AN ABSTRACT OF THE DISSERTATION OF

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Title: Distribution, Decay, and Quantification of Fecal Source-Tracking Markers.

Abstract approved:

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Waterborne fecal contamination poses serious risks to human health and can disrupt aquatic ecosystems. Molecular marker methods are widely used to identify and, in some cases, quantify the sources of contamination and guide management decisions regarding water resource protection. However, methods to detect some likely sources of fecal contamination such as birds are not available. Furthermore, the effects of environmental variables, such as water type and light exposure, on marker decay could change quantitative interpretations of source tracking results. Additionally, sample interference can be a significant source of variability and error during sample analysis could prevent accurate quantification of fecal sources. Methods that are robust to sample-to-sample variability and consider variable marker decay are needed to accurately quantify molecular markers in environmental samples.

We characterized the geographic and species distribution of three novel molecular markers based on rRNA gene sequences from bird fecal bacteria for the detection of bird feces in the environment. The distribution of the markers differed across geographic location and host species. Two markers were found mainly in gulls,

but one occurred in many bird species. The wide geographic distribution of the markers suggested that they will be useful in many areas where birds are suspected contributors to aquatic fecal contamination. We developed two quantitative PCR (qPCR) assays using two of the markers and determined their limits of detection of in natural water matrices. Both qPCR assays detected down to 100 ng feces/100 ml. Although one assay was designed to detect gull contamination, the marker occurred in sheep feces at low levels ($2.9x10^1 \pm 9.6 x10^1$ copies/ng DNA), but high concentrations of sheep feces (>0.2 g/100 ml) would be required for detection. The high specificity and sensitivity of the assays make them excellent tools for the quantification of aquatic avian fecal contamination.

We compared the decay of human-targeted *Bacteroides* markers in marine and fresh water under light and dark conditions using microcosms. Markers persisted about 2-3 days longer in marine water than in fresh water, suggesting that differential persistence of molecular markers might justify different standards between marine and fresh water bodies. Sunlight limited the persistence of DNA and RNA markers. Significant correlation between the decay of *Bacteroides* DNA and RNA markers suggested that most of the markers detected were bound within cells. This finding is important because the persistence of extracellular DNA could complicate estimation of fecal contaminants with molecular methods. The decay rates of *Bacteroides* markers differed despite their close phylogenetic relationship. Differences in physiological responses between *Bacteroides* clades to stresses may translate into different decay rates or persistence times in the environment.

We developed a novel method based on the spike and recovery of a genetically modified *Escherichia coli* strain to describe and limit the variability in marker quantification caused by sample interference. The spike-and-recovery approach accurately reflected low recovery of *Bacteroides* genomic DNA in low salt extractions and *Bacteroides* qPCR assay inhibition by a common carry-over reagent, ethanol. We used multivariate Z-scores to identify amplification deviants and showed that this new statistical method was more sensitive to ethanol and humic acid inhibition of qPCR than other widely used analysis methods using Ct values. These methods are useful for

detection of sample interference, not only in fecal source identification, but also in most environmental applications of qPCR.

Distribution, Decay, and Quantification of Fecal Source-Tracking Markers

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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.

Hyatt C. Green, Author

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CONTRIBUTION OF AUTHORS

In Chapter 2, Linda K. Dick performed subtractive hybridization and sequencing. Kate Field performed sequence analysis and primer design. Hyatt Green performed and contributed text on conventional and quantitative assay development and testing.

In Chapter 3, Orin Shanks performed all cloning and sequencing. Mano Sivaganesan performed Bayesian statistical analyses on qPCR data calibration and decay curve modeling. Hyatt Green performed all other work and contributed all of the text.

In Chapter 4, Hyatt Green performed all of the work and contributed all of the text.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure Page

LIST OF TABLES

Table Page

LIST OF APPENDIX TABLES

Table Page

CHAPTER 1

GENERAL INTRODUCTION & LITERATURE REVIEW

Hyatt C. Green

Risks of aquatic human fecal contamination

Despite technical advances in waste management and increased regulation of fecal waste management practices, waterborne pathogen outbreaks due to fecal contamination cause thousands of illnesses a year in the United States alone, and far more worldwide (Corso et al., 2003; Pond, 2005; O'Reilly et al., 2007; Jonathan S. Yoder et al., 2008; Schets et al., 2010). Upon infection, pathogens present in feces can cause a variety of acute and chronic symptoms, resulting in over $12 billion in economic losses per year (Shuval, 2003). Further economic impacts arise from closures of recreational beaches (e.g., Doheny Beach, CA $3.3 million loss annually (Pond, 2005)) or shellfish harvesting waters (e.g., Tillamook Bay, OR (Busse, 1998) and Samish Bay, WA (Haley, 2010)).

The World Health Organization (WHO) and US Environmental Protection Agency (EPA) have developed strategies to prevent or mitigate the effects of unsafe recreational waters (WHO, 2003), some of which also apply to shellfish harvesting waters. The strategies propose both sanitary inspection and microbial water quality assessment via monitoring to estimate the human health risk associated with a water body. The primary focus of sanitary inspection is to identify all potential sources of fecal pollution, while microbial water quality assessment relies heavily on monitoring of fecal indicator bacteria (FIB) or pathogens. The strategies have been adopted by the European Union, New Zealand, and Australia, and are likely to be adopted, at least in part, by the United States (Ashbolt et al., 2010). This framework guides most of the current research being conducted on methods to mitigate human health impacts from aquatic fecal contamination.

Source Identification

Determining the source of contaminants is important, not only for the estimation of human health risk, but also to guide management efforts in limiting contamination events. Common sources of contaminants include malfunctioning septic and sewage systems, agricultural runoff, storm water overflow events, and wildlife such as birds, deer, or elk. Importantly, the ecological and human health consequences vary between

these contaminant sources in terms of overall fecal load and the number and types of human pathogens present. Human derived contaminants, such as sewage or septic, carry far more bacterial and viral pathogens infectious to humans than other sources such as pig or bird feces, and pose greater human health risk (WHO, 2003; Soller et al., 2010). Therefore, identifying the source of contaminants in a water body is essential in defining this risk. Furthermore, no matter what the risk, reducing fecal loads cannot occur until the major contaminant sources are identified.

Culture-based measurements of FIB are usually unhelpful in source identification (however, see (McLellan and Salmore, 2003)). Commonly used FIB, *Escherichia coli* and enterococci, occur in all mammals and birds, albeit at varying concentrations. Phenotypic traits of FIB from different sources are not easily distinguished, and have led investigators to use analysis of antibiotic resistance patterns, but these methods can be unreliable at source identification (Ebdon and Taylor, 2006). Genotyping of FIB isolates has shown more success (Samadpour et al., 2005; Meays et al., 2006), but the survival and growth of FIB outside the intestine complicate source identification (Grant et al., 2001). Furthermore, culturing may lead to a bias towards more cultivable FIB strains.

Some success has been shown with chemical tracers such as caffeine (Buerge et al., 2003, 2006) and laundry brighteners (Sinton et al., 1998), but their recalcitrance and persistence in the environment may confound identification of fecal sources and needs to be further investigated (Eganhouse and Sherblom, 2001).

PCR-based Bacteroides *Source-Identification Methods*

The most widely used approach for determining sources of fecal contaminants is the use of bacterial and viral genetic markers. Molecular methods, such as polymerase chain reaction (PCR), can differentiate closely related bacterial strains that otherwise appear identical using phenotypic methods. As an added advantage, molecular methods permit identification of difficult-to-culture organisms, such as *Bacteroides* spp., and expand the range of potential target organisms for source identification compared to culture-based methods. For these reasons, molecular methods have gained attention as tools for source discrimination.

The *Bacteroides* group is attractive for source tracking because it is often the dominant bacterial group in the mammalian gut, occuring at high concentrations in fecal matter (Qin et al., 2010; Shanks et al., 2011). These bacteria's ability to metabolize otherwise indigestible polysaccharides, such as xylan and pectin, and produce short chain fatty acids, vitamins B_{12} and K (Hooper et al., 2002), and amino acids (Metges, 2000) provides the basis for their mutualistic relationship with a wide range of hosts (Xu et al., 2003). Despite their relatively uniform occurrence across mammalian hosts, physiologically and genetically distinct *Bacteroides* clades have emerged due to host diet adaptation (Ley et al., 2008).

In the past decade, many *Bacteroides*-targeted assays have been developed for the detection of human (Bernhard and Field, 2000a; Layton et al., 2006; Kildare et al., 2007; Shanks et al., 2009; Haugland et al., 2010), cattle (Bernhard and Field, 2000a; Shanks et al., 2006a; Kildare et al., 2007), dog (Dick et al., 2005a; Kildare et al., 2007), elk (Dick et al., 2005a), horse (Dick et al., 2005b), and pig feces (Dick et al., 2005b; Lamendella et al., 2009). At first, cloning and sequencing were used to identify unique markers within host-adapted clades (Dick et al., 2005a,b), but methods to enrich unique 16S ribosomal and metagenomic gene fragments before sequencing have been employed more recently (Dick et al., 2005a; Shanks et al., 2006a; Lu et al., 2007; Shanks et al., 2007). After unique fragments are identified, many methods can be used for their detection in the environment. The most commonly used methods involve probe hybridization, such as PCR or dot-blots. Interestingly, direct high-throughput sequencing to search for unique fragments has not been used for source identification but may be useful as advances in sequencing technology continue.

PCR assays targeting *Bacteroides* spp. have been used in large- (e.g., (Colford et al., 2007)) and small-scale studies (e.g., (Lee et al., 2008)) and have been incorporated into management guidelines (USEPA, 2005). In the Tillamook Bay watershed (Oregon, USA), application of these methods has led to measurable improvements in water quality (Johnson, 2009;York Johnson, personal communication). The assumptions of this approach are the focal point of this research and are discussed in later sections of this thesis.

Other Molecular Methods for Source Identification

Other PCR-based non-*Bacteroides* methods for source identification have been proposed and tested to varying degrees. For instance, PCR assays targeting mammalian mitochondria have been used to identify sources of fecal contamination. These methods may have an advantage over those targeting *Bacteroides* because mitochondria cannot be horizontally transferred between hosts, nor can they multiply in the environment. However, host cells are shed at much lower concentrations than *Bacteroides* per gram of feces and require high contaminant concentrations in water for detection (0.2 g/100 ml water sample) (Martellini et al., 2005; Kortbaoui et al., 2009).

Library-dependent methods require a reference "library" composed of patterns or strains collected from fecal samples of known origin. Since the reference fecal samples are usually collected in the same area as the water samples, similarities between the two sample types can be used to identify sources of contaminants. Furthermore, library-dependent methods, such as DNA fingerprinting, use pattern comparisons to identify the source and are not greatly affected by the absence of one or two bacterial community members. However, with these methods, comprehensive source identification is dependent on the size of the library, which can be relatively time-consuming to create (Field and Samadpour, 2007). Other methods to identify fecal contamination are reviewed elsewhere (Field and Samadpour, 2007; Santo Domingo et al., 2007).

Although some consider PCR methods of source identification to be library-independent (Field and Samadpour, 2007), analysis of a reference fecal collection is still necessary to evaluate an assay's utility in environmental applications by two important measures. Sensitivity is a measure of a marker's prevalence *among* the target population of individual hosts. Specificity is a measure of a marker's prevalence in individual hosts *outside* the target population. Assays are not useful in areas where the marker is found in low proportions, or is absent in local populations (low sensitivity). Likewise, a marker that is prevalent outside its intended target group also may not be useful (low specificity). Many factors can affect an assay's sensitivity or specificity. Commensal microbial community composition varies as a function of diet

(Ley et al., 2008; Turnbaugh et al., 2009; Shanks et al., 2011) or antibiotic treatment (Dethlefsen et al., 2008; Jernberg et al., 2010). Furthermore, host lifestyles such as cohabitation, domestication, or migration may affect sensitivity and specificity by facilitating intra- and inter-species transfer of host bacteria. For many PCR methods, these factors are not considered, and marker prevalence and geographic distribution are unknown. However, estimates of both sensitivity and specificity over a wide geographic range are necessary because they are used to decide in which locations or scenarios a particular source identification tool may be useful. Currently, not enough is known to predict marker distributions accurately, so their prevalence must be tested directly.

An assay's utility is also determined by its limit of detection in an environmental scenario; assays that can detect low concentrations are most useful. So that PCR-based source tracking methods can be evaluated in the context of regulatory standards, the most effective and relevant measure of an assay's limit of detection should be in terms of commonly used indicator bacteria concentrations. Expression of limits of detection in terms of FIB facilitates integration of relatively unusual source-tracking data into current management frameworks. For instance, an assay that can detect only samples that violate regulatory standards by orders of magnitude does not aid managers in identifying a low-level, but frequent, contaminant source. Assays that can detect down to low FIB levels allow source identification of a wide range of contaminant concentrations. Practical limits of detection can also be expressed in terms of grams feces per volume water. Expression of limits of detection in terms of purified fecal DNA mass or copy number are most helpful in judging assay chemistry performance during the optimization stage, but have little relevance in application.

The presence of multiple fecal sources could potentially interfere with reliable source identification and assay limits of detection; however, this possibility is frequently neglected in assay development and application. Even though PCR assays have both flexibility in primer design and the power of sensitive genetic discrimination to a single nucleotide difference, specificity testing and database searching can never exclude the possibility that markers are present in co-occurring non-target sources. Many assays target the bacterial 16S rRNA gene, which experiences relatively little

sequence divergence over long time periods (Fox et al., 1980): all bacteria share some sequence identity. Limits of detection of newly developed assays should be determined under realistic environmental scenarios, including those with more than one contaminant.

Fluorescent detection technologies, such as quantitative PCR (qPCR), have made lower limits of detection (as low as 10 marker copies/100 ml) and estimates of marker quantities possible. Marker quantification could potentially identify the sites that are most highly contaminated and could facilitate source identification. However, until marker decay and sample interference in the quantification process are understood, interpretations of quantitative source-tracking data remain unclear. Simple qualitative data (presence or absence) in the context of land use information have been used to improve water quality previously (Shanks et al., 2006b). Currently, much work is being done to understand, test, or bypass the assumptions that support quantitative source identification.

In areas with multiple sources of fecal contaminants it may be necessary to rank sources by their contribution to the total fecal load in order to prioritize restoration efforts. Using qPCR, some researchers have proposed methods of apportioning fecal sources from a single water sample (Silkie and Nelson, 2009; Wang et al., 2010). According to this approach, the concentration of source-specific *Bacteroides* markers as a percentage of general *Bacteroidales* markers is identical to its source inputs (Equation 1):

Equation 1:

$$\%\ Bacteroides_{Env.sample}^{Human} \equiv \left(\frac{Bacteroides_{Human} : Bacteroidales_{Env.sample}}{Bacteroides_{Human} : Bacteroidales_{sewage}}\right) \times 100\%$$

However, the untested assumption that all markers decay at equal rates could threaten the validity of this theory (Wang et al., 2010). In addition, the differential decay of general *Bacteroidales* from different hosts and inconsistent specific:general marker ratios within hosts may prohibit this approach. Physiological or structural differences in some *Bacteroides* spp. could allow for extended survival in natural waters. For

instance, differential gene expression of oxidative defense proteins, superoxide dismutase and catalase, between *Bacteroides* spp. (Brioukhanov and Netrusov, 2004) could translate into differential survival times. The assumption that *Bacteroides* spp. decay at equal rates must be tested in order to validate these potentially useful methods of source identification.

The Role of Molecular Methods in Water Quality Monitoring

Development of a routine monitoring capacity is a much desired and more complex application of PCR-based methods compared to simple source identification. Routine monitoring of source-specific marker concentrations would add information regarding contaminant type where current monitoring methods (e.g., culturable *E. coli* and enterococci) fall short. The appeal of molecular methods in estimating human health risk not only comes from their power of genetic discrimination, but also from their rapid sample-to-answer turn around time: culture methods take at least 18 hours, while molecular methods require as little as three hours. In theory, this rapidity could improve response time after hazardous contamination events, thereby reducing human exposure to pathogens. Some work has been done to increase the speed of sample processing (Haugland et al., 2005) and data acquisition (Shanks et al., 2010) to further reduce the response time. However, in addition to assumptions about assay specificity and sensitivity, specific markers' correlation with pathogens and illness are not understood but are necessary to relate their concentrations to human health risk (Field and Samadpour, 2007; Ashbolt et al., 2010). In theory, markers that correlate well with pathogens are good indicators of health risk (USEPA, 2005).

Marker Correlation with Pathogens and Illness

Even if a marker correlates well with pathogens or illness, the extent to which this correlation holds across environmental variables is unknown. Currently, there is an assumption that marker/pathogen relationships are the same in marine and fresh water. From a regulatory perspective, differential marker/pathogen correlation between these water types could prevent the use of marker concentrations as uniform standards for water quality: markers may be useful in some areas but not in others. The

correlations between markers and pathogens over a variety of environmental conditions are under-investigated and their investigation precedes the use of molecular methods in risk monitoring.

Marker correlation with pathogens has been investigated in both decay studies and environmental surveys. Only a few decay studies have been able to compare decay rates of markers and pathogens under environmental conditions (Walters et al., 2009; Klein et al., 2011). More common are surveys that estimate the predictive power of indicators or markers in reference to pathogens or illness over a large number of samples (Colford et al., 2007). Both methods allow evaluation of indicators or markers; however, illness surveys can directly relate indicator concentrations to human illness rates, while microcosm decay studies enable controlled hypothesis testing of selected environmental variables.

Effects of Environmental Variables on Marker Decay

Clearly, the effects of environmental variables on molecular marker decay are important in both fecal source identification and in marker monitoring. The effects of salinity (Okabe and Shimazu, 2007; Schulz and Childers, 2011), temperature (Okabe and Shimazu, 2007; Dick et al., 2010; Klein et al., 2011), particulate size and concentration (Bell et al., 2009), sediment presence (Dick et al., 2010), sunlight (Bae and Wuertz, 2009; Walters and Field, 2009; Walters et al., 2009; Dick et al., 2010), and protozoan predation (Kreader, 1998; Okabe and Shimazu, 2007; Bell et al., 2009; Dick et al., 2010) on bacterial marker decay have been investigated through microcosm studies previously. Of these factors, temperature and predation have been shown to have the greatest effect on marker decay, while there are conflicting observations on the effects of sunlight (Bae and Wuertz, 2009; Walters and Field, 2009; Walters et al., 2009).

Bacteroides spp. inoculated in filtered water without predatory protozoa decay slower than in unfiltered water (Kreader, 1998; Okabe and Shimazu, 2007; Bell et al., 2009; Dick et al., 2010). Furthermore, addition of cyclohexamide, a compound that inhibits growth of predatory protozoa, to sewage seeded microcosms extended the persistence of *B. distasonis* PCR markers by 12 days (Kreader, 1998). There are still

many questions regarding protozoan predation rates as a function of the types of predators, prey selectivity, and other variables. Rates of marker disappearance could differ between marine and fresh waters due to differences in abundance or composition of predatory populations. Furthermore, physiologically distinct *Bacteroides* clades may be ingested or degraded at different rates.

The effects of sunlight on bacterial decay have been intensely studied with culture-based methods; however, similar studies of molecular markers are infrequent and often arrive at conflicting conclusions (Bae and Wuertz, 2009; Walters and Field, 2009; Walters et al., 2009). Components of sunlight, UVA, UVB, and visible light, can damage bacterial DNA, proteins, and lipid bilayers (Ravanat et al., 2001; Cho et al., 2010). Previous studies found rapid decreases in concentrations of culturable cells in natural water microcosms when exposed to sunlight (Sinton et al., 1999; Sinton et al., 2002). Furthermore, shoreline culturable enterococci concentrations closely follow a diurnal cycle and are negatively-correlated with UVB intensity (Boehm et al., 2009). However, the decay of molecular markers may be slower than that of their culturable carriers, because detectable DNA persists within non-culturable and dead cells and can persist outside of dead cells for days or weeks (Dupray et al., 1997). The role of extracellular DNA and viable but non-culturable cells in marker decay must be established if markers are going to be used to estimate health risk.

Occurrence, Risk, and Detection of Aquatic Avian Fecal Contamination

For some time, birds have been thought to be an important source of aquatic fecal contamination (Gould and Fletcher, 1978; Benoit et al., 1993), but their contributions relative to obvious sources of fecal contaminants, such as human septic and livestock waste, are unknown. Recreational beach closures due to gulls damage local economies in southern California and the Great Lakes; only recently has the risk of human exposure to gull feces been investigated (Schoen et al., 2010). Avian flocks comprised of species such as Canada geese (*Branta canadensis*) and Mallard ducks (*Anas platyrhinchos*) have been implicated in the transport of pathogenic *E. coli* (Samadpour et al., 2002) and have been identified as leading contributors of fecal

indicator bacteria in multiple watersheds (Meays et al., 2006). Chicken fecal waste, frequently applied to agricultural fields as fertilizer, can be a source of bacterial and viral pathogens to recreational water bodies (Altekruse et al., 1997), especially after rainfall events. Furthermore, genetic analysis of chicken feces and natural water sources found considerable overlap in *Campylobacter* strains, highlighting chickens as significant sources of this waterborne pathogen (Van Dyke et al., 2010). Until recently, the contribution of birds to fecal contamination has been largely ignored, but these findings support investigations into the extent they affect water quality conditions in the US and elsewhere.

The impacts of gulls on water quality have received special attention in the past few years. Although in some areas, aquatic fecal contamination can be attributed to gulls, they represent a lower health risk than cattle, pigs, and raw sewage (Soller et al., 2010). Many bacterial and eukaryotic pathogens found in birds are host-adapted and unlikely to cause human illness (Zhou et al., 2004; Feng et al., 2010; Soller et al., 2010). For instance, less than 8% of fecal *Campylobacter* strains in gulls have been known to cause human infection (Lu et al., 2011). Pathogenic viruses found in sewage at high concentrations (Katayama et al., 2008) are absent in gulls. Ongoing studies aim to further define the risk of exposure to gull feces given their close association with recreational beaches, but cheap, rapid, and reliable methods are needed to investigate the link between avian fecal contamination and human illness.

Methods to Detect Avian Fecal Contamination

Methods to detect or quantify contaminants from birds are time-intensive and may not be reliable. Avian fecal identification using bacterial antibiotic resistance patterns has been used previously (Edge et al., 2007; Edge and Hill, 2007). This approach is based on the assumption that wild birds are not exposed to the diversity and abundance of antibiotics prescribed to humans and livestock. However, bacteria resistant to antibiotics can be disseminated over large geographic distances and between a diversity of hosts (Sjölund et al., 2008). Moreover, genes conferring resistance can be transferred between bacteria via transformation, conjugation, or phage transduction. Depending on the scenario, these mechanisms of antibiotic

resistance propagation may confound reliable source identification (Field and Samadpour, 2007), and, in fact, the method has performed poorly in comparative studies (Griffith et al., 2003; Moore et al., 2005; Samadpour et al., 2005). Further, gulls may ingest relatively high concentrations of human waste products, given their close association with sewage outfalls in some areas (Monaghan et al., 1985). DNA fingerprinting methods such as ribotyping have been used to identify avian sources (Samadpour et al., 2005), but, in one study, as many as 67% of *E. coli* banding patterns from gulls matched those from sewage or trash (Nelson et al., 2008), suggesting that this method may be just as unreliable as antibiotic resistance methods. Ideally, "library-independent" methods that are specific to gulls or other birds are needed to estimate their role in aquatic fecal contamination and PCR-based methods offer an attractive solution.

Quantification of Bacterial 16S Genes in Natural Waters with qPCR

In the context of water quality, qPCR offers unparalleled advantage over other methods. All variations of qPCR involve monitoring the progression of the chain reaction, or the accumulation of short clonal segments of DNA (amplicons), with a fluorescent probe or dye. Fluorescent detection, provided by an intercalating dye or fluorescent oligonucleotide conjugate, is very sensitive and can reflect small changes in amplicon concentration. Furthermore, monitoring the reaction progression at each cycle allows examination of the early exponential phase of amplicon replication, which can be an accurate indicator of marker starting concentration.

Sample Interference

Quantification with qPCR can be complicated when analyzing samples that vary in physical, chemical, and biological composition and complexity. In contrast to a lab setting, samples taken from the environment contain a wide variety of compounds that can interfere with sample processing and PCR amplification (Loge et al., 2002; Jiang et al., 2005). Components of decaying organic matter, such as humic acids, form colloidal particles that are thought to sequester nucleic acids, primers, and Taq polymerase (Baar et al., 2011). Methods have been developed to remove these

compounds from certain types of samples, but complete separation can be difficult without reducing or biasing recovery of nucleic acids (Delmont et al., 2011). Furthermore, nucleic acid extraction performance will vary between samples depending on their attributes (Zhou et al., 1996). Therefore, methods to detect extraction loss and PCR inhibition are necessary for both absolute and relative environmental quantification.

Detection of PCR Inhibition

Despite efforts to remove PCR inhibitors from samples, the complexity and variability in a single environmental sample set complicates complete removal of all such compounds. Thus, each sample extract should pass some quality criterion in order to be included in analyses. Three main approaches are used to detect or quantify PCR inhibition.

The simplest approach is to serially dilute nucleic acid extracts in PCR grade water prior to qPCR (e.g., Schriewer et al., 2011). As the extract is diluted, effects of PCR inhibition may be relieved. If no change is seen in the reported quantity after accounting for the dilution factor, no inhibitors are believed to be present. The disadvantage in this method is obvious: dilution prevents detection of low concentrations of target. In cases where target viruses or cells are at low concentrations, this method is not an option.

A second approach uses an exogenous, or spiked, control template that is mixed with an unknown DNA extract and amplified. Fluorescence data from this reaction can be compared with that of a reference sample that is free of inhibitors (e.g., distilled water) to determine the degree of inhibition in the unknown extract (e.g., Shanks et al., 2008). While this approach is common, considerable effort is needed to develop the control template and qPCR assay. Furthermore, some have suggested that inhibitory compounds affect control and experimental markers (i.e., host-associated *Bacteroides* 16S rRNA genes versus controls) unequally (Boehm et al., 2009; Silkie and Nelson, 2009), perhaps because of differences in amplicon length, %GC content, or fluorescence reporting methods (i.e., SYBR Green® dye, TaqMan® probes, etc.).

An alternative method that has received more attention in eukaryotic gene expression than in environmental qPCR applications is based on the observation that inhibitory compounds decrease qPCR amplification efficiency (Ramakers et al., 2003; Kontanis and Reed, 2006). Theoretically, at the end of each amplification cycle each amplicon should produce two identical daughter amplicons (100% efficiency). Significant reduction in efficiency due to sequestration of primers or other amplification reagents could indicate the presence of PCR inhibitors, thereby eliminating the need for an exogenous control (Tichopad et al., 2010). While this method requires modeling and more data processing time, it seems highly applicable in the field of water quality, where small changes in qPCR amplification curves can result in large differences in estimates of human health risk. However, some have suggested that amplification efficiency may not always be affected by inhibition. Instead, inhibition may cause an initial lag in amplification without a change in amplification efficiency (Schriewer et al., 2011). Furthermore, presence of inhibition cannot be assessed in samples that do not amplify because there is no means of estimating amplification parameters without an amplification curve. Therefore, some combination of these methods and the use of an exogenous control may be most effective.

Specificity of PCR-based Methods in the Environment

Given that we know carry-over compounds, such as humic acids or polysaccharides, can have a clear effect on PCR amplification kinetics (Boehm et al., 2009; Schriewer et al., 2011), it is surprising that almost no one has investigated their effects on qPCR assay specificity. It is probable that salts or other commonly used extraction reagents could change primer-binding kinetics if not removed from DNA extracts prior to PCR. The chance of mis-priming unintended targets is increased with added, untested genetic diversity in environmental samples, which competes with the intended marker sequence. The assumption that an assay maintains its intended specificity when taken from the lab to the environment should be tested.

Summary

Although there is much excitement surrounding the use of molecular methods to improve water quality, critical research needs hinder their application and effectiveness in estimating human health risks. Tools to detect some sources of fecal contaminants are not available. Although birds are thought to be significant sources of aquatic fecal contaminants, estimation of the risk posed by these types of contaminants is not possible without methods to differentiate them from of other fecal sources. Furthermore, little is known about the decay of molecular markers in the environment. New standards for the regulation of water quality that incorporate molecular methods are being drafted: however, differential decay of *Bacteroides* in marine and fresh waters suggests that drafters of regulatory standards should consider water type. Additionally, the extracellular persistence of DNA could invalidate the use of molecular markers in a monitoring application; however, the role of extracellular DNA in environmental marker detection with qPCR is unknown. Finally, sample interference may limit the accuracy of the qPCR method and may lead to misinterpretations of health risks in some cases. Methods that account for poor DNA extraction and qPCR inhibition are needed.

Accordingly, the objectives of this research were largely to develop and test molecular tools to eliminate major areas of uncertainty in the application of molecular source-tracking and monitoring methods. In the following chapters, I describe the development and testing of PCR and qPCR assays for the detection of aquatic fecal contaminants originating from birds. I also investigate the decay of human-targeted Bacteroides markers in marine and fresh waters using microcosms. Finally, I develop tools to estimate extraction efficiency and indicate qPCR inhibition using a spike-and-recovery approach. The results have widespread theoretical impacts on central topics in microbiology, such as the distribution of bacteria and the interrelation between bacteria and their predators.

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CHAPTER 2

GENETIC MARKERS FOR RAPID PCR-BASED IDENTIFICATION OF GULL, CANADA GOOSE, DUCK, AND CHICKEN FECAL CONTAMINATION IN WATER

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Abstract

Avian feces contaminates waterways, but contributes fewer human pathogens than human sources. Rapid identification and quantification of avian contamination would therefore be useful to prevent over-estimation of human health risk, when one source of contamination is avian feces. We used subtractive hybridization of PCR-amplified gull fecal 16S RNA genes to identify avian-specific fecal rDNA sequences. The subtracters were rRNA genes amplified from human, dog, cat, cow, and pig feces. Recovered sequences were related to *Enterobacteriaceae* (47%), *Helicobacter* (26%), *Lactococcus*/*Catellicoccus* (11%), *Fusobacterium* (11%), and *Campylobacter* (5%). Three PCR assays, designated GFB, GFC, and GFD, were based on unique recovered sequence fragments. Quantitative PCR assays for GFC and GFD were developed using SYBR Green. GFC detected down to 100 µg gull feces/100 ml, corresponding to 2 gull enterococci most probable number (MPN)/100 ml. GFD detected down to 100 µg chicken feces/100 ml, corresponding to 87 coliform MPN/100 ml or 13 *E.coli* MPN/100 ml. Fecal samples from humans, dogs, cats, cows, pigs, rodents, sea mammals, geese, ducks, gulls, and chickens were used to establish the host prevalence, sensitivity, and specificity of each assay. GFB and GFC were 97% and 94% specific to gulls respectively. GFC cross-reacted with 35% of sheep samples, but occurred at about 100,000 times lower concentrations in sheep ($2.9\times10^{1} \pm 9.6\times10^{1}$ copies/ng DNA) compared to gulls ($2.0\times10^{6} \pm 2.8\times10^{6}$ copies/ng DNA). GFD was 100% avian specific, and in addition to gulls, occurred in geese, chickens, and ducks. A geographic survey of samples from the US, Canada and New Zealand suggested that although the three markers differed in their geographic distributions, they were found across the range tested. These assays detected four important bird groups contributing to fecal contamination of waterways: gulls, geese, ducks, and chickens. Marker distributions across North America and in New Zealand suggest that they will have broad applicability in other parts of the world as well.

Introduction

Contamination from gulls, Canada geese, ducks, and other birds negatively impacts water quality(Gould and Fletcher, 1978; Benton et al., 1983; Benoit et al., 1993; Wither et al., 2005; Edge and Hill, 2007; Shibata et al., 2010). Their feces are sources of fecal coliforms, enterococci and *Escherichia coli,* and their presence is correlated with elevated fecal indicator bacteria (FIB) and beach closures (Alderisio and DeLuca, 1999; McLellan et al., 2003; Genthner et al., 2005; Ge et al., 2010). Pathogenic *E. coli*, *Campylobacter*, *Salmonella*, *Giardia,* and *Cryptosporidium* spp. occur in bird feces (Fallacara et al., 2001; Samadpour et al., 2002; Fallacara et al., 2004; Devane et al., 2005) and can infect domestic poultry and humans (Handeland et al., 2002; Refsum et al., 2005) and contaminate shellfish (Albarnaz et al., 2007). Bird feces are also a source of antibiotic resistance genes (Middleton and Ambrose, 2005; Literak et al., 2010; Rocha Simões et al., 2010). Recently, because of avian influenza, concerns have risen about pathogen movement due to bird migration (Kilpatrick et al., 2006; Jourdain et al., 2007; Ellström et al., 2008; Boyce et al., 2009; Delogu et al., 2010).

In spite of pathogens in bird feces, however, exposure to bird feces is considered less harmful to humans than exposure to other sources of fecal contaminants, especially that of humans (Schoen and Ashbolt, 2010; Soller et al., 2010). For example, molecular evidence indicates that genotypes of certain parasites in birds, such as *Giardia* and *Cryptosporidium,* are host-adapted and cannot cross-infect among different hosts (Zhou et al., 2004; Feng et al., 2007). The relative human health risks of bird and human fecal contamination will be more amenable to measurement once reliable methods are developed to distinguish them quantitatively.

The ability to rapidly identify and quantify fecal contamination from birds will thus improve our ability to estimate human health risk from contaminated waters. Although reliable methods can identify human fecal contamination in water without cultivating indicator bacteria (Bernhard and Field, 2000a), tools for bird fecal source identification are less widely tested (Hamilton et al., 2006; Devane et al., 2007; Lu et al., 2007; Lu et al., 2008; Lu et al., 2009; Fremaux et al., 2010).

We previously developed PCR-based fecal source tracking assays that target 16S rRNA gene sequences from fecal anaerobes in the order *Bacteroidales* (Bernhard and Field, 2000b, a; Dick and Field, 2004; Dick et al., 2005b; Dick et al., 2005a). However, an analysis of gull feces uncovered many gull *Bacteroidales* sequences that were closely related to sequences from human, dog, and cat feces, suggesting horizontal acquisition of bacteria between hosts and affording no useful targets for source tracking (Dick et al., 2005b). Cloning and sequencing of near full-length 16S rRNA fragments generated using general bacterial primers suggests that the *Bacteroidales* group accounts for only a small fraction of gull fecal bacteria (Lu et al., 2008).

We previously showed that microplate subtractive hybridization (Zwirglmaier et al., 2001) could empirically identify unique fecal sequences that differentiate between very closely related hosts (cow vs. elk), and between hosts that live in close contact and undergo horizontal transfer of fecal bacteria (human vs. dog) (Dick et al., 2005a). In microplate subtractive hybridization, subtracter DNA is fixed to the bottom of a microplate well. Target DNA is added in solution to the microplate and allowed to hybridize to the attached subtracter DNA. Unhybridized target fragments are removed from the microplate well, amplified, cloned, and sequenced. The method is reported to enrich for unique sequences not present in the subtracters (Zwirglmaier et al., 2001).

Since 16S rRNA genes provide attractive and well-studied targets for molecular identification, we performed microplate subtractive hybridization to identify unique 16S rRNA gene fragments found in gull feces but not in other species, including humans, dogs, cats, cows, and pigs. These sequences formed the basis of three new PCR assays that identify fecal contamination from gulls, ducks, geese, and chickens. We modified two of the assays for quantitative PCR (qPCR) and tested their ability to quantify avian contaminants in natural water sources.

Materials and Methods

Sample collection and DNA preparation.

We utilized fecal samples or fecal DNA collected around Columbus, OH, Seattle, WA, Corvallis, OR, and the Oregon Coast, in New Zealand around Christchurch, from the Institute for Environmental Health (Lake Forest Park, WA) collections, or donated by collaborators in the US (California, Texas, Florida and North Carolina), and Canada (British Columbia, Alberta, Saskatchewan, and New Brunswick). Marine mammal samples came from Marine Mammal Center, CA and Monterey Bay Aquarium, CA. Many fecal samples had been identified by host common name only. Gull fecal samples included Western, California, Herring, Laughing, and Ring-billed gulls; geese fecal samples included Canada, Cackling, Brant, and domestic geese; duck fecal samples included Mallard, Black, Wood, and domestic ducks; chicken samples were from broiler and egg operations. Donated gull fecal samples from California were used with Oregon samples in the target for subtractive hybridization; otherwise, fecal and DNA samples donated by outside collaborators were used for prevalence and geographic distribution assays only, and integrity of fecal DNA samples that did not amplify was checked by reamplifying with rRNA gene primers 27F (bacterial) and 1492R (bacterial/archaeal) (Lane, 1991). DNA samples that did not amplify with these rRNA gene primers were excluded from the study. In the authors' laboratories, fecal samples were collected in sterile containers and stored at –80º C. The FastDNA kit for Soils (Q-Biogene, Carlsbad, CA) was used to extract DNA from the fecal samples used in the initial subtractive hybridization. We used the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) for subsequent DNA isolation, and the Powerwater DNA kit (MoBio Laboratories, Inc., Carlsbad, California) for DNA extractions from chicken fecal dilutions.

Subtractive hybridization and sequence identification

The target was a gull fecal DNA pool containing an equal mixture of DNA from 12 Oregon and 12 California gull samples (3 ng/ml). For the subtracter, fecal DNA extracts from 10 to 30 individual samples from human, dog, cat, cow and pig (from Oregon) were combined and mixed in equal amounts (3 ng/µl) for each species. DNA was quantified by Picogreen assay (Molecular Probes, Inc., Eugene, OR). The experimental design was adapted from a study by Zwirglmaier and colleagues

(Zwirglmaier et al., 2001) and was performed as described in a previous paper (Dick et al., 2005a), except that instead of using fecal *Bacteroidales* 16S rRNA genes, we used fecal rRNA genes amplified with 27F and 1492R primers (Lane, 1991) extended with *Aci*I restriction sites: *Aci*27F (5' AAT ATA AAC CGC AGR GTT TGA TYM TGG CTC AG) and *Aci*1492R (5' AAT ATA AAC CGC GGT TAC CTT GTT ACG ACT T). After amplification, subtracter and target amplicons were separately digested with *Aci*1 and ligated to S1/S2 and P1/P2 linkers respectively, excess linkers were removed, and P1 and S1 linkers were used as PCR primers to reamplify target and subtracter ligation products, as previously described (Dick et al., 2005a). PCR products were quantified by Picogreen assay (Molecular Probes). Subtracter fragments were heat denatured, then dried to microplate wells (Maxisorp; MalgenNunc, Naperville, IL); target DNAs were heat denatured, added, and allowed to hybridize for two hours. After hybridization, 2 µl of subtracted (non-hybridized) target DNA fragments were removed from the supernatant, diluted 10,000-fold, and reamplified with linker P1 as previously described (Dick et al., 2005a). Two different-sized PCR products were separately gel purified (QiaQuick Gel Purification Kit; Qiagen, Inc.) and cloned (TOPO TA, Invitrogen, Carlsbad, CA). Five clones from each amplified band were randomly selected for sequencing on an ABI 3730 capillary sequence machine. Sequences were identified and aligned with related sequences using NCBI/BLAST (Altschul et al., 1990). Short (60 bp) overlapping regions within each sequence were analyzed separately in order to uncover chimeric sequences. Sequences that met the minimum size criteria of 200 bases were deposited in GenBank (JN084061-JN084064). The remaining sequence set, including sequences shorter than 200 bases, is available upon request.

Assay design, optimization, and performance testing

Unique regions in sequences were identified by comparison to related sequences, and used to design PCR primers. Primer sequences were tested *in silico* using NCBI/BLAST, Oligo Analyzer 3.0 (Integrated DNA Technologies, Coralville, IA) and the Probe Match program of the Ribosomal Database Project (Cole et al., 2007). Primers were optimized for annealing temperature and magnesium concentration and

tested for host specificity using PCR beads (Institute for Environmental Health, Lake Forest Park, WA) containing buffer, *Taq* DNA polymerase, dNTPs, trehalose, and 1.0mM $MgCl_2$. PCR reactions contained one PCR bead, 2.5 µM each primer, additional $MgCl_2$ as determined for each primer pair by optimization, and 2 µl DNA in a 25 µl reaction volume. Cycling parameters were as follows: 4°C for 2 min, 95°C for 10 min, followed by 35 cycles of 95ºC for 10 sec, the primer-specific annealing temperature for 30 sec, and 72°C for 20 sec (Table 2.1). Host "pools" were constructed from fecal DNA samples from 5 to 12 individuals of the same species; these were used for initial testing of host specificity. Once primers were optimized, fecal DNA samples were tested individually.

We used the GFC and GFD primer sets for SYBR Green® qPCR. 25 µL reactions consisted of 2.0 mM $MgCl_2$, PCR Buffer I (ABI, Foster City, CA), 2 mM each dNTP, 100 nM each primer, 0.04 µg/µL bovine serum albumin, 0.625 U Taq (ABI, AmpliTaq Polymerase), 50 µM ROX dye® (Invitrogen), 0.1X SYBR Green nucleic acid gel stain® (supplied at 10,000X by Invitrogen), and 2 µL template. SYBR Green reactions were cycled for 95ºC for 2 min followed by 40 cycles of 95ºC for 15 sec and primer dependent annealing temperature for 32 sec (Table 2.2). Melt curve analysis with a resolution of 0.3 ºC was used to determine amplification specificity. We tested reference fecal DNA pools, as described above, to ensure that the host range of qPCR assays did not change due to modifications in reaction chemistry. The fluorescence threshold was set at 0.8. Reactions were cycled on an ABI 7300 Real-time PCR system. Triplicate standard curves were used to convert Ct values to copy numbers for each run.

We measured the limits of detection (LOD) of each new conventional PCR assay and GFC and GFD qPCR assays as: (1) target copy number, using 11 replicate marker-specific plasmid dilutions; and (2) wet weight feces in either marine or fresh water, with accompanying FIB counts. LOD was defined as the lowest number of plasmids or fecal dilution at which all PCR replicates amplified. For plasmid LODs, plasmids containing marker fragments were purified using the QIAprep Spin Miniprep Kit (Qiagen) and quantified with NanoDrop-1000 (Thermo Scientific, Wilmington, DE, USA). Serial dilutions from 10^6 to 1 copies/µl were used as PCR templates.

For gull fecal dilutions, equal weights of 12 different fresh fecal samples from Oregon coast gulls were combined and 1 g of the mix was diluted in 1 L of seawater and stirred vigorously. This emulsion was serially diluted in either marine water or marine water containing human sewage. Dilution blanks with sewage contained 119 enterococci MPN/100 ml before the addition of gull feces. One hundred ml of each dilution was filtered through 0.22 µm pore size filters (Supor 200; Pall Life Sciences, Ann Arbor, MI) in parallel for a total of six filtration replicates for each dilution. We extracted DNA using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's directions, and eluted DNA in 100 µl AE buffer. These dilutions were used to find LODs of conventional GFB and GFD assays as well as both conventional and quantitative GFC assays.

For chicken fecal dilutions, equal weights of 10 fresh individual fecal samples from chicken were mixed and 1 g of the mix was diluted in 1 L of stream water (Oak Creek, Corvallis, OR) and stirred vigorously. This emulsion was serially diluted prior to filtration in duplicate. DNA was extracted using the PowerWater Kit® (MoBio, Carlsbad) and eluted in 100 µl supplied elution buffer. We substituted this extraction method to decrease variance between extraction replicates. These dilution extracts were used to find LODs of the GFD qPCR assay.

We enumerated MPN enterococci (the fecal indicator recommended for marine waters (USEPA, 1986)) in the gull fecal dilutions (Enterolert; Idexx Laboratories, Westbrook, ME) and MPN coliforms and *E. coli* (the fecal indicator recommended for fresh waters (USEPA, 1986)) (Colilert-18; Idexx) in chicken fecal dilutions in order to relate the PCR LODs to FIB. FIB MPN are rounded to the nearest whole cell.

ANCOVA was performed using R (R Core Development Team, 2010).

Results

Sequences recovered from subtractive hybridization

After subtractive hybridization targeting gull feces, we obtained two different sized bands, which were separately eluted, cloned and sequenced. Sequences from the

smaller band ranged from 227 to 303bp; sequences from the larger band ranged from 303 to 459bp.

Six of the ten sequences were chimeras. We separated the chimeric sequences into their component sequences when possible, resulting in 19 separate sequences. Sequence diversity was low. Sequences fell into 5 groups: 9 related to Enterobacteriaceae (47%), 5 related to *Helicobacter* (26%), 2 perfectly matched *Catellicoccus marimammalium* (11%), 2 were related to *Fusobacterium* (11%), and one was related to *Campylobacter* (5%).

Eleven of the sequences recovered were perfect matches to sequences found in the GenBank database, including members of the Enterobacteriaceae such as *E. coli*, *Shigella*, *Salmonella*, *Enterobacter*, *Klebsiella*, and *Serratia*; and *C. marimammalium* (*Lactobacillales*, *Firmicutes*). Some of these were components of chimeras, but three sequences that matched *Enterobacteriaceae* and one matching *C. marimammalian* comprised an entire clone sequence.

Assay development and performance testing

Potential primer pairs were designed based on unique sequences. These were first tested *in silico* to search for matches to known sequences; unique primer pairs (those exclusively found in their target bacterial sequences) were optimized. Conventional PCR assays termed GFB, GFC, and GFD were based on 16S rRNA sequence fragments from *Fusobacterium* spp., *C. marimammalium*, and *Helicobacter* spp. respectively (Table 2.1), and did not occur in GenBank outside of their target sequences. In tests for specificity against fecal DNA pools, GFB, GFC, and GFD amplified fecal DNA from birds but not from human, dog, cat, cow, horse, deer, pig, rodent, or sea mammals. Both GFB and GFC detected gull fecal dilutions in marine water when the fecal enterococcus concentration in the dilution was 30 MPN/100ml, well below the regulations for recreational water

Table 2.1 Target bacterial groups, primer sequences, conventional PCR conditions and limits of detection (LOD) by conventional PCR for GFB, GFC, and GFD.

Assay	Target Bacterial Group	Primer sequence	$MgCl_2$[a] (mM)	Annealing temp. ºC	Product size (bp)	Plasmid LOD[b] (copies/rxn)	Gull Fecal LOD[c] (mg feces/100 ml)	enterococci LOD (MPN/100 ml)[d]
GFB	Unclassified *Fusobacterium* sp.	F 5'-TCA TGA AAG CTA TAT GCG CCA AAA R 5'-TCC ATT GTC CAA TAT TCC CCA C	1.5	64	176	2000	1	30
GFC	*Catellicoccus marimammalium*	F 5'-CCC TTG TCG TTA GTT GCC ATC ATT C R 5'-GCC CTC GCG AGT TCG CTG C	2.0	69	162	20	1	30
GFD	Unclassified *Helicobacter* sp.	F 5'-TCG GCT GAG CAC TCT AGG G R 5'-GCG TCT CTT TGT ACA TCC CA	1.0	57	123	20	10	194

[a] Final $MgCl_2$ in the reaction.

[b] defined as the lowest number of plasmids at which all PCR replicates amplified

[c] defined as the lowest fecal dilution at which all PCR replicates amplified

[d] FIB in the lowest fecal dilution at which all PCR replicates amplified

We developed quantitative assays for GFC and GFD, based on detection with SYBR Green. We did not use GFB for qPCR because its sensitivity for gull fecal DNA samples (0.26) was lower than that of GFC and GFD (0.64 and 0.58, respectively), and because the GFB assay formed primer dimers, interfering with SYBR Green detection. qPCR performance characteristics based on plasmid dilutions are shown in Table 2.2. In marine water and marine water with added human sewage (119 enterococci MPN/100 ml), GFC qPCR consistently detected down to 0.1 mg gull feces/100 ml, which corresponded to 2 gull enterococci MPN/100ml (Figure 2.1 & Table 2.3). In dilutions with 0.01 mg gull feces the marker was detected in 10 of 12 qPCR replicates. Analysis of covariance indicated that the addition of human sewage did not significantly change the assay's ability to quantify gull feces in marine water within fecal LODs ($p > 0.10$). In addition, estimated marker quantities within each dilution were not significantly different between marine water and marine water with human sewage, as shown by one- or two-sided t-tests ($p > 0.068$). In freshwater, GFD detected down to 0.1 mg chicken feces/100 ml, corresponding to 87 coliform MPN/100 ml or 13 *E.coli* MPN/100 ml (Table 2.3).

Because with conventional PCR, we were able to detect gull feces at a ten times more dilute concentration with GFC than GFD (Table 2.1), we used the GFC and GFD qPCR assays to estimate marker concentrations in individual fecal samples. On average, the GFC marker occurred in Oregon gull fecal samples at about 100 times the concentration of GFD per mg DNA (Figure 2.2).

Table 2.2. GFC and GFD qPCR assay performance characteristics based on 11 standard curves separately run over a six-month period.

Assay	Slope Range	Intercept Range	Efficiency[a] Range	r^2 Range	ROQ[b] (copies)
GFC	-4.06, -3.34	34.55, 39.54	0.88, 1.00	0.990, 0.996	20 - 2×10^5
GFD	-3.51 -3.02	32.52, 38.26	0.96, 1.07	0.993, 0.999	200 - 2×10^5

[a] defined as $10^{(1/-slope)}/2$.

[b] plasmid concentration range that remained linear to Ct values (r^2 >0.98) in all 11 standard curves over the six-month period.

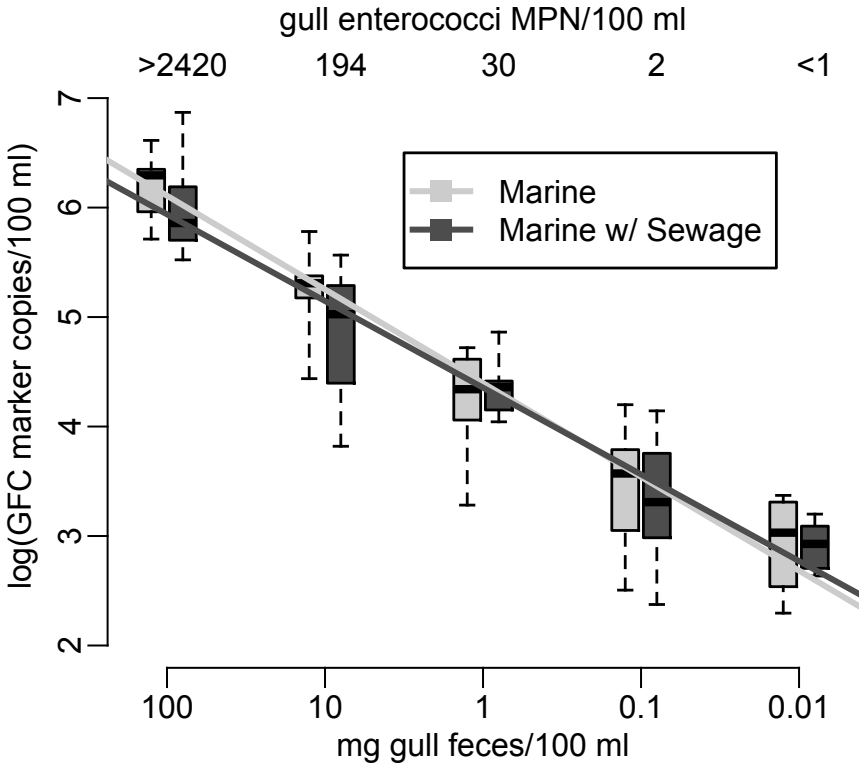

Figure 2.1. GFC qPCR performance on gull fecal dilutions. GFC qPCR assay performance on gull fecal dilutions in marine water and marine water with added sewage. Duplicate PCR reactions were performed on six replicate filters for each dilution. Regression lines were formed for each matrix using fecal concentrations within the fecal limit of detection (LOD). Boxplot whiskers extend to data extremes. The number of enterococci (MPN/100 ml) measured in each dilution is shown across the top.

Table 2.3. GFC and GFD qPCR performance on fecal dilutions. For GFC LOD determination, gull feces was diluted in unfiltered marine water. For GFD LOD determination, chicken feces was diluted in unfiltered stream water.

Assay	Fecal Source	Slope	r^2	Fecal LOD (mg feces/ 100ml)	Indicator LOD (MPN indicator/ 100 ml)
GFC	Gull [a]	0.86	0.89	0.1	2 enterococci
GFD	Chicken[b]	1.01	0.96	0.1	87 fecal coliforms or 13 *E. coli*

[a] gull feces diluted in unfiltered marine water

[b] chicken feces diluted in unfiltered stream water

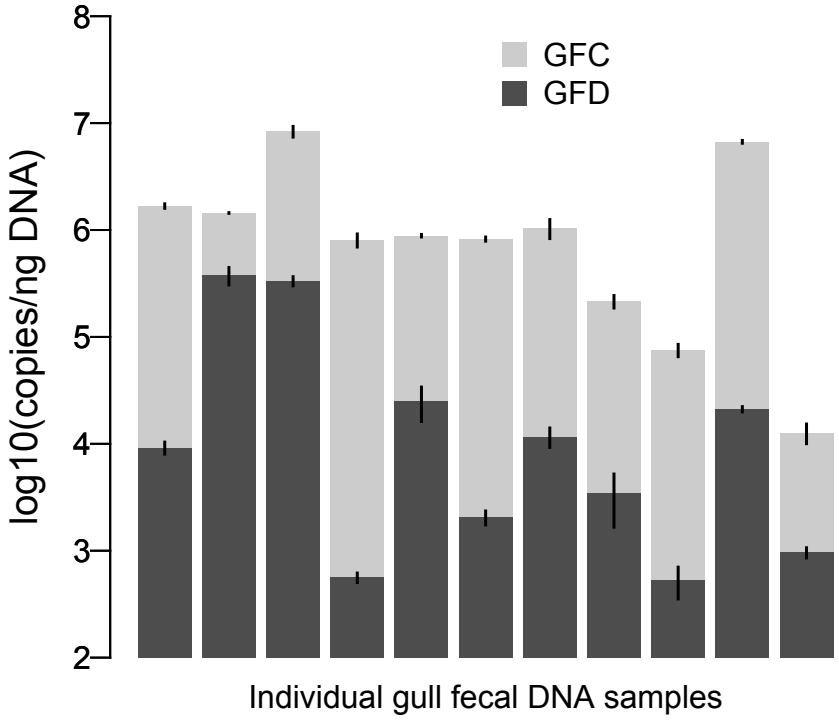

Figure 2.2. GFC and GFD marker abundance per nanogram Oregon gull fecal DNA from individual samples. Error bars represent standard deviations of qPCR replicates.

Species prevalence and geographic distribution

We used DNA from 635 individual fecal samples to establish the prevalence of the markers within host species (Table 2.4). Although the markers were designed from sequences recovered from gull feces, all three occurred in fecal DNA from other species. Two (GFB and GFC) were far more common in gulls than in other species tested (97% and 94% specificity, respectively). GFB amplified 2 out of 12 New Zealand rabbit samples. GFC was present in 1 of 12 New Zealand sheep and 11 of 22 Oregon sheep. The third marker, GFD, was 100% avian specific, and amplified fecal DNA from gulls, geese, ducks, and chickens, as well as from a variety of other seabirds. GFB and GFC each occurred in several beach/seaside bird species besides gull, although the number of individual samples

tested was very low. GFB detected only 26% of gull samples tested, whereas GFC and GFD had 64% and 58% gull sensitivity, respectively, and GFD detected 57% of all bird fecal DNA samples. The total percent of individual avian samples detected with at least one assay was 70% for gulls, 69% for geese, 78% for ducks, and 45% for chickens.

Table 2.4. Species distribution of markers, showing the number of samples from each source that amplified with the specified marker, followed by the percentage (in parentheses). "Total" shows the total number of each source that was detected by at least one marker, followed by the percentage (in parentheses). For each sample type, "*n"* is the total number of samples; "*n* (NZ*)*" is the number of samples from New Zealand.

			Amplified			
Fecal Source	*n*	*n* (NZ)	GFB	GFC	GFD	Total
Gull	**73**	**12**	**19 (26)**	**47 (64)**	**43 (59)**	**51 (70)**
Goose	**106**	**12**	**1 (1)**	**3 (3)**	**72 (68)**	**73 (69)**
Duck	**76**	**12**	**0 (0)**	**3 (4)**	**58 (76)**	**59 (78)**
Chicken	**98**	**8**	**6 (6)**	**4 (4)**	**42 (43)**	**44 (45)**
Human	11		0 (0)	0 (0)	0 (0)	0 (0)
Sewage	11	11	0 (0)	0 (0)	0 (0)	0 (0)
Cat	9	4	0 (0)	0 (0)	0 (0)	0 (0)
Dog	16	12	0 (0)	0 (0)	0 (0)	0 (0)
Cow	24	12	0 (0)	0 (0)	0 (0)	0 (0)
Horse	18	6	0 (0)	0 (0)	0 (0)	0 (0)
Sheep	34	12	0 (0)	12 (35)	0 (0)	12 (35)
Goat	12	12	0 (0)	0 (0)	0 (0)	0 (0)
Deer	9		0 (0)	0 (0)	0 (0)	0 (0)
Pig	5		0 (0)	0 (0)	0 (0)	0 (0)
Rabbit	12	12	2 (17)	0 (0)	0 (0)	2 (17)
Rodent	3		0 (0)	0 (0)	0 (0)	0 (0)
Possum	12	12	0 (0)	0 (0)	0 (0)	0 (0)
Sea Lion	22		0 (0)	0 (0)	0 (0)	0 (0)
Dolphin	9		0 (0)	0 (0)	0 (0)	0 (0)

			Amplified			
Fecal Source	*n*	*n* (NZ)	GFB	GFC	GFD	Total
Elephant seal	10		0 (0)	0 (0)	0 (0)	0 (0)
Harbor seal	1		0/0	0 (0)	0 (0)	0 (0)
Godwit	2		1 (50)	1 (50)	0 (0)	2 (100)
Sandpiper	13		1 (8)	3 (23)	4 (31)	7 (54)
Coot	2		1 (50)	0 (0)	2 (100)	2 (100)
Pigeon	13		1 (8)	0 (0)	2 (15)	3 (23)
Cormorant	3		0 (0)	0 (0)	2 (67)	2 (67)
Egret	3		0 (0)	0 (0)	2 (67)	2 (67)
Pelican	4		0 (0)	1 (25)	4 (100)	4 (100)
Tern	3		0 (0)	0 (0)	1 (33)	1 (33)
Crow	1		0 (0)	0 (0)	1 (100)	1 (100)
Swan	8	8	4 (50)	1 (13)	8 (100)	8 (100)
Pukeko	10	10	0 (0)	9 (90)	0 (0)	9 (90)
Avian Specificity			**0.99**	**0.98**	**1.00**	
Avian Sensitivity			**0.08**	**0.17**	**0.57**	
Gull Specificity			**0.97**	**0.94**	**0.64**	
Gull Sensitivity			**0.26**	**0.64**	**0.58**	

Because the GFC conventional PCR assay amplified some sheep fecal DNA, we used qPCR to measure the relative concentrations of the marker in Oregon gull and sheep fecal samples. In sheep where we could not detect the marker, we assumed that it was present at concentrations just below limits of detection. The GFC marker occurred at concentrations approximately 100,000 times lower in those sheep with detectable GFC ($2.9\times10^{1} \pm 9.6\times10^{1}$ copies/ng DNA) compared to gulls ($2.0\times10^{6} \pm 2.8\times10^{6}$ copies/ng DNA).

Although the number of samples from some locations was very small, we found evidence suggesting that the three markers differed in their geographic distributions (Figure 2.3). GFB occurred in 50-100% of gulls from the West Coast (British Columbia,

Washington, Oregon and California), but was rare or absent in gulls from Ohio, Florida, and New Brunswick. GFC occurred in 71-100% of gull samples from the West Coast and Ohio, but in 38% on the Gulf Coast and was absent in New Brunswick samples. GFD occurred in 90-100% of gull samples from British Columbia, Oregon, and Ohio, at an 18-52% frequency in Washington, California, and Florida, and was absent in gulls in New Brunswick. GFD was a good indicator for both geese and duck feces in all the samples, occurring in 68% and 76% of these samples, respectively, and was found in about half of chicken samples in most areas.

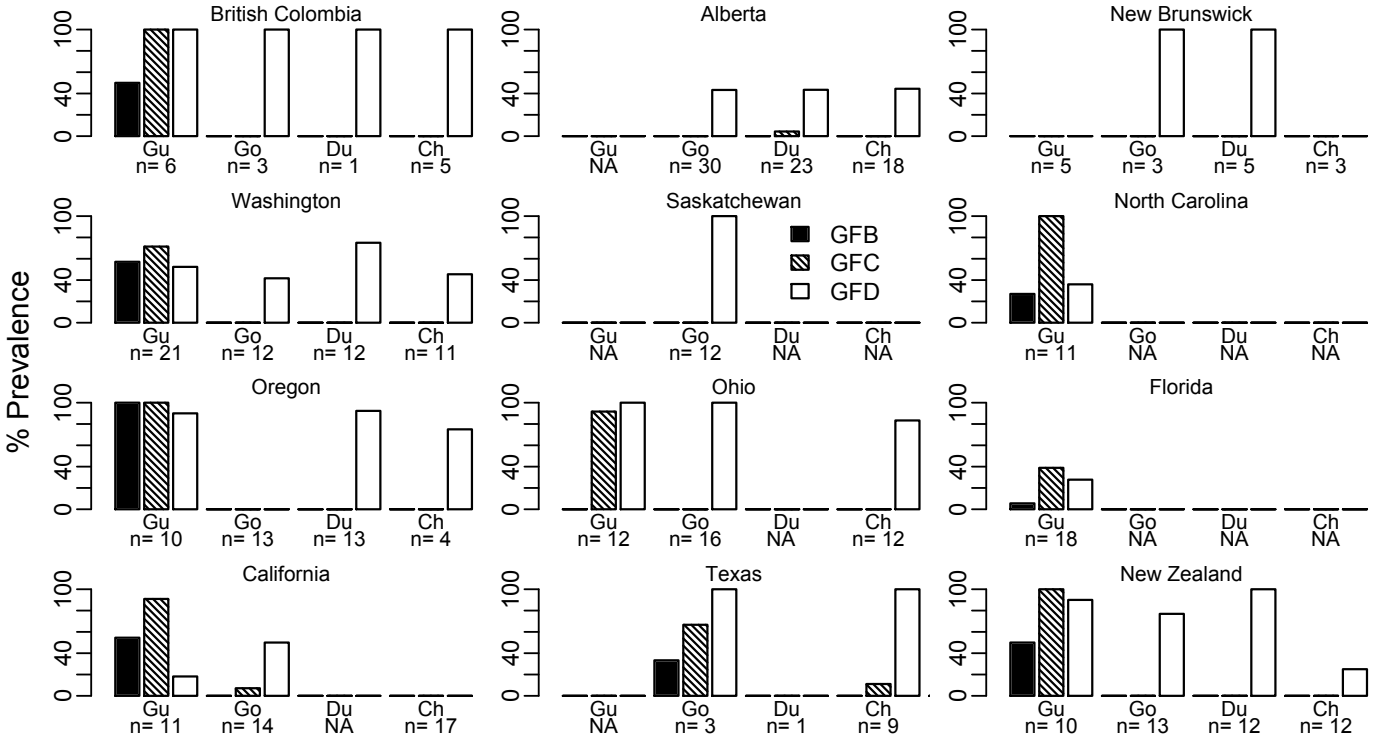

Figure 2.3. Marker prevalence and geographic distribution. Prevalence and geographic distribution of 3 bird fecal PCR markers in gull (Gu), goose (Go), duck (Du), and chicken (Ch) fecal DNA samples in the US, Canada, and New Zealand. BC, British Columbia, CAN; AB, Alberta, CAN; NB, New Brunswick, CAN; WA, Washington, US; SK, Saskatchewan, CAN; NC, North Carolina, US; OR, Oregon, US; OH, Ohio, US; FL, Florida, US; CA, California, US; TX, Texas, US; NZ, New Zealand. *n* = number of individual samples tested. The distribution of the three markers in New Zealand gull, goose, and duck samples was very similar to their distributions on the West Coast of the US and Canada. GFD occurred at a somewhat lower frequency in New Zealand chickens (25%) than in US/Canada chickens and was absent in samples of California chickens. GFC occurrence in sheep was 50% in Oregon (n=22) but 8% in New Zealand (n=12).

Discussion

The PCR assays described here detected fecal DNA from 70% of all gulls, 69% of all geese, 78% of all ducks, and 45% of all chickens. Combining quantitative GFC and GFD assays could provide information on relative contributions of gulls versus other birds. For example, a sample negative for GFC, but positive for GFD, would contain avian contamination, but from something other than gulls. These new assays allow rapid and sensitive detection over a wide geographic range of the most important avian groups contaminating environmental waters.

The GFC assay was highly specific for gulls, with the ability to detect gull fecal contamination at a level representing only 2 enterococci MPN/100 ml. The identification of a *Helicobacter* spp. sequence common to gulls, geese, ducks and chickens was a serendipitous result of the gull subtractive hybridization, allowing design of the GFD assay to detect all of these groups. The GFD assay detected 68% and 76% of geese and duck samples, respectively, and showed 100% specificity for avian fecal samples.

Differences between LOD of plasmid copy number and feces (wet weight) of GFC and GFB (Table 2.1) in conventional PCR suggested that each targeted bacterial group occurred at different concentrations in feces. Similarly, the GFD assay had the same plasmid copy number limit of detection as GFC, but its limit of detection in feces was 10 times higher. Quantitative comparison of GFC and GFD in individual gull fecal samples (Figure 2.2) demonstrated that GFC occurred at a higher concentration.

Many assays to identify the sources of fecal contamination have been based on *Bacteroides* and relatives (Bernhard and Field, 2000a; Layton et al., 2006; Kildare et al., 2007), as the *Bacteroides* group is common in mammalian feces, and amenable to detection in the environment. However, past studies have demonstrated both the relative paucity of *Bacteroides* in gulls (Lu et al., 2008) and the likelihood of horizontal transfer from human to gull (Dick et al., 2005b). We did not recover any sequences in this group following subtractive hybridization, and thus utilized *Fusobacterium, Catellococcus*, and *Helicobacter* sequences for our assays. The

finding of unique and widespread *Helicobacter* sequences in birds suggests functional differences between the GI tracts of birds and mammals.

Since the ability to accurately apportion fecal contamination among sources is dependent on similar persistence of host group-specific fecal markers (Green et al., 2011), it is important to measure the persistence of these new avian assays, especially in comparison with FIB and *Bacteroides* markers and with pathogens.

Previous isolation and investigation of *C. marimammalium* found the organism in marine mammals (Lawson et al., 2006). Marine mammal fecal samples from this and another study (Lu et al., 2008) did not contain detectable amounts of *C. marimammalium.* In this study, marine mammal fecal samples were collected from confined animals, which could have limited horizontal acquisition from gulls in comparison to their wild counterparts. Further investigations into the occurrence of *C. marimammalium* in wild marine mammals may be necessary if they are to be ruled out as contributors of fecal bacteria in recreational waters.

Geographic and species distributions of the markers were inferred from low numbers of samples in some cases, as noted in Figure 2.3, and should be repeated with larger sample sizes. We utilized donated samples, a few of which had been extracted in other laboratories, and eliminated samples that could not be amplified with 16S rRNA gene primers. This could have led to an under-estimate of the markers' prevalence, if a sample was sufficient to amplify 16S rRNA but not to amplify the less common markers.

In addition, we noted very large differences in DNA quantities obtained from Canada goose feces depending on the time of year and diet, underlining the importance of surveys of seasonal/temporal prevalence of these markers. Since the substrates for fecal bacteria are dietary compounds, proportions of fecal bacteria in a given host species will vary according to diet (Turnbaugh et al., 2009; Shanks et al., 2011), which for birds could change both regionally and seasonally.

The limits of detection of fecal source tracking assays have been reported in plasmid copy numbers (Lu et al., 2008; Shanks et al., 2009) or amounts of fecal DNA (Lu et al., 2008), units that are not informative to field applications. We expressed our assays' limits of detection in terms of mg of feces, but tied these to counts of indicator

bacteria, which are more useful to regulators. These FIB counts supported the compatibility of the GFC and GFD assays with environmental applications, as their limits of detection were below the FIB cut-offs for recreational waters. However, because FIB counts in bird feces are expected to vary according to diet, season, geographic location, and bird species, our estimates of FIB should be considered approximations.

The number of chimeric sequences was high, suggesting that the subtractive hybridization procedure systematically produced, and then selected for, chimeric sequences. Analyses of the chimeras did find sequences that were expected to occur in subtracters (e.g., perfect matches to *E. coli* and *Enterobacter*). In future studies, using linkers that contain the appropriate overhang to ligate to the target fragments, but that do not contain the entire restriction recognition sequence, would protect the connection between linkers and target fragments, allowing chimeras to be removed with a second restriction digestion.

The subtractive hybridization technique used by Shanks and colleagues (Shanks et al., 2006a; Lu et al., 2007; Shanks et al., 2007) to enrich for host-specific sequences for source tracking did not appear to produce chimeras. However, because their studies targeted metagenomic sequences, many of which are likely to be single copy genes or pseudogenes, whereas we targeted multicopy 16S rRNA, our assays are likely to have a lower limit of detection.

Our subtractive hybridization technique provided enough unique sequence data to obtain three different host-associated assays with a very small, targeted amount of sequencing compared to clone library analysis. Recent improvements in sequencing coupled with reduced costs suggest that massive sequencing may be a practical approach to marker development. However, improved sequencing will not negate the necessity of analyzing the resulting sequences, so methods that target unique sequences remain attractive.

Modification of the GFC and GFD conventional PCR assays to a qPCR platform permitted more favorable limits of detection and quantification of these molecular markers. The assays consistently detected down to levels below recreational FIB cutoffs in natural water sources. The presence of multiple contaminant sources might

be expected to decrease the accuracy of molecular discrimination tools by introducing interfering particulates, organics, or bacteria previously untested for specificity (Green et al., 2011). We showed that the addition of sewage did not significantly affect our estimates of gull contamination. However, we did observe unexplained variability over the expected linear range (r^2=0.89). This variability was reduced in the chicken fecal dilution experiment, where we used an improved extraction protocol (r^2=0.96). Optimized extraction protocols and accurate methods to account for nucleic acid processing loss may increase method precision in future studies. Despite this variability, the ability of the qPCR assays to detect down to traditional FIB levels well below common recreational water quality standards supports their utility as source-tracking markers in areas potentially impaired by avian fecal contamination. However, an understanding of marker survival (Green et al., 2011), as well as site-specific information on sensitivity and specificity (Kildare et al., 2007) is needed in order to better interpret quantitative results.

Some sheep in the US and New Zealand tested positive for the GFC marker. However, the very low concentration of GFC in sheep feces means that sheep feces could not be detected at less than 0.2 g sheep feces/100 ml, assuming negligible extraction loss. Based on previous estimates of enterococci concentrations in sheep feces (Moriarty et al., 2011), we calculated that initial sheep contaminant inputs would have to contain about 1.6×10^5 sheep enterococci MPN /100 ml to be detected by the GFC assay. In contrast, high concentrations of the marker in gulls allow as low as 2 gull enterococci MPN/ 100 ml (0.1 mg gull feces /100 ml) to be detected. The low limit of detection and the high dosage of this marker in feces make it a valuable tool for estimating gull fecal inputs.

In conclusion, this paper describes three new assays for bird fecal contamination with broad distributions, both geographically and among bird species. Together, these assays detect four of the most important bird groups contributing to fecal contamination of waterways: gulls, geese, ducks, and chickens. Although additional studies are needed to validate these assays across a range of conditions, the assays are useful across North America and in New Zealand to estimate amounts of bird feces,

even in water with low amounts of traditional fecal indicator bacteria. This distribution suggests that they will have broad applicability in other parts of the world as well.

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CHAPTER 3

DIFFERENTIAL DECAY OF HUMAN FECAL *BACTEROIDES* IN MARINE AND FRESH WATER

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Abstract

Genetic markers from *Bacteroides* and other fecal bacteria are being tested for inclusion in regulations to quantify aquatic fecal contamination and estimate public health risk. For the method to be used quantitatively across environments, persistence and decay of markers must be understood. We measured concentrations of contaminant molecular markers targeting *Enterococcus* and *Bacteroides spp.* in marine and fresh water microcosms spiked with human sewage and exposed to either sunlight or dark treatments. We used Bayesian statistics with a delayed Chick–Watson model to estimate kinetic parameters for target decay. DNA- and RNA-based targets decayed at approximately the same rate. Molecular markers persisted (could be detected) longer in marine water. Sunlight limited the survival of cultured indicators and the persistence of molecular markers. Within each treatment, *Bacteroides* markers had similar decay profiles, but some *Bacteroides* markers significantly differed in decay rates. It appeared that the persistence of extra-cellular DNA did not play a role in marker detection. Because conditions were controlled, microcosms allowed the effects of specific environmental variables on marker persistence and decay to be measured. While marker decay profiles in more complex environments would be expected to vary from those observed here, the differences we measured suggest that water type is an important factor affecting quantitative source tracking and microbial risk assessment applications.

Introduction

Waterborne human fecal contaminants harbor many pathogens, pose serious health risks to humans (Haile et al., 1999), cause economic losses, and may disrupt aquatic ecosystems (van der Putten et al., 2007; Stewart et al., 2008). In recent years, researchers have developed specific methods of fecal contaminant detection and identification using *Bacteroides* targeted polymerase chain reaction (PCR) and sensitive and quantitative methods using quantitative real-time PCR (qPCR) (Dick and Field, 2004; Layton et al., 2006; Kildare et al., 2007; Shanks et al., 2008; Converse et al., 2009; Shanks et al., 2009). Compared to culturing methods, qPCR offers advantages for estimating bacterial and viral concentrations, both because of its speed (same day results) and because it can detect difficult-to-cultivate organisms. Application of these methods could therefore reduce uncertainty in fecal source identification and associated risk assessment. Nevertheless, in order to interpret quantitative molecular data for risk assessment, it is necessary to understand marker decay in environmental matrices (Wade et al., 2006; Field and Samadpour, 2007; Santo Domingo et al., 2007).

Decay of culturable fecal indicator bacteria (FIB) in natural water sources has been studied and reviewed extensively over the last 50 years (Gainey and Lord, 1952; Chamberlin and Mitchell, 1978; McCambridge and McMeekin, 1981; Sinton et al., 1999; Noble et al., 2004; Boehm et al., 2009). Far less is known about how genetic markers from indicators and pathogens behave in the environment (Leach et al., 2007), both within, and when released from, the cell, although the fate of qPCR targets under environmental conditions is receiving increased attention. Temperature, particulate concentration, particulate size, predation, salinity, and sunlight all affect marker decay (Kreader, 1998; Okabe and Shimazu, 2007; Bae and Wuertz, 2009b; Bell et al., 2009; Walters and Field, 2009; Walters et al., 2009; Dick et al., 2010; Klein et al., 2011; Schulz and Childers, 2011). Although sunlight contributes most to the deactivation of culturable bacteria (Davies-Colley et al., 1994), observations on the effects of sunlight on the decay of molecular markers have been mixed (Bae and Wuertz, 2009b; Walters and Field, 2009; Walters et al., 2009). Studies that have compared indicator and

pathogen decay provide insights into indicator/pathogen relationships (Walters et al., 2009; Klein et al., 2011). An indicator that correlates highly with an infectious pathogen or group of infectious pathogens through the environment is a more accurate predictor of human health risk (USEPA, 2005).

Because DNA can persist in metabolically inactive or dead cells, and in the environment after cell lysis, its detection does not directly indicate viability of environmental bacteria (Masters et al., 1994; Deere et al., 1996; Keer and Birch, 2003). Two methods have been used to estimate the extent of detection of extra-cellular DNA. The first, propidium monoazide treatment before PCR, causes only membrane enclosed DNA to be detected (Nocker et al., 2007; Bae and Wuertz, 2009a). When this method was used in decay studies, authors reported that extra-cellular DNA accounted for much of the signal in the environment (Bae and Wuertz, 2009b). Alternatively, significant presence of ribosomal RNA (rRNA) suggests viable or dead cells with intact cell membranes, because rRNA is actively degraded by cellular mechanisms under conditions of starvation or cold shock (Chen and Deutscher, 2005) and deteriorates faster than DNA when liberated from the cell (Novitsky, 1986). Furthermore, cellular ribosome content is correlated with growth and metabolic rate (Kemp et al., 1993; Kerkhof and Ward, 1993; Poulsen et al., 1993; Wawer et al., 1997) and is used as a proxy for cell activity in microbial ecology studies using fluorescent in situ hybridization and community sequence analysis (Mills et al., 2004, 2005; Gentile et al., 2006; Akob et al., 2007; Gaidos et al., 2011). Quantification of rRNA with reverse transcriptase quantitative PCR (RT-qPCR) has previously been used to suggest presence of membrane enclosed cells in human fecal *Bacteroides* decay studies (Walters and Field, 2009).

Microcosms are often used to study environmental processes, because they allow the effects of isolated environmental variables to be studied under highly controlled conditions (e.g., see (Kreader, 1998; Okabe and Shimazu, 2007; Bae and Wuertz, 2009b; Bell et al., 2009; Walters and Field, 2009; Walters et al., 2009; Dick et al., 2010; Klein et al., 2011; Schulz and Childers, 2011)). We investigated the decay of culturable enterococci and molecular markers from *Bacteroides* and *Enterococcus spp.*, in marine and fresh water microcosms in sunlight and dark treatments. To

address the correlation of molecular and culturable indicators with pathogens, we monitored decay of *Campylobacter* molecular markers with qPCR using a published assay (Lund et al., 2004). We extracted nucleic acids from microcosms over a period of 21 days. A delayed Chick-Watson (DCW) model, previously used for pathogen decay (Sivaganesan et al., 2003) and *Nitrosomonas europaea* disinfection studies (Wahman et al., 2009), was used to estimate lag times (*Z*) and decay rates (*k*) of both rRNA genes (rDNA) and rRNA. We compared marker decay using *Z* and *k*, and also compared marker persistence, the length of time that markers remained above the limit of quantification (LOQ). Independent of DCW model analysis, we also calculated human-specific to general *Bacteroidales* ratios, as these ratios have been suggested as a means to estimate contributions from human sources.

Results

Decay curves fit a delayed Chick-Watson model.

Assay performance characteristics obtained from standard curves are reported in Table 3.1. Regression lines obtained from measured versus predicted values suggested that the data set as a whole fit a DCW model, with a lag phase followed by pseudo-first order decay, better than standard Chick-Watson (CW) ($r^2_{DCW} = 0.92$, $r^2_{CW} = 0.76$). Therefore all comparisons between molecular data sets were made using DCW unless otherwise stated. For clarity, a subset of the data is used here to convey significant study results and conclusions drawn using the entire data set. Estimates from model fitting and persistence times for all DNA and RNA markers are shown in Appendix Tables 6.1 and 6.2.

Table 3.1. Assay performance characteristics. qPCR reactions used either TaqMan Fast Universal PCR Master Mix ("FastMix"), TaqMan Universal PCR Master Mix ("Universal"), or SYBR Green® in-house Master Mix ("SYBR"). Calibration equations were obtained using either the master calibration curve method (M) or a single standard curve (S). Amplification efficiency is equal to $10^{(1/-slope)}/2$. Range of quantification (ROQ) refers to the range in which the logarithm of copies per reaction maintains a linear relationship with Ct. Assay limits of quantification (LOQ) are defined as the lowest target concentration within the ROQ. Method LOQs are the lower limits of quantification per 100 mLs water sample and are defined as the assay LOQ times the processing dilution factor (50 for DNA and 213.3 for RNA). Percent coefficient of variation (%CV) indicates the average precision in measuring standard concentrations across the range of quantification.

Assay	Target	Reagent	Fluor. Threshold	Cal. Equation	Cal. method	Amplification Efficiency (%)	Range of quantification	%CV across range of quant.	Reference
BsteriF1	Human *Bacteroides* 16S	FastMix	0.02	y = 38.08 - 3.303x	M	100.4	10^1-10^5	1.13	(Haugland et al., 2010)
BuniF2	Human *Bacteroides* 16S	FastMix	0.02	y = 38.07 - 3.425x	M	97.9	10^1-10^5	1.08	(Haugland et al., 2010)
GenBac3	*Bacteroidales* 16S	Universal	0.02	y = 39.26 - 3.416x	M	97.1	10^1-10^5	1.30	(Seifring et al., 2008)
HF183 Taq	Human *Bacteroides* 16S	FastMix	0.02	y = 37.48 - 3.393x	M	98.6	10^1-10^5	1.11	(Seurinck et al., 2005)
HF134/303R	Human *Bacteroides* 16S	SYBR	0.8	y = 38.00 - 3.261x	M	101.3	10^2-10^5	1.24	(Bernhard and Field, 2000a), this study

Table 3.1 Continued

Assay	Target	Reagent	Fluor. Threshold	Cal. Equation	Cal. method	Amplification Efficiency (%)	Range of quantification	%CV across range of quant.	Reference
HF183/303R	Human *Bacteroides* 16S	SYBR	0.8	y = 39.01 - 3.349x	M	99.4	10^2-10^5	1.48	(Bernhard and Field, 2000a), this study
HumM2	*Bacteroides*-like functional gene	Universal	0.02	y = 39.26 - 3.416x	M	98.1	10^1-10^5	1.14	(Shanks et al., 2009)
Entero1 Multiplex	*Enterococci* and internal amplification control	Universal	0.02	y = 37.98 - 3.383x	M	98.8	10^1-10^5	1.13	(Ludwig and Schleifer, 2000)
Camp	*Campylobacter spp.*	FastMix	0.02	y = 37.44 - 3.336x	M	99.7	10^1-10^5	1.24	(Lund et al., 2004)
groEL	*C. jejuni gro*EL gene	Universal	0.02	y = 40.901 - 3.414x	S	98.1	10^1-10^5	1.82	(Love et al., 2006)
mapA	*C. jejuni map*A gene	SYBR	0.8	y = 41.658 - 3.967x	S	89.3	10^1-10^5	1.73	(Price et al., 2006)
pAW 109	pAW 109 RNA (ABI)	FastMix	0.02	y = 38.591 -	S	97.6	10^1-10^5	0.80	(Cook et al., 2004)
PAO	*Pseudomonas aeruginosa* strain PAO-T7	SYBR	0.8	y = 36.75 - 3.568x	M	95.3	10^2-10^5	1.54	this study, (Hoang et al., 2000)

Molecular targets persisted longer in marine water than in fresh water.

Despite decay curve variations dependent on assay or light treatment, there was a highly significant difference in the length of the lag phase (*Z*) between water types: on average the lag phase was 3.1 days ($p < 0.005$) longer in marine water than in fresh water treatments. However, post-lag decay (*k*) was faster in marine water ($p < 0.05$). The lack of post-lag data points restricted model fitting and estimate comparisons on decay curves obtained from marine water microcosms exposed to sunlight. On average, DNA markers persisted above the method limit of quantification 2.5 days longer in marine water compared to fresh water (Table 3.2; $p < 0.01$).

Table 3.2. Persistence of BsteriF1, BuniF2, and GenBac3 rDNA and rRNA within the method LOQs. Values represent the number of days post-seeding that markers were detected at concentrations above the assay LOQ on all three filters for that day. Sampling did not occur on days 8, 10, 12, and 14-19.

	rDNA		rRNA	
BsteriF1	Fresh	Marine	Fresh	Marine
Light	5	7	5	7
Dark	6	7	6	9
BuniF2				
Light	5	7	5	7
Dark	6	7	6	7
GenBac3				
Light	7	11	7	9
Dark	9	20	11	13

Sunlight had a small effect on the decay of DNA and RNA markers.

Differences in marker decay attributable to light were small (Figure 3.1). ANOVA of rDNA decay estimates of lag time and decay rate resulted in no significant differences between light treatments ($p > 0.2$) in either matrix, but in fresh water

GenBac3 and BuniF2 markers exhibited a significantly higher decay of rDNA markers in light compared to dark treatments when comparing estimate credible intervals (Table 3.3). The effects of light in freshwater microcosms were more noticeable on rRNA than on rDNA (Figure 3.1). BsteriF1, BuniF2, Entero1, and GenBac3 all showed significantly higher decay rates of rRNA in light versus dark fresh water treatments (Table 3.3). Both rDNA and rRNA markers displayed biphasic decay in dark treatments (Figure 3.1) and had shorter persistence times in light (Table 3.2, paired t-test $p < 0.05$).In fresh water microcosms, we also estimated decay rates of culturable enterococci and compared them with rDNA and rRNA decay rates, using a CW decay model. In this case, the CW model fit the data better than DCW (Appendix Table 6.1), providing a more accurate comparison of decay rates. In dark treatments, analysis of covariance indicated that decay rates for rRNA and culturable cells were not significantly different from that of DNA ($p>0.2$), suggesting that a similar set of factors determined their decay rates. Under light conditions, DNA decay rates were lower than that of culturable enterococci ($p < 0.05$), but were not significantly different than RNA decay rates ($p=0.053$) (Table 3.4) when using a standard cutoff for significance.

Decay rate dependence on molecular target.

Weighted one-way ANOVA was used to analyze the difference in lag phase Z and decay rate k between targeted clades, independent of treatment. The Entero1 marker experienced a shorter lag phase than BsteriF1, BuniF2, GenBac, and HF 183 Taq ($p < 0.05$) and slower decay than Buni and HF 183 Taq ($p < 0.05$) (Table 3.5).

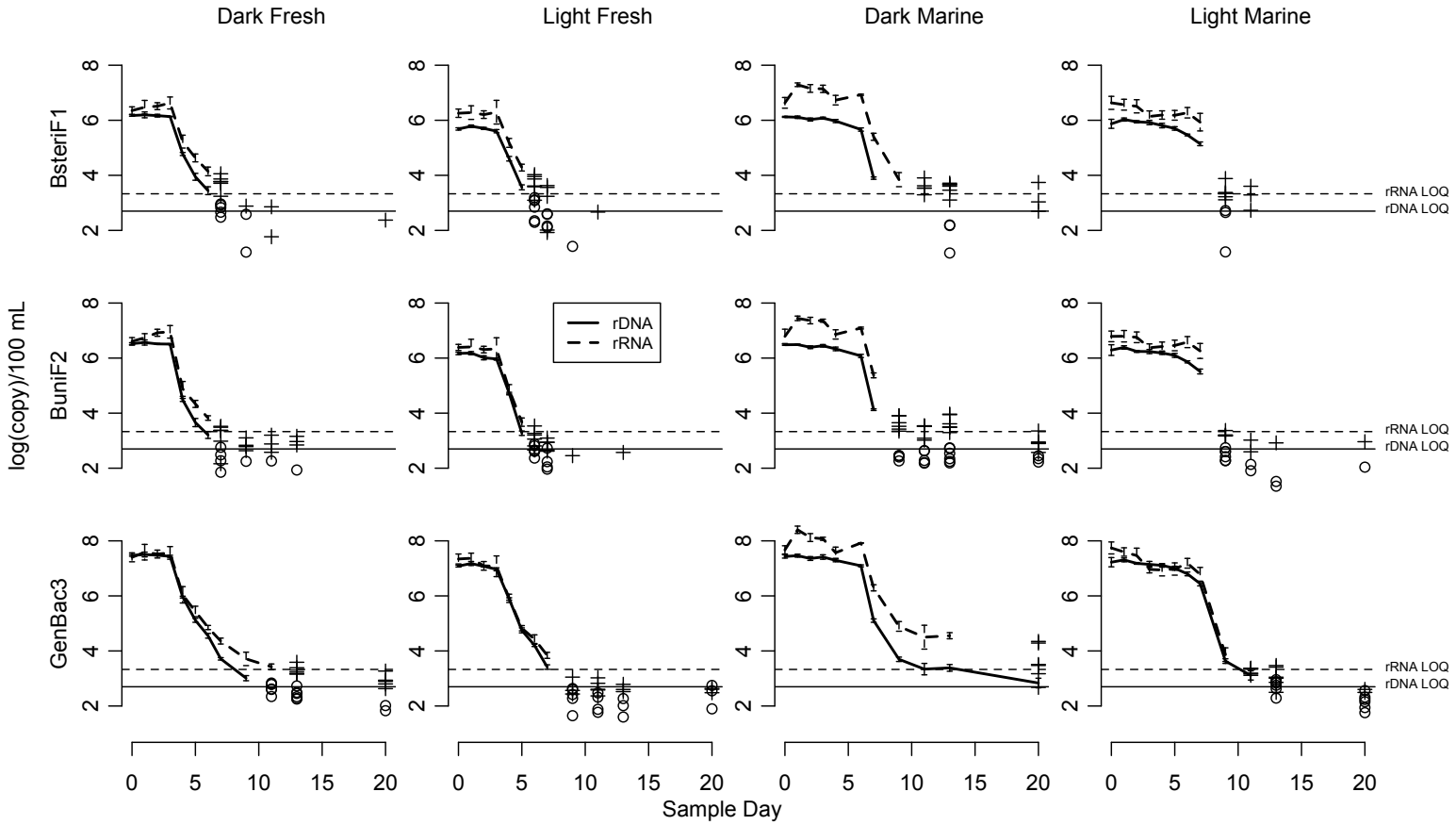

Figure 3.1 Decay profiles of BsteriF1, BuniF2, and GenBac3 rDNA and rRNA. Thick solid and dashed lines represent rDNA and rRNA marker concentrations within the method LOQ, respectively. Circles and crosses represent rDNA and rRNA marker detects below the method LOQ, respectively. Horizontal solid and dashed lines represent method LOQs for rDNA and rRNA analysis, respectively. Error bars represent the standard deviation of six Ct values.

Table 3.3. Comparison of decay rates between BsteriF1, BuniF2, and GenBac3. Comparison of decay rates (k_{DCW}) and their lower (LCI) and upper (UCI) 95% credible intervals between BsteriF1, BuniF2, and GenBac3 rDNA and rRNA in dark fresh (DF) and light fresh (LF) treatments. Data sets that fit DCW with r^2 values >0.90 are shown. Estimates with credible intervals that overlap are not significantly different at the 95% significance level.

		DF			LF		
		k_{DCW}	LCI	UCI	k_{DCW}	LCI	UCI
BsteriF1							
	rDNA	-0.89	-1.00	-0.78	-1.03	-1.15	-0.95
	rRNA	-0.78	-0.93	-0.63	-1.04	-1.36	-0.80
BuniF2							
	rDNA	-1.07	-1.26	-0.88	-1.35	-1.45	-1.26
	rRNA	-0.97	-1.18	-0.75	-1.35	-1.67	-1.11
GenBac3							
	rDNA	-0.72	-0.79	-0.64	-0.88	-0.94	-0.82
	rRNA	-0.49	-0.57	-0.43	-0.79	-0.90	-0.66

Table 3.4. Comparison of enterococci decay using molecular and culture-based methods. Results of analysis of covariance on decay rates of rDNA, rRNA, and cultured cells of enterococci (most probable number (MPN)) in fresh water. p-values represent the significance in decay rate differences when compared to DNA markers.

	Dark			Light		
Method	estimate	r^2	p-value	estimate	r^2	p-value
rDNA	-0.306	0.918		-0.152	0.926	
rRNA	-0.234	0.943	0.241	-0.542	0.955	0.053
MPN	-0.275	0.873	0.606	-1.012	0.996	0.007

Table 3.5. Comparison of enterococci and *Bacteroides* marker decay. Significance matrix of p-values from weighted ANOVA testing estimate differences between assays. Estimates from all treatments were used.

Z

	BsteriF1	BuniF2	Entero1	GenBac3	HF 183 Taq
BsteriF1		0.87	**0.02**	0.92	0.80
BuniF2	0.87		**0.02**	0.82	0.66
Entero1	**0.02**	**0.02**		**0.04**	**0.01**
GenBac3	0.92	0.82	**0.04**		0.93
HF 183 Taq	0.80	0.66	**0.01**	0.93	

k

	BsteriF1	BuniF2	Entero1	GenBac3	HF 183 Taq
BsteriF1		0.42	0.08	0.97	0.10
BuniF2	0.42		**0.02**	0.39	0.30
Entero1	0.08	**0.02**		0.08	**0.01**
GenBac3	0.97	0.39	0.08		0.09
HF 183 Taq	0.10	0.30	**0.01**	0.09	

Despite the close genetic relatedness of groups targeted by *Bacteroides* assays, we observed differences in post-lag decay in some treatments, particularly in freshwater (Table 3.3). These differences led to changes in the ratios between markers. Independent of model estimates, initial and final human-specific:GenBac3 ratios were significantly different in most cases (Table 3.6). Results of parametric and non-parametric analyses agreed: the human specific:GenBac3 ratios increased in those

human specific assays with lower decay rates (BsteriF1 & HumM2), while BuniF2 had a higher decay rate and the BuniF2:GenBac3 ratio declined.

Table 3.6. Comparison of human-specific:GenBac3 marker ratios. Initial ratios are from day 0. Final ratios are from the last sample day concentrations of human-specific markers were quantifiable. Means of filter triplicates were used to calculate ratios. p-values were obtained assuming unequal variances in one-tailed t-tests and represent the significance level when comparing initial and final ratios with each marker within each treatment.

	Initial ratio	Final ratio	p-value
BsteriF1			
DF	0.05	0.08	0.068
LF	0.04	0.066	0.032
DM	0.049	0.063	0.011
LM	0.047	0.05	0.393
BuniF2			
DF	0.114	0.046	0.001
LF	0.122	0.034	0.002
DM	0.113	0.106	0.102
LM	0.125	0.119	0.427
HF 183 Taq			
DF	0.074	0.035	< 0.001
LF	0.05	0.027	0.038
DM	0.076	0.075	0.158
LM	0.069	0.082	0.546
HumM2			
DF	0.005	0.01	0.015
LF	0.008	0.01	0.032
DM	0.005	0.007	0.031
LM	0.006	0.008	0.136

Camp marker increased in concentration in marine water.

We used a published qPCR assay (Camp) to monitor *Campylobacter* decay (Lund et al., 2004). While Camp markers remained below quantification limits in fresh

water treatments throughout the study, concentrations of both DNA and RNA Camp markers increased in marine water. Camp marker concentrations reached maximums at about day 6 but decreased during a rapid decay phase similar to that observed in the decay of other markers (Figure 3.2). We retested microcosm DNAs that tested positive for Camp with *C. jejuni* specific assays mapA (Price et al., 2006) and groEL (Love et al., 2006), and both were below assay limits of detection. A search for database sequences matching Camp primers and probe found no exact matches outside the genus *Campylobacter* (NCBI nr/nt). To identify the cells that grew in the marine microcosms and tested positive with the Camp assay, we cloned ~1300 bp fragments produced when DNA or cDNA from Day 6 dark marine treatment was amplified with the Camp reverse primer paired with a universal rRNA primer (27F, (Weisburg et al., 1991)). We screened the resulting 27F/CampR2 clones with the original Camp qPCR assay. Sequences from clones testing positive with the Camp assay revealed that almost all clones clustered near or within *Kordiimonadales*, *Sphingomonadales*, or elsewhere within the *Alphaproteobacteria* (Figure 3.3). There were no *Campylobacter* sequences. Almost all of the sequence fragments contained mismatches corresponding to the 3' end of CampF2 or CampP2 oligo sequences. An unclassified clade of *Alphaproteobacteria* designated as microcosm clone group E, comprising nine DNA clones and 20 cDNA clones, represented a common actively growing group of bacteria unintentionally identified by the Camp assay.

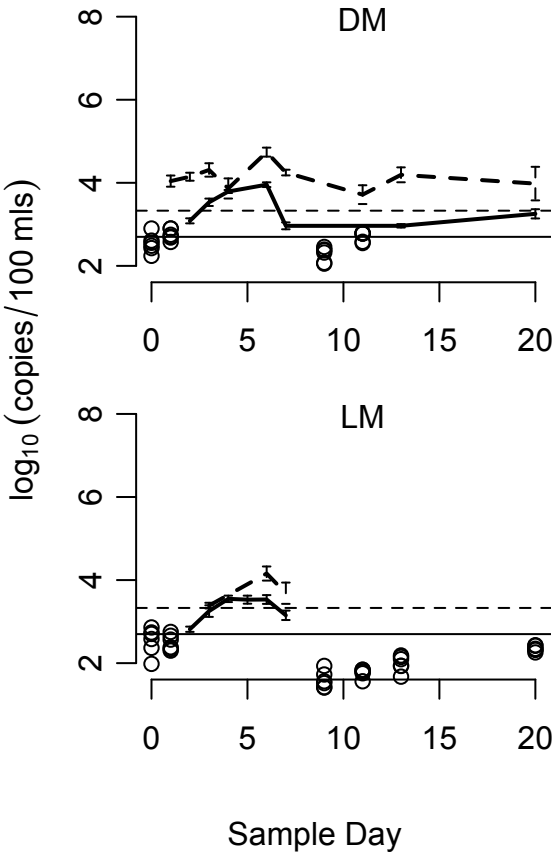

Figure 3.2. Decay/growth profiles of Camp rDNA and rRNA in marine treatments. DM, dark marine; LM, light marine. Solid and dash-dot lines represent rDNA and rRNA marker concentrations within the method LOQ, respectively. Circles and crosses represent rDNA detects below the method LOQ. rRNA detects below the method LOQ were omitted for clarity. Horizontal dotted and dashed lines represent method LOQs for rDNA and rRNA analysis, respectively. Error bars represent the standard deviation of six Ct values.

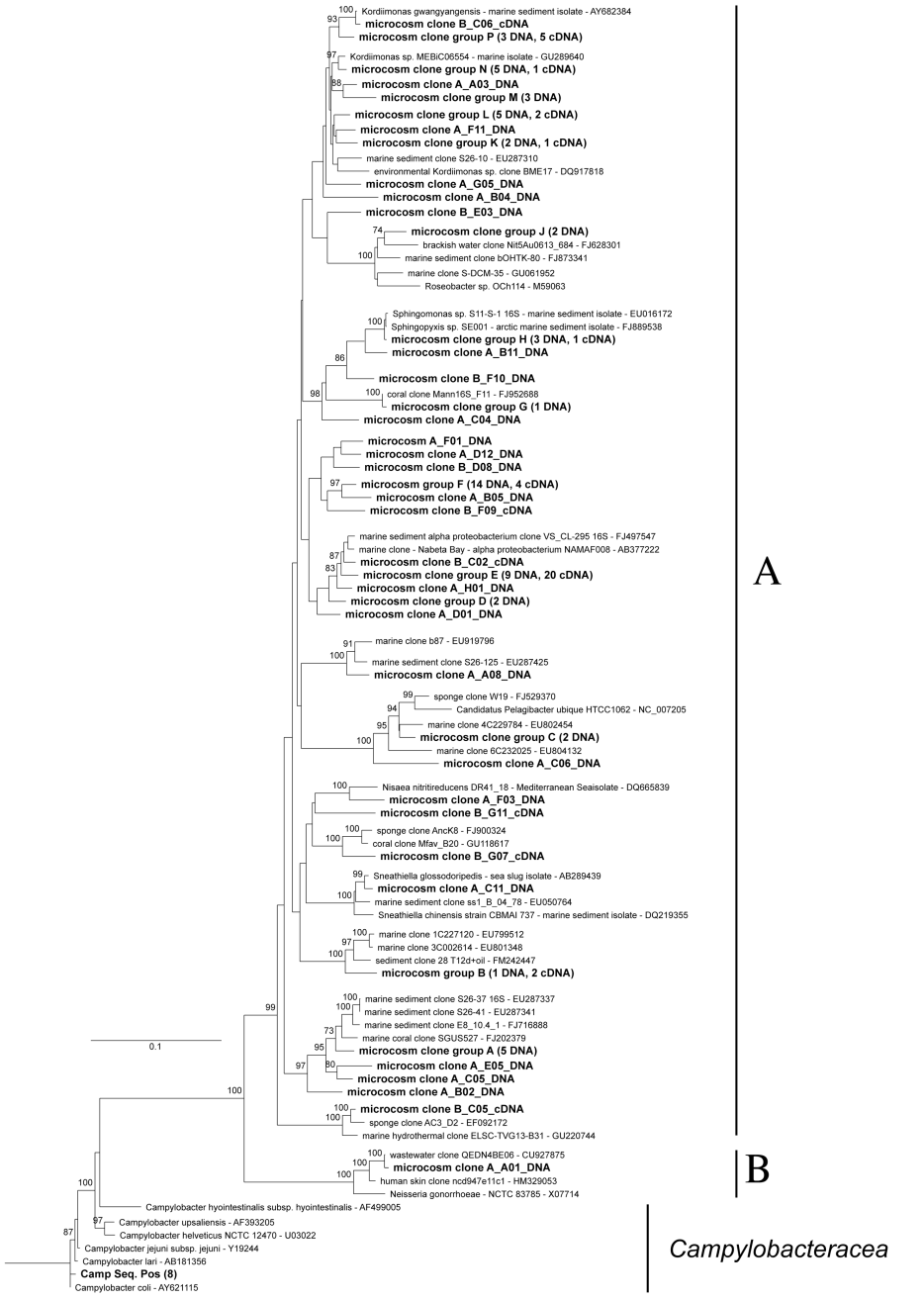

Figure 3.3. Phylogenetic tree of sequences testing positive with the Camp assay.

Figure 3.3. Phylogenetic tree of sequences testing positive with the Camp assay. Clones were created using amplification products from both DNA and cDNA. Representatives were selected from groups with 97% similarity and clustered using a maximum likelihood approach. “A”, *Alphaproteobacteria*; “B”, *Betaproteobacteria*

Matrix Effects on Sample Processing and qPCR.

Matrix specific compounds did not significantly affect DNA recovery or qPCR amplification efficiency. Variability in estimated DNA marker concentrations within the assay limits of quantification between triplicate microcosm water samples was very low (CV=1.2%). Estimated DNA marker concentrations for all assays on day 0 did not significantly differ ($p > 0.3$) between marine and fresh water microcosm samples. Internal amplification control (IAC) (plasmid) and an engineered strain of *Pseudomonas aeruginosa* strain PAO-T7 (PAO) (genomic) controls, used to indicate the presence of carry-over compounds, did not have significantly different Ct values ($p > 0.5$) between marine and fresh water microcosm DNA extracts.

In contrast, estimates of recovery of the RNA processing control pAW 109 RNA (ABI) through DNase treatment and reverse transcription steps were significantly higher in marine water (p-value < 0.005) compared to fresh water RNA. Variability in RNA processing from marine water limited our ability to draw conclusions from these datasets.

Discussion

Delayed Chick-Watson model

The DCW model describes experimental data when a shoulder (lag phase) occurs before pseudo-first order decay. In disinfection studies, the lag phase is usually interpreted as representing a survival period before the disinfectant has reached concentrations that cause cell death (e.g., see (Wahman et al., 2009)). Our decay curves suggest a period of survival of *Bacteroides* cells, followed by cell death, cell lysis, and destruction of nucleic acids.

Effects of water matrix on decay of molecular markers.

The decay profiles in this study were similar to those of *Bacteroides fragilis* (Okabe and Shimazu, 2007). Although the exact conditions of the latter experiment were unclear, we showed that similar decay profiles extend to complex and genotypically diverse *Bacteroides* communities encountered during contamination

events. Walters and Field inoculated human feces into fresh water microcosms and observed a similar 4-day lag phase in *Bacteroides* DNA decay (Walters and Field, 2009). Highly similar fresh water decay profiles in all three studies imply that biological and chemical differences between the freshwater sources did not greatly affect *Bacteroides* decay.

We suspect that the differences between lag-phase durations in marine and fresh microcosms are largely due to differences in predator populations. Microcosm conditions before addition of sewage likely reflect "bottom-up" regulatory conditions that are the result of relatively low nutrient availability. In these conditions, bacterial populations remain relatively low and thus restrict growth of predator populations. However, with the influx of sewage (~10^{10} bacteria), predatory organisms are no longer limited by prey scarcity and their populations expand, resulting in a transition to a "top-down" regulated, or predator-controlled, bacterial community. In this study, the end of lag phase and the beginning of post-lag decay may mark a rapid increase in predator abundance and bacterial mortality. Thus, the length of lag phase would correspond to the time required for predator population growth to reach a level that results in rapid bacterial decay. In this framework, several factors could explain why the lag phase was shorter in fresh water microcosms. The fresh water used in microcosms could contain a more abundant and/or faster growing predator population, resulting in an earlier onset of rapid decay. Alternatively, increased salinity may delay predator growth in marine water (Okabe and Shimazu, 2007; Schulz and Childers, 2011).

Viral lysis causes the decline of abundant bacterial hosts that are susceptible to viral infection. Seeded *Bacteroidales*, at concentrations between 10^7-10^8 per liter, present abundant targets for viruses, qualifying as "winners" according to the "kill the winner" phenomenon (Thingstad and Lignell, 1997; Thingstad, 2000). However, in each treatment, post lag decay began simultaneously for genetically distant *Bacteroides*, *Enterococci*, and the range of *Alphaproteobacteria* detected by the Camp assay, suggesting a mechanism of decay less discriminatory than viral lysis. This also suggests that, at least under these conditions, factors causing decay did not greatly

differ according to bacterial growth rate (growing *Alphaproteobacteria* versus stationary *Bacteroides*).

Several lines of evidence demonstrated that matrix specific compounds did not drastically affect DNA recovery or qPCR amplification efficiency. A matrix effect would be expected to increase the variability between extraction and/or qPCR replicates, yet the coefficient of variation was very low between triplicate microcosm water samples. Had DNA extraction efficiency or qPCR amplification kinetics been dependent on matrix, we would expect to see a difference in the starting concentrations on day 0 between marine and freshwater microcosms; such a difference was not seen. In addition, we used both plasmid (IAC) and cellular (PAO) spiked process controls, and neither demonstrated matrix effects.

Since RNA processing includes the additional steps of DNase treatment and reverse transcription, we included the RNA processing control pAW 109 RNA after extraction, but before the DNase step, to estimate potential target loss or matrix effects from this point forward. We used a published qPCR assay with primer and probe sequences, complementary to sequences on pAW 109 RNA, that was originally used to estimate recovery of RNA extracted from human serum samples (Cook et al., 2004). Higher estimates of RNA recovery in marine water compared to fresh water suggested that matrix effects altered the processing efficiency of DNase treatment and/or reverse transcription.

Effects of light on cultured and molecular indicators.

The detrimental effects of UV and visible light on the culturability of indicator bacteria are well documented (Davies and Evison, 1991; Davies-Colley et al., 1994; Sinton et al., 1999; Sinton et al., 2002; Boehm, 2007), but observations on the effects of light on molecular markers targeting *Bacteroides* and enterococci vary (Bae and Wuertz, 2009b; Walters and Field, 2009; Walters et al., 2009). Here, sunlight decreased the length of time *Bacteroides* markers persisted, presumably by killing cells and terminating DNA maintenance mechanisms, or by damaging DNA templates directly or via photosensitized intermediates (reviewed in (Ravanat et al., 2001)). Similar decay profiles of enterococci by culture and molecular markers suggest that a

similar set of factors cause decay of enterococci genomic DNA, ribosomal RNA, and culturable cells under dark conditions in freshwater. Furthermore, the tight linear correlation (Pearson's r=0.933, p=0.002) between culturable enterococci and enterococci DNA markers in dark fresh treatments suggests that the detectable Entero1 markers were not only enclosed by a cell membrane, but also contained within culturable cells. However, under light conditions culturable cells decayed much faster than both ribosomal DNA and RNA, strengthening previous assertions that light has a much greater impact on the culturability of cells than on the persistence of rDNA and rRNA targets.

In dark treatments, we observed a biphasic decay pattern, not only with general *Bacteroidales* as previously observed (Dick et al., 2010), but also with BsteriF1, BuniF2, HF 183 Taq, and Entero1 markers. Biphasic decay may have also occurred to some degree in light treatments, as suggested by some detection below assay LOQs. The onset of decay of these markers was simultaneous and independent of marker concentration. Biphasic decay, or tailing, is typical of heterogeneous populations, owing to genetic variability among organisms targeted by these assays, or to differences in growth phase of contaminant bacteria upon introduction into water (Hellweger et al., 2009).

Previous studies have reached opposing conclusions about the effects of sunlight on molecular markers. In our microcosms and in the environment, UVA radiation is the predominant form of UV light. UVA damages cellular components mostly via photosensitized intermediates versus direct DNA absorption (Sinton et al., 2002). UVA therefore has a greater effect on culturability than on direct modification of nucleic acid and deterioration of the primer/probe target region. It is possible that exposure to a higher level of UVB light could result in a higher decay by direct DNA damage than estimated in this study, for both culturable indicators and molecular markers.

Role of extracellular DNA detection

It has been suggested that extra-cellular DNA often contributes to the signal in environmental qPCR methods (Bae and Wuertz, 2009a). However, the persistence of

RNA outside of the cell is limited to very short time periods (Novitsky, 1986). Therefore, similar decay rates between DNA and RNA in this study suggests that we detected mostly DNA and RNA targets enclosed within a cell membrane; if we had detected large amounts of extra-cellular DNA, we would expect to see RNA concentration fall below DNA. This is a desirable result for the purpose of estimation of risk, as survival of infectious pathogens is likely to be better correlated with indicator cells than with extra-cellular DNA. The difference between our results and previous studies may be due to sample concentration methods; our filtration methods may be less likely to capture extra-cellular DNA. Abiotic features of samples, such as increased concentrations of particles that associate with DNA, could facilitate capture of extra-cellular DNA. Furthermore, bactericidal mechanisms that attack the cell membrane specifically (e.g., viral lysis and membrane oxidation) may be more likely to produce detectable extracellular DNA than other mechanisms of cell death, such as consumption by protozoan predators.

Decay profiles differ by bacterial target group

Bacteria targeted by Entero1 experienced an earlier onset of decay, but slower decay, than targeted *Bacteroides*. Protozoan predators have been shown to prefer prey based on prey outer membrane characteristics (Gonzalez et al., 1990; Tarao et al., 2009), size (Simek and Chrzanowski, 1992), morphology (Justice et al., 2008), and perhaps growth rate (Pernthaler, 2005). Gram positive *Actinobacteria* are notably resistant to grazing (Pernthaler et al., 2001) due to surface layer characteristics (Tarao et al., 2009). Furthermore, *Enterococcus faecalis* mutants lacking genes involved in capsular polysaccharide biosynthesis displayed enhanced susceptibility to phagocytosis, suggesting a defensive role for capsule formation in some *Enterococcus spp*. (Hancock and Gilmore, 2002). Alternatively, higher susceptibility to abiotic factors such as reactive oxygen could also explain the earlier onset of decay of bacteria targeted by Entero1. Indicators and pathogens with cellular similarities, such as cell wall composition, morphology, and resistance to the effects of reactive oxygen, may show higher correlation in environmental waters. While we have not determined the

exact causes, the observed differences in decay between Entero1 and *Bacteroides* markers support separate interpretations of data obtained using these tools.

Silkie and Nelson have suggested using the ratio between host-specific (e.g., BsteriF1, BuniF2) and general *Bacteroidales* markers (e.g., GenBac3) as a means to estimate the proportion of contamination from host sources (Silkie and Nelson, 2009). Similar decay of host-specific and general *Bacteroidales* markers supports the utility of this method and others (Wang et al., 2010), because decay rates would remain out of the equation. However, using the DCW model, we found that host-specific and general *Bacteroidales* markers can have different decay rates. Accordingly, we found that host-specific and general *Bacteroidales* marker ratios changed over time. The ability to reveal different decay rates between diverse lineages of *Bacteroides* may have been aided by the analytical precision offered by qPCR and an appropriate decay model, in contrast to previous studies that used clone library analysis (Schulz and Childers, 2011). In addition, background levels of general *Bacteroidales* markers due to chronic contamination and/or extended persistence in sediments (Dick et al., 2010) could lead to under estimates of source contributions using a ratio approach. Another untested assumption inherent in such approaches is that general *Bacteroidales* markers from different sources decay at the same rate. Information may be gained from ratio approaches, but differential decay should be considered, and targeting markers that decay similarly to general *Bacteroidales* (e.g., BsteriF1) may be more accurate in such approaches.

Both study conditions and results are relevant to Pacific Northwest estuaries that experience chronic contamination from terrestrial sources. Microcosm temperature (12.8 ºC) reflects that of Tillamook Bay, OR (11.6 ± 2.0 ºC) and one of its major tributaries, Wilson River (13.5 ± 3.7 ºC), during the summer months (NOAA, 2011; USGS, 2011) when aquatic fecal concentrations are highest (Shanks et al., 2006). In a molecular source tracking study in this area, researchers observed that the probability of detecting *Bacteroides* human-specific markers, HF183 and HF134, in the saline bay was double the probability of detecting the same markers in rivers (Shanks et al., 2006), despite the rivers being the source of contaminants to the bay.

Our results suggest that the higher occurrence of markers in bay samples could have been due to an accumulation of slowly decaying *Bacteroides* cells.

Camp assay non-specificity.

The *Campylobacter* assay we used is reported to target pathogenic and non-pathogenic *Campylobacter* species, and was previously tested by others for specificity using 63 *Campylobacter* strains and 14 non-*Campylobacter* species (Lund et al., 2004). Cloning and sequencing of partial 16S genes from microcosm organisms detected by the Camp assay revealed that it detected mostly *Alphaproteobacteria*, whose assay target regions only partially match Camp primer and probe sequences. The change from the original protocol (Lund et al., 2004) to an ABI platform using the Fast Universal PCR Master Mix may have caused a decrease in specificity and allowed the assay to detect a diverse range of bacteria in a separate lineage of Proteobacteria. Alternatively, previous testing may have been insufficient to reveal the assay's non-specificity in genotypically complex environmental samples. In future environmental studies using modified qPCR protocols, specificity should be confirmed independently.

Limitations of the study

One important caveat to any microcosm study results is provided by previous experiments with nutrient enriched microcosms, which have resulted in rapidly changing community structures (Schäfer et al., 2001; Allers et al., 2007). These changes may reflect a response to confinement, and thus may not necessarily predict the types and rates of community change in the native setting.

We expect that the decay profiles observed in this study may not perfectly predict those found in the environment, due to microcosm setup or to variables not tested in this study, such as sediments, turbidity, salinity, temperature, and bacterivore concentration. For example, we would expect higher decay rates had we incubated microcosm at higher temperatures. Results from microcosm studies are sometimes criticized because their controlled conditions do not correspond to complex natural ecosystems (Downing et al., 2008). However, because of their lack of complexity, microcosms allow critical factors influencing persistence to be identified (Downing et

al., 2008). Here we showed that both light and water type influenced genetic marker persistence and rate of decay.

Another limitation of this study is its lack of replication. However, although microcosms were not replicated, microcosms of the same water type (e.g., the two freshwater microcosms versus the two marine water microcosms), or light type, displayed similar decay profiles, increasing the confidence in the observed decay profiles. It will be important in future studies to measure the coefficients of variance among replicates.

Concluding remarks

Molecular methods, such as qPCR, have potential to surpass culture-based methods in terms of specificity and sample-to-answer turn around time. However, basic questions concerning viability of cells and extracellular persistence of targets under environmental conditions hamper development of standards for their application and data interpretation. Currently, differential decay of molecular markers under varying environmental conditions is not considered when choosing appropriate molecular monitoring tools or interpreting the data. Divergent decay profiles of *Bacteroides* markers between marine and fresh water, however, suggest that separate sets of standards may be appropriate for *Bacteroides* qPCR when applied to these sample types.

In the presence of sunlight, our study showed that markers may be bound within cells that are non-culturable, but enclosed in a cell membrane. We found that at least in some conditions, nearly all Entero1 molecular markers were contained within culturable enterococci cells, removing the persistence of extra-cellular DNA from the equation and simplifying interpretation.

Despite their phylogenetic relationship, not all *Bacteroides* markers decayed at the same rate. It is unclear whether this variability of survival traits at the level of species or phylotype will affect the utility of these tools in the environment. Small differences observed in this study may be absent or amplified under other conditions not tested.

Characterizing the effects of environmental variables on molecular markers of fecal contamination is the biggest challenge to molecular source tracking and risk assessment. The large number of environmental variables that can dramatically change quantitative interpretations of environmental molecular marker data warrants further investigation. Additional pathogen and illness correlation studies are needed to determine the predictive power of fecal molecular markers across all aquatic environments.

Experimental Procedures

Sewage and Water Samples.

Raw sewage influent was obtained from the Corvallis sewage treatment plant. Marine water was collected from just under the surface three miles off the central Oregon coast (courtesy of Tony Bertagnolli). Fresh water was collected from Canyon Creek, about 30 miles east of Sweet Home, OR. The land use for Canyon Creek catchment is exclusively timber. Water samples had no visible turbidity or sediment.

Microcosms.

Two marine water and two freshwater 15 L microcosms, consisting of plastic buckets, were inoculated with 150 mL raw sewage influent and partially submerged in constant 12.8°C outdoor water baths at the Salmon Disease Lab (Oregon State University, Corvallis, OR), as described previously (Walters and Field, 2009). Continuous airflow was supplied to the bottom of each microcosm with sterile 4 mm tubing and a fish-tank pump to prevent stratification. A four-way valve was used to ensure equal airflow among tanks.

Light and Dark Treatments

To test the effects of ambient light on marker decay, one marine and one fresh water microcosm were individually covered with an opaque lid and the water bath in which they were submerged was also covered with an opaque water bath lid. The water bath lid was left open for the marine and freshwater light treatment microcosms, and individual clear acrylic lids were used to prevent rain accumulation and

evaporation. This allowed 92.5% of the natural light to penetrate the microcosms but prevented evaporation and dilution by rainwater (Walters and Field, 2009). Mean global horizontal solar radiation in August in this location is about 6.5 kWhr m-2 day-1 (http://solardata. uoregon.edu).

Sampling & Culturing.

Five 50 mL samples were taken from each microcosm daily at 7:30am and stored at 4°C until processing. Microcosms were sampled daily for one week, every other day for the following week, and once the next week for a total of 12 sampling time points. From freshwater microcosms, two 50 mL samples were diluted with 50 mL distilled water and used for the quantification of enterococci with Enterolert® (Idexx Laboratories, Westbrook, Maine, USA), according to the manufacturer's instructions. Approximately, thirty minutes elapsed between sampling and culturing.

Filtration.

Triplicate 50 mL samples were filtered simultaneously onto 47 mm 0.2 μM pore Supor-200® (Pall, Port Washington, NY, USA) filters using a filtration manifold and vacuum pump. Filters were placed in tubes containing 700 μL GITC buffer (5M guanidine thiocyanate, 100 mM EDTA, and 0.5% Sarkosyl) as previously described (Shanks et al., 2006). A maximum of 2.5 hours elapsed between sampling and filtration. Tubes with filters were stored at -80°C for two days prior to nucleic acid extraction.

Nucleic Acid Extraction.

DNA and RNA were extracted using the All Prep DNA/RNA Micro Kit® (Qiagen, Valencia, CA) according to the manufacturer's protocol. Total RNA was eluted with 14 μL RNase free water in low-retention 1.7 mL tubes, which resulted in a final elution volume of 12 μL (dead volume = 2 μL). DNA was eluted in 100 μL elution buffer. Extracted nucleic acids were stored at -80°C for no longer than 319 days before DNase treatment and reverse transcription.

Total RNA DNase Treatment and Reverse Transcription.

Each RNA sample was treated with DNase using the TURBO DNA-free kit® (ABI, Foster City, CA, USA). To control for target loss or potential matrix effects during DNase treatment and reverse transcription we spiked an equal amount of control pAW 109 RNA in each RNA extract. pAW 109 RNA is transcribed from a plasmid containing an array of target sequences and supplied at one million copies per microliter (ABI). pAW 109 RNA was mixed with DNase buffer, DNase enzyme, and molecular grade water before distribution to plate wells to equal one million copies per sample and incubated following the manufacturer's protocol. Five microliters of each DNase-treated RNA sample was transferred directly to the reverse transcription reactions. Reverse transcription was performed in 25 µL reactions with the High Capacity RNA-to-cDNA Master Mix®, according to the manufacturer's protocol (ABI). Fifty-five microliters of buffer AE was added to each sample for a final volume of 80 µL and stored at -20°C until qPCR analysis. GenBac3 qPCR analysis on reverse transcriptase negative samples indicated contaminant DNA concentrations below limits of detection for all samples.

qPCR.

Assay chemistries and threshold settings are listed in Table 3.1. Twenty-five microliter reactions were run on an ABI StepOne Plus® real-time thermalcycler. SYBR green® PCR reactions consisted of 3.5 mM $MgCl_2$, 1X PCR Buffer I (ABI), 2 mM each dNTP, 100 nM each primer, 1 µg bovine serum albumin, 4% w/v acetamide, 4% v/v glycerol, 0.625 U Taq polymerase (ABI, AmpliTaq), 50 µM ROX dye, 0.1X SYBR Green® nucleic acid stain, and 2 µL template. SYBR Green® reactions were thermal cycled for 95ºC for 2 min and 40 cycles of 95ºC for 15 sec and 60ºC (64ºC for mapA) for 32 sec. Melt curve analysis with a resolution of 0.3 ºC was used after cycling to determine amplification specificity. TaqMan® reactions were performed as described previously (Shanks et al., 2009) using either Fast or Universal TaqMan Master Mix® (ABI). Reactions were cycled under the "Fast" or "Standard" default parameters stored by ABI StepOne Plus® software depending on the assay chemistry (Table 3.1). Primers, probes, BSA, acetamide, and SYBR Green® dye were stored in

single-use aliquots. Only DNA samples were analyzed by SYBR Green® qPCR. Microcosm nucleic acid extracts were processed in batches to eliminate the impact of repeated freeze-thaw cycles. Microcosm DNA and cDNA samples were stored at 4°C between reaction setups (maximum storage time of 30 hours). All microcosm samples were analyzed in duplicate. Standard curves were run in triplicate.

qPCR Standards, Controls, and Quality Criteria.

Bulk standard and control DNA extracts were quantified with PicoGreen® (Molecular Probes, Eugene, OR), serially diluted, and stored in single use aliquots in 0.65 mL low-retention tubes. At least five reaction wells on each plate contained positive control template for the appropriate assay. At least three wells on each plate were designated as no template controls (NTC). qPCR inhibition was monitored by two qPCR assays; a plasmid internal amplification control (IAC) multiplexed with the Entero1 assay and a SYBR Green® assay that targets genomes of an engineered strain of *Pseudomonas aeruginosa* strain PAO-T7 (PAO) (Hoang et al., 2000). In each Entero1/IAC reaction, 50 copies of IAC linearized plasmid template were added prior to amplification of microcosm DNA. For the non-competitive inhibition control, 500 genomes of PAO were added to reactions with 2 µL microcosm DNA and amplified using SYBR Green® chemistry to test for co-extraction of PCR inhibitors. PAO-T7 is a lab strain of *Pseudomonas aeruginosa* originally designed for integration of single copy genes into the chromosome but used here as an inhibition control. Capitalizing on the integration of human generated sequence, we amplified the region spanning the junction between PAO native and human derived sequences knowing that finding this strain in the environment is unlikely. PAO-F (5'-GAG TGG TTT AAG GCA ACG GT) and PAO-R (5'-ATG GAA ACA TCA ATG AAA ACA GCA) were used to prime amplification of the *attP*/*B* region (Hoang et al., 2000). As criteria for inhibition, we established bounds based on Ct values obtained from control amplification in molecular grade water at 2 standard deviations above the mean (Ct of 32.57+0.90 and 26.86+0.82 for IAC and PAO, respectively). We concluded that nucleic acid extracts were free of inhibitors if mean Ct values for each extract fell below the bound for both IAC and PAO assays. The mean IAC Ct for one of the 288

nucleic acid samples (144 DNA+144 cDNA) fell just above the predefined bound and was omitted from data analysis (Figure 3.4). In the final data set none of the 617 NTCs from all TaqMan runs showed amplification within the assay LOQ. Melt curve analysis indicated that of the 15 of 188 NTCs from SYBR runs that had Ct values within assay LOQs, none were contaminated with target DNA templates and positive amplification in these wells was assumed to be a product of primer-primer interactions. Melt curve analysis on microcosm DNA amplification reactions that were positive showed melt peaks corresponding to the proper melt peak for each assay.

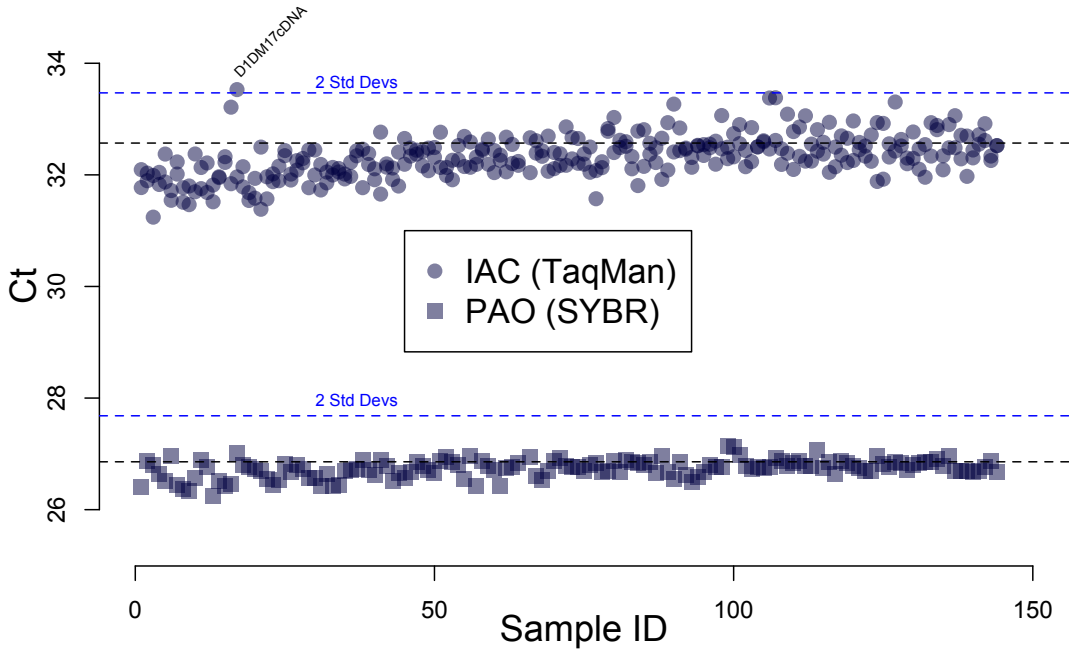

Figure 3.4 Results of tests for qPCR inhibition. Two controls were used to test for inhibition; PAO and an internal amplification control (IAC) multiplexed with Entero1. The bounds for inhibition (or facilitation) were placed at 2 standard deviations from mean Ct values obtained from using 2 µL of laboratory grade water substituted for microcosm sample DNA. One sample, D1DM17cDNA, may have displayed some effects of inhibition as indicated by the IAC and was removed from the data set before analysis.

Camp target sequence analysis.

We identified targets amplified by the Camp qPCR assay through cloning and sequence analysis. Amplicon libraries were constructed from both DNA and cDNA extracts from day six of the dark marine treatment using the primers 27F (5'-AGR GTT TGA TYM TGG CTC AG) and CampR2 (5'- GGC TTC ATG CTC TCG AGT

T) in 30 cycles of PCR. Products from three identical parallel PCR amplifications were pooled and incorporated into the pCR4-TOPO plasmid vector as directed by the manufacturer (Invitrogen; Carlsbad, CA). Ninety four percent of clones containing inserts tested positive with the Camp assay. Sequencing of both strands of inserts was performed on an ABI PRISM 3730XL DNA Analyzer (ABI). High quality sequences were paired and queried against the NCBI-nr/nt database using BLAST (Altschul et al., 1990). Phylogenetic analysis was performed with Bosque (Ramírez-Flandes and Ulloa, 2008). These sequence data have been submitted to the GenBank databases under accession numbers HQ216233:HQ216358.

Copy Number Calculation

The master calibration curve method (Sivaganesan et al., 2008) was used to estimate the copy numbers in unknown samples for all assays except groEL, mapA, and pAW 109. For these assays, a single standard curve was used to estimate copy numbers. Assay limits of quantification are defined as the lowest target concentration within the ROQ (Table 3.1).

Delayed Chick-Watson Model

qPCR data collected for sample days that were above the method limit of quantification (all 6 Ct values > assay LOQ) was used for model fitting. Model fitting was performed on 32 DNA (8 assays × 4 treatments) and 20 RNA (5 assays × 4 treatments) data subsets. 26 DNA and 8 RNA data subsets had r^2 values greater than 0.90. Estimates from data subsets that fell below the 0.90 threshold were excluded from further statistical analysis.

The scatter plot of $\log_{10}(N_t/N_0)$ vs. day showed a clear delayed phase before any post-shoulder decay (an example in Figure 3.5), where N_t and N_0 are respectively the estimated copy numbers on day t and day 0. A delayed Chick-Watson model was used to estimate the lag time Z (in days), and the post-shoulder decay rate constant k ($\log_{10}$(copies/100 mL)/day). The Bayesian regression model for a given data set with n data points, is given by:

$$Y_i=\log_{10}(N_t/N_o)_i=\mu_i+\varepsilon_i, \quad i=1,\ldots,n$$

where,

μ_i=0 if $(day)_i \leq Z$

μ_i=-k[$(day)_i$-Z] if $(day)_i > Z$

Normal distribution with mean 0 and variance 10^4 was considered as the non-informative priors for k (>0). As Z could be anywhere in the range of the number of days, a uniform prior was assumed for Z between 0 and the maximum number of days. In the equation above, ε_i values are independent and identically distributed normal random variables with mean 0 and variance σ^2. A diffuse Inverse-Gamma (0.0001, 0.0001) prior was used for σ^2. Thus Y_i values were all independent normal random variables with mean μ_i and variance σ^2. According to Bayes' theorem, the posterior distribution of the model parameters k , Z, and σ^2 given the data $y_1, ..., y_n$ is proportional to the product of the normal densities (or likelihood) of all Y_i values evaluated at $y_1, ..., y_n$ (given μ_i, σ^2) and prior distributions of these parameters. This posterior distribution was used to estimate the rate constant k, Z, and σ^2. Estimates of k and Z from data sets with r^2 values <0.90 were excluded from further statistical analysis. Weighted one-way ANOVA (weight=1/standard error of estimate) was used to compare estimates between conditions or between assays.

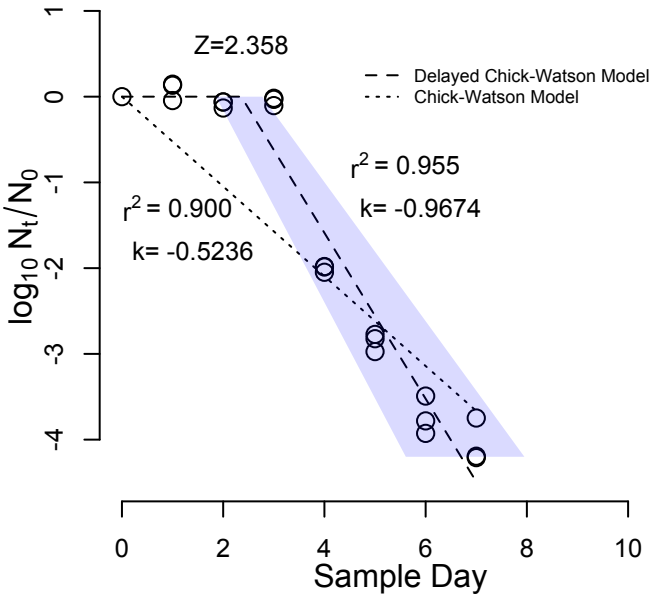

Figure 3.5. Chick-Watson (CW) and delayed Chick-Watson (DCW) model comparison. HF183/303R decay under dark fresh. Decay rates (k) are calculated in both models. The change point (Z) indicates the beginning of rapid decay and is estimated through DCW only. The shaded area represents the 95% credible region estimated by DCW.

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CHAPTER 4

SENSITIVE DETECTION OF SAMPLE INTERFERENCE IN QUANTIFICATION OF HUMAN FECAL MOLECULAR MARKERS

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Abstract

The effects of sample interference in environmental applications of quantitative PCR (qPCR) can prevent accurate estimations of molecular markers in the environment. We developed a spike-and-recovery approach using a mutant strain of *Escherichia coli* that contains a chromosomal insertion of a mutant GFP gene. This approach, along with new statistical modeling methods (multivariate Z-scores), allowed sensitive detection of PCR inhibition by humic acids, compounds often found in environmental samples that inhibit qPCR. The currently used method of measuring inhibition by using Ct values did not reveal qPCR inhibition by humic acids. Although a human fecal *Bacteroides* assay (HF183) was more sensitive to the effects of qPCR inhibitors than the control, outlier identification methods correctly identified inhibition of both control and HF183 assays in samples containing as little as 0.1 ng humic acids per reaction. Using these methods, we were able to simultaneously identify qPCR inhibited reactions and estimate recovery of nucleic acids in environmental samples using a single control assay. While we demonstrate the methods in the context of water quality regulation, they will be useful in all areas of environmental research that use qPCR.

Introduction

Quantification of genetic markers in environmental samples using quantitative PCR (qPCR) is difficult because the samples often contain complex biomolecules, such as humic acids, that inhibit downstream enzymatic processes (Tsai and Olson, 1992; Schriewer et al., 2011), and complex organics or sediment that can reduce nucleic acid recovery (Rajal et al., 2007a). For water quality regulation, qPCR results must be compared to a predetermined acceptable level, but unknown amounts of sample interference may limit their comparability. Detection of sample interference in rapid water quality monitoring applications is particularly important, because qPCR is used to estimate the human health risk associated with a water body; if not accounted

for, sample interference can cause underestimation of health risk (Santo Domingo et al., 2007).

Currently, there are a wide range of approaches and methods to deal with sample interference in qPCR. Many methods use two separate controls: one to estimate extraction recovery, and another to test for the presence of inhibitors. One method uses spiked salmon DNA to estimate recovery of nucleic acids (Shanks et al., 2010); however, because purified DNA is added, this method does not account for inefficient cell lysis. Furthermore, controls for inhibition can be quite complex, and the methods can be difficult or expensive to reproduce (Shanks et al., 2008). Simple methods that can account for both DNA recovery and PCR inhibition are needed.

Recently introduced statistical modeling methods have been used in eukaryotic gene expression studies to detect qPCR inhibitors (Tichopad et al., 2010). Instead of reducing an amplification curve down to a single value (Ct), the curve is fit to a sigmoidal model, allowing the progression of a qPCR amplification reaction to be described in detail. Derivation of Z-scores by multivariate analysis allows identification of outlier amplification curves (inhibited samples). While these data analysis methods have been shown to be highly sensitive to qPCR inhibitors, they have not been applied to complex environmental samples.

Molecular markers from human fecal *Bacteroides* are commonly used to indicate the presence of aquatic human fecal contamination (Shanks et al., 2006), and their amplification is affected by inhibitory compounds (Boehm et al., 2009). In this study, we developed a full-process control that, when analyzed with multivariate methods, was sensitive to both inefficient DNA extraction and qPCR inhibition. To validate the control, we separately reduced extraction efficiency or added a PCR inhibitor (humic acids). We compared the effects of inefficient DNA extraction and qPCR inhibition on control and *Bacteroides* quantification.

Methods

Cell counting

Escherichia coli AF504 was chosen as a control, not only because it grows rapidly and can be quantified with routine methods, but also because it carries a single-copy mutant *gfp* insertion that is not typically found in the environment and can be targeted by qPCR. *E. coli* strain MG 1655 λ attB::bla-Prib-RBSII-gfpmut3*-T0-T1 (AF504) (Folkesson et al., 2008) was grown in 180 µL LB broth with 20 µL mineral oil in 96-well plates (Greiner Bio-One, Monroe, North Carolina, #655 098). Optical density readings (595nm) and aliquots for flow cytometry were taken at roughly 30 min intervals. Cell aliquots were immediately diluted 1:2 in filter-sterilized fixation buffer (0.37% formaldehyde in phosphate buffered saline), vortexed, and stored overnight at room temperature. Cell aliquots were counted using a Guava EasyCyte® (Millipore, Billerica, MA, USA). Aliquots of 10^4 cells/µL were stored in filter-sterilized 15% glycerol solution (pH 7.5) at -20 ºC for future use.

qPCR

A qPCR assay was developed for AF504 using previously published SYBR Green dye (Invitrogen, Carlsbad, CA) chemistry and cycling conditions (Green et al., 2011). Primers (mut3F-5'-CGG TTA TGG TGT TCA ATG CTT TGC GAG ATA CCC, mut3R-5'-ATG GCA CTC TTG AAA AAG TCA TGC CGT TTC) were designed to target the mutated region of *gfp*, with an annealing temperature of 63 ºC. Nucleic acids extracted from marine water, fresh water, and sewage influent all tested negative with the AF504 assay. The efficiency of the AF504 assay was 89 ± 0.01%. Assay performance characteristics for two human fecal *Bacteroides* qPCR assays based on HF134/303R and HF183/303R (Bernhard and Field, 2000) were presented in a previous publication (Green et al., 2011). Thresholds for calculating Ct values were set at 0.8.

Estimating Extraction Recovery

We diluted raw sewage influent 1:10,000 in marine water and filtered 100 ml of this mixture through 0.22 µm filters. Filters were rolled and placed in 10 ml cryo-tubes

with 500 µl GITC buffer (5M guanidine thiocyanate, 100 mM EDTA, and 0.5% Sarkosyl) as previously described (Shanks et al., 2006). 5 µl AF504 cryostock ($5x10^4$ cells) were added to each tube. We extracted DNA from filters using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA), in which DNA in a high-salt buffer, AL, is bound to a silica membrane. To test and compare the effects of reduced extraction efficiency, we reduced the efficiency of DNA binding to the silica membrane during extraction by diluting buffer AL. This buffer also contains a lysis reagent that could have an additional effect on extraction efficiency, but does not contain reagents that aid inhibitor removal. Eight hundred µl of 100%, 75%, 50%, and 25% buffer AL solutions (diluted in molecular grade water) were added to each tube before vortexing; the rest of the extraction was according to the manufacturer's directions. Triplicate extractions were performed at each buffer AL concentration.

Inhibition by Humic Acids

To test the effects of humic acids inhibition of AF504 and HF183 qPCR amplification, we mixed equal parts DNA extracts from 75% and 50% buffer AL treatments to use as template (30 µl each). We dissolved 100 mg humic acid powder (#53680, Sigma Aldrich, St. Louis, MO) in molecular grade water, centrifuged at 5,000g for 1 min to remove undissolved particles, and further diluted so that when added to separate PCR master mixes before amplification, the final concentrations in the reactions were 250, 125, 25, 12.5, and 2.5 ng/µl. Sixteen qPCR replicates were performed at each humic acid concentration for each assay.

Environmental Sample Analysis

Sixty-nine 500 ml environmental water samples were collected from a variety of freshwater sites in a single watershed draining into a bay in Washington State, including agricultural drainage ditches, small streams and rivers. Five µl AF504 cryostock was added to 100 ml of each sample and they were filtered as described above. DNA was extracted using the PowerWater DNA Isolation kit (MoBio, Carlsbad, CA) according to the manufacturer's directions. All DNA extracts in addition to filtration and extraction blanks were analyzed on the same day with the AF504 assay in duplicate.

Data Analysis and Statistical Methods

Data were analyzed using the *qpcR* package (Ritz and Spiess, 2008) with R (R Development Core Team, 2010). Of four-, five-, six-, and seven-parameter models the seven-parameter model fit with the least residual error. The amplification curves were not averaged between replicates and weights from other models besides the seven-parameter model were not applied. Curves with r^2 values < 0.999 after fitting were excluded from analysis.

For the humic acids experiment, estimates from model fitting were used to assign Z-scores to each amplification curve. This statistical modeling method was taken directly from Tichopad and colleagues (Tichopad et al., 2010) and is restated here. The relationship between t_1, the maximum of the first derivative and t_2, the maximum of the second derivative, is described by

$$t_2 = t_1 \times b + a + \tau$$

where b and a are linear coefficients and τ is the residual error independent of t_2. At least 10 control reactions without qPCR inhibitors are used to produce t_1 and t_2 pairs for regression analysis. After regression modeling, τ is estimated

$$\tau = t_2 - \widehat{t_2}$$

where $\widehat{t_2}$ is a prediction of t_2 as a function of the linear regression given t_1. t_1 and τ are normalized by subtracting the mean and dividing by the standard deviation:

$$t_{1_{norm}} = (t_1 - \bar{t}_1)/\sigma_{t_1}$$

$$\tau_{norm} = (\tau - \bar{\tau})/\sigma_{\tau}$$

where $\bar{t}_1$ and $\bar{\tau}$ are the means and σ_{t_1} and σ_{τ} are the standard deviations. $t_{1_{norm}}$ and τ_{norm} are used in the calculations of Z-scores.

$$Z = t_{1_{norm}}^2 + \tau_{norm}^2$$

Thus, each curve was assigned a Z-score that was used to identify amplification deviants. Z-scores were calculated only for curves with negative τ values. Amplification curves with Z-scores in the 99th percentile of the χ^2 distribution for two degrees of freedom (9.210) were considered amplification outliers.

We modified the methods of Tichopad and colleagues to estimate recovery from environmental samples that did not display high levels of inhibition. Whereas

previously described multivariate methods assume the same initial concentration between samples, marker recoveries may differ between samples; therefore, one variable, τ_{norm} , may be used to indicate the rate of amplification and the degree of inhibition. Where τ_{norm} is less than 5.22 for both amplification replicates we assumed no qPCR inhibition and used the following equation to estimate recovery for each sample:

$$\%\ Recovery_{sample} = \frac{Qty_{AF504}}{5\times10^4}$$

where Qty_{AF504} is the quantity of AF504 recovered from the 100 ml sample estimated by standard curve quantification using mean Ct values.

Results

Estimation of DNA Recovery

AF504 cell concentration had a significant linear relationship to cell OD within the exponential growth phase ($p<0.01$, $r^2=0.96$), described by the function $cells/ml=10^{(\log_{10}(OD)\ x\ 1.21\ +\ 10.12)}$. We used known quantities of spiked AF504 to estimate extraction recovery.

Although the variance was large between extraction replicates, reducing buffer AL concentration clearly reduced recovery (Figure 4.1). Importantly, there was no significant difference in response among the three assays over the range of AL concentrations tested (Figure 4.2). Confidence intervals for regression line slopes (HF134, 0.66-1.76; HF183, 0.63-1.29) indicated that slopes (shown on plot) were not significantly different from one.

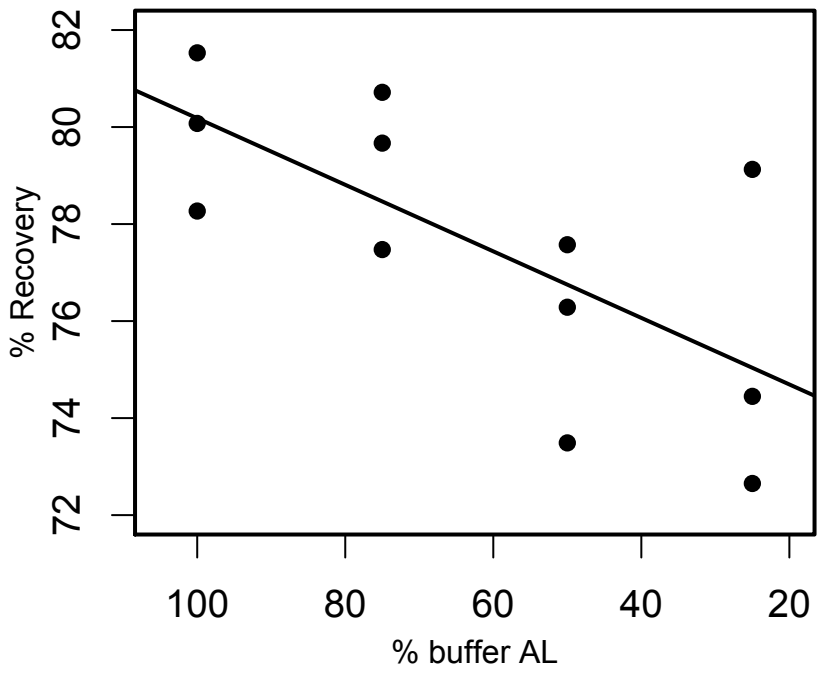

Figure 4.1 Effect of decreased concentration of buffer AL on AF504 recovery. Recovery was calculated as the number of AF504 marker copies measured by qPCR divided by the number of cells added to each extraction. The data fitted to a linear regression model had a p-value of 0.01, an r^2 of 0.48, and a slope of 0.068.

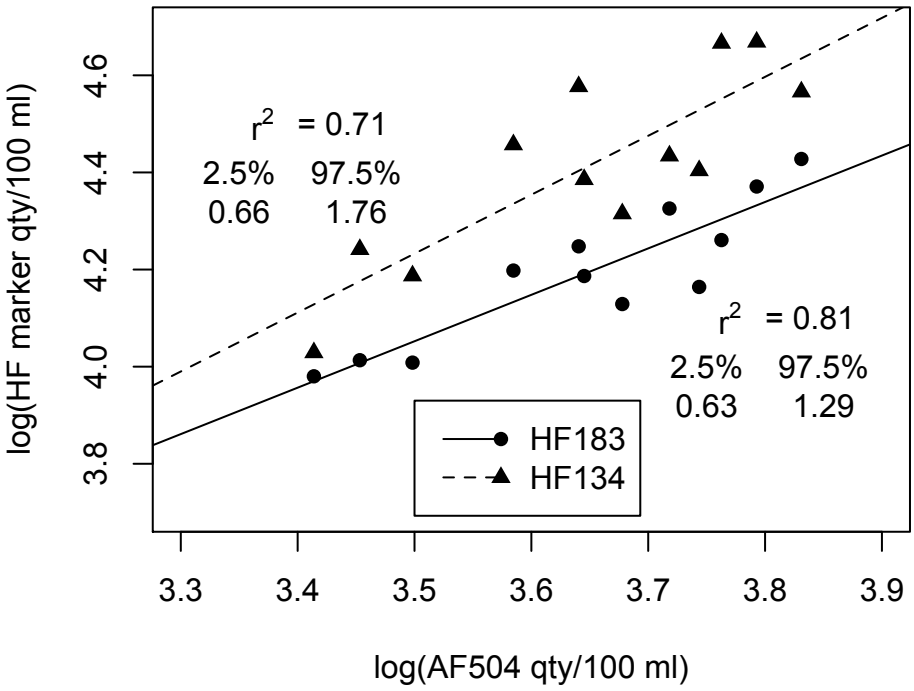

Figure 4.2 Correlation between recovery of AF504 and HF183 and HF134 marker DNA. Regression lines were formed by plotting each HF marker Ct as a function of AF504 Ct. The relationships were significant ($p<0.05$).

Detection of qPCR Inhibition by Humic Acids

In qPCR with no humic acids, t_1, the maximum of the first derivative and t_2, the maximum of the second derivative, of the AF504 amplification curves had a predictable linear relationship (Figure 4.3, inset). Addition of humic acids caused a shift in the relationship between t_1 and t_2 (Figure 4.3, Figure 4.4). Curve derivative maxima had a predictable relationship in both the absence and presence of humic acids, and reactions containing 5 ng humic acids lay outside the expected range predicted from reactions without humic acids.

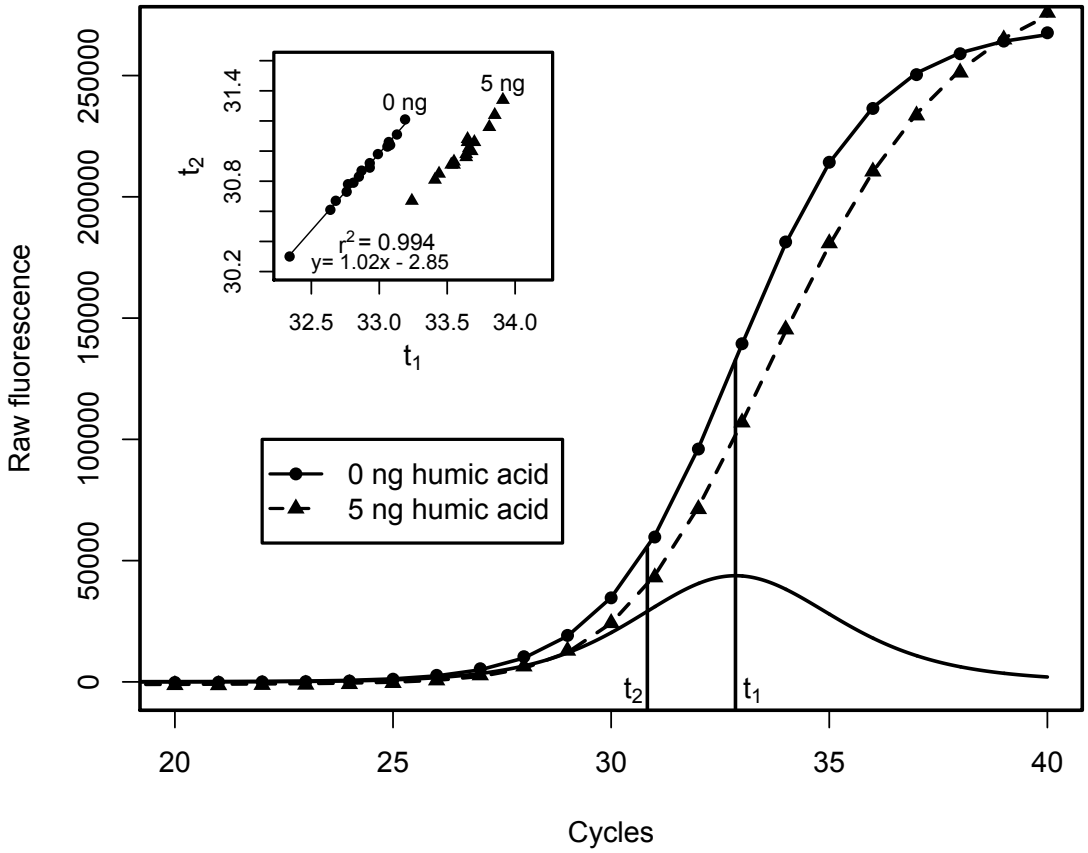

Figure 4.3 AF504 amplification curves of reactions containing 0 and 5 ng humic acids. The solid curved line without points at the bottom of the plot represents the first derivative of the 0 ng humic acid amplification curve. Maxima of first (t_1) and second (t_2) derivatives are depicted as solid lines without points. Inset: relationship between first and second derivative maxima for each curve after replication.

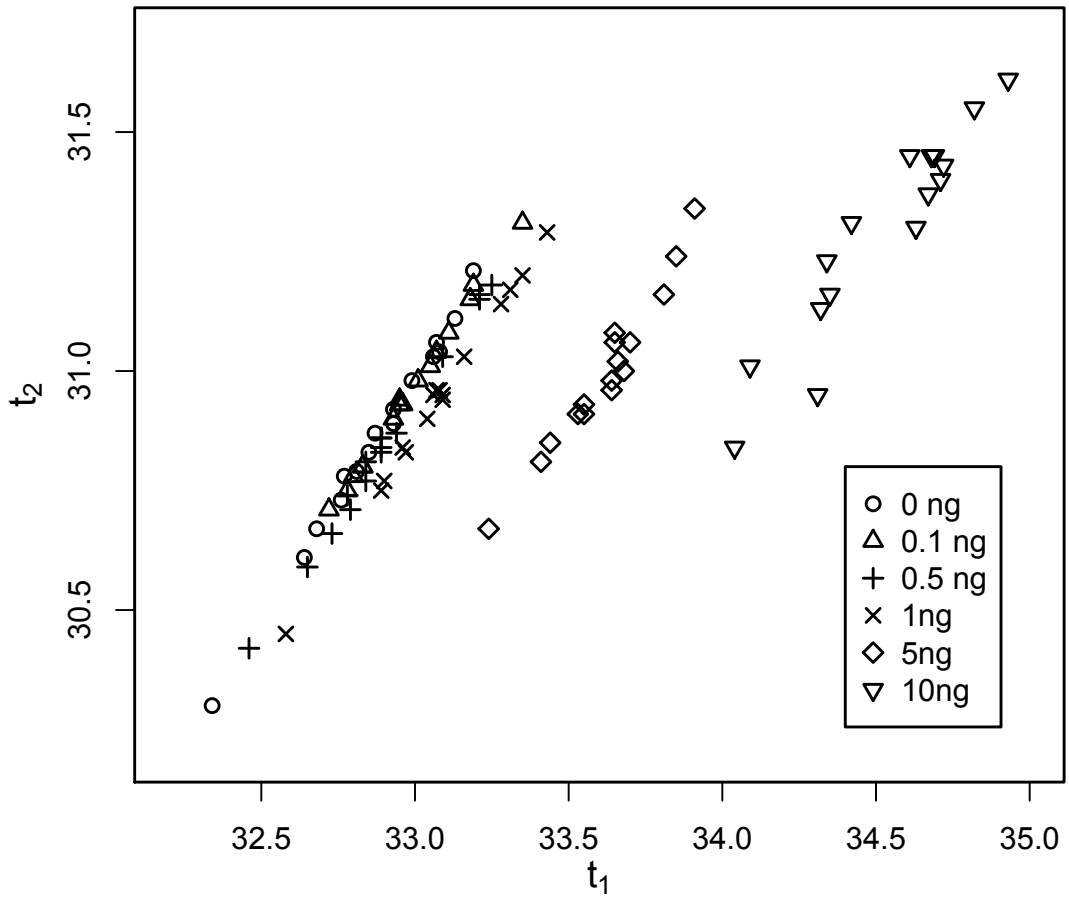

Figure 4.4 The relationship between the maxima of the first (t_1) and second (t_2) derivatives of qPCR amplification curves when humic acids were added to amplification reactions. Each reaction was replicated 16 times.

The calculation of Z-scores using t_1 and t_2 allowed correct identification of reactions that contained 0.1 ng humic acid or higher for both AF504 and HF183 assays. However, using univariate Z-scores calculated using Ct values, AF504 reactions containing up to 10 ng humic acid were not properly identified as containing inhibitors (Table 4.1).

Table 4.1 Detection of amplification outliers using multivariate and univariate Z-scores. The number of reactions out of 16 that were identified as inhibited is shown.

Outlier Method	Assay	Humic acids per reaction					
		0 ng	0.1 ng	0.5 ng	1 ng	5 ng	10 ng
Multivariate Z-scores	AF504	0	13	13	13	16	16
	HF183	0	14	15	15	16	16
Univariate Z-score (τ)	AF504	0	0	0	14	16	16
	HF183	0	0	5	16	16	16
Univariate Z-scores (Ct)	AF504	0	0	0	0	0	0
	HF183	0	0	0	0	0	16

Estimation of qPCR inhibition and DNA recovery in environmental samples

The observation that τ_{norm} could be used as an indicator of qPCR inhibition by humic acids, independent of t_1, suggested that the AF504 assay could be used to simultaneously rule out the presence of high concentrations of inhibitors and estimate extraction recovery. Extraction recoveries could be estimated only in samples where inhibition could be ruled out because τ_{norm} values were in the expected range. Out of 138 reactions (69 samples run in duplicate), 101 fit sigmoidal models and had τ_{norm} values below 5.99 (95% cutoff). Extraction recoveries among samples for which *both* qPCR replicates passed these quality criteria ranged from 4.5% to 65%. Recovery from 25 samples could not be estimated either because both amplification curves did not fit a sigmoidal model or because qPCR amplification was inhibited as determined by τ_{norm} values.

Discussion

Estimation of extraction recovery

Bacteria have a wide range of cell envelope compositions and structures that could affect the efficiency of cell lysis and DNA extraction. We compared recoveries of markers amplified from *E. coli* strain AF504 and *Bacteroides* cells following inefficient DNA extractions, and found that recovery of AF504 DNA reflected the recovery of *Bacteroides* DNA under our test conditions, and that AF504 is therefore a good surrogate organism in estimating the recovery of *Bacteroides*. However, both

AF504 and *Bacteroides* are gram-negative bacteria. Extraction efficiencies may differ for bacteria that have envelope structures that are particularly difficult to lyse. Recoveries of AF504 and other bacterial targets should be compared experimentally before application of these methods, particularly when the target is widely different in structure from *E. coli*.

It is notable that for the environmental samples analyzed here, which were collected and analyzed under identical conditions, at the same time and by the same operator, estimated recoveries varied widely (4.5% to 65%). The most likely explanation for these differences is that they are related to sample characteristics such as amount of suspended sediments, tannins, humic acids, and other cells present. This result highlights the necessity for controlling for recovery when using qPCR to estimate important water quality parameters. In our data set, we used DNA recoveries to adjust source-specific marker data and to estimate sample limits of detection as others have done (Rajal et al., 2007b) (data not shown). An alternative would be to exclude samples that fall below a defined threshold for extraction recovery from analysis. This approach would bypass assumptions about differential effects of sample interference among assays, but would discard valuable data. Importantly, the methods presented are amenable to both approaches and should be flexible to differences in sample type and study design.

Modeling methods for the detection of qPCR inhibition

Better detection of inhibition by the new statistical modeling methods, compared to currently used methods, suggests that they will be useful in defining acceptable levels of qPCR inhibition. In our studies, we found that the inhibition caused by <5 ng humic acids was inconsequential when using Ct calibration methods. This finding supports the use of Ct analysis methods in environmental qPCR applications where qPCR inhibition may be a problem. How much qPCR inhibition is acceptable should be defined to prevent exclusion of valuable samples from analysis when using sensitive sigmoidal modeling methods.

Many compounds can inhibit qPCR (Opel et al., 2010). In this study we found that humic acids had a greater effect on the rate of amplification than on the start of

amplification (lag phase). The ability of sigmoidal modeling methods to separate these two parameters enables differentiating the effects of sample interference on extraction and qPCR. The underlying assumption is that if inhibition delays lag phase it will always be accompanied with an outlying τ_{norm} value. However, other compounds found in the environment could potentially affect qPCR by other mechanisms and could have a greater effect on amplification lag phase than on amplification rate. These types of effects would limit the ability to estimate extraction efficiency using a single full-process control because it would be difficult to distinguish poor extraction recovery from qPCR inhibition.

Assay specific response to inhibitors

qPCR amplification with both AF504 and HF183 responded similarly to humic acids; however, all three outlier identification methods indicated that HF183 was slightly more sensitive to the effects of inhibition than AF504. The higher annealing temperature of the AF504 assay (63ºC) may limit colloidal particle formation by humic acids and thus limit reagent sequestration (Baar et al., 2011). Other assay differences such as amplicon length or primer base pair composition could also have an effect. However, the small differences observed here in assay response to humic acids are unlikely to change quantitative interpretations to a large degree.

Concluding remarks

We showed that a single cellular control is effective at indicating both low extraction recovery and qPCR inhibition of important environmental markers. Furthermore, the ability to use one control assay as both a means of indicating inhibition and estimating recovery was enabled by new data analysis methods. In addition, these techniques were more sensitive in detecting qPCR inhibition than currently used methods. Implementation of these methods could increase the accuracy in predicting human health risk and restrict human illness by avoiding under-estimation of contaminants. While we demonstrated these methods in the context of water quality, they will be useful in most environmental qPCR applications.

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CHAPTER 5

GENERAL CONCLUSION

Hyatt C. Green

Newer molecular methods have been developed to improve the estimation of human health risks associated with water bodies; however, fundamental unanswered questions regarding their application limit their effectiveness and preclude their integration into water quality regulatory standards (Field and Samadpour, 2007; Santo Domingo et al., 2007). While *Bacteroides*-targeted PCR applications have led to measurable increases in water quality in some areas (Johnson, 2009), methods to identify all potential fecal contaminant sources have not been developed. Where methods have been developed, the distribution of markers over a range of hosts and geographic distance is poorly defined in most cases. Research gaps concerning the survival of target organisms and the persistence of molecular markers under various environmental conditions remain. Organisms that grow outside of the host and markers that persist in the environment for long time-periods are not good indicators of recent contamination events. Information regarding marker correlation with pathogens or illness is limited (Field and Samadpour, 2007; Santo Domingo et al., 2007). Practical concerns such as high analysis costs and data analysis and interpretation are additional barriers to the integration of molecular methods into current regulations (Santo Domingo et al., 2007).

We developed methods to identify aquatic fecal contamination originating from birds. The quantitative methods can detect low amounts of contaminants and, based on their wide geographic prevalence, are likely to be useful across North America, New Zealand, and, perhaps, world-wide. From a practical point of view, the methods are beneficial because they are cheap, rapid, and publically available. These new tools will be extremely helpful in coastal areas where gulls have long been suspected as the major source of fecal bacteria to recreational waters (Gould and Fletcher, 1978; Benton et al., 1983; Rocha Simões et al., 2010), but because the source could not be identified, accurate estimates of human health risk were not possible. These methods also play an effective role in implementing World Health Organization sanitary survey strategies (WHO, 2003).

Our findings show that applications and interpretations of *Bacteroides*-targeted PCR may differ depending on water matrix. These results will inform the creation of

regulatory standards using *Bacteroides* molecular markers. However, marker correlation with pathogens across a range of environmental scenarios will also be important if the markers are to be used in a monitoring capacity. Our observation that extracellular DNA does not contribute significantly in the detection of molecular markers supports monitoring applications for molecular markers; pathogens are less likely to correlate with extracellular DNA than intact cells. Our decay studies also found that differential decay and persistence of *Bacteroides* markers complicates quantitative source apportioning.

Sample interference in DNA isolation and qPCR confounds accurate estimates of marker concentrations in the environment. Our observations of high variance in the quantification of gull markers and an effect of marine water on downstream enzymatic processes spurred development of a full-process control that could quantify nucleic acid losses and matrix effects on qPCR amplification. The method, using a genetically modified *E. coli* strain, was highly sensitive to the effects of inhibition and would be easily transferrable to other labs with basic laboratory equipment. Multivariate Z-scores properly identified PCR inhibited samples that other widely used methods could not. In the context of water quality, these methods will be beneficial in both source identification and monitoring applications, by eliminating false negatives due to sample interference.

An appropriate role for molecular methods in water quality has been debated intensely in the past decade. Their role as a tool to identify non-point sources of contaminants has been established; qualitative PCR is currently used around the world to help improve water quality. Our work in avian marker development strengthens this role by relating unfamiliar qPCR units (copies/100 ml) to units of fecal indicator bacteria (FIB) used to monitor regulated waters. This approach will provide water managers with some idea of what concentrations of FIB are necessary before molecular source identification methods are informative. In addition, levels of FIB contributed by a particular source can be estimated from marker quantities. We identified some factors that make quantitative source tracking more difficult to interpret (e.g., differential decay of *Bacteroides* markers and differential decay of markers due to environmental factors). We were able to remove some of the

uncertainty in the quantification process and are now confident that estimating and comparing marker quantities within and between samples is justified. However, given the differential decay of markers in environmental waters, interpreting these quantities will remain a challenge to the implementation of these tools and a focus of future research.

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APPENDIX

Table 6.1 Decay rates and persistence times for all DNA molecular markers.

	DNA markers																		
	DCW model estimates							Persistence (days)											
	Z	LCI	UCI	k_{DCW}	LCI	UCI	r^2	0	1	2	3	4	5	6	7	9	11	13	20
BsteriF1																			
Fresh																			
Light	2.945	2.839	3.117	-1.032	-1.151	-0.955	0.993	6	6	6	6	6	6	4	0	0	0	0	0
Dark	2.695	2.419	2.931	-0.888	-0.996	-0.779	0.973	6	6	6	6	6	6	6	3	0	0	0	0
Marine																			
Light	4.352	3.626	4.841	-0.271	-0.357	-0.188	0.905	6	6	6	6	6	6	6	6	0	0	0	0
Dark	5.735	5.589	5.856	-1.767	-2.018	-1.510	0.967	6	6	6	6	6	6	6	6	0	0	0	0
BuniF2																			
Fresh																			
Light	2.878	2.784	2.980	-1.350	-1.454	-1.260	0.993	6	6	6	6	6	6	2	0	0	0	0	0
Dark	2.550	2.097	2.908	-1.066	-1.256	-0.877	0.946	6	6	6	6	6	6	6	1	0	0	0	0
Marine																			

Light	4.264	3.484	4.757	-0.278	-0.361	-0.192	0.910	6	6	6	6	6	6	6	6	1	0	0	0
Dark	5.787	5.685	5.877	-1.945	-2.161	-1.728	0.977	6	6	6	6	6	6	6	6	0	0	0	0
Entero1																			
Fresh																			
Light	1.521	1.226	1.777	-0.465	-0.522	-0.410	0.976	6	6	6	6	6	6	2	0	0	0	0	1
Dark	1.578	0.951	2.291	-0.391	-0.495	-0.313	0.920	6	6	6	6	6	6	6	3	1	1	3	1
Marine																			
Light	2.281	1.596	2.884	-0.283	-0.345	-0.227	0.929	6	6	6	6	6	6	6	6	0	1	3	0
Dark	5.658	5.401	5.854	-1.164	-1.427	-0.902	0.921	6	6	6	6	6	6	6	6	3	1	0	1
GenBac3																			
Fresh																			
Light	2.673	2.475	2.845	-0.878	-0.938	-0.816	0.989	6	6	6	6	6	6	6	6	2	0	0	1
Dark	2.159	1.747	2.561	-0.716	-0.792	-0.643	0.966	6	6	6	6	6	6	6	6	6	5	1	0
Marine																			
Light	5.506	4.994	5.952	-0.816	-0.932	-0.704	0.954	6	6	6	6	6	6	6	6	6	6	5	0
Dark	1.774	0.293	3.319	-0.319	-0.383	-0.264	0.811	6	6	6	6	6	6	6	6	6	6	6	6

HF183 Taq																			
Fresh																			
Light	3.089	2.913	3.289	-1.387	-1.593	-1.221	0.988	6	6	6	6	6	6	4	0	0	0	0	0
Dark	2.872	2.666	3.055	-1.339	-1.521	-1.156	0.973	6	6	6	6	6	6	4	2	0	0	0	0
Marine																			
Light	4.332	3.519	5.012	-0.251	-0.359	-0.164	0.869	6	6	6	6	6	6	6	6	2	0	0	0
Dark	5.844	5.770	5.911	-2.024	-2.200	-1.852	0.986	6	6	6	6	6	6	6	6	4	2	4	0
HF134/303R																			
Fresh																			
Light	2.895	2.801	3.027	-1.398	-1.528	-1.295	0.994	6	6	6	6	6	6	3	0	0	0	0	0
Dark	2.627	2.267	2.916	-1.192	-1.370	-1.009	0.962	6	6	6	6	6	6	6	5	1	0	0	0
Marine																			
Light	3.025	2.279	3.638	-0.202	-0.255	-0.152	0.909	6	6	6	6	6	6	6	6	5	1	0	0
Dark	4.886	4.287	5.440	-0.918	-1.132	-0.729	0.926	6	6	6	6	6	6	6	6	6	5	4	2
HF183/303R																			
Fresh																			

Light	2.735	2.548	2.903	-1.143	-1.262	-1.030	0.986	6	6	6	6	6	6	6	5	0	0	0	1
Dark	2.358	1.858	2.771	-0.967	-1.124	-0.814	0.955	6	6	6	6	6	6	6	6	0	0	0	0
Marine																			
Light	2.840	1.959	3.591	-0.202	-0.266	-0.149	0.909	6	6	6	6	6	6	6	6	5	3	0	0
Dark	3.240	1.951	4.335	-0.459	-0.569	-0.356	0.851	6	6	6	6	6	6	6	6	6	5	6	4
HumM2																			
Fresh																			
Light	2.741	2.540	2.905	-0.904	-1.071	-0.721	0.963	6	6	6	6	6	0	0	0	0	0	0	0
Dark	3.639	3.008	3.925	-5.666	-16.350	-1.232	0.387	6	6	6	6	6	2	0	0	0	0	0	0
Marine																			
Light	4.387	3.462	5.229	-0.236	-0.368	-0.147	0.855	6	6	6	6	6	6	6	6	0	0	0	0
Dark	3.464	2.170	5.366	-0.143	-0.308	-0.041	-0.012	6	6	6	6	6	6	6	3	0	0	0	0

Table 6.2. Decay rates and persistence times for all RNA molecular markers

	RNA markers																		
	DCW model estimates							Persistence (days)											
	Z	LCI	UCI	k_{DCW}	LCI	UCI	r^2	0	1	2	3	4	5	6	7	9	11	13	20
BsteriF1																			
Fresh																			
Light	3.043	2.686	3.418	-1.042	-1.363	-0.800	0.925	6	6	6	6	6	6	5	2	0	0	0	0
Dark	2.929	2.527	3.261	-0.779	-0.928	-0.631	0.925	6	6	6	6	6	6	6	5	0	0	0	0
Marine																			
Light	0.750	0.030	2.143	-0.108	-0.157	-0.075	0.356	6	6	6	6	6	6	6	6	2	1	0	0
Dark	4.788	4.158	4.995	-0.588	-0.738	-0.419	0.778	6	6	6	6	6	6	6	6	6	4	5	1
BuniF2																			
Fresh																			
Light	2.945	2.667	3.253	-1.354	-1.665	-1.110	0.956	6	6	6	6	6	6	1	0	0	0	0	0
Dark	2.809	2.281	3.187	-0.969	-1.181	-0.750	0.907	6	6	6	6	6	6	6	2	0	0	0	0
Marine																			

Light	1.056	0.050	2.866	-0.085	-0.138	-0.051	0.278	6	6	6	6	6	6	6	6	0	0	0	0
Dark	4.346	2.276	4.986	-0.361	-0.685	-0.064	0.307	6	6	6	6	6	6	6	6	5	4	5	0
Entero1																			
Fresh																			
Light	0.884	0.332	1.434	-0.514	-0.632	-0.418	0.925	6	6	6	6	6	6	3	2	0	0	0	0
Dark	1.037	0.399	1.643	-0.405	-0.492	-0.331	0.903	6	6	6	6	6	6	6	0	0	0	0	0
Marine																			
Light	0.221	0.006	0.723	-0.291	-0.336	-0.256	0.747	6	6	6	6	6	6	6	6	0	0	0	0
Dark	1.402	0.484	2.451	-0.242	-0.309	-0.190	0.811	6	6	6	6	6	6	6	6	6	3	5	2
GenBac3																			
Fresh																			
Light	2.273	1.723	2.684	-0.786	-0.901	-0.664	0.958	6	6	6	6	6	6	6	6	0	0	0	0
Dark	1.733	0.989	2.405	-0.495	-0.566	-0.425	0.917	6	6	6	6	6	6	6	6	6	6	2	1
Marine																			
Light	4.318	1.863	4.981	-0.710	-0.941	-0.354	0.683	6	6	6	6	6	6	6	6	6	3	2	0
Dark	4.349	3.194	4.969	-0.428	-0.507	-0.339	0.859	6	6	6	6	6	6	6	6	6	6	6	4

HF 183 Taq																					
Fresh																					
Light	2.609	0.616	3.810	-2.441	-10.750	-0.482	0.074	6	6	6	6	6	4	1	0	0	0	0	0		
Dark	1.043	0.038	3.536	-0.981	-4.775	-0.406	0.197	6	6	6	6	6	5	3	0	0	0	0	0		
Marine																					
Light	0.227	0.006	0.799	-0.183	-0.220	-0.152	0.496	6	6	6	6	6	6	6	6	1	0	0	0		
Dark	4.820	4.405	4.995	-0.715	-0.816	-0.597	0.913	6	6	6	6	6	6	6	6	6	3	5	1		