45 s, repeated for the cycle numbers described below; with a final extension step at 72 ºC for 7 min. The annealing temperature of Bacterial 27F (AGAGTTTGATCMTGGCTCAG) (Suzuki and Giovannoni, 1996) paired with Universal primer 338R (GCTGCCTCCCGTAGGAGT) (Suzuki and Giovannoni, 1996) was 55° C. The annealing temperature of the *Bacteroidales*-specific primer pair Bac32F (AACGCTAGCTACAGGCTT) (Bernhard and Field, 2000b) and 6-carboxyfluorescein (6-FAM)-labeled Bac708R

(CAATCGGAGTTCTTCGTG) (Bernhard and Field, 2000b) was 53° C. For human-specific *Bacteroidales* primers HF134 (GCCGTCTACTCTTGGCC) (Bernhard and Field, 2000a) and HF183 (ATCATGAGTTCACATGTCCG) (Bernhard and Field, 2000a) paired with 6-FAM labeled 708R, annealing temperature was 63° C. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide (Sigma), using a UVP gel imager (UVP, Upland, CA), or by GeneScan analysis.

GeneScan analysis

6-FAM labeled PCR products were diluted by an empirically determined amount to avoid saturating the fluorescent detector, as follows: 15 cycles of PCR, no dilution; 20 cycles of PCR, 1:2; 25 cycles of PCR, 1:10; 30 cycles of PCR, 1:10. Aliquots (2 μl) of diluted PCR products were submitted to the Central Services Laboratory at Oregon State University for resolution by a model ABI 3100 capillary sequencer and GeneScan software (ABI: Applied Biosystems Inc., Freemont, Calif.). GENESCAN500-ROX (ABI) was added as an internal size standard with each sample where the expected fragment length was less than 400 bp; MAPMARKER1000 was added as an internal size standard in samples where fragments were expected to be greater than 400 bp in length. Fragment sizes were estimated using the Local Southern Calling Method provided in GeneScan, version 3.1 (ABI).

BrdU labeling of B. vulgatus

To test the ability of bacteria in the order *Bacteroidales* to take up and incorporate BrdU, we used pure cultures of *B. vulgatus* and labeled with BrdU as

described above. To detect *B. vulgatus* BrdU-labeled DNA, we used 30 cycles of PCR with 16S rRNA gene primers 27F and 338R and visualized products by gel electrophoresis as described above.

Establishing conditions under which immunocapture separates BrdU-labeled DNA from unlabeled DNA

Because we observed background amplification of unlabeled control DNA, we tested the ability of the immunocapture method to accurately separate BrdU-labeled from unlabeled DNAs. Mixtures of *F. pelagi* and *B. vulgatus* DNAs were used in varying proportions as templates, producing different-length fragments that could be distinguished and quantified by LH-PCR.

(i) Quantitative cycle number

To establish PCR conditions that yielded the same proportion of PCR fragments in the product as in the template, we combined 1:1, 1:10, and 10:1 proportions of *F. pelagi*:*B. vulgatus* genomic DNAs. PCR of 15, 20 or 25 cycles was carried out as above using 2 µl (5 ng/µl) of these mixtures as template with primers 6-FAM labeled 27F and 338R. PCR products were submitted for GeneScan analysis within 24 hours. The quantitative cycle number was determined by comparing the initial template ratios to the recovered fragment abundances calculated using ratios of relative fluorescence units (rfu). The cycle number producing fragment abundances with ratios equal to the initial template ratios was considered quantitative.

(ii) Determining immunocapture using LH-PCR detection

We combined 1:1, 1:10, and 10:1 proportions of unlabeled *F. pelagi*:BrdU-labeled *B. vulgatus* genomic DNAs. Pre-immunocapture LH-PCR was carried out

using 2 µl (5 ng/µl) of these mixtures; the remaining DNA was used in immunocapture reactions as above. Detection was by LH-PCR with 15, 20 and 25 cycles, using 6-FAM labeled 27F with 338R.

Growth in sewage samples

To test whether *Bacteroidales* cells grew in sewage incubations, incubations were labeled with BrdU, DNA was extracted, immunocapture was carried out as described above, and detection was by PCR with general or human-specific *Bacteroidales* primers, at cycle numbers determined to be quantitative.

(i) General Bacteroidales quantitative cycle number

To determine a quantitative PCR cycle number using *Bacteroidales*-specific primers Bac32F and 6-FAM labeled Bac708R (Bernhard and Field, 2000b), we used varying quantities of *B. vulgatus* template DNA and quantified PCR products by GeneScan after 15, 20, 25 and 30 cycles of PCR. Template DNA was added to PCR in the following quantities, measured by PicoGreen assay (Molecular Probes Inc.): 0.5 ng, 1 ng, 5 ng, and 10 ng. PCR products were submitted for GeneScan analysis within 24 hours. Quantitative cycle number was determined as above.

(ii) Human-specific Bacteroidales quantitative cycle number

Similarly, we determined the quantitative PCR cycle number for *Bacteroidales* human-specific primers HF134 and HF183 (Bernhard and Field, 2000a) in combination with 6-FAM labeled Bac708R (Bernhard and Field, 2000b). Cloned plasmid DNA was added to PCR in the following amounts: 23 ng, 11.65 ng, 2.33 ng,

1.17 ng. PCR products were quantified by GeneScan after 15, 20, 25 and 30 cycles. Quantitative cycle number was determined as above.

(iii) Detection of growth and persistence in sewage samples

Recovered bead supernatants and immunocaptured fractions from sewage incubations from each time point (4, 8, 12, and 24 h) were added to PCR in unknown concentrations, at a volume of 2 µl per reaction. Analyses were performed on all bead supernatants and immunocaptured DNA for both BrdU-labeled samples and unlabeled controls. PCRs were as described above. Samples were submitted for LH-PCR analysis from products obtained after PCR 15 cycles using primer pairs Bac32F-Bac708R, HF134-Bac708R, and HF183-Bac708R; PCR with primers 27F and 338R was included as a labeling control.

Limits of detection at the quantitative cycle number

We measured the limit of detection of target sequences with each of the primer pairs at its quantitative cycle number. Genomic *B. vulgatus* DNA was added to PCR primed with Bac32F and 6-FAM labeled Bac708R in the following amounts: 0.5 pg, 1 pg, 5 pg, 0.01 ng, 0.05 ng, 0.1 ng, 0.5 ng, 1 ng, and 5 ng, and allowed to amplify through 15 cycles as described above. Plasmid DNA containing the human-specific sequences was added to PCR and primed with either HF134 or HF183 in combination with 6-FAM labeled Bac708R in the following amounts: 5 pg, 0.01 ng, 0.05 ng, 0.1 ng, 0.5 ng, 1 ng, and 5 ng (BioSpec-1601, Shimadzu Corp., Kyoto, Japan); amplification was arrested after 15 cycles as previously stated. Detection was by fluorescent fragment analysis as described above.

Immunocapture with fluorescent PCR: reproducibility

We used *B. vulgatus* and *Escherichia coli* to measure reproducibility and establish the lower limits of detection of the assay in mixed populations.

(i) BrdU labeling

Pure cultures of *B. vulgatus* and *E. coli* were subjected to BrdU-labeling as described above. DNA was extracted according to the DNeasy (Qiagen) protocol (see above) and quantified using PicoGreen (Molecular Probes Inc.). DNAs were diluted to obtain a concentration of 10 ng/µl.

(ii) Immunocapture

Labeled *B. vulgatus* and *E. coli* DNAs were combined in the following proportions: 1:100, 1:10, 1:1, 10:1, and 100:1, with a final template concentration of 1 µg. Immunocapture was performed on three sets of replicate samples as previously described. Each set of replicate samples was processed independent of other replicates. DNA pellets were dissolved in 8 µl (immunocaptured) and 20 µl (supernatant) molecular biology grade water.

(iii) Fluorescent PCR and GeneScan analysis

Fifteen cycles of PCR with Bac32F and 6-FAM labeled Bac708R were performed using pre-immunocapture DNA mixtures, bead supernatants, and immunocaptured fractions recovered from the labeling and immunocapture procedure, for all three replicates. Each fluorescently labeled PCR product (2 µl) was submitted for GeneScan analysis.

Results

To determine whether *Bacteroidales* fecal bacteria can grow under environmental conditions, we labeled cultures of *B. vulgatus* and sewage influent with BrdU and used immunocapture (Borneman, 1999; Urbach et al., 1999; Artursson and Jansson, 2003) to recover labeled DNA. Labeled (immunocaptured) or unlabeled (supernatant) DNA was used as template in PCR with primers specific for the order *Bacteroidales* or human-specific *Bacteroidales* fecal bacteria. Amplification of unlabeled DNA demonstrated that there was marker persistence, whereas amplification of BrdU-labeled DNA demonstrated that cell growth occurred.

BrdU immunocapture and 30-cycle PCR

We successfully amplified *B. vulgatus* genes from immunocaptured DNA, indicating that *B. vulgatus* took up BrdU during growth, and incorporated it into newly synthesized DNA. However, we obtained inconsistent results using 30 cycles of PCR to detect BrdU-labeled DNA. Frequently, amplification occurred in the immunocaptured fraction of the unlabeled control (Figure 4.1); if the antibody technique isolates BrdU-labeled DNA only, the immunocaptured unlabeled control should not amplify. Occasional amplification in the immunocaptured fraction of the unlabeled controls occurred even when a variety of blocking agents, including herring sperm DNA, Roche blocking agent, Tween-20, and Denhart's reagent, were evaluated alone or in combination (data not shown).

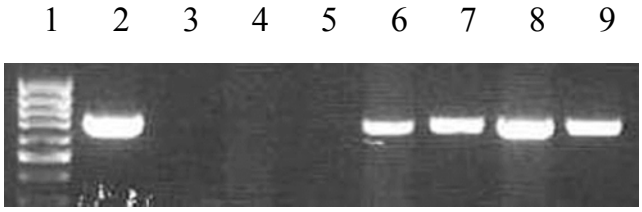

Figure 4.1. Agarose gel electrophoresis following 30 cycles of 27F and 338R primed PCR on immunocaptured fractions of *B. vulgatus* BrdU-labeled and unlabeled DNA, showing robust amplification of the unlabeled control (lane 9). Lanes: 1, 100 bp ladder as size standard; 2, PCR positive control; 3, PCR negative control; 4, No DNA bead supernatant; 5, No DNA immunocaptured fraction; 6, BrdU-labeled bead supernatant; 7, BrdU-labeled immunocaptured DNA; 8, unlabeled bead supernatant; 9, unlabeled immunocaptured DNA.

Establishing conditions under which immunocapture separates BrdU-labeled DNA from unlabeled DNA

The observed amplification in immunocaptured fractions of unlabeled controls could have been caused by the high sensitivity of PCR amplifying background level fragments of unlabeled DNA, or by the lack of specificity in the assay. In order to use the BrdU assay to detect growth, it was necessary to distinguish between these hypotheses. To test the ability of the antibody assay to separate BrdU-labeled from unlabeled DNA, we used a more sensitive technique, LH-PCR (Suzuki et al., 1998). Following PCR with 16S rRNA primers 27F and 338R, LH-PCR revealed a naturally occurring 37-bp difference in length between amplicons derived from pure cultures of *B. vulgatus* (352 bp) and *Fulvimarina pelagi* (315 bp), caused by insertions and deletions within rRNA genes (Suzuki et al., 1998).

First, we empirically established PCR conditions that were quantitative; that is, ratios of amplified PCR products were the same as their ratios in the initial template

mixtures. When we added known proportions of *F. pelagi* and *B. vulgatus* unlabeled DNAs to PCR, and arrested cycling at 15, 20, and 25 cycles, amplification bias occurred as cycle number increased. With 15 cycles of PCR the ratio of fragments in the products corresponded to the original template proportions (Table 4.1); when more than 20 cycles were used (Table 4.1), this relationship diminished.

Table 4.1. Ratios of relative fluorescence units resulting from LH-PCR products, following 15 and 20 cycles of PCR, where known template ratios of unlabeled *F. pelagi* and BrdU-labeled *B. vulgatus* DNA were amplified using the 27F and 338R primer pair.

***F. pelagi*:*B. vulgatus* ratio in template**	***F. pelagi*:*B. vulgatus* ratio in PCR products (15 cycles)**	***F. pelagi*:*B. vulgatus* ratio in PCR products (20 cycles)**
1:10	0.0597	0.2540
1:1	0.5660	0.7162
10:1	5.060	6.261

We subjected unlabeled *F. pelagi* and BrdU-labeled *B. vulgatus* DNAs mixed in known proportions to immunocapture. We analyzed bead supernatants, immunocaptured eluates, and mixtures prior to immunocapture using LH-PCR at 15, 20, and 25 cycles. 15 cycles of LH-PCR produced fragment abundances proportional to fragment abundance in the original template mixtures. We observed amplification bias at 20 cycles: amplification shifted from linear to logarithmic, approaching a 1 to 1 final amplicon composition, consistent with previously published data (Suzuki and Giovannoni, 1996) (Figure 4.2).

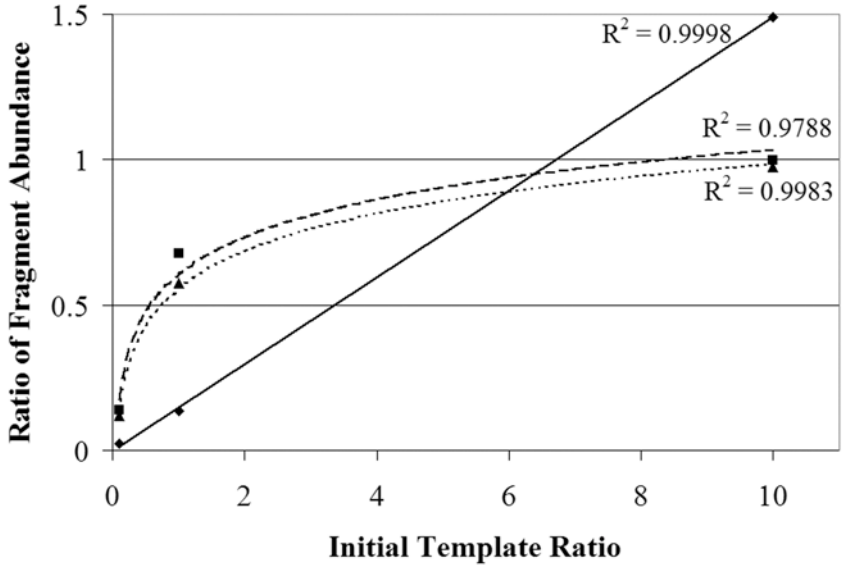

Figure 4.2. As cycle number increases the proportion of initial template concentrations (*F. pelagi*:*B. vulgatus*) relative to the ratio of fragment abundance shifted from linear to logarithmic. ——15 cycles, – – –20 cycles, ·····25 cycles; 15 cycles linear, 20 and 25 cycles logarithmic. Fragments were amplified using 27F and 338R.

Immunocapture followed by 15-cycle LH-PCR on bead supernatants, as well as immunocaptured fractions, revealed that immunomagnetic separation enriched for BrdU-labeled DNA (Figure 4.3). In repeated experiments, unlabeled *F. pelagi* DNA was never identified in the immunocaptured fraction when detected with 15 cycles of PCR (data not shown). These results demonstrated that immunocapture was specific for BrdU labeled DNA; however, at high cycle number unlabeled "background" DNA was occasionally amplified as a result of blocker leakage or PCR sensitivity. We eliminated amplification of background DNA using fluorescent detection of PCR amplicons following 15 cycles.

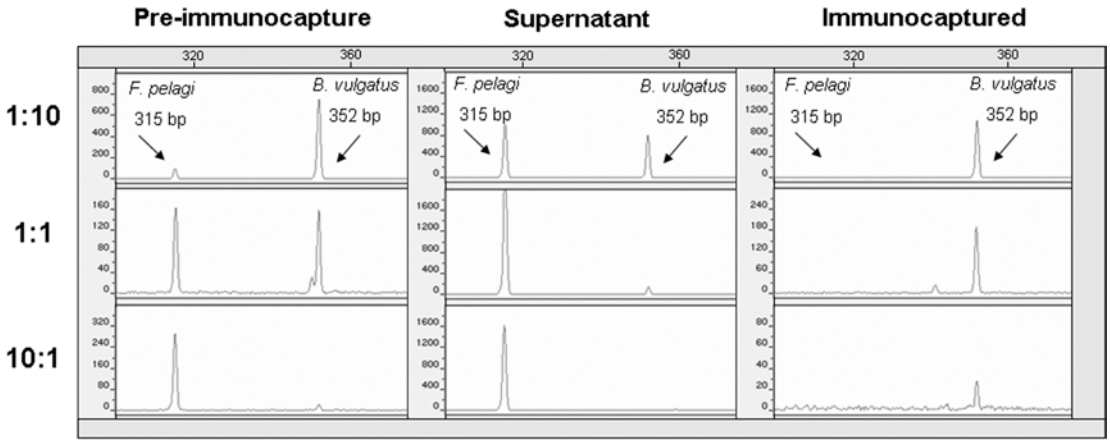

Figure 4.3. LH-PCR electropherogram after 15 cycles of PCR depicts unlabeled *F. pelagi* and BrdU-labeled *B. vulgatus* proportions before and after immunocapture, when amplified using 27F and 338R.

BrdU labeling and immunocapture of sewage influent

We labeled sewage with BrdU and incubated for up to 24 hours to measure growth of *Bacteroidales* cells and persistence of *Bacteroidales* molecular markers in sewage, using immunocapture followed by fluorescent PCR detection. To detect with general and human-specific *Bacteroidales* primers, we first established conditions under which these primer pairs were quantitative, by varying template concentrations and establishing the cycle number in which product concentration was proportional to template concentration. Plotting the log of fragment abundance (rfu) versus the log of the initial template concentrations indicated that a quantitative PCR, for all *Bacteroidales*-specific primer pairs used in this study, was achieved using 15 cycles of amplification (Figure 4.4). At 20 cycles and above we observed bias in sample results

from LH-PCR fragment analysis, with slopes approaching zero as cycle number increased (Figure 4.5).

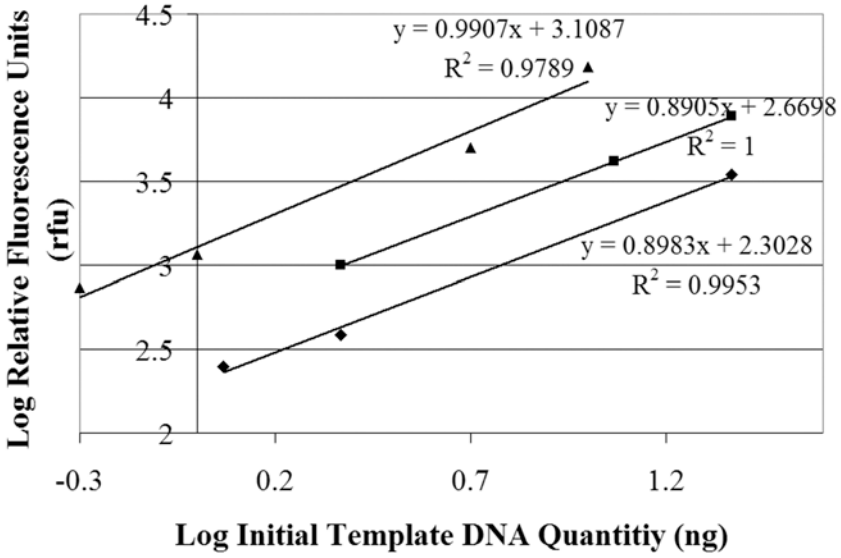

Figure 4.4. 15 cycles of PCR revealed an increase in amplicon fluorescence that was directly proportional to an increase in initial template concentration. ♦General *Bacteroidales*, ■HF134, ▲HF183. Equations of the line are given in point slope form.

Fluorescent fragment analysis of PCR products obtained following 15 cycles did not detect the *Bacteroidales* human-specific markers in either supernatants or immunocaptured fractions, even when using ten times the concentration of labeled sewage DNA in the immunocapture. Either the human-specific markers did not persist or grow, or fluorescent detection was not sensitive enough using 15 cycles of PCR. We estimated the limits of detection of fluorescent fragment analysis following 15 cycles of PCR using decreasing concentrations of template DNA. We detected as little as 0.01 ng of genomic *B. vulgatus* DNA, corresponding to 1.24×10^4 copies of the 16S

rRNA gene, and 0.1 ng of human-specific *Bacteroidales* plasmid DNA, corresponding to 2 x 10^7 copies of the gene.

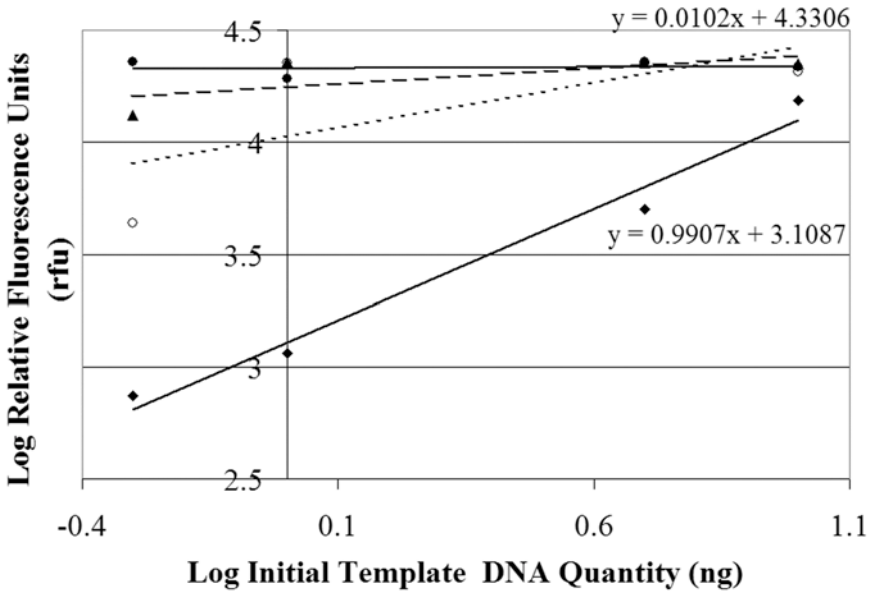

Figure 4.5. PCR using the general *Bacteroidales* primers (32F/708R) at 15, 20, 25, and 30 cycles. Above 15 cycles amplification bias was observed as amplicon fluorescence reached constant concentrations, and slope values approached zero. ♦15 cycles, ○20 cycles, ▲25 cycles, ●30 cycles; line equations are given for 15 cycles and 30 cycles.

To examine the reproducibility of the assay, as well as to establish the lower limit of BrdU-labeled target DNA that could be detected in a mixed population of BrdU-labeled DNAs, we conducted three replicate immunocaptures using five different proportions of BrdU-labeled *B. vulgatus* and *E. coli* DNAs. We reliably detected down to 0.5 ng, and occasionally detected as little as 0.01 ng of BrdU-labeled template DNA (Table 4.2) using the general *Bacteroidales* primers.

Table 4.2. Results of fluorescent PCR efficiency, at 15 cycles, when known quantities of BrdU-labeled target DNA are immunocaptured among a community of BrdU-labeled DNA. +/- indicates weak positive result with rfu values below those of statistical significance.

Starting Proportions (*E. coli* + *B. vulgatus*)	Detection of *B. vulgatus* before immunocapture			Detection of *B. vulgatus* following immunocapture		
	Rep#1	Rep#2	Rep#3	Rep#1	Rep#2	Rep#3
0.99 + 0.01 ng	+	+	+/-	-	-	+
0.9 + 0.1 ng	+	+	+	-	+	+
0.5 + 0.5 ng	+	+	+	+	+	+
0.1 + 0.9 ng	+	+	+	+	+	+
0.01 + 0.99 ng	+	+	+	+	+	+

Discussion

A major limitation to studying the ecology of allochthonous organisms is the inability to cultivate the majority of them under laboratory conditions. BrdU-labeling of environmental samples or natural assemblages, followed by immunocytochemical separation, can allow detection of actively growing members within a population, and in the case of pollution source-tracking, enables a distinction to be made between persistence of a molecular marker and growth of the indicator organism(s).

Our data demonstrate that growth analysis by BrdU immunocapture and PCR detection at 30 cycles is not reliable, due to innate background, commonly observed in immunological assays, that can subsequently be amplified by PCR. Previous studies using 30 cycles of PCR to detect BrdU labeled sequences failed to show PCR results from unlabeled controls (Borneman, 1999; Artursson and Jansson, 2003). Unlabeled *Roseobacter* and lake water DNAs can be seen in LH-PCR electropherograms (Urbach

et al., 1999) from a study using PCR to detect BrdU-labeled *Alteromonas* mixed with unlabeled *Roseobacter* DNA following immunocapture. In order to eliminate PCR detection of unlabeled DNA, we used LH-PCR to verify enrichment for BrdU labeled DNA in a mixed community, and PCR with fluorescent detection to determine a quantitative cycle number that reliably amplified only BrdU labeled DNA. By comparing known template proportions to fragment abundances recovered following PCR at the quantitative cycle number, we showed that the BrdU immunocapture technique enriched for BrdU-labeled DNA and did not amplify unlabeled DNA. We optimized our analyses by analyzing dilutions of PCR products from each sample. Thus, the observed bias is not due to overloading the sequencer with excessive PCR product. Peak amplitudes between 100 – 5000 rfu are deemed most reliable using the ABI 3100 sequencer; therefore we retained data for peaks within this range.

Though highly reproducible, using PCR with fluorescent detection at the low number of cycles required to be quantitative decreased the sensitivity of this assay; we could not detect the human-specific *Bacteroidales* group in bead supernatants or immunocaptured fractions from BrdU-labeled sewage. This may be due to low cell densities. The smallest amount of BrdU-labeled DNA that could consistently be detected was 0.5 ng, and detection of 0.1 ng and 0.01 ng of BrdU-labeled template was erratic (in contrast, standard PCR with 30 cycles routinely detects 100 gene copies using these primers; Shanks et al., unpublished data). Because the human-specific clades are rare in sewage compared to *Bacteroidales* in general, it is possible that cycle number for detection of these targets could be increased slightly without compromising quantitative results; we did not test anything between 15 and 20 cycles.

We do not believe the inability to detect was due to inhibition. It is possible that impurities carried over in the extraction of DNA from sewage interfered with antibody binding; however, we routinely detected growth of general *Bacteroidales* cells in sewage, suggesting that interference with antibody binding was negligible in these experiments. A third possibility is that we did not detect cells from the *Bacteroidales* human marker clade because they did not grow, although other *Bacteroidales* cells did. This would suggest that different *Bacteroidales* marker types may exhibit differential survival.

We demonstrated that *Bacteroidales* fecal bacteria grew up to 24 hours in sewage, when incubated aerobically at the *in situ* temperature of sewage, and that the marker(s) persisted at least 24 hours under the same conditions. This is in agreement with the findings of Kreader (Kreader, 1998) showing that the molecular signal for *Bacteroides distasonis* persists up 14, 5, or 2 days at 4 º, 14 º, and 24 ºC respectively, in unfiltered river water. Additionally, Seurinck and colleagues (Seurinck et al., 2005) found that the human-specific marker (HF183) persisted up to 24 days at 4 º and 12 ºC, and up to 8 days at 28 ºC in fresh river water (Seurinck et al., 2005).

It is known that at least some bacteria in the *Bacteroidales* group are not obligately anaerobic. *Bacteroides fragilis*, previously deemed an obligate anaerobe, requires nanomolar concentrations of oxygen for growth, and possesses an O_2-dependent cytochrome (Baughn and Malamy, 2004). These findings are consistent with the observation that *Bacteroidales* cells exhibit some degree of oxygen tolerance when manipulated in the laboratory. In addition, sewage influent often contains flocculent material. Bacteria colonize small particles, creating anaerobic microniches

within an overall aerobic environment (Tay et al., 2002) such as surface water. Finally, we did not shake or otherwise aerate our sewage incubations. When we added the oxygen sensitive, colorimetric indicator resazurin to 10 ml sewage samples and incubated them aerobically for 4 hours, the mixture stratified such that the top third of the tube was pink, indicating the presence of oxygen, but the lower two-thirds were clear, indicating anaerobiosis (data not shown). This observation, combined with our data demonstrating growth of *Bacteroidales* cells during aerobic incubation of sewage influent, suggests *Bacteroidales* fecal bacteria may be able to persist and grow in low oxygen refugia within streams, lakes, estuaries, and bays.

When employing commensal bacteria as source markers for fecal pollution, a reliable means of quantifying marker abundance and growth is necessary in order to implement policy involving human health risk assessment and diagnosis of watershed quality. Studies exploring growth of indicator organisms should employ quantitative methods at the time of sampling in order to examine the extent of growth in the environmental matrix. Factors influencing the growth of allochthonous organisms include predation, ambient water temperature, UV radiation, and sediments as refugia (Hood and Ness, 1982; Fish and Pettibone, 1995; Menon et al., 2003; Anderson et al., 2005).

Our findings may have significant impacts for microbial ecology studies using PCR of immunoseparated, BrdU-labeled DNA to detect active populations. Because of the kinetic biases inherent in some primer pairs (Suzuki and Giovannoni, 1996) an unequal proportion of amplicon concentration to initial template concentration can result from high cycle number PCR, such that unlabeled background DNA amplicons

may reach a detectable concentration, and appear as active members of a population or community when in fact they are not, leading to inaccurate conclusions about metabolic or biogeochemical processes within a population or community. The sensitivity of PCR is so great that in order to overcome the problem of amplifying unlabeled background DNA, a quantitative assay should be incorporated into this protocol. Here we demonstrate LH-PCR and PCR followed by fluorescent fragment analysis can be used at low cycle number to overcome the problem of background amplification in these assays.

BrdU-labeling and immunocapture followed by a low number of PCR cycles and fluorescent detection is an attractive method for identification of growing cells based on phylotype because it circumvents the need for direct cultivation and isolation, is non-radioactive, and is relatively simple and inexpensive. However, for the purposes of studying growth of uncommon or rare species in aquatic environments this method is likely not sensitive enough to verify growth of slow growing organisms or organisms growing at low cell densities. Thus, using PCR to detect growing populations at a low, quantitative cycle number could cause ecologically significant populations that have longer generation times, and comprise a small proportion of the overall community, to be overlooked.

Other methods for detection of viable or active cells from specific bacterial groups include rRNA-targeted hybridization using specific oligonucleotide probes and microautoradiography. Detection methods that target RNA sequences can be used quantitatively and are reasonable predictors of cell viability because of rapid cellular RNA degradation following cell death. Fluorescence in situ hybridization (FISH) is a

non-radioactive method that uses phylogenetic group-specific probes targeting rRNAs to selectively visualize cells, preserving cell size, morphology, and bacterial aggregate composition. Like the BrdU assay, FISH may miss slow growing or starving cells, due to a decrease in rRNA content. In addition, the power of FISH is limited by available sequence data and sequence specificity. However, the sensitivity of FISH assays can be heightened by using multiple probes that are specific for the same target organism(s) (Lee et al., 1993), by the use of helper probes (Fuchs et al., 2000), or by inclusion of a catalyzed reporter deposition (CARD) (Pernthaler et al., 2002). *Bacteroidales* fecal marker organisms have nearly 100 percent sequence identity (Dick et al., 2005a), making it difficult to design a probe or probe set specific to each host-specific marker, and limiting the applicability of the FISH technique in our system. Microautoradiography is used to visualize active microbial cells metabolizing a radioactive substrate. When used with 16S rRNA-targeted hybridization, microautoradiography offers the advantage of phylogenetically detecting active cells and deciphering group-specific substrate utilization by these bacteria (Lee et al., 1999; Ouverney and Fuhrman, 2001). However, microautoradiography depends on cells' ability to both grow in culture and take up the supplemented substrate. To follow the studies reported here, we are using RNA and DNA hybridization techniques to quantitatively survey the persistence and growth of host-specific *Bacteroidales* bacteria in mesocosms simulating a variety of environmental parameters.

In microbial ecology the questions posed are often three-fold: who is there, how many, and what are they doing? When used alone, FISH provides information on identity and abundance of microbial cells but does not discern the function of

microbial populations within a community or provide information about growth. FISH paired with BrdU-labeling can determine cell growth and viability of specific microbial species within communities (Pernthaler et al., 2002). Combining methods that employ 16S rRNA-specific probe hybridizations with microautoradiography allows inferences about the viability and function of environmental populations (Lee et al., 1999; Rossello-Mora et al., 2003), without direct cultivation. Recent advances have broadened our ability to implement multiple detection techniques simultaneously. It appears no single method is best for examining the activity and viability of aquatic microorganisms; thus to gain maximum insight about the growth, viability, and function of microbial communities the experimental approach should include multiple methodologies.

Acknowledgements

This work was supported, in part, by the Charles E. and Clara Marie Eckelman Graduate Assistantship, Oregon Sea Grant 04-016, and the Nucleic Acids and Protein Service Core of the Environmental Health Sciences Center, Oregon State University, NIH Grant P30 ES00210, National Institute of Environmental Health Sciences, National Institutes of Health.

We thank Jakob Pernthaler, Stephen Giovannoni, Linda Dick, and Malcolm Lowry for thoughtful discussion and valuable input.

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CHAPTER 5

SURVIVAL AND PERSISTENCE OF HUMAN AND RUMINANT-SPECIFIC FECAL *BACTEROIDALES* IN FRESHWATER MICROCOSMS

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Applied and Environmental Microbiology
Manuscript in preparation

Abstract

The standard indicators of fecal pollution in water, *Escherichia coli* and fecal enterococci, grow and persist in extra-intestinal environments, confounding attempts to mitigate human exposure to water polluted with feces and fecal pathogens. Alternative, source-specific indicators using PCR of host-specific markers from the *Bacteroidales* group of fecal anaerobes have been proposed as a rapid diagnostic tool for identifying fecal pollution and fecal source discrimination. To validate the usefulness of these indicators we followed persistence and survival of human and ruminant-specific fecal *Bacteroidales* markers and marker organisms, compared to *E. coli* and enterococci, in freshwater microcosms incubated at 13 °C; exposure to natural light was included as a variable. Numbers of *E. coli* (MPN) were above established limits for recreational water for the duration of the experiment. Enterococci survival was dependent on fecal source and exposure to natural light but fell below established limits by 14 days in both human and ruminant fecal microcosms. Ruminant-specific fecal *Bacteroidales* markers displayed differential persistence and survival profiles. The CF128 ruminant-specific marker survived and persisted through 14 days. The CF193 marker was detected by PCR until day 3 in microcosms exposed to natural light and day 6 in microcosms where light was excluded; persistence and survival as determined by QPCR was until day 7 and 14. The survival and persistence profiles for the human-specific fecal *Bacteroidales* markers and marker organisms were identical. The HF134 and HF183 marker organisms and genetic markers

survived and persisted 6 and 7 days; no effect of natural light was observed. These results support use of host-specific fecal *Bacteroidales* markers as indicators of recent fecal pollution. The survival and persistence profiles are consistent with survival profiles for several fecal pathogens.

Introduction

Fecal contamination of water used for drinking, recreation, and fishing activities is a growing problem world wide. Fecal pollution in aquatic environments not only presents human health risks but also causes significant environmental and economic losses (Prüss, 1998; Fleming et al., 1999; Anderson et al., 2002; Johnson et al., 2003; Molbak and Scheutz, 2004; Rabinovici et al., 2004; Cornish, 2005; Dorfman, 2006). Implementation of the U.S. Environmental Protection Agency (USEPA) standard methods of diagnosing fecal pollution in water has reduced the number of human illnesses associated with exposure to feces in water. However, the standard methods do not identify sources of contamination thereby making remediation efforts ineffective. In addition, current standard methods of identifying fecal pollution rely on cultivation based assays that require 18-24 hour incubations. Due to the time required to grow these indicator organisms human exposure often occurs before a diagnosis is made. Because fecal bacteria rapidly lose the ability to be cultivated using traditional microbiological techniques, yet may retain metabolic functions (Menon et al., 2003) and

pathogenicity (Pommepuy et al., 1996; Rahman et al., 1996), these methods may underestimate potential health risks.

In order for an indicator to be an effective measure of recent fecal pollution it should not grow well outside of the animal host. To adequately assess human health risks from exposure to pathogens of fecal origin, an indicator should survive and persist only as long as fecal pathogens and be detectable when pathogens are present (Tamplin, 2003). One drawback to use of current standard methods is the ability of the standard indicator organisms, *Escherichia coli* and enterococci, to survive and grow in extra-intestinal environments such as soil, beach sand, sediments, and bodies of water in general (Hardina and Fujioka, 1991; Fujioka et al., 1999; Byappanahalli et al., 2003; Wheeler Alm et al., 2003; Whitman et al., 2003; Anderson et al., 2005; Byappanahalli et al., 2006).

Recent research has focused on development of innovative techniques for fecal source identification using alternative methods. For example, antibiotic resistance analysis (ARA) (Parveen et al., 1997; Hagedorn et al., 1999; Wiggins et al., 1999) and carbon utilization profiles (CUP) (Hagedorn et al., 2003; Wallis and Taylor, 2003) are phenotypic, library dependent methods used to infer host sources of fecal pollution using *E. coli* and enterococci isolates from each potential contributor. Genotypic, library dependent methods include ribotyping (Parveen et al., 1999; Carson et al., 2001; Scott et al., 2004), repetitive extragenic palindromic polymerase chain reaction (rep-PCR) (Dombeck et al., 2000; Carson et al., 2003; McLellan et al., 2003), and amplified fragment length polymorphism (AFLP) (Guan et al., 2002). These methods require construction of libraries from each

geographic location (McLellan et al., 2003), and because they rely on isolation *E. coli* and enterococci they are subject to the same limitations as current cultivation based methods of detection.

We have proposed using PCR amplification of *Bacteroidales* host-specific 16S rRNA gene sequences for fecal source identification. This is a less cumbersome genotypic, library independent method, that provides a diagnosis in under 5 hours post sampling (Bernhard and Field, 2000a; Bernhard and Field, 2000b; Bernhard et al., 2003; Dick and Field, 2004; Shanks et al., 2006). *Bacteroidales* genetic markers make excellent candidates for indicators of fecal pollution. *Bacteroidales* bacteria are found in large numbers in mammalian intestines and have been shown to correlate with the presence of certain fecal pathogens (Walters et al., 2006, in press). Because bacteria from this group possess anaerobic metabolisms, survival of host-specific *Bacteroidales* marker organisms is likely to be restricted in extra-intestinal environments (Kreader, 1995).

Traditional PCR reliably provides a measure of marker persistence and quantitative PCR (QPCR) provides absolute quantification of genetic markers. Due to the resistant nature of the DNA molecule these are inadequate methods for evaluating survival and growth of the marker-containing cells. Methods used to survey survival, growth, and persistence of extant fecal indicator bacteria typically rely on cultivation (Hood and Ness, 1982; Davies et al., 1995; Fish and Pettibone, 1995; Byappanahalli et al., 2003; Menon et al., 2003; Wheeler Alm et al., 2003; Whitman et al., 2003; Anderson et al., 2005). However, strains containing the

markers proposed as host-specific indicators have not been isolated in culture; for this reason, examining their survival cannot be accomplished by cultivation.

Alternative non-culture based, molecular approaches have proven useful for studying metabolically active, non-culturable populations of bacteria. One approach to studying metabolically active members of a population uses DNA labeling. Because bacterial growth requires DNA replication, DNA labeling techniques can be used to describe species of actively growing bacteria. Radioactive nucleotides or the thymidine analog, bromodeoxyuridine (BrdU), are taken up by replicating bacterial cells and incorporated into the nascent DNA molecule. In the case of BrdU labeling, a magnetic immunocapture procedure separates BrdU-labeled DNA from unlabeled DNA and PCR is used to identify metabolically active members of a population (Borneman, 1999; Urbach et al., 1999; Artursson and Jansson, 2003). However, because it is PCR based, this method is sensitive to minute amounts of blocker leakage, resulting in false positives (Walters and Field, 2006). Lowering the sensitivity to remove false positives may miss active groups when the organisms of interest are minor constituents within complex communities (Walters and Field, 2006).

It is generally accepted that molecular techniques exploiting RNA provide reliable estimates of bacterial survival, growth, and physiological status (Weller and Ward, 1989; Kemp et al., 1993; Wagner, 1994). RNA degrades rapidly after cell death. Therefore, detection of particular RNA sequences identifies organisms that are living at the time of sampling. Methods of RNA based detection include fluorescent in situ hybridization (FISH) which can be combined with

autoradiography (Amann et al., 1995; Lee et al., 1999; Ouverney and Fuhrman, 2001; Pernthaler et al., 2002) or live/dead staining (Savichtcheva et al., 2005); RNA dot blot hybridization using chemiluminescent or radioactive probes (Sahm et al., 1999); and reverse transcription PCR (RT-PCR) (Miskin et al., 1999; Duineveld et al., 2001).

Currently, little is known regarding survival and persistence of human and ruminant-specific fecal *Bacteroidales* bacteria and genetic markers in the environment. The human-specific HF183 marker was detected in river water by QPCR for 8 – 24 days depending on temperature (Seurinck et al., 2005). Using BrdU labeling and immunocapture, followed by a low, quantitative number of PCR cycles, Walters and Field (Walters and Field, 2006) demonstrated growth of general *Bacteroidales* marker organisms up to 24 h in aerobically incubated sewage influent but were unable to definitively conclude how long human-specific markers grew in sewage influent. Here we describe a study concurrently evaluating persistence and survival of human and ruminant-specific fecal *Bacteroidales* markers and marker organisms in freshwater microcosms, incubated in the dark or allowed exposure to natural light, compared to the standard indicators *E. coli* and enterococci. Natural light was included as a variable because sunlight appears to be an important factor influencing survival of sewage bacteria and rates of sunlight inactivation vary with respect to different fecal indicator bacteria (Davies-Colley et al., 1994; Sinton et al., 1999; Sinton et al., 2002). In all microcosms, *E. coli* grew and persisted in exceedance of established guidelines for recreational water (USEPA, 2004), through the last day of sampling. Survival and

persistence followed the trend human-specific *Bacteroidales* < ruminant-specific *Bacteroidales* < enterococci < *E. coli*/fecal coliforms.

Materials and Methods

Collection of Host Feces

(i) Cow Feces

Fresh cow feces were collected from the Oregon State University Dairy Research Facility, Corvallis, OR. Ten individual patties were collected from 3 separate enclosures and transported back to the laboratory in sterile containers on the day of inoculation and were kept at 4°C until used as inoculum.

(ii) Human Feces

Fresh human feces were collected from 8 healthy adult volunteers. Samples were collected within 24 hours of use, in sterile containers and kept at 4°C until used as inoculum.

Preparation of Inocula

Three grams of each individual fecal sample (cow and human) were weighed out and combined separate Ziploc® bags. The contents were mechanically homogenized inside the bag. A corner of the bag was removed and 20 g of each (cow and human) fecal mixture was weighed into sterile 100 ml bottles. Nanopure water (100 ml) was added to each bottle containing the fecal mixtures and vortexed extensively to homogenize the fecal slurry.

Microcosms

Microcosm experiments were conducted at the outdoor facilities at the Salmon Disease Laboratory (SDL), Oregon State University, Corvallis, OR. Microcosms consisted of 5 gallon buckets, each containing 15 L of fresh river water, with an airstone at the bottom of each bucket to facilitate water circulation. Buckets were placed on top weighted supports and submerged to within 5 cm from the top of a 3 foot holding tank to control microcosm temperature. Water piped in to the holding tank was maintained at a constant temperature of 13°C, the experimental temperature in these experiments. Water in the holding tank was on continuous flow.

Two cow and two human fecal *Bacteroidales* survival microcosms were constructed. All variables were the same among the microcosms including the natural water source, volume, temperature, mixing, and quantity of fecal inoculum (50 ml of the appropriate fecal slurry). In order to test the effects of ambient light on survival of the fecal indicator organisms, one of the human and one of the cow fecal microcosms were completely covered and never exposed to ambient light throughout the duration of the experiment. The other microcosms remained uncovered allowing light penetration into the water column. On days when there was rain, the "light" microcosms were covered with clear acrylic (1/8"), allowing light penetration without dilution from rain water accumulation.

(i) Source River Water

Fresh river water was collected from the upper south fork of the McKenzie River, Blue River, Oregon on 29 October, 2006 and was stored in the dark at 4 °C,

overnight before being added to the microcosms.

(ii) Inoculation of Survival Microcosms

On the afternoon of 30 October 2006 fecal slurries were transported to the microcosm location at the Salmon Disease Laboratory on ice. Each human fecal microcosm received approximately 50 ml of the human fecal slurry, measured using a serological pipet. Because of large particles in the fecal matter the pipet tip was removed to assist in transfer of the fecal slurry to the microcosms. Each cow fecal microcosm was inoculated in the same manner as the human, receiving approximately 50 ml of slurried feces using a serological pipet with the tip removed.

Sampling

Sampling was carried out daily for the first 8 days and then every other day until one sampling day past the last day the host-specific fecal *Bacteroidales* markers were no longer detected using conventional PCR. Sample volume varied between human and cow microcosms because of differences in the numbers of enterococci and fecal coliforms among humans and cows, as well as differences between light and dark incubations.

E. coli and enterococci enumeration

E. coli and enterococci were quantified using the commercially available Colilert®-18 and Enterolert™ enumeration kits (IDEXX Laboratories, Westbrook, ME), according to the manufacturer's directions. One sample from each day was

tested for *E. coli* and enterococci; quadruplicate samples were not included since these are EPA approved methods and reproducibility has already been established. Depending on host-species and time of incubation, different dilutions were used in these assays (Table 5.1). The Colilert or Enterolert reagent was added to each sample and dissolved. Samples were placed in individual Quanti-tray®/2000 (IDEXX Laboratories) packages and sealed with the Quanti-tray® Sealer (IDEXX Laboratories). Colilert and Enterolert tests were incubated for 18 to 24 hours at 35° and 41°C respectively. The most probable number (MPN) of *E. coli*, coliforms, or enterococci was determined using the MPN table provided by the company.

Table 5.1. Dilution factors used in Colilert-18® and Enterolert™ assays on microcosm samples.

Sample Day	**Enterolert**				**Colilert**			
	Cow		Human		Cow		Human	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
0	100	100	100	100	100	100	100	100
1	100	100	100	100	100	100	100	100
2	10	10	100	100	100	100	100	100
3	1	20	100	100	100	100	1000	1000
4	1	1	100	100	100	10	1000	1000
5	1	1	100	100	100	100	10000	10000
6	1	1	100	100	100	100	1000	1000
7	1	1	10	10	100	100	100	100
8	1	1	1	10	10	10	100	100
10	1	1	1	1	10	10	10	10
12	1	1	1	1	10	10	10	10
14	1	1	1	1	10	10	10	10

Nucleic Acid Extraction

(i) Source River Water

River water used in the microcosms was subjected to nucleic acid extraction to verify the absence of ruminant and human-specific fecal *Bacteroidales* markers before inoculation. A 100 ml water sample was filtered onto 0.2 µm Supor-200 filters (Pall Gelman Laboratory, Ann Arbor, MI) under vacuum of at least 15 lb/in^2. The filter was placed in a 15 ml conical tube containing 500 µl GITC buffer (5 M guanidine isothiocyanate, 100 mM EDTA [pH 8.0], 0.5% *N*-lauroyl sarcosine; Sigma, St. Louis, MO), vortexed for 60 s to saturate the filter and stored at -80°C. The DNeasy Tissue Kit (Qiagen, Valencia, CA) was used to recover DNA from the filter according to the methods of Walters and Field (Walters and Field, 2006) with one modification. Briefly, after thawing, the sample was vortexed an additional 60 s, 500 µl of AL buffer was added to the sample and vortexed for 60 s, then 500 µl of 100% ethanol was added and the sample was again vortexed for 60 s. The lysate was transferred to a DNeasy spin column using a sterile transfer pipet and the filter was discarded. Following the third AW2 wash step, DNA was eluted from the column twice by adding 25 µl of buffer AE, warming the preparation to 65°C for 10 min, and then centrifuging it at 10,000 rpm for 60 s.

(i) Feces

Fecal DNA and RNA were isolated from cow and human feces using the RNA/DNA Mini Kit (Qiagen) according to manufacture's instructions with the following modifications. First, the pooled human (200 mg) and cow feces (220

mg) were added to individual 5 ml polyethylene cryotubes. Each tube of feces received 200 µl of 5 mg/ml lysozyme diluted in Tris-EDTA, pH 8.0 (Sigma). The samples were vortexed for 60 s and incubated at room temperature for 10 min. Next, 600 µl of lysis buffer QRL1 (Qiagen) plus 0.01% β-mercaptoethanol (Sigma) was added to each sample and vortexed again. Samples were stored at -80°C until extraction. After thawing, fecal samples received 3 ml dilution buffer QRV2 and were vortexed to homogenize the mixture. The mixtures were then separated into 2- 2ml screw cap centrifuge tubes and centrifuged at 8,000 rpm for 5 min at 4°C to pellet the cellular debris. Lysates were added to 2 separate gravity columns to bind RNA and flow through was collected in separate tubes and set aside for DNA preparation. This process was repeated until all debris had been pelleted between the two tubes and all lysates had been loaded onto the columns. The extraction and isolation procedure was then followed according to manufacture's instructions, and pellets were combined during the ethanol precipitation step. RNA was resuspended in 200 µl of RNase-free water with warming to 65°C and vortexing. DNA was resuspended in 100 µl AE buffer with warming to 65°C and vortexing. RNA was stored at -80°C and DNA at -20°C for downstream assays.

(i) Microcosm Samples

Four replicate samples were collected from each microcosm, at each sampling time. Aqueous samples from microcosms were centrifuged at 15,000 rpm, for 20 min, at 4°C, in a Sorvall® Centrifuge holding an SS-34 rotor (Sorvall, Thermo Electron Corp., Asheville, NC) in 50 ml Nalgene™ Oak Ridge

polypropylene centrifuge tubes (Fisher Scientific, Pittsburg, PA). Sample volumes from microcosms used for nucleic acid extractions varied depending on the sampling day (Table 5.2). In instances where sample volume was 50 ml, 40 ml of each sample was centrifuged and supernatants were discarded, then the additional volume was added to the tubes and centrifuged again, producing one pellet per sample. Pellet supernatants were carefully discarded so not to disturb the pellet. The RNA/DNA Mini Kit (Qiagen) was used to isolate DNA and RNA from microcosm samples according to manufacture's instructions with several modifications. First, all samples received 5 mg/ml lysozyme diluted in Tris-EDTA, pH 8.0 (Sigma), were vortexed for 60 s, and then allowed to sit at room temperature for 10 min. Samples from day 0 – 6 received 100 µl of the lysozyme solution, samples from day 7, 8, 10, and 12 received 50 µl. Following incubation with lysozyme, lysis buffer QRL1 plus 0.01% β-mercaptoethanol (Sigma) was added to each sample at a volume 3 times greater than the volume of lysozyme solution. Samples were vortexed for 60 s and stored at -80°C until extraction. After thawing, 1 – 1.35 ml dilution buffer QRV2 was added to each tube and the samples were vortexed an additional 60 s. Samples were then transferred from the centrifuge tubes to 2 ml screw-cap microcentrifuge tubes and centrifuged at 8,000 rpm for 5 min. at 4 °C. The supernatants were applied to individual gravity filtration columns and the remainder of the isolation was carried out according to the manufacture's specifications. RNA and DNA pellets were resuspended, following ethanol precipitation, in RNase-free water or AE buffer (Qiagen)

respectively. Resuspended volumes varied between sampling day and were based on nucleic acid pellet size (Table 5.2).

Table 5.2. Volumes of reagents used in DNA and RNA extractions from microcosm samples. CD, CL, HD, and HL refer to cow dark, cow light, human dark, and human light microcosms respectively. * Light bucket samples, † Dark bucket samples, § Cow samples only, ‡Resuspension volume.

Sample Day	Sample Vol. (ml)	Lysozyme /TE Vol. (µl)	QRL1+β ME Vol. (µl)	QRV2 Vol. (ml)	RNA‡ Vol. (µl)				DNA‡ Vol. (µl)
					CD	CL	HD	HL	
0	25	100	300	1	200	200	100	100	50
1	25	100	300	1	100	100	50	100	50
2	25	100	300	1	100	100	100	100	50
3	25	100	300	1	50	50	100	100	50
4	25	100	300	1	100	100	100	100	50
5	30	100	300	1.1	50	50	50	50	50
6	35*	100	300	1.1	50	50	50	50	50
6	30†	100	300	1.1	50	50	50	50	50
7	30	50	150	1.3	50	50	50	50	50
8	40	50	150	1.3	50	50	50	50	50
10	50	50	150	1.3	50	50	50	50	100
12	50	50	150	1.3	50	50	50	50	50
14	50	50	150	1.3	50	50			50§

DNA and RNA Quantification

Quantification of total DNA and RNA was accomplished using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

PCR amplification of host-specific Bacteroidales 16S rRNA genes

(i) PCR presence/absence detection of host-specific sequences

Each PCR reaction mixture consisted of 1X PCR buffer containing 2.0

mM $MgCl_2$ (TaKaRa BIO Inc., Otsu, Shiga, Japan), 0.2 mM dNTPs, each primer at a concentration of 0.2 μM, 0.08% BSA, and 0.025 U Ex *Taq* DNA polymerase (TaKaRa BIO Inc.). The amplification conditions included an initial denaturation step of 94°C for 2 min, followed by 94°C for 45 s, the annealing temperature specific for each primer pair for 45 s, 72°C for 45 s for 30 cycles, with a final extension step at 72°C for 7 min. For human-specific *Bacteroidales* primers HF134 (GCCGTCTACTCTTGGCC) and HF183 (ATCATGAGTTCACATGTCCG) (Bernhard and Field, 2000a) paired with Bac708R (CAATCGGAGTTCTTCGTG) (Bernhard and Field, 2000b) the annealing temperature was 63°C. For the ruminant-specific *Bacteroidales* primers CF128 (CCAACYTTCCCGWTACTC) and CF193 (TATGAAAGCTCCGGCG) (Bernhard and Field, 2000a) paired with Bac708R the annealing temperature was 62°C. PCR products were separated by electrophoresis on 1.5% agarose gels, and the results were recorded with a UVP gel imager (UVP, Upland, CA).

(ii) QPCR quantification of Bacteroidales 16S rRNA genes

Each QPCR reaction mixture consisted of 1 X *Power* SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), containing ROX™ as a passive reference dye, and 0.2 μM each primer. Two microliters of DNA preparation from each microcosm sample was added to the QPCR. The amplification conditions included and initial denaturation step of 94 ºC for 2 min; followed by 40 cycles of 94 ºC for 15 s and 60 ºC for 32 s, for all primer pairs used. Dissociation curves were created following amplification to check for non-target amplification. Standard curves were created using sequence specific plasmid

DNA. Amplification efficiency was calculated using the formula: efficiency = $-1+10^{(-1/slope)}$. Host-specific primers HF134, HF183, CF128, and CF193 (Bernhard and Field, 2000a) were paired with 265R (TACCCCGCCTACTATCTAATG) (Seurinck et al., 2005). QPCR amplification and analysis was accomplished using an Applied Biosystems 7500 Real-Time PCR System and the 7500 system software (Applied Biosystems). QPCRs were performed in duplicate for each DNA and cDNA sample, average values are reported in the results.

Detection and quantification of Bacteroidales 16S rRNA

(i) DNase treatment of RNA samples

Residual DNA was removed from each RNA sample using the TURBO DNA-*free*™ kit (Ambion, Austin, TX) according to manufacture's instructions for a 96-well plate format. Each 50 µl reaction consisted of 1X TURBO DNase buffer, 2 U of TURBO DNase, and 44 µl of RNA. The reactions were incubated for 30 min at 37 °C. Following incubation with the DNase inactivation reagent, 96-well plates were centrifuged for 5 min at 2000 x g. RNA supernatants were transferred to fresh 96-well plates. RNAs were quantified following DNase treatment.

(ii) Reverse transcription of cDNA

cDNA was transcribed using the High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems) according to manufacture's instructions. Each 20 µl reaction consisted of 1 X RT buffer, 4 mM dNTPs, 1 X RT random primers, 1 U MultiScribe™ reverse transcriptase, 1 U RNase inhibitor (Applied

Biosystems), and 10 µl of RNA. The amplification conditions consisted of 25 °C for 10 min, 37°C for 120 min, and DNase inactivation at 85 °C for 5 s.

(iii) QPCR detection and quantification of Bacteroidales 16S cDNA

Following reverse transcription, cDNA was amplified according to the QPCR protocol outlined above. Plasmid standards were included in each set of reactions to estimate template quantity and dissociation curves were created following amplification.

Results

To estimate how long the human and ruminant-specific *Bacteroidales* fecal marker organisms survive and the genetic markers persist in an extra-intestinal environment we constructed freshwater survival microcosms using a defined set of parameters. We included natural light as a variable possibly affecting survival. After extracting total nucleic acid from microcosm samples collected over a two week period, we used PCR and QPCR to assess persistence of each marker and estimate the quantity of target molecules obtained from each sample. Next we utilized reverse transcription to create cDNA molecules from RNA samples obtained from each microcosm, on each sampling day, to estimate survival. Using QPCR to estimate the quantity of target rcDNA molecules in each sample we estimated the survival time of each host-specific *Bacteroidales* marker organism in our microcosms. We concurrently compared survival and persistence of the fecal

Bacteroidales markers and marker organisms to survival of *E. coli* and enterococci.

Differential persistence of human and ruminant-specific fecal Bacteroidales markers

We did not observe a difference in persistence of the two human-specific *Bacteroidales* markers among samples obtained from the light and dark microcosms (Table 5.3). In fact, the persistence profile of the HF134 and HF183 genetic markers was nearly identical; exposure to natural light did not produce an effect. Neither human-specific marker was detected by conventional PCR after day 7.

There were striking differences in detection of the ruminant-specific *Bacteroidales* markers over the course of the experiment using conventional PCR. The CF128 genetic marker was more robust and persisted longer in both the light and dark microcosm incubations, compared to the CF193 genetic marker (Table 5.3). Regardless of the presence of natural light, the CF128 marker was detected using conventional PCR until day 14, in both light and dark incubations, although amplification was weak in samples collected after day 7. In addition, there was a marked difference between detection in the light and dark microcosms for CF193; CF193 was only detected through day 3 in the microcosm exposed to natural light, but was detected until day 6 in the dark microcosm. This finding provides evidence for a negative effect of natural light on persistence of the CF193 marker.

Table 5.3. *Bacteroidales* host-specific marker occurrence on each sampling day measured by conventional PCR after 30 cycles. +/- indicates weak positive result. Numbers in parentheses indicates the number of samples with that result.

Day	Light Microcosms				Dark Microcosms			
	CF128	CF193	HF134	HF183	CF128	CF193	HF134	HF183
0	+	+	+	+	+	+	+	+
1	+	+ (2), ± (2)	+	+	+	+	+	+
2	+	± (3), - (1)	+	+	+	+	+	+
3	+	± (3), - (1)	+	+	+	+	+	+
4	+	-	+	+	+	+	+	+
5	+	-	+	+	+	+	+	+
6	+	-	+	+ (3), - (1)	+	± (3), - (1)	+	+ (3), - (1)
7	+	-	± (1), - (3)	± (3), - (1)	+	-	± (3), - (1)	± (3), - (1)
8	±	-	-	-	±	-	-	-
10	± (1), - (3)	-	-	-	±	-	-	-
12	-	± (1), - (3)	-	-	± (2), - (2)	-	-	-
14	±	-			±	-		

Persistence trends of human and ruminant-specific Bacteroidales genetic markers

We used QPCR to detail persistence profiles for each of the human and ruminant-specific *Bacteroidales* genetic markers. We used dilutions of plasmids containing the target sequences to create standard curves for each primer pair. We estimated target template quantities based on these curves. The efficiency of amplification was between 90-100% for each primer pair, with the exception of CF128.

Persistence profiles of the human-specific *Bacteroidales* markers were similar in the light and dark microcosms, with the highest occurrence on day 0 and 4 post-inoculation (Figure 5.1); there was no clear relationship between the presence of natural light and persistence of the human-specific markers. There was a sharp decline in the number of marker molecules from day 0 to 1. The decline in numbers on day 1 was followed by a steady increase from day 2 through 4, to a level comparable or higher than the level on day 0. Another sharp drop occurred after day 4; no increase was observed following the decline between day 4 and 5. The number of human-specific markers continued to decrease from day 5 through 10. The number of target molecules detected corresponded to less than one cell/ml after day 7. Both markers, regardless of light conditions, experienced approximately a 4-log reduction between day 4 and day 10.

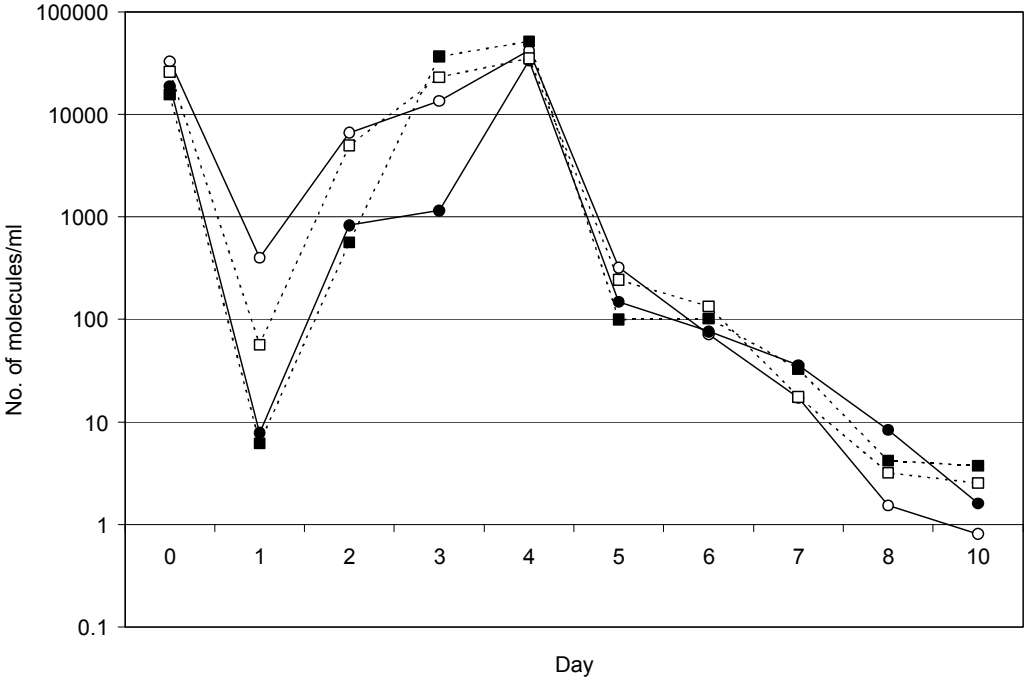

Figure 5.1. Average numbers of human-specific *Bacteroidales* 16s rRNA genes in microcosm samples over time, estimated using QPCR, for light and dark incubations. □HF134 (light), ■HF134 (dark), ○HF183 (light), ●HF183 (dark).

The ruminant markers had an entirely different pattern of persistence. They did not exhibit the early drop followed by an increase seen in the human markers. Initial measurements of ruminant-specific marker sequences indicate a higher proportion of the CF128 marker in cow feces; the number of CF128 markers was greater than CF193 in samples obtained on the day of microcosm inoculation. The CF128 markers experienced a sharp decline in numbers between day 0 and 3, with a slight increase from day 4 to 5, before steadily decreasing from day 6 through the remainder of the experiment. No difference in CF128 marker persistence was observed among samples from the light or dark incubations between day 0 and 8. However, there was a 1-log

difference between CF128 marker abundance from day 10 through 14, indicating that natural light exposure affected persistence of this marker (Figure 5.2).

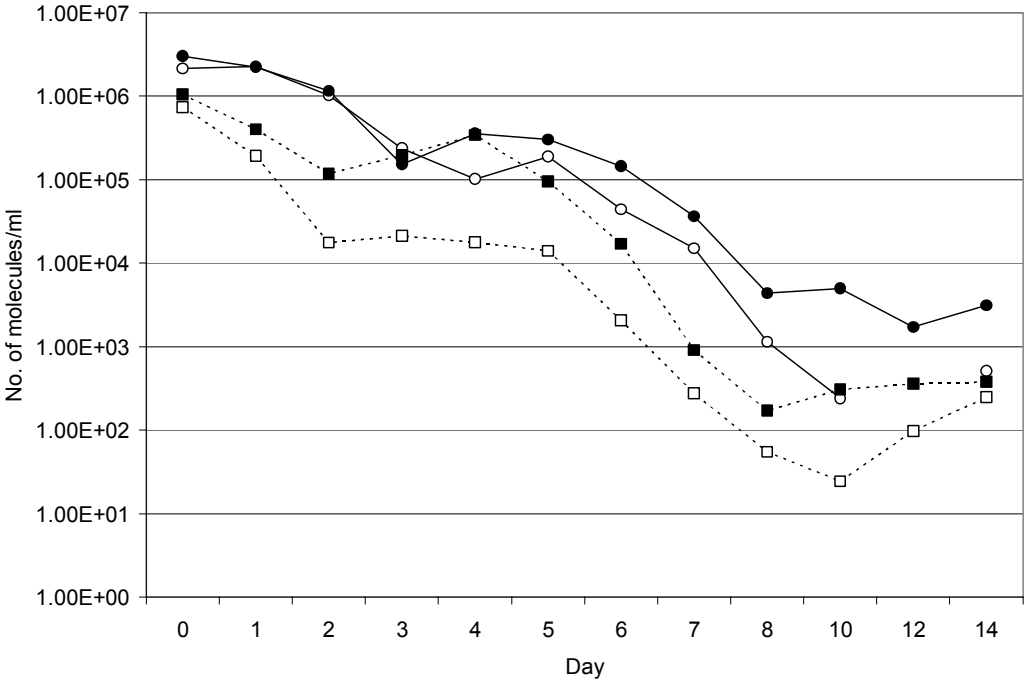

Figure 5.2. Average numbers of ruminant-specific *Bacteroidales* 16s rRNA genes in microcosm samples over time, estimated using QPCR, for light and dark incubations. □CF193 (light), ■CF193 (dark), ○CF128 (light), ●CF128 (dark).

An initial drop in the number of target molecules/ml was observed for the CF193 marker from day 0 through day 2, at which point the number of markers began to rise in the dark incubation but continued to decline in the light incubation. The number of CF193 markers remained constant from day 2 through 5 in the light incubation and then declined approximately 3-logs over the next 5 days. In the dark incubation, the number of CF193 markers increased from day 2 through 4 and then experienced a reduction of approximately 3-logs over the next three days. By day 14

the number of CF193 molecules was nearly equal in the light and dark incubations. This marker appeared to be more sensitive to changing environmental conditions than the CF128 marker, but exposure to natural light played only a minor role.

Differential survival of human and ruminant-specific Bacteroidales marker organisms

We estimated survival of *Bacteroidales* cells containing the markers by QPCR amplification of cDNAs obtained by reverse transcription of sample RNAs. The survival profiles of the human markers were the same for both markers in the light and dark incubations (Figure 5.3). Numbers of human-specific marker rcDNAs dropped below 1000/ml for all samples, regardless of light presence, by day 6. An extremely conservative estimate of 1000 ribosomes/cell would indicate the human-specific marker organisms did not survive past day 6, but it is more likely they ceased to survive after day 5 when rcDNA numbers fell between 750 – 10,000/ml. There was nearly a ten-fold difference in cDNA abundance for both the HF134 and HF183 markers on day 10 between the light and dark incubations. However, the number of molecules corresponding to these data would produce an estimate of <1 marker organism/ml, making these values relatively equal.

The ruminant-specific fecal *Bacteroidales* markers survived longer than the human-specific markers. Using the conservative estimate of 1000 ribosomes/cell, both ruminant-specific marker organisms survived through day 14 in both light and dark microcosms (<1 – 10 cells/ml) but dropped below the limit of detection for the assay on days 6 – 8 (CF128) and day 7 (CF193) (Figure 5.4). There were no obvious differences in the survival profiles of the CF128 marker organisms between the light

and dark incubations. The number of CF193 target rcDNA molecules detected was consistently 2 to 3-logs higher in the dark microcosm incubation than in the light, from day 2 through day 7, at which point the difference in number decreased; there was an approximate 1-log difference between the light and dark incubations by day 10. These data suggest that exposure to natural light has some effect on survival of CF193 marker organisms.

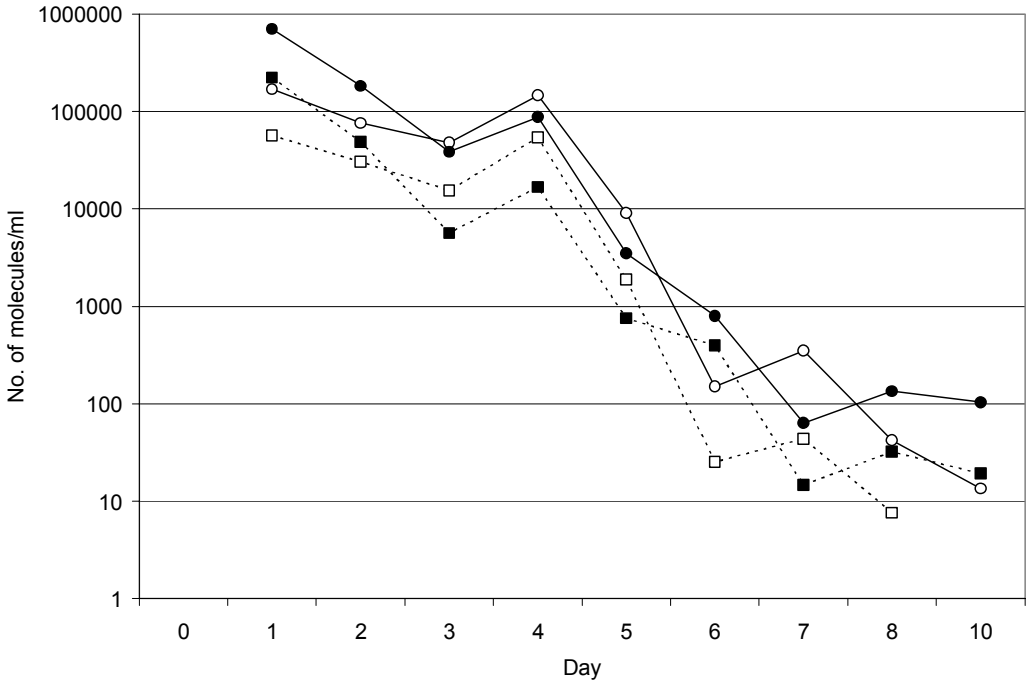

Figure 5.3. Average numbers of human-specific *Bacteroidales* 16s rRNA molecules in microcosm samples over time, estimated using QPCR following reverse transcription. □HF134 (light), ■HF134 (dark), ○HF183 (light), ●HF183 (dark).

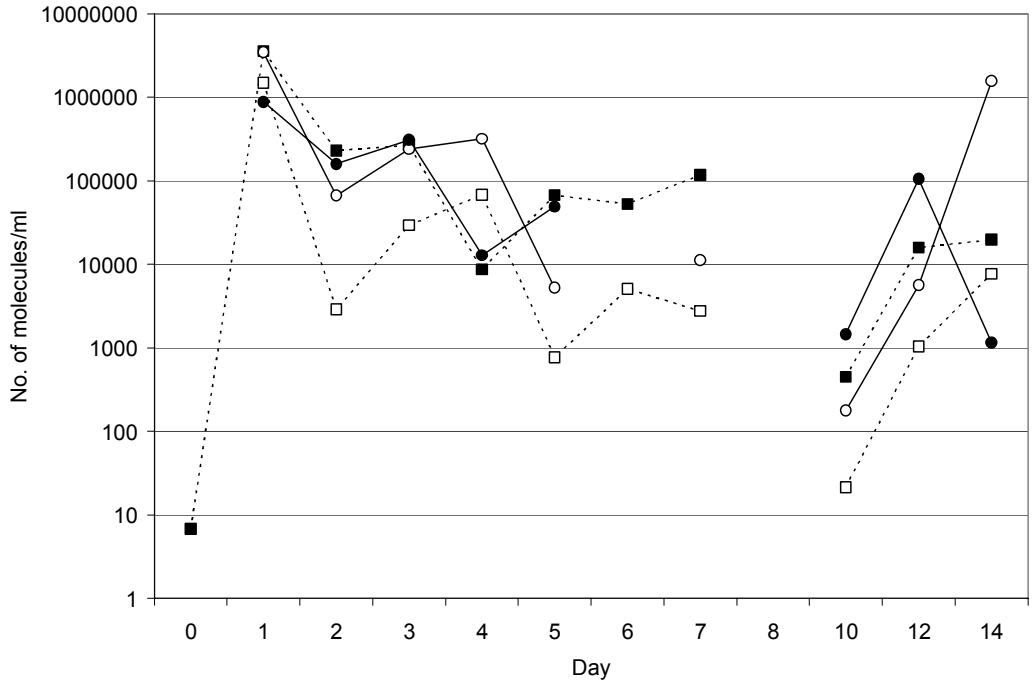

Figure 5.4. Average numbers of ruminant-specific *Bacteroidales* 16s rRNA molecules in microcosm samples over time, estimated using QPCR following reverse transcription. □CF193 (light), ■CF193 (dark), ○CF128 (light), ●CF128 (dark). Values equal zero where lines break.

Differential survival of the standard indicators E. coli and enterococci

The number of *E. coli* in the human and cow fecal microcosms was above the water quality standard of 126 MPN/ml throughout the entire sampling period. Survival of *E. coli* and fecal coliforms in the microcosms was not affected by the presence of natural light in either human or cow microcosms. The greatest difference in numbers of *E. coli* between light and dark microcosms was observed on days 7 and 8 when there was a 1-1.5-log difference in MPN observed between the light and dark microcosms in the cow and human microcosms (Figure 5.5 and 5.6). The survival profile for fecal coliforms followed the same trend as that of *E. coli* (data not shown).

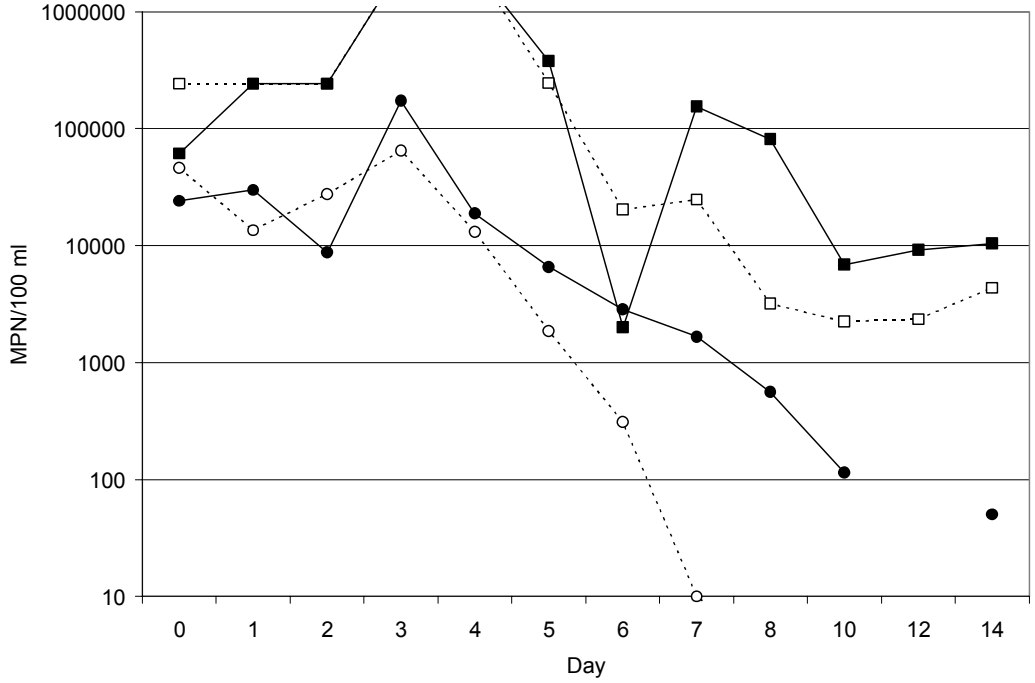

Figure 5.5. Numbers of *E. coli* and enterococci (MPN/100 ml) in human fecal microcosms incubated under both light and dark conditions. Data missing for day 12. □*E. coli* (light), ■*E. coli* (dark), ○enterococci (light), ●enterococci (dark).

Exposure to natural light influenced survival of enterococci more than *E. coli* (Figure 5.5 and 5.6). In the human fecal microcosms, numbers of enterococci increased slightly at first, but began to decrease in both the light and dark microcosms after day 3. Enterococci MPN remained relatively equal between the light and dark microcosms until day 4. After day 4 numbers of enterococci dropped more rapidly in the light incubation than in the dark, with a 1-log difference by day 6 and more than 2-logs on days 7 and 8. By the end of the experiment numbers of enterococci decreased 3 to 4-logs from peak concentration, in the dark and light microcosms respectively. Enterococci numbers fell within the established guidelines for freshwater (33 MPN/ml) by day 7 in the light incubation and day 14 in the dark incubation.

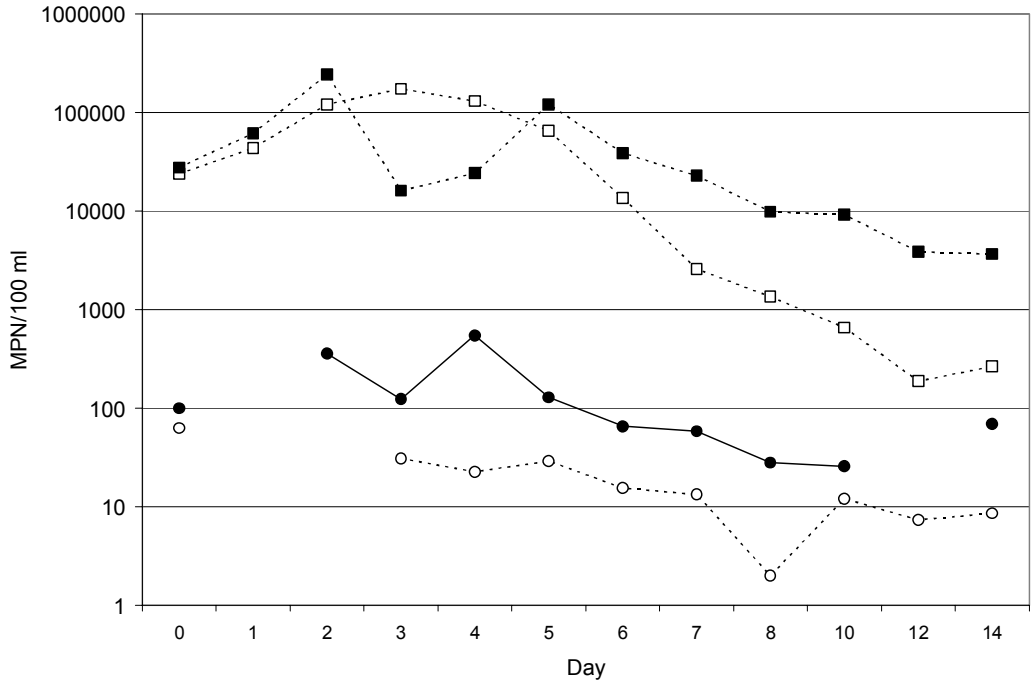

Figure 5.6. Numbers of *E. coli* and enterococci (MPN/100 ml) in cow fecal microcosms incubated under both light and dark conditions. Line breaks indicate missing data. □*E. coli* (light), ■*E. coli* (dark), ○enterococci (light), ●enterococci (dark).

Numbers of enterococci in the cow microcosms were substantially lower than those in the human microcosms. Enterococci MPN were below the established water quality limit in the light microcosm throughout the entire experiment, except for the day of inoculation at which time the number of enterococci was 63 MPN/ml (Figure 5.6). In the dark microcosm, numbers of enterococci increased substantially over the first 4 days and began declining on day 5; enterococci MPN fell within established limits by day 6 and were borderline throughout the rest of the experiment, but exceeded water quality limits again on day 12. There was an average difference of approximately 1-log between enterococci numbers in the light and dark cow

microcosms throughout the experiment, indicating that the presence of natural light affects the survival of this indicator.

Discussion

This is the first study evaluating persistence and survival of *Bacteroidales* human and ruminant-specific fecal markers, and marker organisms, in simulated natural environments. Factors influencing survival and persistence of enteric bacteria include solar irradiation, temperature, the presence of predators, and availability of nutrients (Barcina et al., 1997; Sinton et al., 2002; Menon et al., 2003). Protein synthesis is highly dependent on temperature (Ryals et al., 1982) and, as a result changes in temperature affects growth rate, metabolic activity, and microinvertebrates predation rates (Sherr et al., 1988; Barcina et al., 1997).

We chose 13 °C as our microcosm incubation temperature primarily for ease of conducting the experiment. However, this temperature provides biologically useful information regarding the persistence and survival of allochthonous bacteria in aquatic ecosystems and is an environmentally relevant temperature for rivers, lakes, and streams in the Pacific Northwest. For example, the mean water temperature of the Wilson River in the Tillamook watershed is 13.2 °C, and the mean temperature of the watershed, including all rivers and the bay is approximately 13 °C in June and September (Shanks et al., 2006).

Here, we present results from a study that sought to determine how long human and ruminant-specific fecal *Bacteroidales* marker organisms and genetic markers survive and persist in non-filtered freshwater microcosms, maintained at a

constant temperature, in the presence or absence of natural light, while making direct comparisons to the behavior of the standard indicators *E. coli* and enterococci. Our results show that in freshwater microcosms incubated at 13 °C both fecal *Bacteroidales* human-specific markers persisted for 7 to 10 days as determined by conventional PCR and QPCR respectively and the marker organisms survived approximately 6 days. Previous studies evaluated persistence of PCR detectable *B. distasonis* from human feces (Kreader, 1998), and persistence of the HF183 genetic marker using QPCR (Seurinck et al., 2005). *B. distasonis* was detected for 4 – 5 days in 14 °C river water (Kreader, 1998); the HF183 marker was detected up to 24 days in freshwater incubated at 4 ° and 12 °C (Seurinck et al., 2005).

There are several possible explanations for the observed differences between our findings and those previously reported. First, the sources of river water varied among comparable experiments. The headwaters of the McKenzie River originate from an underground source in the Oregon Cascade Mountain range. It is possible that the upper south fork of the McKenzie River contains fewer predators than the canal Coupure (Gent, Belgium) (Seurinck et al., 2005) or the Ohio River (Kreader, 1998). In addition, it is unclear if previous experiments evaluating persistence of human fecal *Bacteroides* spp. in river water included mixing. Here, we used an airstone in each microcosm to create movement and mixing within the water column of the microcosms. It is possible that stratification of the water column occurs in the absence of mixing, providing a reduced oxygen environment below the water surface that can lead to prolonged survival and subsequent persistence of the markers; this

phenomenon occurs when sewage influent is incubated in test tubes (Walters and Field, 2006).

Exposure to natural light did not affect survival of *E. coli* or fecal coliforms in these experiments. This contrasts with previous findings that sunlight adversely affects the survival of *E. coli* and other fecal coliforms (Barcina et al., 1997; Sinton et al., 2002). However, the start of our microcosm experiments was almost concomitant with the onset of the rainy season. We did not measure UV transmittance but most days were cloudy with low light, during the time of year when daylight hours are short in the Pacific Northwest. Our results show a more rapid decline in numbers of enterococci than *E. coli* among all of the microcosms regardless of whether light was excluded. This observation is consistent with other experiments comparing survival of *E. coli* and enterococci (Sinton et al., 2002). Anderson and colleagues (Anderson et al., 2005) used ribotyping to follow survival of different *E. coli* phylotypes in freshwater microcosms. *E. coli* survival varied depending on phylotype. The hardiest phylotype survived the duration of the experiment (96 hours); other phylotypes exhibited shorter survival times. Contrary to our findings, numbers of *E. coli* did not increase. Data from this study clearly demonstrates how the number of fecal indicator bacteria can differ between hosts. For example, identifying fecal pollution using enterococci in watersheds polluted by cow feces can lead to misinterpretation of the relative health risks since numbers of enterococci are lower in cow feces than human feces.

Some variation exists among PCR data obtained via conventional PCR and QPCR of the human and ruminant-specific fecal *Bacteroidales* markers. This is most

likely due to the number of PCR cycles used to detect target sequences increasing from 30 cycles (conventional PCR) to 40 cycles (QPCR), making the QPCR assay more sensitive. The QPCR assays used for these experiments were not optimized for host specificity and should not be used in fecal source tracking. Because we were not trying to discriminate among hosts, and these assays could be run under the same amplification conditions, the assays were suitable for our uses. The QPCR primer pairs did not amplify *E. coli* or cultivated *Bacteroides* spp. under the defined parameters (data not shown). The reported estimates for numbers of CF128 markers are underestimated. QPCR using the CF128 primer produced efficiencies of ~65%. This is most likely because of the low T_m of the CF128 primer (55 °C). Furthermore, CF128 is a degenerate primer and not an ideal choice for QPCR using SYBR Green® detection. The 265R primer also hits fewer *Bacteroidales* sequences in the ribosomal database than Bac303R, suggesting it is a less sensitive primer.

Reports of fecal pathogen survival in freshwater ecosystems are underrepresented in the literature. However, Wang and Doyle (Wang and Doyle, 1998) reported survival of *E. coli* O157:H7 in lake water incubated at 8 ° C for at least 13 weeks; survival was 49 – 84 days at 15 ° and 25 °C. In three out of four freshwater sources tested, a 10^6 cell/ml inoculum of *Salmonella typhimurium* decreased to 0 – 10 cells/ml after 17 days (Maki and Hicks, 2002). In 20 °C incubations, coxsackievirus was undetectable after 4 and 10 days, poliovirus after 8 and 10 days, and echovirus after 10 and 3 days, in freshwater impacted by sewage outfall and in non-polluted freshwater respectively (Hurst and Gerba, 1980). Our results demonstrating persistence and survival of human and ruminant-specific *Bacteroidales* fecal markers

are comparable to the survival of *S. typhimurium*, and survival and persistence of the human-specific markers is comparable to infectious enteric viruses.

Our findings hold promise for use of PCR-based detection of host-specific fecal *Bacteroidales* markers as an effective tool for source-specific diagnosis of fecal contamination and health risk assessment (Bernhard and Field, 2000b; Bernhard et al., 2003; Field et al., 2003; Shanks et al., 2006; Walters et al., 2006, in press). However, several questions have yet to be addressed. Members of the *Bacteroidales* group of anaerobic bacteria frequently colonize surfaces and form biofilms (Macfarlane et al., 2005; Macfarlane and Macfarlane, 2006; Olapade et al., 2006). These strategies can enhance environmental survival and persistence so it will be important to examine survival of the host-specific indicators in various types of natural sediments. Previous data suggest salinity plays a role in differential persistence profiles among the ruminant-specific *Bacteroidales* fecal markers (Bernhard et al., 2003). Therefore, it will be necessary to evaluate persistence and survival of the human and ruminant-specific *Bacteroidales* fecal markers under conditions of varying salinity. Finally, water quality standards for tropical waters may require modification or different indicators altogether. Because of the strong correlation between water temperature and depreciated survival time, *Bacteroidales* host-specific fecal markers, *E. coli*, and enterococci may not sufficiently predict the presence of fecal pathogens in tropical waters. Even so, detection of the *Bacteroidales* markers in tropical coastal water may better indicate recent pollution than current standard indicators which grow and persist in tropical soils (Hardina and Fujioka, 1991; Fujioka et al., 1999) and are currently undergoing evaluation in Hawaii river and coastal water sources.

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CHAPTER 6

GENERAL CONCLUSION

Sarah P. Walters

This study used statistical models to examine the usefulness of host-specific fecal *Bacteroidales* to predict the presence of fecal pathogens in freshwater sources. Although we did not find a significant relationship between the occurrence of pathogenic *Salmonella* spp. and any of the host-specific *Bacteroidales* markers we showed that presence of the ruminant-specific CF128 marker was predictive of *E. coli* O157:H7 presence. We also found an increase in the odds of the presence of *Campylobacter* spp. when the human-specific *Bacteroidales* markers were detected. These findings provide support for continued evaluation of *Bacteroidales* genetic markers for use in fecal source tracking and health risk assessment. Because none of the markers tested in our study adequately predicted the presence of *Salmonella* spp., and because *Salmonella* is frequently isolated from a broad range of hosts, these results obviate the need for further marker development, particularly development of markers for avian fecal sources.

Retrospective studies provide a statistic useful for making statements about the odds of pathogen presence when a particular marker is present. Because our sample sites were not randomly chosen the results from our study are only applicable to the dataset used in the presented analysis. More analyses such as this are required to evaluate *Bacteroidales* host-specific marker correlation with other pathogens and should include enteric viruses and protozoa. An ideal study design should include extensive sampling from sample site locations chosen at random and an estimate for the minimum number of samples necessary for strong statistical support should be calculated beforehand. By including a prospective study design, one would not be limited to making statements about odds or comparisons of odds ratios, but instead

allow for estimation of the proportion of water samples that test positive for the pathogen among those that have the marker (Ramsey and Schafer, 2002). Using QPCR to correlate marker numbers to the presence of fecal pathogens would broaden the applicability of *Bacteroidales* host-specific markers in health risk analyses.

We examined persistence and survival of human and ruminant-specific fecal *Bacteroidales* markers. To date, representatives from these groups have not been cultured. In order to overcome the need for direct cultivation in assessing survival, we initially used bromodeoxyuridine (BrdU) labeling of sewage influent to measure persistence and survival of general and human-specific fecal *Bacteroidales* markers and marker organisms. We found PCR to be sensitive to blocker leakage after repeatedly recovering rDNA sequences among immunocaptured, unlabeled controls. To trounce this limitation we modified the protocol to use a low, empirically determined, quantitative number of PCR cycles, followed by fluorescent amplicon detection. This modified technique enabled detection of BrdU-labeled cells without detecting unlabeled, background DNA but sensitivity was substantially decreased. We caution that experiments using 30 cycles of PCR to detect BrdU-labeled DNA may identify inactive bacteria. However, due to the loss of sensitivity by reducing the number of PCR cycles, our method is likely to miss active and ecologically significant populations, particularly in very dilute environments or very complex communities, where organisms of interest are relatively few in number. Using our method in sewage we could detect growth of general *Bacteroidales* cells but not human-specific *Bacteroidales*; human-specific *Bacteroidales* are estimated to comprise approximately 1/1000 of the general *Bacteroidales* in sewage (unpublished data).

Freshwater microcosms inoculated with human and cow feces were used to follow persistence and survival of ruminant and human-specific *Bacteroidales* in water. We determined persistence using PCR and constructed decay curves for each marker using QPCR. To measure survival of the unculturable human and ruminant-specific *Bacteroidales* organisms we used reverse transcription of sample RNAs followed by QPCR quantification of rcDNA copy number. This provided an estimate of survival and allowed survival curves be made for each marker. The ruminant-specific fecal markers survived and persisted longer than either human fecal marker. This observation could be due to the large amount of undigested plant matter found cow feces. This detritus is a source of nutrition that provides a surface for biofilm formation. The production of biofilm could produce anaerobic micro-niches conducive to the metabolism of *Bacteroidales* bacteria (Tay et al., 2002).

Additional survival studies evaluating survival of fecal pathogens in freshwater are needed. If natural water sources are used, pathogen survival experiments must be done alongside the *Bacteroidales* markers for valid survival comparisons. Inclusion of additional environmental parameters will be fundamental in enhancing our understanding of the factors influencing survival of the host-specific markers. It is essential that survival is evaluated in a variety of climates including tropical, sub-arctic, alpine, semi-desert, and desert regions if these markers are to become standard diagnostics of fecal pollution and source identification.

In watersheds impacted by multiple sources it is useful to be able to determine the percent contribution from each host. Information of this type would assist in health risk assessment since certain fecal pathogens are associated with particular hosts. Such

comparisons rely on the assumption that the clonal composition of fecal bacteria remains constant between the primary (feces) and secondary (aquatic) environments (Gordon, 2001). The genotypic composition of *E. coli* populations can vary significantly between primary and secondary sources (Gordon et al., 2002). Experiments are currently underway, using data and samples from this study, to determine the initial ratios of human-specific and ruminant-specific markers relative to the general *Bacteroidales* marker, in the feces of contributors. These ratios will be compared to ratios obtained from host-specific markers relative to general markers, in the microcosm samples.

A multi-tiered approach to fecal pollution and source identification based health risk analysis is the most likely means of effectively mitigating human exposure to fecal pollution. An array of pathogens can cause waterborne disease, including helmiths, protozoa, bacteria, and viruses. Each pathogen behaves differently in the environment. Some possess life cycles requiring non-human hosts; others are able to encyst when subjected to inclement environmental conditions. These survival mechanisms introduce confounding factors to the problem of health risk assessment using indicator organisms. Thus, a suite of indicators would facilitate assessments of aquatic health, human health risks, and sources of fecal pollution.

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