FACTORS ASSOCIATED WITH CONCENTRATIONS OF PERSISTENT ENTERIC MARKERS IN WATER QUALITY SAMPLES AND SEDIMENT CORES FROM THE LAKE ST. CLAIR WATERSHED

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Microbiology and Molecular Genetics-Doctor of Philosophy

2015

ABSTRACT

FACTORS ASSOCIATED WITH CONCENTRATIONS OF PERSISTENT ENTERIC MARKERS IN WATER QUALITY SAMPLES AND SEDIMENT CORES FROM THE LAKE ST. CLAIR WATERSHED

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Molecular methods to monitor water quality can address current and historical pollution. Molecular measurements of enteric markers from sediments can aid in the evaluation of historical water quality using a singular index and be used to analyze correlations to climate and human impact. However, the stability of the markers in defined storage conditions and durations is uncertain. The goals of this dissertation were to: 1) investigate how storage conditions and duration affected concentrations of enteric molecular markers in water samples; and 2) evaluate the correlations of historical anthropogenic and climate variables with the deposition of persistent enteric markers in sediment cores from the Lake St. Clair watershed.

Autoclaved water from the Red Cedar River was seeded with 10% (vol/vol) raw sewage and stored in liquid suspension (LS) or attached to a solid matrix (SM). Enterococci (ENT) *23S rDNA*, *Escherichia coli* (EC) *uidA*, and *Bacteroides thetataiotaomicon* (BT) *1,6 alpha-mannanase* were measured with quantitative polymerase chain reaction (qPCR) in order to evaluate their persistence for up to 28 and 366 days at 4º (long term and short term studies), and 27º and 37ºC (short term study only). Five linear and non-linear best-fit models were fit to the indicator concentrations. Persistence of the indicators was enhanced on SM ($p < 0.001$), and decreased with time ($p < 0.001$). Persistence was also dependent on indicator species in the short term and long term studies ($p < 0.001$, and $p = 0.001$, respectively). The least to most persistent indicators were: BT < EC < ENT. The time needed for 90% decay of the indicators, T_{90},

calculated with the best-fit models in the short term study (and long term study) ranged from 1 day for BT in LS at 37˚C to > 28 days for ENT and EC on SM at all temperatures (and 35.8 days for EC in LS to 164 days for ENT on SM at 4°C). At 4°C, the T_{90} values were greater in the long term study compared to the short term study. This study suggests that storage of water samples at 4°C attached to a solid matrix can increase the persistence of markers from fecal indicators.

Surface sediments from Anchor Bay, northwestern Lake St. Clair (AB), and the mouth of the Clinton River (CR) were spiked with EC and *Enterococcus faecium*. qPCR measurements of ENT *23S rDNA* and EC *uidA* extracted from 17 DNA extraction methods were compared. Within each location, Kruskal-Willis tests confirmed few significant differences between the concentrations of the indicators. The optimal method included a bead beating step with a DNA sorption blocker followed by centrifugation. This method evaluated the concentrations of ENT *23S rDNA* and EC *uidA* in sediment cores from AB and CR, representing the years c.1757 - 2012, and c.1895 - 2012, respectively. EC concentrations in the AB and CR cores increased with year, and ranged from 1.42×10^6 to 16.9×10^6 cell equivalents (CE) per g-dry wt, and 1.81×10^6 to 8.46×10^6 CE per g-dry wt, respectively. ENT concentrations in the CR core increased with year, and ranged from 3×10^3 to 990×10^3 CE per g-dry wt. The ENT concentrations in the AB core experienced two steady states: ~1×10^4, and ~2×10^5 CE per g-dry wt during c.1757 – c.1878, and c.1902 – c.2010, respectively. ENT concentrations in both cores were correlated to river discharge ($p = 0.046$), while EC concentrations were correlated to air temperature ($p = 0.018$), and total nitrogen and total carbon concentrations ($p = 0.038$, and 0.029, respectively). Also, ENT and EC concentrations were significantly correlated to population in watershed ($p = 0.003$ and 0.023, respectively). This study offers a novel analysis of ≥ 100 years of the management of the Clinton River watershed.

To my supportive family, both given and chosen. Thank you for always being there for me.

ACKNOWLEDGMENTS

I would like to acknowledge the following people that have helped me complete my dissertation: Joan Rose for her guidance during this project; Rebecca Ives for all of her help and dedication in the laboratory; and my collaborators Asli Aslan, Tiong Gim Aw, Mark Baskaran, Melissa Baustian, Jade Mitchell, Bharathi Murali, Nathaniel Ostrom and Sushil Tamrakar. Finally, I would like to thank my labmates. This dissertation would not have been possible without your help.

This research was funded by the following organizations: NOAA, NSF, and the Graduate School and Department of Microbiology and Molecular Genetics at Michigan State University.

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CHAPTER 1. WATER POLLUTION AND WATER QUALITY

1.1 Fecal indicator organisms

Access to clean water is a basic human right (United Nations, 2010). However, contact with and ingestion of polluted water can cause respiratory, gastrointestinal, ear, and eye illnesses. Therefore, water quality monitoring is essential in order to mitigate the risk of waterborne disease in drinking water and recreational water. Water quality monitoring is achieved by measuring concentrations of fecal indicator organisms in water. Fecal indicators are enumerated in place of pathogens because culturing multiple pathogens is difficult and time consuming (USEPA, 2012a). The World Health Organization (WHO) defined the following ideal characteristics of fecal indicator organisms: 1) non-pathogenic viruses and bacteria that are universally found in animal and/or human excreta; 2) do not grow in the environment; 3) have similar persistence compared to pathogens; 4) are in higher concentrations than pathogens; and 5) have methods that are easy and cost-efficient to numerate (World Health Organization, 2011). A variety of fecal indicators are recommended by the WHO, and they include: total coliforms, fecal coliforms, sulfite-reducing clostridia, F-specific RNA bacteriophages, somatic coliphages, total bacteriophage, *Bacteroides fragilis* bacteriophages, *Escherichia coli* and enterococci (World Health Organization, 2003, 2011). However, there is no single fecal indicator that meets all of the characteristics listed above. Below are descriptions of four fecal indicators.

1.1.1 Total coliforms and fecal coliforms

Total coliforms are a group of bacteria that are associated with fecal pollution (USEPA, 2011). They include taxonomically unrelated species that are gram-negative, facultative anaerobes, non-spore forming, rod shaped, grow at 36°C, and produce gas while fermenting

lactose (Madigan, Martinko, Dunlap, & Clark, 2009). Not all total coliforms represent fecal pollution. Some species have natural habitats in the environment.

Within the total coliform group, is a subset called fecal coliforms. They produce acid in lactose fermentation when grown at 44 ± 0.5°C, grow in the presence of bile salts, and are oxidase negative (Doyle & Erickson, 2006). However, some species are found naturally in the environment (USEPA, 2012a).

1.1.2 Escherichia coli

Escherichia coli is a species within the fecal coliform group. It is a popular fecal indicator for recreational water and drinking water (World Health Organization, 2011) because its primary environment is in the intestinal tract of warm blooded animals (Doyle & Erickson, 2006). Its mutualistic relationship provides the host with vitamin K_2, while the host provides a nutrient rich and stable habitat (Suvarna, Stevenson, Meganathan, & Hudspeth, 1998). Although most *E.coli* strains are beneficial, there are seven pathogenic groups including: enteropathogenic, enterotoxigenic, enteroinvasive, enteroaggregative, diffusely adherent, and shiga toxin producing (Brenner & Farmer III, 2005). The pathogenic strains can cause urinary tract infections, pneumonia, respiratory illnesses and diarrhea (Centers for Disease Control, 2011). Also, some pathogenic strains are multi-drug resistant (Centers for Disease Control, 2011).

Many standard methods to enumerate culturable *E.coli* in water have a single carbon source, β–glucuronide, in the media. β–glucuronide is metabolized by an enzyme specific to *E.coli*, β–D-glucuronidase (Hansen & Yourassowsky, 1984), that is encoded by *uidA* (USEPA, 2002). In its natural habitat, β–D-glucuronidase hydrolyzes mammalian gut bile intermediates, β–glucuronides, into alcohols and glucuronic acid for cellular metabolic energy (USEPA, 2002).

1.1.3 Enterococci

There are 28 enterococci species. They are facultative anaerobes, gram-positive, cocci, catalase-negative, form no gas with lactose fermentation, and form pairs or short chains (Facklam, Carvalho, & Teixeira, 2002). Enterococci can hydrolyze esculin, and can optimally grow in media at 42.7˚C with 40% bile salts and 6.5% NaCl (Fisher & Phillips, 2009; G. Klein, Pack, Bonaparte, & Reuter, 1998). Some enterococci naturally inhabit plant matter (Fisher & Phillips, 2009), while others are commensal to the human oral cavity, the human vagina, and animal gastrointestinal tracts and fecal matter (Jett, Huycke, & Gilmore, 1994). *Enterococcus faecalis* and *Enterococcus faecium* are the first and second most common enterococci isolated from humans, respectively, but are in lesser prevalence in livestock (Franz, Holzapfel, & Stiles, 1999). Enterococci have been associated with community-acquired and nosocomial infections like urinary tract infections, wound infections, bacteraemia, sepsis, and endocarditis (Poh, Oh, & Tan, 2006). Enterococcal infections originate in contaminated food, the person's own microflora, and infect immuno-compromised people (Murray, 1990). Treating enterococcal infections are difficult because many strains are multi-drug resistant (Ryan, Ray, Ahmad, Drew, & Plorde, 2010).

The standard methods to enumerate culturable enterococci contain two carbon/energy sources, esculin and its chromogenic analog, which select for enterococci growth (USEPA, 2009). The incubation temperature, 41°C, also selects for its growth (USEPA, 2009).

A molecular method, Method 1611, is recommended by the United State Environmental Protection Agency (EPA) to enumerate enterococci *23S rRNA*, Entero1, in water samples within 3-4hrs with quantitative polymerase chain reaction (qPCR, USEPA, 2012b). The primer and

probe sequences are: GAGAAATTCCAAACGAACTTG, CAGTGCTCTACCTCCATCATT, and [6-FAM]-5'-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA, respectively (USEPA, 2012b). *23S rRNA* is a bacterial ribozyme, peptidyltransferase, in the large subunit of the ribosome (Snyder & Champness, 2007). The gene is highly conserved and is essential for bacterial growth and development (Snyder & Champness, 2007). Although, the copy number of *23S rRNA* is dependent on the species and the metabolic state, the suggested copy number is four, which is the average copy number of *Enterococcus faecalis 23S rRNA* (USEPA, 2012b). The detection limit is about 27 cell equivalents (CE) or ~10^2 Entero1 sequences per reaction (Haugland, Siefring, Wymer, Brenner, & Dufour, 2005).

There are many disadvantages of assessing water quality with enterococci and *E.coli*. Their culturable methods need an incubation period between 18-24 hrs, which may not allow for fast response to unacceptable concentrations. Also, false-negatives and false-positives can occur with the culturable and qPCR methods. False-negatives in the culturable methods can be attributed to the lack of a functional target enzyme in subpopulations of the fecal indicators, while false-negatives obtained in qPCR methods may be attributed to qPCR inhibitors or the detection limit. The false-positives obtained in culturable methods may be due to targeted enzyme activity from other species. There are few reported false-positive results in qPCR methods (Griffith, Weisberg, & McGee, 2003).

Another limitation of culturable and qPCR methods to enumerate *E.coli* and enterococci represent general pollution and do not identify the host. *E.coli* and enterococci can also persist and even grow in the environment (Pote et al., 2009; Wheeler Alm, Burke, & Spain, 2003). Growth of fecal indicators in the environment may warrant unnecessary beach warnings and

closures. Additionally, concentrations of fecal coliforms and enterococci were not associated with concentrations of *Salmonella* spp., and *Cryptosporidium* spp. in wastewater effluent and freshwater (Lemarchand & Lebaron, 2003).

1.2 Fecal indicators and their associations to health outcomes in recreational waters

Contact with recreational waters can cause various types of adverse health outcomes affecting the following areas of the human body: gastrointestinal tract, ear, respiratory tract, eyes, and skin. During 2009-2010, there were 81 water associated disease outbreaks in the United States with a total of 1,326 cases affecting 28 states and Puerto Rico that were reported to the Centers for Disease Control (CDC, Hlavsa et al., 2014). However, the actual number of water associated disease outbreaks in the United States is suspected to be larger as local public health departments voluntarily report outbreaks to the CDC (Blackburn et al., 2004).

Beginning in the 1950s, epidemiological studies began to investigate the relationship of swimming in recreational waters to adverse health outcomes. For example, one study determined that swimmers were at a higher risk to experience gastrointestinal, skin, respiratory, eye, and ear illnesses compared to non-swimmers in the following freshwater and marine beaches: Lake Michigan, Illinois; Ohio River, Kentucky; and Long Island Sound, New York (Stevenson, 1953).

Further studies have examined the dose-response relationship between health outcomes of swimmers and the water quality of recreational water experiencing various sources and levels of pollution. For example, culturable *E.coli* and enterococci were associated with the incidence of gastrointestinal illness from beaches on Lake Erie, Pennsylvania and Keystone Lake, Oklahoma (Dufour, 1984). Meta-analyses of freshwater and marine water determined that there was a

significant association between concentrations of enterococci to gastrointestinal illness incidence (Wade, Pai, Eisenberg, & Colford, 2003). Compared to concentrations of total coliforms and fecal coliforms, enterococci concentrations were better associated with the risk of gastroenteritis in marine beaches in New York polluted with point sources (Cabelli, Dufour, McCabe, & Levin, 1982). Meta-analyses of recreational freshwaters also determined that concentrations of *E.coli* were shown to be a better indicator of risk of gastrointestinal illness compared to concentrations of enterococci and fecal coliforms (Prüss, 1998; Wade et al., 2003). A comparison of qPCR methods determined that measurements of Entero1 were better correlated than *Bacteroidetes 16S rRNA* to waterborne illness incidence on beaches at Lake Michigan, Michigan and Indiana; and Lake Erie, Ohio (Wade et al., 2006). Additionally, compared to concentrations of culturable enterococci, measurements of Entero1 had a stronger correlation to gastrointestinal illness incidence of children visiting beaches near point sources of treated sewage at Lake Michigan, Michigan and Indiana; and Lake Erie, Ohio (Wade et al., 2008). In marine beaches experiencing non-point pollution at Mission Bay, California, the incidence of adverse health outcomes was better associated to the concentration of male-specific coliphage compared to culturable enterococci, Entero1, total coliforms and fecal coliforms (Colford et al., 2007). The risk of adverse health outcomes reported from a marine beach in Santa Monica Bay, California, with untreated storm runoff inputs was associated to concentrations of either culturable *E.coli* and enterococci (Haile et al., 1999).

1.3 Criteria and standards of fecal indicators concentrations in the United States

Decades of epidemiological studies investigating the association between adverse health outcomes and fecal indicator concentrations have guided the formation of the water quality

criteria for recreational waters in the United States. The EPA established criteria for the maximum acceptable concentrations of *E.coli* and/or enterococci concentrations in recreational freshwater and marine water. Recommended criteria were set to estimated illness rates of 32 or 36 cases per 1000 people that have primary contact with recreational waters. The recommended 30 day geometric mean for an estimated illness rate, 32/1000 (or 36/1000), is 100 colony forming units (or 126 colony forming units, CFU) of *E.coli* per 100ml in freshwater and 30 CFU (or 35 CFU) of enterococci per 100ml in freshwater and marine water (USEPA, 2012c). The criteria are enforced by the states, territories and tribal governments. Also, the states, territories, and Native American tribal lands can strengthen the EPA's water quality criteria because regional areas can have unique epidemiology situations that affect the association between risk of adverse health outcomes and fecal indicator concentrations in recreational waters (López-Pila & Szewzyk, 2000). Specifically, Michigan monitors the water quality of its recreational waters at least five times during a 30 day period during peak recreational usage (May-October). Water samples are taken from waist deep water in at least three locations in a beach area. The highest acceptable fecal indicator concentrations for full body exposure is set at a geometric average of 130 CFU of *E.coli* per 100ml over a 30 day period, or 300 CFU of *E.coli* per 100ml for measured in a single day (MDEQ, 2012). Unacceptable concentrations of fecal indicators the can cause beach closures or beach advisories. In 2013, there were 413 beaches that were monitored in Michigan (MDEQ, 2013). Only 3.3% of all of the samples taken at these beaches exceeded the water quality standard for a single day measurement (MDEQ, 2013).

1.4 Microbial source tracking

Conventional fecal indicator organisms cannot identify the source of fecal pollution in water samples. Identifying the species of fecal waste in recreation water is important because different origins of pollution are suggested to pose varying risks of adverse health effects (Soller, Schoen, Bartrand, Ravenscroft, & Ashbolt, 2010). Therefore, Microbial source tracking (MST) methods were developed in order to identify the source of fecal pollution. Host specific library independent methods are a subset of MST methods. These methods target genetic elements or chemical markers such as sterols, pharmaceuticals, detergents, and other chemicals associated with anthropogenic pollution (Santo Domingo, Bambic, Edge, & Wuertz, 2007). The presence of host associated markers in library independent MST methods is confirmed by polymerase chain reaction (PCR), while qPCR enumerates host-specific markers. qPCR methods follow a basic protocol, which includes collection of a water sample, filtration, DNA extraction, and qPCR quantification (Shanks et al., 2010). Once host-specific markers are identified, they undergo a validation process that determines the specificity, sensitivity, and quantities of the marker in non-host and host fecal matter (Harwood, Staley, Badgley, Borges, & Korajkic, 2014). However, there is lack of evidence that any marker has been completely validated.

qPCR methods to measure host-specific pollution are preferred over conventional methods for many reasons. qPCR methods have a low detection limit, high specificity, and quickly return results (Santo Domingo et al., 2007). A single sample can be analyzed for multiple indicators. The DNA extracts can be stored and used for another indicator analysis as long as DNA degradation can be accounted for (Santo Domingo et al., 2007). qPCR methods can quantify host-specific pollution independent of viability. However, many of the human “specific” markers have cross-reactivity to other species (Shanks et al., 2010). Additionally, a negative result does

not give explicit proof that the sample doesn't contain the host-specific pollution, but that its concentration is undetectable due to the detection limit, die-off, or inhibitors (Field & Samadpour, 2007). Additionally, the concentrations of some human-specific markers in primary sewage effluent can be as low as 1,000 copies per ng-DNA (Shanks, Kelty, Sivaganesan, Varma, & Haugland, 2009), which can be further diluted to undetectable concentrations in recreational waters.

Bacteroides spp. is a popular genus for genetic markers enumerated in library-independent MST methods. The genus belongs to the phylum *Bacteroidetes*, class *Bacteroidia*, order *Bacteroidales* and family *Bacteroidaceae* (Boone et al. 2001). The genus is made up of 42 known obligate anaerobic gram-negative species. *Bacteroides* spp. live in the intestinal tract of some birds and most mammals, and are excreted in fecal matter (Madigan et al., 2009). They are the dominant commensal organisms in the large intestines of humans because of their ability to ferment sugars and proteins (Madigan et al., 2009). Markers have been developed to measure human (Shanks et al., 2009), bovine (Layton et al., 2006), porcine (Mieszkin, Furet, Corthier, & Gourmelon, 2009), and bird associated pollution (Green, Dick, Gilpin, Samadpour, & Field, 2012). **Table 1.1** outlines the name and gene target of a selection of human associated *Bacteroides* spp. markers. Many of the markers highlighted in **Table 1.1** target human associated *Bacteroides* spp. *16S rRNA* (Converse, Blackwood, Kirs, Griffith, & Noble, 2009; Layton et al., 2006; Reischer, Kasper, Steinborn, Farnleitner, & Mach, 2007; Seurinck, Defoirdt, Verstraete, & Siciliano, 2005).

There are many advantages to using *Bacteroides* spp. markers to measure host associated pollution. For example, *Bacteroides* spp. do not experience extensive growth in the environment

(Ballesté & Blanch, 2010). One study suggested that fecal associated *Bacteroides* spp. *16S rRNA* are of higher epidemiological importance because they better correlate to waterborne illness incidence in four Great Lakes beaches located near treated wastewater outflows (Wade et al., 2008, 2010). Human associated *Bacteroides* spp. markers such as Human-Bac1 show promise because their concentrations better predicted the presence of *Salmonella* spp., and *E.coli* O-157 compared to concentrations of fecal coliforms and total coliforms in freshwater receiving various types and concentrations of point and non-point pollution (Savichtcheva, Okayama, & Okabe, 2007).

A description of the primer and probe sequences, and amplified sequence lengths of two human associated *Bacteroides* spp. markers, HF183 and BT-am, are in **Table 1.2**. HF18 and BT-am further described below.

Table 1.1: Marker name, species, and gene target of a selection of human associated *Bacteroides* spp. markers that are used in microbial source tracking and qPCR methods.

Marker name	**Gene target**	**Reference**
HumM2	*Bacteroides* spp. hypothetical protein, BF 3236	Shanks et al. (2009)
HumM3	*Bacteroides* spp. putative RNAP sigma factor	
HuBac	*Bacteroides* spp. *16S rRNA*	Layton et al. (2006)
HF183		Seurinck et al. (2005)
Human-Bac1		Okabe, Okayama, Savichtcheva, & Ito (2007)
BacH		Reischer et al. (2007)
BFD		Converse et al. (2009)
BT-am	*Bacteroides thetaotaiomicron α-1-6 mannanase*	Yampara-Iquise, Zheng, Jones, & Carson (2008)
Bf	*Bacteroides fragilis gyrB*	Lee & Lee (2010)

Table 1.2: Sequences and length of two human associated *Bacteroides spp.* markers.

Marker Name	Primer and Probe Sequences (5'-3')	Sequence length (bp)	Reference
HF183	ATCATGAGTTCACATGTCCG	82	Seurinck et al. (2005)
	TACCCCGCCTACTATCTAATG		
	SYBR Green I		
BT-am	CATCGTTCGTCAGCAGTAACA	63	Yampara-Iquise et al. (2008)
	CCAAGAAAAAGGGACAGTGG		
	FAM-ACCTGCTG-NFQ		

One popular marker is *Bacteroides* spp. *16S rRNA*, HF183 (Seurinck et al. 2005). The species origin of HF183 is unknown (Seurinck et al., 2005). *16S rRNA* is a highly conserved gene that encodes a non-protein that helps shape the ribosome, and initiate protein synthesis (Madigan et al. 2009). *Bacteroides* spp. *16S rRNA* has multiple copies per genome (Xu et al., 2003), and thus improves the detection limit of HF183. Although HF183 is associated with fecal pollution of human origin, it was amplified from chicken, and dog feces (Seurinck et al., 2005; Shanks et al., 2010). In freshwater microcosms spiked with human wastewater and stored at 25°C, HF183 persistence decreased with sediment presence and sunlight, and was enhanced at 15°C storage and reduced predation (Dick, Stelzer, Bertke, Fong, & Stoeckel, 2010). HF183 decayed faster than cultivable *E.coli* in freshwater microcosms spiked with human feces (Liang et al., 2012), which indicates that HF183 may only represent recent fecal pollution. However, culturable enterococci decayed faster than HF183 in marine water microcosms spiked with sewage (Walters, Yamahara, & Boehm, 2009).

It is suggested that reliable genetic markers for MST methods are from genes that are involved in a specific host-microbe interaction (Carson et al., 2005), such as *Bacteroides thetataiotaomicron α-1-6 mannanase*, BT-am (Yampara-Iquise et al., 2008). *B.thetataiotaomicron* is dominant in human fecal matter, but present in smaller concentrations in animals (Kreader, 1998). Bt-am is a single genome copy number (Yampara-Iquise et al., 2008).

Also, *α-1-6 mannanase* encodes a glycosylhydrolase that metabolizes plant based dietary mannose polysaccharides into mannose monosaccharides for host consumption (Xu et al., 2003). BT-am has cross reactivity in gulls, swine, and cat feces, but has greater host specificity than HF183 (Aslan & Rose, 2013). Concentrations of BT-am were significantly associated to culturable *E.coli* and enterococci throughout wastewater treatment processes (Srinivasan, Aslan, Xagoraraki, Alocilja, & Rose, 2011). However, few studies have evaluated its persistence in the environment.

1.5 Factors affecting the concentration of enteric bacteria in the environment

1.5.1 Factors in environmental waters

Accurately and precisely measuring fecal indicators in environmental waters is paramount to monitoring the water quality of recreational waters. Therefore, it is crucial to investigate the factors that affect the survival of enteric bacteria in the environment. For example, fecal coliforms survived longer in freshwater-sediment mesocosms spiked with wastewater, compared to enterococci, while the opposite was true for seawater-sediment mesocosms (Anderson, Whitlock, & Harwood, 2005). The inactivation of total coliforms increased with salinity, while salinity did not affect the survival of fecal coliforms (Okabe & Shimazu, 2007). The host origin of enterococi and fecal coliforms affected their decay rates in freshwater and saltwater mesocosms (Anderson et al., 2005). Salinity increased the inactivation rate of enterococci in mesocosms inoculated with dog feces, contaminated soil, and wastewater (Anderson et al., 2005). The survival of enterococci and *E.coli* decreased in the presence of autochthonous microorganisms in freshwater microcosms inoculated with raw sewage (Medema, Bahar, &

Schets, 1997). The inactivation rate of *E.coli* in freshwater microcosms spiked with raw sewage was dependent on the strain (Anderson et al., 2005).

Researchers have also evaluated the persistence of enteric markers in the environment. For example, the persistence of human associated *Bacteroidetes 16S rRNA* markers, HF183 and BacHum, were significantly different than general Bacteroides *16S rRNA*, Allbac, in freshwater-sediment microcosms spiked with raw sewage (Dick et al., 2010). The persistence of Entero1 wasn't altered in the presence of sunlight in seawater microcosms spiked with human, dog and bovine feces, while the survival of culturable enterococci decreased significantly in sunlight (Bae & Wuertz, 2009). Additionally, general *Bacteroides 16S rRNA*, GenBac, persisted longer in marine water than freshwater (Green, Shanks, Sivaganesan, Haugland, & Field, 2011).

1.5.2 Factors in sediments

It is commonly accepted that fecal pollution can affect the water quality of recreational waters. However, many indicators and pathogens do not decay in the water column. For example, *E.coli* and *Salmonella* spp. can excrete extracellular polymeric substances that bind to suspended particulate matter in order to facilitate deposition to benthic sediment (Droppo et al., 2009). Thus, sediments tend to have larger concentrations of fecal indicators such as *E.coli*, total coliforms, and enterococci compared to the water column (Pote et al., 2009). High organic carbon content and small sediment particle size were associated with increased survival of culturable *E.coli* ATCC 25922 inoculated in microcosms of coastal sediments that were submersed in estuarine water and stored in various temperatures (D L Craig, Fallowfield, & Cromar, 2004). A decrease in temperature (24° vs. 4°C) resulted in a 15-fold increase of first-order inactivation rates of culturable *E.coli* in the sediment portion of water-sediment microcosm

spiked with dairy manure (Garzio-Hadzick et al., 2010). Naturally occurring enterococci and *E.coli* decreased 2 logs in the first 30 days of storage followed by a subsequent < 1 log decrease during 30 – 90 days of storage in microcosms of surface sediments obtained near an outlet from a wastewater treatment plant in Geneva, Switzerland, and stored at 4°C (Haller, Poté, Loizeau, & Wildi, 2009). The results of a study that evaluated the persistence of fecal indicators in sediments suggested that disruption of sediments can resuspend enteric bacteria into the water column (Pote et al., 2009). Another study determined that enterococci survived longer in sediment microcosms spiked with wastewater compared to *E.coli* and total coliforms (Haller, Amedegnato, Poté, & Wildi, 2009). Concentrations of naturally occurring fecal coliforms in sediments were correlated to rainfall events in a marine beach and an estuary in Southern Australia (D.L. Craig, Fallowfield, & Cromar, 2002). Concentrations of naturally occurring fecal coliforms in sediments in a marina in Southern Australia increased in colder months with less sunlight (D.L. Craig et al., 2002). Another study determined that *E.coli* in manure could leach through soils (Brennan, O'Flaherty, Kramers, Grant, & Richards, 2010). Despite the large wealth of knowledge of the factors that are associated with the persistence of enteric bacteria in sediments, there are no recommended criteria for the monitoring of fecal indicators in sediments.

1.6 Scientific Needs

qPCR measurements of host associated and general pollution in recreational waters are being adopted by many public health departments. Currently, the storage of water samples slated qPCR is the same as conventional methods. There is a lack of knowledge of how the persistence of enteric markers is affected by storage conditions, DNA extraction methods, and storage temperatures over short term and long term storage durations. Such investigations will allow for

insight into the parameters to effectively store water samples in order to efficiently monitor the water quality of recreational waters.

It is accepted that fecal indicators persist longer in sediments than the water column. Studies have evaluated various factors that have affected the persistence of fecal indicators in sediments. However, few studies have evaluated the anthropogenic and climate factors that affect the concentrations of general pollution markers over larger time scales. Such investigations would allow for meaningful, multi-disciplinary exploration into what factors are significant influences to persistent fecal indicators using a singular index.

1.7 Research Objectives

1.7.1 Goal 1

Water quality monitoring with qPCR methods are not efficient because of the lack of optimization of the storage methods. Therefore, water quality samples spiked with raw sewage were stored in three temperatures, and in two storage conditions, i.e. liquid suspension and attached to a solid matrix, for short term and long term durations up to 28 and 366 days, respectively. DNA extraction methods were evaluated in order to determine how these methods affected indicator persistence patterns. The samples were assayed for the concentrations of three enteric markers, and mathematical relationships of the persistence of three genetic markers over time were evaluated with linear and non-linear models.

The specific objectives were to: 1) identify the mathematical relationships of enteric markers measured in water samples over short term and long term storage durations; 2) describe and compare the persistence pattern of general and human-associated enteric

markers stored over short and long durations; and 3) evaluate how temperature, attachment to a solid surface vs. liquid suspension, DNA extraction method, indicator species, and storage duration affected the persistence of DNA over time.

1.7.2 Goal 2

The Lake St. Clair watershed is small and highly populated. As part of the Great Lakes, it has a long history of water quality and anthropogenic disruptions. Fecal indicators were spiked into surface sediment from the Lake St. Clair watershed, and eighteen DNA extraction methods were evaluated in order to compare DNA extraction efficiency and identify the optimal method. Additionally, few studies have evaluated the water quality in the watershed over larger time scales. Sediment cores were collected in the watershed and assayed for fecal indicators concentrations, and sedimentary nutrients concentrations in order to determine how climatic and anthropogenic variables were associated with historical fecal pollution over $\geq$ 100 years.

The specific objectives were to: 1) compare the concentrations of fecal indicator markers spiked into sediments and extracted with various DNA extraction methods; 2) identify the optimal DNA extraction method to extract DNA from sediments in the Lake St. Clair watershed; and 3) determine how time, climate, i.e. air temperature, and discharge rate, and anthropogenic attributes, i.e. nutrient loading and population, were associated with concentrations of general pollution markers over large time tables in Lake St. Clair sediment cores.

CHAPTER 2. SHORT TERM PERSISTENCE ENTERIC BACTERIA IN WATER SAMPLES STORED ON A SOLID MATRIX AND IN LIQUID SUSPENSION

2.1 Introduction

Rapid and sensitive detection of fecal indicator bacteria (FIB) in drinking, recreational, ambient waters, and wastewater is critical for addressing pollution, providing adequate treatment solutions, and estimating potential public health risks upon exposure. Culturable methods for FIB have known limitations. For example, some FIB have no association with concentrations of *Salmonella* and *Cryptosporidium* in wastewater effluent and freshwater (Lemarchand & Lebaron, 2003), and ≥ 18 hr delays in reporting results (Wade et al., 2006). USEPA has published an alternative rapid method, Method 1611, which is culture independent, and uses quantitative polymerase chain reaction (qPCR) to measure enterococci (ENT) concentrations in recreational waters (USEPA, 2012b). Also, qPCR has been used to measure *Escherichia coli* (EC) (Frahm & Obst, 2003), and general *Bacteroidales* (USEPA, 2010b) in recreational water regardless of metabolic state. Therefore, the persistence of FIB cells as measured by their target genes is particularly important for recreational water monitoring. *Bacteroidales 16S rRNA* markers, bac-pre1, human-bac1 and pig-bac2, were shown to have increased persistence in environmental water microcosms as temperature decreased and salinity increased (Okabe & Shimazu, 2007), while the persistence of *Bacteroidales 16S rDNA* markers, GenBac3, BuniF2, and HF183, decreased in sunlight in freshwater and seawater microcosms spiked with sewage (Dick et al., 2010; Green et al., 2011). *Bacteroidales 16S rDNA*, Allbac, showed increased persistence with decreased dissolved oxygen concentration and decreased river water temperatures (Ballesté & Blanch, 2010). The persistence of ENT via the *23S rDNA* was also negatively correlated with temperature, while EC (*23S rDNA)* decayed rapidly on either side of its optimal growth temperature (37°C) in manure (M. Klein, Brown, Ashbolt, Stuetz, & Roser, 2011).

In comparative studies, Dick et al. (2010) showed that culturable EC, HF183, and BacHum had a similar persistence, with 99% reductions in concentration (T_{99}) at 2.0, 2.2, and 1.7 days, respectively, in freshwater microcosms stored at 25°C (Dick et al., 2010). Additionally, BacHum and culturable ENT quantified from sewage spiked freshwater microcosms in sunlight had comparable T_{90} (removal of 90% of initial concentration) at 1.8, and 1.0 day, respectively (Walters et al., 2009).

The first purpose of this study was to use qPCR to describe and compare the persistence of *Bacteroides thetataiotaomicron alpha mannanase* (BT-am), enterococci *23S rRNA* (ENT-23), and *E.coli uidA* (EC-uidA) measured from sewage spiked river. In a controlled bench scale study, the second purpose was to evaluate if immobilization of cells on a solid surface such as a membrane filter may extend the persistence of FIB markers. BT-am was chosen as a target because it was shown to be specific to human fecal contamination (Aslan & Rose, 2013). ENT-23 was chosen because the USEPA recommended this target for monitoring water quality in recreational waters (USEPA, 2012b). EC-uidA was selected because it has a species specific gene that is the basis of USEPA conventional cultivatable enumeration methods (USEPA, 2002). The influence of storage temperature on FIB persistence over time was also analyzed. Four degrees Celsius was chosen to mimic winter as well as an ideal storage temperature, while 27˚C mimicked storage temperature at room temperature. Also, 37°C represented an extreme storage temperature such as storage in a car without ice during the summer in a remote. Investigating the effects of storage temperature and transit time on the persistence of genetic markers from FIB measured in water samples may improve the efficiency of sample collection and storage from remote areas or low resource settings where storage on ice is not an option or extended time in transit is necessary.

The specific objectives of this study were to:

i) Identify the mathematical relationship between time (up to 28 days), and FIB persistence as measured by qPCR in sewage spiked river water stored at 4°C, 27°C and 37°C.

ii) Compare how cell attachment to a solid matrix (SM) versus liquid suspension (LS) affect persistence calculated from the experimental data, and calculate the time for 90% of FIB decay (T_{90}).

iii) Compare how DNA extraction methods affect the analysis of DNA persistence over time.

2.2 Methods

An overview of the experimental design is shown in **Figure 2.1** and described below.

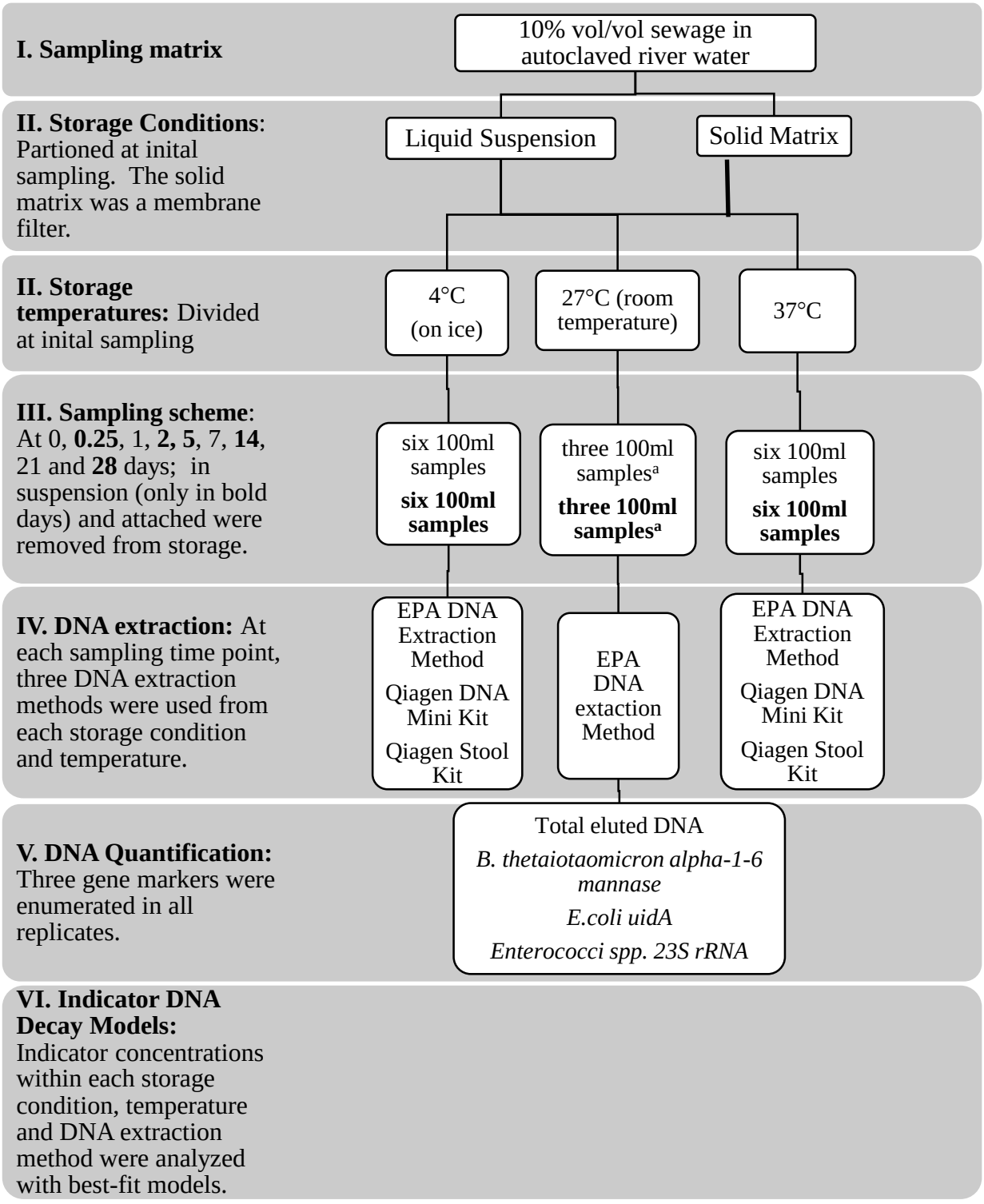

Figure 2.1: Overview of experimental design.
[a]At 27˚C, only one DNA extraction method, EPA DNA extraction method, was performed with three replicates from each storage condition.

2.2.1 Water Sample Storage

Water was collected from the Red Cedar River (East Lansing, MI) on June 6, 2011 and October 28, 2011. The water temperature, turbidity, conductivity, and pH values from the June (and October) samples were 20.5°C (10.5˚C), 8.67 NTU (2.29 NTU), 1970 µS (855 µS), and 6.95 (and 7.78), respectively. The water was autoclaved and seeded to a final concentration of 10% (vol/vol) raw sewage (City of East Lansing Sewage Treatment Plant, MI). After seeding, the samples were set up as bench scale experiments under two conditions. In the first set, the cells were left naturally in suspension (LS) as 100 ml aliquots in seventy-five sterile plastic bottles, and the second set consisted of 100 ml of seeded river water (SM) filtered onto 120 Nucleopore Track Etch polycarbonate membrane filters (0.45 µm pore size, 47 mm diameter, Whatman Inc., Piscataway, NJ). The filters were removed from the housing wet, folded in half and individually stored in sterile 50 ml centrifuge tubes, and moisture from the filter was able to collect in the tube. The LS (and SM) samples were divided into three temperature groups, 4°, 27,° and 37°C, that included 30 bottles (and 48 filters), 18 bottles (and 24 filters), and 30 bottles (and 48 filters), respectively (**Figure 2.1**). The storage temperatures were measured daily. The average daily temperatures with one standard error were 37±0.5˚C (dark incubator), 27 ± 2°C (room temperature in the dark), and 4 ± 2°C (storage in a cooler with ice packets replaced every 24 hrs).

2.2.2 Sample processing and DNA extraction

Cells from the liquid suspension (LS) samples were filtered using 0.45 um Nucleopore Track Etch membrane filters after days 0, 0.25, 2, 5, 14 and 28 days from all storage temperatures. Cells were recovered and DNA was extracted in the same way as the SM samples.

Fifteen solid matrix (SM) filters were also processed on days 0, 0.25, 1, 2, 5, 7, 14, 21 and 28. At the two extreme temperatures (4°C and 37°C), duplicate filters were folded four times aseptically with sterile forceps, and placed into the extraction tubes for the following methods: Qiamp Stool DNA Mini Kit®, STOOL (Qiagen Inc, Valencia, CA); Qiamp DNA Mini Kit, MINI (Qiagen Inc, Valencia, CA); and USEPA Method 1611, EPA-DNA (USEPA, 2012b). Triplicate filters from 27°C were folded four times aseptically and placed into extraction tubes of EPA-DNA. A preliminary elution was incorporated for MINI in order to detach DNA from the filter by first transferring the filter into a sterile tube with 50 ml phosphate buffered saline (PBS) and were vortexed for 2 min at 3200 rpm. The supernatant was centrifuged for 20 min at 8000 x g and 48 ml of the supernatant was discarded. The remaining supernatant (2 ml) was transferred into a sterile microfuge tube and proceeded with MINI according to the manufacturer's instructions. EPA-DNA and STOOL methods followed the published and manufacturer's protocols, respectively. The MINI, EPA-DNA and STOOL final DNA elution volumes were 400, 350 and 200 µL, respectively. Total DNA concentrations were measured with Nano-Drop ND-1000 (Thermo Fisher Scientific Inc, Waltham, MA) for all DNA extracts before storage at -80°C.

2.2.3 qPCR quantification of three indicators

Concentrations of EC-uidA, ENT-23 and BT-am were measured in all DNA extracts with qPCR using the Roche LightCycler® 480 Instrument (Roche Applied Science, Indianapolis, IN). When inhibition was noted in the qPCR results (i.e. analytical replicates differed in marker concentration by 1 log or an analytical replicate wasn't quantifiable), the DNA extracts were diluted 5x and rerun. Each qPCR reaction volume, including the standards, was 20 µl (5 µl-sample and 15 µl qPCR reagents). The reagents included 10 µl LightCycler 480 Probes

MasterMix (Roche, Indianapolis, IN), indicator probes sequences, reverse and forward primers **(Table 2.1)**, 2 mg/ml bovine serum album (BSA), 1 mM $MgCl_2$ (EC-uidA only), and sufficient nuclease free water to bring reaction volume to 20 µl. The BSA concentrations in BT-am, ENT-23 and EC-uidA were: 2, 1 and 1 µl, respectively. The BT-am qPCR program was previously described (Srinivasan et al., 2011). The EC-uidA qPCR program started with a 10 min cycle at 95°C, then 40 cycles of 30 sec at 95°C, 30 sec at 58°C, and 10 sec at 72°C. The ENT-23 qPCR program started with a 10 min cycle at 95°C then 40 cycles of 10 sec at 95°C, 30 sec at 60°C, and 15 sec at 72°C. Duplicates of positive controls (ATCC cells or DNA extracts depending on the assay), method blanks (sterile phosphate buffered saline water as template for each storage time interval), and no template control (nuclease free water as template) were analyzed in each qPCR run. All samples were analyzed in duplicates.

The indicator concentrations were calculated from their respective qPCR standard curves. Genomic DNA was extracted from overnight cultures of *Enterococcus faecalis* ATCC strain 19433 and *E.coli* ATCC strain 15597 using MINI. The eluted DNA was serially diluted 1:10 to create 6 dilution steps for the ENT-23 and EC-uidA standard curves. The standard curve for *B. thetataiotaomicron* was prepared from genomic DNA (ATCC, 29148) with 1:10 serial dilutions of the DNA to create 6 dilution steps. Each dilution was analyzed in triplicate. A new standard curve run was prepared after every four qPCR runs. The average efficiencies, r^2 values, and the threshold cycle values of the lowest detected amplification (and representative copies/rxn) of the qPCR standard curves of ENT-23, EC-uidA and BT-am are described in **Table 2.2**.

EC-uidA and BT-am have one copy per genome. Therefore, each quantified sequence represented one *E.coli* and *B. thetataiotaomicron* cellular equivalent (CE), respectively. There

are an estimated average four copies of ENT-23 per *Enterococcus faecalis* cell (USEPA, 2010a), and represented one CE in this study.

The qPCR analytical replicates from each sample were averaged and converted into cell equivalents (CE) per 100 ml-water sample. Specifically, the qPCR measurements of the 5 µl template were a fraction of the elution volume. The concentration of the marker in copies/1 µl was multiplied to the elution volume, which in the case of EPA-DNA and STOOL represented the 100 ml sample. In the case of MINI, it represented 10 ml of the sample, and then the concentration in the elution volume was multiplied by 10 to represent the 100 ml sample.

Table 2.1: Fecal indicator species, targeted gene, primer sequences, probe sequences, and amplified sequence length (bp) of the qPCR genetic markers in this study.

FIB Species	**Gene**	**Primer and probe sequences (5'-3')**	**Primer (and probe) concentration in qPCR reaction matrix**	**Amplified sequence size (bp)**	**Reference**
B.thetataiotaomicron (BT)	*α-1-6 mannanase* (BT-am)	CATCGTTCGTCAGCAGTAACA CCAAGAAAAAGGGACAGTGG FAM-CAGCAGGT-NFQ[a]	0.3 µM (0.1 µM)	63	Yampara et al. (2008)
enterococci (ENT)	*23SrRNA* (ENT-23)	AGAAATTCCAAACGAACTTG CAGTGCTCTACCTCCATCATT 6FAM- GGTTCTCTCCGAAATAGCTTTAGGGCTA- TAMRA	0.5 µM (0.2 µM)	91	Frahm and Obst (2003)
E.coli (EC)	*uidA* (EC-uidA)	CAATGGTGATGTCAGCGTT ACACTCTGTCCGGCTTTTG 6FAM- TTGCAACTGGACAAGGCACCAGC-BBQ	0.5 µM (0.2 µM)	163	Srinivasan et al (2011)

[a]Roche Universal Probe Libraries (UPL) Probe 62.

Table 2.2: Average efficiency, R^2, lowest detected dilution's CT (respresentative copies/5 μl), and DNA extraction method specific detection limits from EC-uidA, ENT-23, and BT-am from *E.coli*, enterococci and *B.thetataiotaomicron*, respectively.

Genetic marker (Species)	**R^2 values**	**Average efficiency (95% confidence interval)**	**Lowest detected dilution's CT value (representative copies/5 μl)**	**DNA extraction method specific detection limits (and indicator persistence as measured by Log N/N_0) [a]**
BT-am (BT)	0.96	95.7% (± 5.00%)	37.60 (183)	MINI: 3.05 x 10^3 copies/100 ml (Log N/N_0 = -1.56) EPA-DNA: 2.67 x 10^2 copies/100 ml[b] (Log N/N_0 = -2.31) STOOL: 1.53 x 10^2 copies/100 ml (Log N/N_0 = -2.29)
ENT-23 (ENT)	0.98	97.33% (± 2.88%)	36.31 (3.72)	MINI: 7.16 x 10^3 copies/100 ml (Log N/N_0 = -3.46) EPA-DNA: 6.27 x 10^2 copies/100 ml[b] (Log N/N_0 = -3.87) STOOL: 3.58 x 10^2 copies/100 ml (Log N/N_0 = -1.83)
EC-uidA (EC)	0.93	97% (± 1.50%)	35.85 (8.95)	MINI: 1.46 x 10^5 copies/100 ml (Log N/N_0 = -1.61) EPA-DNA: 1.28 x 10^4 copies/100 ml[b] (Log N/N_0 = -2.49) STOOL: 7.32 x 10^3 copies/100 ml (Log N/N_0 = -1.91)

[a]The detection limits from a 100 ml water sample are based on the Qiagen Qiamp Mini Kit (MINI), USEPA Crude DNA Extraction Method (EPA-DNA) and Qiagen Qiamp DNA Stool Mini Kit (STOOL) final elution volume and the initial concentrated sample for MINI.

[b]DNA extracts from EPA-DNA were diluted 5x prior to qPCR, the BT-am, ENT-23 and EC-uidA detection limits were increased to $1.34x10^3$, $3.14x10^4$, and $6.4x10^4$ representative copies/1 μl, respectively.

2.2.4 Statistical Analyses and Persistence Modeling

A two-way ANOVA and post-hoc Tukey's HSD test using SPSS 17.0 (SPSS, Inc., Chicago, IL) were performed on the total DNA concentrations from samples stored at 4˚C to determine if DNA extraction method and/or attachment status affected total DNA concentrations over time. The low temperature experiments were used to minimize the effects of other variables. Total DNA concentrations from all 4°C samples were transformed to ng/ 100 ml-sample. Subsequently, total DNA concentrations within each treatment were transformed into log (C_t/C_o), where C_t was the DNA concentration after t days of storage and C_o was the DNA concentration at initial sampling (t = 0 days).

After evaluation of the total DNA concentrations and linear regression analysis, the EPA-DNA eluted samples were chosen to evaluate the genetic markers' persistence patterns for all storage conditions and temperatures. Explanation of why EPA-DNA was chosen is given in the Results section. Reporting the non-detect data at their detection limit was chosen because it cannot be reasonably assumed that a value below the detection limit equals zero concentration of the indicator. Additionally, the USEPA has advised that non-detect data be reported at the detection limit (USEPA, 1991) in order to best represent the measurements.

The fractional persistence of the target concentration at time, t, to initial concentration, Log_{10} (N/N_0), where N was the CE/ 100 ml-sample after **t** days in storage, and N_o was the CE/ 100 ml- sample at t = 0 was calculated for each experimental condition (attachment status), temperature, and indicator. These data were fit to established decay models using maximum likelihood estimation in a modelling program in R 3.0.1 (R Development Core Team, 2013) that was provided by Drs. Kyle Enger and Jade Mitchell. Since there are few studies to inform the selection of appropriate decay models for qPCR CE data, seventeen models that are commonly

used to model bacterial decay under various conditions were initially evaluated (http://qmrawiki.canr.msu.edu). **Table S2.1** highlights the equations and references of all models employed in the data analysis tool. Using the Bayesian information criterion (BIC), these models were narrowed down to five that produced adequate fits across the data sets in order to produce tractable comparative information. BIC values were used to evaluate the best fit models because it takes into account the number of observed data points, the number of parameters of the model, the observed data, and the maximum likelihood of the function.

The evaluated best fit models were: first-order exponential decay model (Chick, 1908), biphasic exponential decay (Corradini, Normand, & Peleg, 2007), two-stage (Juneja, Huang, & Marks, 2006), log-logistic (Juneja, Marks, & Mohr, 2003), and Gompertz 3-parameter (Gil, Miller, Brandão, & Silva, 2011). **Table 2.3** describes these models and their properties. Differences less than 2 in BIC values are not considered strong evidence for model selection. Therefore, if the smallest BIC was < 2 units from the BIC of the biphasic model, then the biphasic model was chosen. The predicted time needed for 1 $\log_{10}$ reduction (T_{90}) was also calculated by substituting $-1.0 = \text{Log}_{10}(N/N_0)$, and solving for t in each equation. The standard error values of the T_{90} values were not calculated as they were not provided in the model.

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Table 2.3: List of best fit models represented in the datasets, their equations, and properties.

Equation Name	Equation [a]	Eqn. Properties	Reference
First-order exponential decay model (ep)	$Log(^{N}/_{N_0}) = Log(e^{-kt})$	Linear, negative slope	Chick (1908)
Biphasic exponential decay model (bi3)	**for 0 ≤ t <x:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-k_1 t})$ **for t ≥ x:** $Log(^{N}/_{N_0}) = Log(e^{-k_1 t + k_2 * (t-x)})$	Linear, negative slope, slope changes at t=x, the break point	Carret et al. (1991)
Two-stage model (jm1)	$Log(^{\mathrm{N}}/_{\mathrm{N}_0}) = Log(1 - (1 - e^{-k_1 t})^{k_2 t})$	Nonlinear, concave	Juneja et al (2006)
Log-logistic model (jm2)	$Log(^{\mathrm{N}}/_{\mathrm{N}_0}) = -Log(1 + e^{\,(k_1 + k_2 * ln\,(t))})$	Nonlinear, convex	Juneja et al (2003)
Gompertz- 3 parameter (gz3)	$\mathrm{Log}\left(^{\mathrm{N}}/_{\mathrm{N}_0}\right) = \mathrm{Log}\left(10^{(\mathrm{k_1} * \mathrm{e}^{(-\mathrm{e}(^{-\mathrm{k_2} * \mathrm{e}^{1}(\mathrm{k_3} - \mathrm{t})}/_{\mathrm{k_1} + 1}))})}\right)$	Nonlinear	Gil et al. (2011)

[a] K_n = decay constant; t = time in hours; N/N_0 = persistence ratio.

Multiple linear regression analyses of data sets containing all CE for all targets: a) fractional persistence of all experimental data, b) fractional persistence separated by temperature, and c) fractional persistence separated by indicator species, were completed in order to determine which independent variables significantly affected the observed fractional persistence, Log_{10} (N/N_0). These analyses were completed using SPSS 17.0 (SPSS, Inc., Chicago, IL). Linear regression evaluated the following equation:

$$Log_{10}\left(N/N_0\right) = b_S \times S + b_E \times E + b_C \times C + b_T \times T + b_I \times I + b_o,$$

where b_0 = Log_{10} (N/N_0) intercept, b with a letter subscript are coefficients of the following independent variables: S = storage time (0 – 28 days), E = extraction method (MINI, EPA-DNA and STOOL), C = storage condition (membrane filter, MS or liquid suspension, LS), T = storage temperature (4°, 27° and 37°C), and I = indicator (EC-uidA, BT-am and ENT-23). The class variables, E, C, and I, were transformed into to numerical representations starting with 1 for the first case listed and n+1 for the remaining cases in the order that they appeared in the text above. Log_{10} transformation of the dependent variable was chosen because N/N_0 was shown to have major deviations from normal in the probability plot of the linear regression standardized residuals. Additionally, equal variance of the independent variables was observed with box plots. Non-significant variables were not removed from the dataset in order to obtain a complete descriptive analysis of the dataset.

2.3 Results

2.3.1 Summary of results

Water and sewage were collected on two dates. The concentrations of BT, EC and ENT measured from EPA-DNA samples at t = 0 days at 4°/ 37°C (and 27°C), and were: 1.71 x 10^5

(8.06×10^5), 2.85×10^7 (2.54×10^8), and 1.44×10^7 CE/ 100 ml (3.92×10^7 CE/ 100 ml), respectively. Inhibition from qPCR was only noted in samples extracted with EPA-DNA. After dilution, the detection limit increased 5 x. The detection limits measured in cells/ 100 ml and Log (N/N_0) were dependent on the DNA extraction method (**Table 2.2**). The DNA extraction methods affected the persistence of the genetic markers measured in Log N/N_0 (**Table 2.4**). Because the samples were 10% sewage, the percentage of samples below detection limit for ENT-23 and EC-uidA was low regardless of the temperature, attachment status or extraction method, reaching only as high as 4% of the samples. However, between 0 - 45% of the samples were below the detection limit for BT-am as the original concentration in the samples were lower, and non-detects prevailed at the higher temperatures and longer incubation times.

2.3.2 Comparison of concentrations of total DNA after storage at 4°C.

The influence of time and attachment status on concentrations of total DNA was examined for the three extraction methods (**Figure 2.2A-B**) at 4°C storage. There appeared to be increases of total DNA concentrations measured from EPA-DNA (and MINI) at t = 7, and 21 days (t=14 days). The largest decrease in total DNA concentrations over time was seen in liquid suspension and on solid matrix when extracted with STOOL (**Figure 2.2A-B**). Compared to the initial sampling, the average percent of the concentrations of total DNA (and standard error, SE) from the remaining storage interval time points from each of the three extraction methods, MINI, EPA-DNA and STOOL, (combining the LS and SM treatment data) were 96% (20 SE), 63% (11 SE), and 11% (36 SE), respectively. There was no significant difference between the average total DNA concentrations based on attachment status ($p = 0.984$), but there was a significant difference in the average total DNA concentrations between extraction methods ($p = 0.026$). The

average total DNA concentrations using STOOL was significantly smaller than MINI ($p = 0.024$), but not EPA-DNA ($p = 0.207$). This indicated that the persistence data could not be pooled for analysis across extraction methods.

EPA-DNA experienced 0, 6, and 0% of non-detects from BT-am, ENT-23 and EC-uidA, respectively. The method was also relatively easy, provided a low detection limit **(Table 2.2)**, and the total DNA concentrations appeared to be statistically equivalent to the other extraction methods in the ANOVA analysis. The one drawback was inhibition, which was handled with 5x dilution for these sets of experiments. Thus, the results below were focused on the samples extracted using EPA-DNA.

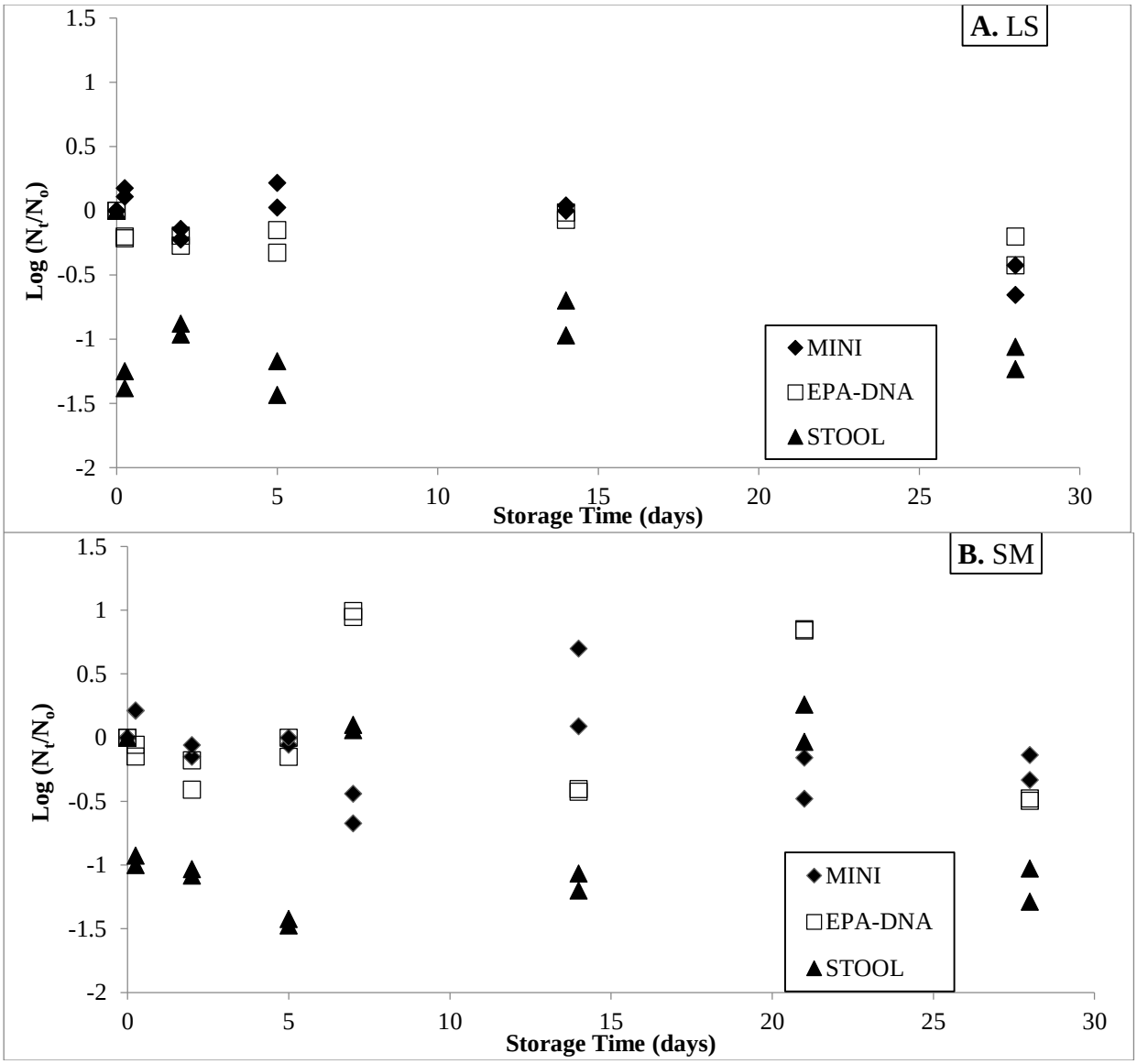

Figure 2.2A-B: Total DNA concentrations [a] from the 4˚C storage conditions, A) liquid suspension (LS) and B) solid matrix (SM), and eluted from three DNA extraction methods [b].
[a] DNA concentrations were reported in log N_t/N_o, where N_t was the DNA concentration normalized to ng/100 ml-sample after t days in storage and N_o was the DNA concentration in ng/100 ml-sample at initial sampling
[b] Qiamp Stool DNA Mini Kit (STOOL, ▲), Qiamp DNA Mini Kit (MINI, ♦), and the EPA DNA Crude Extraction Method (EPA-DNA, □).

2.3.3 Model comparisons for analyzing FIB DNA persistence

The parameters of the best fit models for all treatments are outlined in **Table 2.4**, along with the corresponding BIC values, and T_{90} values of the best fit models. The equations with the

parameters specific to the chosen best fit models are in **Table S2.2**. **Table S2.3** highlights the BIC values of the rejected models. The following models were fit: biphasic exponential decay model (bi3), first-order exponential decay model (ep), two-stage model (jm1), log-logistic model (jm2), and Gompertz 3-parameter model (gz3), representing 61.5, 11, 5.5, 5.5, and 5.5% of the 18 data sets, respectively (**Table 2.4)**. No single model could was chosen as the best fit for all of the data. However, two linear models, bi3 and ep, fit 72.5% of the data, excluding the following datasets: 4°C BT in LS (gz3), 27°C ENT in SM (jm1), and 37°C EC in LS (jm2).

Table 2.4: Selected models, T_{90} values (days) and persistence parameters for *E.coli* (EC), enterococci (ENT), and *B.thetataiotaomicron* (BT) stored at 4°, 27°, and 37°C under two storage conditions, in suspension (LS) or attached (SM).

Temp (°C)	Indicator	Storage condition	Model[a]	BIC value[b]	predicted T_{90} (days)	Parameters		
						k_1	k_2	k_3 (or break point, x, in days[c])
4	EC	SM	bi3*	14.3	> 28[d]	-0.01	-0.12	(14.0)
		LS	bi3*	13.5	12.4	0.19	0.20	(16.8)
	ENT	SM	bi3	2.7	> 28[d]	-0.02	-0.31	(20.4)
		LS	ep	11.5	> 28[d]	0.06	-	-
	BT	SM	bi3	6.0	27.0	-0.04	-0.23	(11.8)
		LS	gz3	11.2	9.6	-2.48	0.27	5.9
27	EC	SM	bi3*	5.2	> 28[d]	0.08	0.07	(14.0)
		LS	bi3*	6.5	7.1	0.78	0.69	(2.4)
	ENT	SM	jm1	2.6	> 28[d]	0.04	0.24	-
		LS	bi3*	-1.8	8.4	1.98	1.83	(0.6)
	BT	SM	bi3	-25.8	18.0	0.21	0.17	(9.4)
		LS	bi3	0.3	1.8	1.32	1.27	(4.1)
37	EC	SM	ep	27.9	> 28[d]	0.17	-	-
		LS	jm2	12.0	6.0	-0.78	1.66	-
	ENT	SM	bi3*	13.9	> 28[d]	0.02	-0.09	(14.0)
		LS	bi3	-10.5	6.5	0.50	0.32	(3.6)
	BT	SM	bi3*	19.5	3.2	0.72	0.66	(5.3)
		LS	bi3*	20.0	1.0	2.30	2.23	(2.1)

[a]The models' abbreviations: first-order exponential decay (ep), biphasic exponential decay (bi3), two-stage (jm1), log-logistic (jm2), and Gompertz 3-parameter (gz3).
[b]Value of the Bayesian information criterion (BIC) for the model. *Denotes if the BIC value of bi3 was ± 2 of the smallest reported BIC value of the available models.
[c]The breakpoint, t = x, is starting point when the slope of the biphasic exponential decay equation (bi3), $Log(N/N_0) = Log(e^{-k_1 t + k_2 * (t-x)})$, included both decay constants, k_1 and k_2.
[d]The experiment duration was 28 days.

2.3.4 Comparison of the three FIB and effects of attachment and temperature.

FIB persisted longer when attached (SM) than in suspension (LS) for all indicators and temperatures **(Figure 2.3A-F)**. The least persistent indicator was BT regardless of the storage condition or temperature. The most to least persistent markers in LS for all temperatures were: ENT > EC > BT.

The T_{90} values were calculated from all models (**Table 2.4**). Overall, the data showed that larger T_{90} values were observed for CE stored on a SM, although the effect was modulated by temperature. At 4°C, ENT had $T_{90} > 28$ days in LS and on a SM. EC and BT had $T_{90} > 28$, and 27 days, respectively, on a SM, and in LS, the $T_{90} = 12.4$, and 9.7 days, respectively.

At 27°C, the $T_{90} > 28$ days for EC, and ENT on a SM, while BT had $T_{90} = 18$ days. If the samples were stored in LS at 27°C, $T_{90} = 8.4$, 7.1 and 1.8 days for ENT, EC and BT, respectively. At 37°C, ENT, EC and BT on a SM had $T_{90} > 28$, > 28, and 3.2 days, respectively, and when in LS T_{90} was 6.5, 6.0, and 1.0 days, respectively.

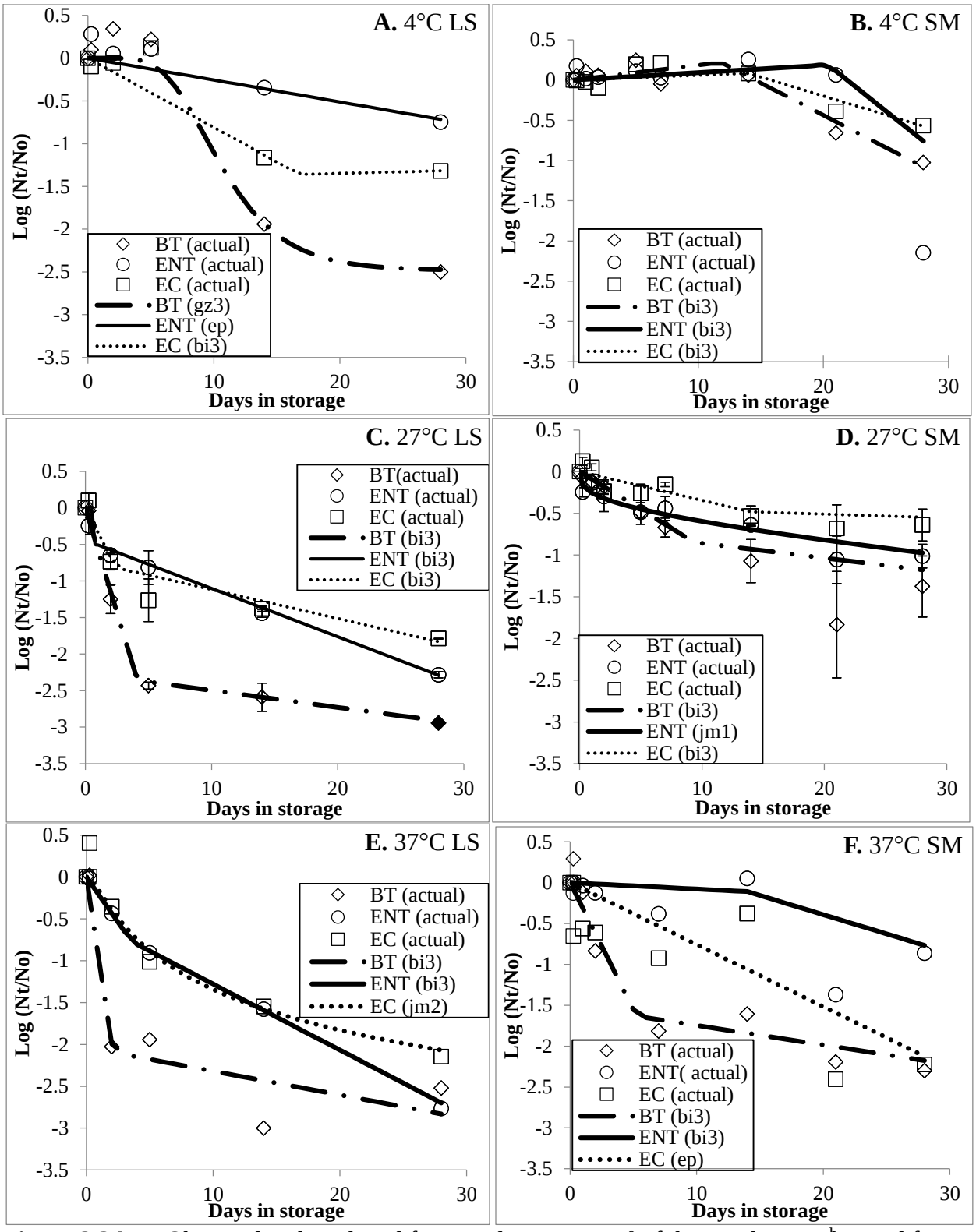

Figure 2.3A-F: Observed and predicted fractional persistence [a] of three indicators [b] stored for up to 28 days at 4˚C **(A-B)**, 27˚C **(C-D)**, and 37˚C **(E-F)** storage temperatures in two storage conditions [c].

Figure 2.3 (cont'd).
[a] The persistence is measured in log (N/N_o), where N is the concentration of the indicator after t days in storage, and N_0 is the initial concentration of the indicator.
[b] The diamond (◊), circle (○), and square (□) labels represent the observed BT, ENT, and EC log (N/N_o) persistence, respectively. The data points below the detection limit are filled in. Duplicate replicates for each indicator were measured in the 4˚ and 37˚C storage treatments, and the averages of each time point are displayed. In the 27˚C treatments, the indicators were measured in triplicate, and the averages were displayed in the graphs along with bars that represent one standard error. The lines represent the following best fit models: first-order exponential decay (ep), exponential decay (bi3), two-stage (jm1), log-logistic (jm2), and Gompertz 3-parameter (gz3).
[c] The graphs on the left side represent in suspension storage condition (LS), while the right represents attached storage condition (SM).

2.3.5 Multiple Linear Regression Analyses.

Linear regression analyses evaluated the statistical significance of storage time, extraction method, indicator type, temperature, and storage condition on the fraction of persistence of the indicators, Log_{10} (N/N_0) (**Table 2.5**). The three analyses, complete dataset (n = 585), indicator specific (n = 195 for each indicator), and temperature specific (n = 117 for 4° and 37°C; and n = 234 for 27°C) sub-datasets showed that the independent variables did not exhibit colinearity, thus indicating statistical independence. Analysis of the complete dataset determined that increasing storage time and storage in LS decreased fractional persistence ($p < 0.001$). The coefficients of the linear regression equations and their standard errors for each dataset are listed in **Table 2.6,** while the R^2 values of the equations are listed in **Table 2.5**. The fractional persistence of EC and ENT datasets were significantly affected by time ($p_{EC} < 0.001$; $p_{ENT} < 0.001$), extraction method ($p_{EC} = 0.047$; $p_{ENT} < 0.001$), and storage condition ($p_{EC} = 0.027$; $p_{ENT} = 0.022$). Additionally, the fractional persistence of BT was significantly affected by time ($p_{BT} < 0.001$), attachment ($p_{BT} < 0.001$), and temperature ($p_{BT} = 0.001$). For the 4°C dataset, time ($p_{4C} = 0.001$) significantly affected the fractional persistence. For 27°C and 37°C sub-datasets, time

($p_{27C} < 0.001$; $p_{37C} < 0.001$), attachment ($p_{27C} < 0.001$; $p_{37C} < 0.001$), and type of indicator ($p_{27C} < 0.001$; $p_{37C} < 0.001$) significantly affected fractional persistence.

Table 2.5: Multiple linear regression analyses determined how the observed fractional persistence ratios [a], were influenced by: storage time measured in hours, storage conditions [b], extraction method [c], genetic markers [d] and storage temperature [e].

		Independent variables' correlation strength (and p-value)				
Data set (sample size)	**R^2**	**Storage time- *S***	**Extraction method- E**	**Storage attachment- C**	**Storage temperature- T**	**Indicator- I**
All data (n = 585)	0.227	-0.393 ($p < 0.001$)	0.056 ($p = 0.142$)	-0.189 ($p < 0.001$)	-0.033 ($p = 0.385$)	0.174 ($p < 0.001$)
4˚C (n = 234)	0.058	-0.220 ($p_{4C} = 0.001$)	0.042 ($p_{4C} = 0.522$)	-0.070 ($p_{4C} = 0.288$)	N/A	0.049 ($p_{4C} = 0.452$)
27˚C (n = 117)	0.677	-0.605 ($p_{27C} < 0.001$)	N/A	-0.463 ($p_{27C} < 0.001$)	N/A	0.308 ($p_{27C} < 0.001$)
37˚C (n = 234)	0.407	-0.520 ($p_{37C} < 0.001$)	0.108 ($p_{37C} = 0.048$)	-0.219 ($p_{37C} < 0.001$)	N/A	0.276 ($p_{37C} < 0.001$)
BT (n = 195)	0.374	-0.492 ($p_{BT} < 0.001$)	-0.067 ($p_{BT} = 0.260$)	-0.284 ($p_{BT} < 0.001$)	-0.203 ($p_{BT} = 0.001$)	N/A
ENT (n = 195)	0.266	-0.365 ($p_{ENT} < 0.001$)	0.324 ($p_{ENT} < 0.001$)	-0.147 ($p_{ENT} = 0.022$)	0.056 ($p_{ENT} = 0.386$)	N/A
EC (n = 195)	0.165	-0.350 ($p_{EC} < 0.001$)	-0.138 ($p_{EC} = 0.047$)	-0.153 ($p_{EC} = 0.027$)	0.046 ($p_{EC} = 0.508$)	N/A

[a] log N/N_0 (normalized to cell/100 ml-sample), where

[b] in suspension-LS or attached-SM

[c] Qiagen Mini-MINI, EPA Method 1611-EPA-DNA, and Qiagen Stool-STOOL

[d] EC-uidA, ENT-23, and BT-am

[e] 4˚C, 27˚C, and 37˚C

Table 2.6: A list of the coefficients in the linear regression equation [a] that evaluated the correlations of the independent variables to fractional persistence [b].

	Coefficients of the independent variables (and their standard errors) of the linear regression equations					
Data set (sample size)	**Storage time- *S***	**Extraction method- E**	**Storage attachment- C**	**Storage temperature- T**	**Indicator- I**	**Y intercept- b_0**
All data (n = 585)	-0.104 (0.010)	0.225 (0.134)	-1.012 (0.201)	- 0.008 (0.007)	0.552 (0.120)	-1.378 (0.495)
4˚C (n = 234)	-0.028 (0.008)	0.064 (0.99)	-0.427 (0.167)	N/A	0.076 (0.100)	-0.785 (0.383)
27˚C (n = 117)	-0.052 (0.005)	N/A	-0.813 (0.094)	N/A	0.321 (0.056)	0.157 (0.183)
37˚C (n = 234)	-0.057 (0.006)	0.107 (0.073)	-0.508 (0.122)	N/A	0.374 (0.073)	-0.839 (0.288)
BT (n = 195)	-0.015 (0.004)	-0.101 (0.089)	-0.633 (0.132)	-0.015 (0.004)	N/A	0.585 (0.288)
ENT (n = 195)	-0.045 (0.008)	0.546 (0.108)	-0.368 (0.159)	0.005 (0.005)	N/A	-1.143 (0.349)
EC (n = 195)	-0.034 (0.007)	-0.186 (0.093)	-0.311 (0.140)	0.003 (0.005)	N/A	0.211 (0.303)

[a] $Log_{10}\left({}^{N}/_{N_0}\right) = b_S \times S + b_E \times E + b_C \times C + \mathrm{b_T} \times T + \mathrm{b_I} \times I + b_o$,

[b] $Log_{10}\left({}^{N}/_{N_0}\right)$

2.4 Discussion

Our study compared the persistence of CE of three naturally occurring FIB using qPCR from sewage spiked into river water, and the influence of attachment to a solid surface at various temperatures. Bacterial cells affixed to a membrane filter instead of remaining in a liquid suspension exhibited significantly slower CE and DNA target degradation regardless of indicator species or storage temperature. Similarly, culturable FIB immobilized onto soil particles survived longer in aquatic environments (Wheeler Alm et al., 2003). Attachment to solid surfaces possibly protects the cell wall from degradation, and therefore, its DNA target, yet the mechanism, type of matrix, and strength of attachment have not been fully explored.

Historically, a first-order linear exponential model was commonly used to describe the rate of inactivation using cultivation (Liang et al., 2012). More recently, nonlinear models have provided a better fit to bacterial persistence (Coroller, Leguerinel, Mettler, Savy, & Mafart, 2006). Our study is one of the few that have evaluated various linear and non-linear models to best describe the persistence patterns of FIB CE using qPCR in water under various conditions. Linear models, particularly the biphasic exponential decay (bi3) and first-order exponential decay (ep), represented the majority of the treatments in our study (**Table 2.4**), indicating that these models may off a "good enough" fit to model the persistence of genetic markers measured in water samples. Biphasic decay has been observed previously. For example, the term "two-staged model", best described by bi3 in our study, was previously coined to describe data that fit two first-order linear equations with two unique slopes (Crane & Moore, 1986). Two-staged models also described the DNA persistence of *Bacteroidales, Salmonella enterica*, enterococci, and *E.coli* in manure amended soil at 25˚C (Rogers et al., 2011). Klein et al. (2011) combined two first-order exponential decay equations to better evaluate "biphasic persistence". This curve

addressed the persistence of an EC marker in composted manure at 37°C (M. Klein et al., 2011). Rogers et al. (2011) offered reasons for the biphasic behavior: 1) microbes die off at a rapid rate until the carrying capacity of the environment is approached; and 2) the true presence of two sub-populations with different decay rates. More persistence studies using molecular methods in various matrices are needed in order to better analyze the biphasic nature of CE and DNA persistence.

Our study also included gz3, jm1, and jm2 models that evaluated BT in LS at 4°C, ENT on SM at 27°C, and EC in LS at 37°C, respectively. These models are non-linear survival curves frequently used to describe thermal inactivation. Jm1 was derived on the assumption that a cell must be "hit" a number of times, k2, and the probability of being hit is described by the function, exp (-k2 x t) (Juneja et al., 2006). Jm2 was used to describe survival data with an indication that as time increases, decay begins to slow, which can be witnessed in datasets that have "shoulder" or display a "lag effect". Gz3 is an empirical model, and has been shown to be capable of quantifying behavior under non-isothermal conditions (Gil et al., 2011). Gz3 does not assume a constant decay rate, but it can be used to model decay rates that change over time related to variable temperature.

The models in our study were used to calculate the days needed to achieve 90% reduction (T_{90}) in cellular equivalent concentrations. Our values were compared to values from previous studies that measured the persistence of *Bacteroides*, enterococci and *E.coli* in liquid microcosms, and on solid matrices (**Table 2.7**). Previous research also confirmed that enterococci markers persisted longer than *Bacteroides* spp. markers (Rogers et al., 2011). *Bacteroides* spp. markers are relatively less persistent than other markers (**Table 2.7**). The shortened persistence may be a result of the inability of *Bacteroides* spp. to grow in the

environment (Kreader, 1998), or cell degradation due to its sharpened death rate in aerobic conditions. EC persistence in our study agreed with a previous study of *E.coli uidA*, Eco, in composted manure microcosms stored at 20˚ and 37˚C (M. Klein et al., 2011). Enterococci *23S rRNA*, Entero1, had a T_{90} = 9 days in 7% raw sewage spiked seawater stored at 17˚C (Walters et al., 2009). Similarly, T_{90} > 32 days was reported for Entero1 in sewage spiked beach sand drained with seawater at 22°C (Yamahara, Sassoubre, Goodwin, & Boehm, 2012). Increased enterococci persistence on substrates like beach sand have indicated that these substrates may act as aquatic FIB reservoirs (Yamahara, Walters, & Boehm, 2009). Human associated *Bacteroidales 16S rRNA*, HF183, had a calculated T_{90} = 2.59 days, thus faster degradation, in 5% sewage spiked river water stored in a suspension stored in the dark at slightly higher temperature, 14°C, compared to our study (Gilpin et al., 2013).

Table 2.7: Comparison of genetic markers from *Bacteroidales*, *E.coli*, and enterococci in various storage conditions, and T_{90} values from our study and previous studies.

***E.coli* genetic markers**				
T_{90} (days)	**Target gene (marker name)**	**Storage condition**	**Storage temp (˚C)**	**Reference**
56	*23S rRNA* (EPA-EC23S)	Beef manure amended soil, 80% field capacity moisture	25	Rogers et al. (2011)
>28[d]	*uidA* (EC-uidA)	SM[c]: 10% (vol/vol) SPRW	4	Our study
			27	
27	*uidA (*Eco)	Compost manure	20	Klein et al. (2011)
13.19	*uidA* (EC-uidA)	SM: 10% (vol/vol) SPRW	37	This study
12.35		LS[b]: 10% (vol/vol) SPRW	4	
7.08		LS: 10% (vol/vol) SPRW	27	
6.5	*uidA (*Eco)	Compost manure	37	Klein et al. (2011)
6.00	*uidA* (EC-uidA)	LS: 10% (vol/vol) SPRW	37	Our study
1.71	*uidA (*Eco)	Compost manure	50	Klein et al. (2011)
Enterococci genetic markers				
T_{90} (days)	**Target gene (marker name)**	**Storage condition**	**Storage temp (˚C)**	**Reference**
>32[d]	Enterococci *23S rRNA (*Entero1)	Sewage spiked beach sand drained with seawater	22	Yamahara et al. (2012)
>28[d]	Enterococci (*23S rRNA, ENT-23*)	LS: 10% (vol/vol) SPRW	4	Our study
		SM: 10% (vol/vol) SPRW	4	
			27	
			37	
8.35		LS: 10% (vol/vol) SPRW	27	
6.46		LS: 10% (vol/vol) SPRW	37	
4.34	Enterococci (*23S rRNA*, Entero1)	Beef manure amended soil, 80% field capacity moisture	25	Rogers et al. (2011)
***Bacteroidales* spp. genetic markers**				
T_{90} (days)	**Target gene (marker name)**	**Storage condition**	**Storage temp (˚C)**	**Reference**
27.00	*B.thetaiotaomicron alpha- mannanase (*BT-am)	SM: 10% (vol/vol) SPRW	4	Our study

Table 2.7 (cont'd).

>24[d]	human associated *Bacteroidales 16S rRNA (*HF183*)*	LS: 10% (vol/vol) SPRW	4	Seurinck et al. (2005)
17.96	*B.thetaiotaomicron alpha- mannanase (*BT-am)	SM: 10% (vol/vol) SPRW	27	Our study
10	human associated *Bacteroidales 16S rRNA (*HF183*)*	LS: 10% (vol/vol) SPRW	12	Seurinck et al. (2005)
9.60	*B.thetaiotaomicron alpha- mannanase (*BT-am)	LS: 10% (vol/vol) SPRW	4	Our study
5.35	*Bacteroidales 16S rRNA* (GenBac3)	Beef manure amended soil, 80% field capacity moisture	25	Rogers et al. (2011)
3.22	*B.thetaiotaomicron alpha- mannanase (*BT-am)	SM: 10% (vol/vol) SPRW	37	Our study
2.59	human associated *Bacteroidales 16S rRNA (*HF183*)*	LS: 5% SPRW	14	Gilpin et al. (2013)
1.75	*B.thetaiotaomicron alpha- mannanase (*BT-am)	LS: 10% (vol/vol) SPRW	27	Our study
1.00	*B.thetaiotaomicron alpha- mannanase (*BT-am)	LS: 10% vol/vol) SPRW	37	Our study

[a] SPRW = sewage spiked river water
[b] LS = in suspension
[c] SM = attached
[d] T_{90} value was larger than experiment duration.

There are persistence studies that differed from our results. Rogers et al. (2011) found that *E.coli (23S rRNA)* was more persistent than enterococci (*23S rRNA)* in 80% moisture beef manure amended soil at 25˚C. Our study determined that T_{90} = 10 days for BT at 4°C in suspension was less than the calculated T_{90} > 24 days for HF183 at 4°C in 10% (vol/vol) sewage spiked freshwater microcosms (Seurinck et al., 2005). The extended HF183 persistence could be due to the variability of abiotic and/or biotic factors in the freshwater and/or sewage or the gene

itself. Roger et al (2011) also observed that T_{90} values from enterococci and *E.coli 23S rRNA* measured from beef manure amended soil with 80% moisture stored at 25°C were less than half of the T_{90} values in both 27°C and 37°C storage conditions in ENT and EC in our study, excluding EC at 27°C SM. The decreased persistence in Rogers et al. (2011) could have been due to the storage of the manure at -20°C for up to six months before application on soil, thus decreasing the biotic nature of the manure. The variation of persistence of markers in our study and previous investigations indicates that there is currently a lack of understanding of the mechanisms of intra- and inter-species CE/DNA persistence.

The accuracy and precision of evaluations of DNA persistence can be improved with extraction methods that elute high quantity and quality DNA. Our ENT and EC persistence data (**Table 2.5**) agreed with previous research that determined DNA extraction methods produced highly variable qPCR derived marker concentrations due to biased recovery (Inceoglu, Hoogwout, Hill, & van Elsas, 2010). The differences in fractional persistence by extraction method in our study could be due to increased resistance to lysis treatments due to the peptidoglycan structure in enterococci (Mahalanabis, Do, ALMuayad, Zhang, & Klapperich, 2010). Our results supported the use of physical shearing DNA extraction methods without a column purification step (i.e. EPA-DNA) as an efficient approach for gram negative and positive organisms, and environmental water samples (Tang, Gao, Zhu, Chao, & Qin, 2009).

Further exploration of the observed persistence data in our study showed that after 1 day of storage on a SM at 4°C, the observed fractional persistence of BT, ENT and EC from EPA-DNA was 1.29, 1.04, and 0.94, respectively. At 27°C, the observed fractional persistence of BT, ENT and EC stored for 1 day on a SM and extracted with EPA-DNA were 0.76, 0.73, and 1.12,

respectively. These results indicated that storage on SM for up to 1 day at 4°C, and 27°C could maintain the CE with ≤ 25, and ≤ 31% difference, respectively, from initial measurements.

Our results support filtering water and transporting samples to the laboratory on a membrane to increase CE persistence. Storage for 28 days on a solid matrix at low temperature (i.e. 4°C) predicted decreases of BT, ENT, and EC concentrations by 92, 83 and 73%, respectively, of initial CE concentrations. Overall, at higher temperatures, 27°C and 37°C, and on a solid matrix, a 90% decrease in CE concentrations of BT occurred until 11 and 4 days of storage, respectively. Our data suggest that water samples could be extended up to 27 days without a < 90% decrease of CE concentrations of BT, ENT and EC if the samples were initially filtered and stored on a solid matrix at low temperature.

APPENDIX

Table S2.1: Best fit equations and their references that evaluated the persistence of the indicators in this study.

Model	**Equation** [a]	**Reference**
First-order exponential decay (ep)	$Log(^{N}/_{N_0}) = Log(e^{-kt})$	Chick (1908)
One parameter logistic (lg1)	$Log\left(^{\mathrm{N}}/_{\mathrm{N}_0}\right) = \log(2) - log(1 + e^{(k_1 * t)})$	Kamau, Doores, & Pruitt (1990)
Two parameter logistic (lg2)	$Log\left(^{\mathrm{N}}/_{\mathrm{N}_0}\right) = -Log(1 + e^{(k_1 * (t - k_2))})$	Peleg (2006)
Exponential damped decay (epd)	$Log\left(^{N}/_{N_0}\right) = e^{(-k_1 * t * e^{(-k_2 * t)})}$	Cavalli-Sforza, Menozzi, & Strata (1983)
Two-stage (jm1)	$Log(^{\mathrm{N}}/_{\mathrm{N}_0}) = Log(1 - (1 - e^{-k_1 t})^{k_2 t})$	Juneja et al (2006)
Log-logistic (jm2)	$Log(^{\mathrm{N}}/_{\mathrm{N}_0}) = -Log(1 + e^{\,(k_1 + k_2 * ln\,(t))})$	Juneja et al (2003)
Gompertz (gz)	$Log(^{\mathrm{N}}/_{\mathrm{N}_0}) = Log(e^{-{k_1}/{k_2}^{*(e^{k_2 * t})-1}})$	Wu, Hung, & Tsai (2004)
Weibull (wb)	$Log\left(^{\mathrm{N}}/_{\mathrm{N}_0}\right) = Log(10^{(-\left(t/k_1\right)^{k_2})}$	Coroller et al. (2006)
Log-normal (ln)	$Log(^{\mathrm{N}}/_{\mathrm{N}_0}) = \mathrm{Log}(1 - \mathrm{plnorm}(\mathrm{t}, k_1, k_2)$	(Aragao, Corradini, Normand, & Peleg, 2007)
Gamma (gam)	$Log\left(^{\mathrm{N}}/_{\mathrm{N}_0}\right) = Log(1 - pgamma(t, k_1, k_2))$	Hogg & Craig (1978)
Biphasic exponential decay with preset breakpoint at 72 hrs (bi)	**for 0 ≤ t < 72:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-k_1 t})$ **for t ≥ 72:** $Log(^{N}/_{N_0}) = Log(e^{-k_1 t + k_2 * (3 - x)})$	Carret et al. (1991)
Biphasic exponential decay (bi3)	**for 0 ≤ t <x:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-k_1 t})$ **for t ≥ x:** $Log(^{N}/_{N_0}) = Log(e^{-k_1 t + k_2 * (t - x)})$	Carret et al. (1991)
Double exponential decay (dep)	$Log\left(^{\mathrm{N}}/_{\mathrm{N}_0}\right) = Log(\,k_3 * e^{(-k_1 * t)} + (1 - k_3) * e^{(-k_2 * t)})$	Peleg (2006)
Gompertz 3-parameter (gz3)	$\mathrm{Log}\left(^{\mathrm{N}}/_{\mathrm{N}_0}\right) = \mathrm{Log}\left(10^{(\mathrm{k}_1 * \mathrm{e}^{(-\mathrm{e}(^{-\mathrm{k}_2 * \mathrm{e}^1 (\mathrm{k}_3 - \mathrm{t})}/_{\mathrm{k}_1 + 1}))})}\right)$	Gil et al. (2011)
Gompertz-Makeham (gzm)	$Log(^{\mathrm{N}}/_{\mathrm{N}_0}) = Log(e^{-k_3 * t - \left(k_1/k_2\right) * (e^{k_2 * t} - 1)})$	Gavrilov & Gavrilov (1991)

Table S2.1 (cont'd).

Sigmoid-A (sA)	$Log(^{N}/_{N_0}) = Log(10^{^{-k_1*t}/_{((1+k_2*t)*(k_3-t))}})$	Peleg (2006)
Sigmoid-B (sB)	$Log(^{N}/_{N_0}) = LogLog(10^{(^{-k_1*t^{k_3}}/_{k_2+t^{k_3}})})$	Peleg (2006)

[a]K_n = decay constant; t = time in hours; N/N_0 = persistence ratio.

Table S2.2: The best fit models, and the equations that represented the persistence patterns of the three indicators [a] stored at three temperatures, and two storage conditions [b].

Temp (°C)	Indicator	Storage condition	Model [c]	Equation [d]
4	EC	SM	bi3*	**for 0 ≤ t < 14.0:** $Log\ (N/N_0) = Log\ (e^{0.01*t})$ **for t ≥ 14.0:** $Log(N/N_0) = Log(e^{0.01*t-\ 0.12*(t-14.0)})$
		LS	bi3*	**for 0 ≤ t < 16.8:** $Log\ (N/N_0) = Log\ (e^{-0.19})$ **for t ≥ 16.8:** $Log(N/N_0) = Log(e^{-0.19\ *t+\ 0.20\ *(t-\ 16.8)})$
	ENT	SM	bi3	**for 0 ≤ t < 20.4:** $Log\ (N/N_0) = Log\ (e^{0.02*t})$ **for t ≥ 20.4:** $Log(N/N_0) = Log(e^{0.02*t-0.31*(t-20.4)})$
		LS	ep	$Log(N/N_0) = Log(e^{-0.06*t})$
	BT	SM	bi3	**for 0 ≤ t < 11.8:** $Log\ (N/N_0) = Log\ (e^{0.04*t})$ **for t ≥ 11.8:** $Log(N/N_0) = Log(e^{0.04*t-\ 0.23*(t-11.8)})$
		LS	gz3	$\mathrm{Log}\left(N/N_0\right) = \mathrm{Log}\left(10^{(-2.48*e^{(-e(^{-0.27*e^1(5.9-t)}/_{-2.48}+1)})}\right)$
27	EC	SM	bi3*	**for 0 ≤ t < 14.0:** $Log\ (N/N_0) = Log\ (e^{-0.08*\ t})$ **for t ≥ 14.0:** $Log(N/N_0) = Log(e^{-0.08*t+0.07*(t-14.0)})$
		LS	bi3*	**for 0 ≤ t < 2.4:** $Log\ (N/N_0) = Log\ (e^{-0.78*t})$ **for t ≥ 2.4:** $Log(N/N_0) = Log(e^{-0.78*t+0.69*(t-2.4)})$
	ENT	SM	jm1	$Log(N/N_0) = Log(1-(1-e^{-0.04*t})^{0.24*t})$
		LS	bi3*	**for 0 ≤ t < 0.6:** $Log\ (N/N_0) = Log\ (e^{-1.98*t})$ **for t ≥ 0.6:** $Log(N/N_0) = Log(e^{-1.98*t+1.83*(t-0.6)})$

Table S2.2 (cont'd).

	BT	SM	bi3	**for 0 ≤ t < 9.4:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-0.21*t})$ **for t ≥ 9.4:** $Log(^{N}/_{N_0}) = Log(e^{-0.21*t+0.17*(t-9.4)})$
		LS	bi3	**for 0 ≤ t < 4.1:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-1.32*t})$ **for t ≥ 4.1:** $Log(^{N}/_{N_0}) = Log(e^{-1.32*t+1.27*(t-4.1)})$
37	EC	SM	ep	$Log(^{N}/_{N_0}) = Log(e^{-0.17*t})$
		LS	jm2	$Log(^{\mathrm{N}}/_{\mathrm{N}_0}) = -Log(1 + e^{\,(-0.78+1.66*ln\,(t))})$
	ENT	SM	bi3*	**for 0 ≤ t < 14.0:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-0.02*t})$ **for t ≥ 14.0:** $Log(^{N}/_{N_0}) = Log(e^{-0.02*t-0.09*(t-14.0)})$
		LS	bi3	**for 0 ≤ t < 3.6:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-0.50t})$ **for t ≥ 3.6:** $Log(^{N}/_{N_0}) = Log(e^{-0.50*t+0.32*(t-3.6)})$
	BT	SM	bi3*	**for 0 ≤ t < 5.3:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-0.72*t})$ **for t ≥ 5.3:** $Log(^{N}/_{N_0}) = Log(e^{-0.72*t+0.66*(t-5.3)})$
		LS	bi3*	**for 0 ≤ t <x 2.1:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-2.30*t})$ **for t ≥ 2.1:** $Log(^{N}/_{N_0}) = Log(e^{-2.30*t+2.23*(t-2.1)})$

[a] Indicators included: *Bacteroides thetatiaotaomicron*, BT; enterococci, ENT; and *Escherichia coli*, EC.
[b] Conditions included liquid suspension, LS; or attached to a solid matrix, SM.
[c] The models' abbreviations: first-order exponential decay (ep), biphasic exponential decay (bi3), two-stage (jm1), log-logistic (jm2), and Gompertz 3-parameter (gz3).
[d] Time, t, is measured in hours.
*Denotes if the BIC value of bi3 was ± 2 of the smallest reported BIC value of the available models.

Table S2.3: BIC values and the standard deviations of the 17 best-fit models analyzed for best fit of the indicators [a] that were stored in two conditions [b] at three temperatures for up to 28 days.

	BIC values (and standard deviations)					
Model [c]	**4°C**					
	BT		**ENT**		**EC**	
	LS	**SM**	**LS**	**SM**	**LS**	**SM**
ep	19.00 (0.87)	19.26 (0.55)	11.49 (0.47)	18.66 (0.53)	14.25 (0.58)	15.08 (0.44)
lg1	19.08 (0.88)	17.53 (0.50)	11.85 (0.48)	18.17 (0.52)	15.84 (0.67)	14.57 (0.43)
lg2	24.04 (1.15)	11.34 (0.31)	19.40 (0.78)	32.74 (0.92)	16.51 (0.61)	27.60 (0.69)
epd	20.53 (0.86)	12.87 (0.34)	12.99 (0.46)	23.11 (0.54)	12.41 (0.43)	13.68 (0.36)
jm1	20.41 (0.85)	9.30 (0.28)	13.27 (0.47)	5.31 (0.23)	13.07 (0.46)	13.06 (0.35)
jm2	15.20 (0.55)	9.29 (0.28)	12.87 (0.45)	5.31 (0.23)	13.03 (0.59)	13.21 (0.35)
gz	20.80 (0.87)	21.46 (0.55)	13.28 (0.47)	20.85 (0.53)	16.04 (0.46)	13.72 (0.37)
wb	22.62 (0.88)	29.72 (0.87)	13.44 (0.43)	5.35 (0.23)	12.77 (0.59)	13.55 (0.35)
ln	34.05 (2.64)	9.70 (0.29)	12.95 (0.45)	5.31 (0.23)	23.54 (0.46)	13.12 (0.35)
gam	20.63 (0.87)	10.26 (0.30)	13.26 (0.47)	5.32 (0.22)	12.99 (1.10)	13.21 (0.44)
bi	22.66 (0.88)	23.67 (0.55)	15.11 (0.47)	23.05 (0.53)	17.88 (0.46)	19.48 (0.33)
bi3	23.12 (0.10)	5.98 (0.21)	14.29 (0.44)	2.69 (0.17)	13.51 (0.59)	14.31 (0.44)
dep	22.59 (0.87)	14.73 (0.55)	14.29 (0.44)	20.48 (0.53)	13.52 (0.41)	19.46 (0.33)
gz3	11.24 (0.34)	8.03 (0.23)	12.35 (0.37)	25.41 (0.61)	13.73 (0.42)	17.07 (0.10)
gzm	37.27 (2.97)	23.65 (0.55)	15.08 (0.47)	23.05 (0.53)	30.80 (1.73)	19.47 (0.44)
sA	30.25 (2.42)	32.03 (0.88)	14.85 (0.46)	16.90 (0.37)	14.98 (0.46)	24.70 (0.59)
sB	11.26 (0.34)	18.49 (0.57)	14.84 (0.63)	17.27 (0.53)	17.39 (0.82)	19.29 (0.55)
Model [c]	**27°C**					
	BT		**ENT**		**EC**	
	LS	**SM**	**LS**	**SM**	**LS**	**SM**
ep	29.45 (2.09)	12.09 (0.37)	14.03 (0.58)	15.31 (0.44)	18.23 (0.82)	5.50 (0.26)
lg1	30.59 (2.29)	16.71 (0.48)	16.75 (0.72)	17.81 (0.51)	20.10 (0.96)	7.57 (0.29)
lg2	30.67 (1.99)	17.13 (0.43)	12.78 (0.45)	10.42 (0.30)	19.14 (0.76)	20.71 (0.53)
epd	20.00 (0.82)	-19.17 (0.06)	10.60 (0.37)	11.61 (0.32)	11.34 (0.40)	3.55 (0.20)

Table S2.3 (cont'd).

jm1	24.45 (1.19)	2.57 (0.19)	-0.75 (0.15)	2.64 (0.19)	12.67 (0.44)	5.28 (0.23)
jm2	15.45 (0.56)	-13.89 (0.08)	11.90 (0.42)	6.18 (0.24)	6.08 (0.26)	3.99 (0.21)
gz	11.37 (0.40)	14.29 (0.37)	10.25 (0.36)	10.79 (0.31)	20.02 (0.82)	5.22 (0.25)
wb	20.88 (0.88)	-1.77 (0.15)	3.86 (0.22)	3.95 (0.21)	9.85 (0.34)	4.85 (0.21)
ln	20.59 (0.74)	-9.00 (0.11)	8.95 (0.33)	5.67 (0.23)	6.94 (0.28)	3.74 (0.21)
gam	24.00 (1.14)	1.96 (0.19)	-0.27 (0.15)	2.71 (0.19)	12.30 (0.43)	5.24 (0.22)
bi	44.38 (1.00)	16.56 (0.37)	17.67 (0.58)	19.81 (0.45)	21.81 (0.82)	9.95 (0.26)
bi3	0.25 (0.14)	-25.78 (0.04)	-1.81 (0.11)	5.25 (0.20)	6.51 (0.23)	5.23 (0.20)
dep	3.29 (0.17)	-21.15 (0.05)	-1.78 (0.11)	5.16 (0.20)	7.47 (0.25)	5.69 (0.20)
gz3	9.75 (0.30)	-9.91 (0.09)	12.47 (0.38)	14.86 (0.10)	16.94 (0.10)	6.75 (0.22)
gzm	33.03 (2.09)	16.48 (0.37)	5.93 (0.22)	5.31 (0.20)	18.22 (0.61)	9.55 (0.25)
sA	15.36 (0.48)	22.64 (0.52)	6.30 (0.23)	6.66 (0.21)	7.96 (0.26)	15.93 (0.36)
sB	8.35 (0.27)	-20.36 (0.05)	5.43 (0.21)	6.15 (0.21)	9.17 (0.29)	5.791 (0.20)
Model [c]	**37°C**					
	BT		**ENT**		**EC**	
	LS	**SM**	**LS**	**SM**	**LS**	**SM**
ep	31.52 (2.48)	29.53 (1.18)	11.55 (0.47)	12.52 (0.41)	18.49 (0.84)	27.94 (1.07)
lg1	32.43 (2.68)	31.41 (1.33)	15.42 (0.65)	12.00 (0.40)	20.36 (0.98)	28.39 (1.10)
lg2	32.70 (2.36)	30.43 (1.10)	14.21 (0.50)	23.74 (0.72)	21.77 (0.95)	29.66 (1.05)
epd	22.46 (1.00)	21.65 (0.63)	6.56 (0.27)	13.21 (0.37)	13.47 (0.48)	29.86 (1.06)
jm1	26.61 (1.42)	25.01 (0.78)	2.33 (0.19)	13.44 (0.38)	17.71 (0.68)	29.22 (1.02)
jm2	20.74 (0.87)	20.52 (0.59)	10.24 (0.36)	13.32 (0.38)	12.00 (0.42)	30.64 (1.11)
gz	31.80 (2.19)	19.82 (0.57)	6.10 (0.26)	14.60 (0.41)	13.18 (0.46)	30.02 (1.07)
wb	23.92 (1.18)	22.82 (0.69)	0.22 (0.16)	14.20 (0.43)	17.74 (0.58)	29.50 (1.03)
ln	22.65 (1.02)	21.68 (0.64)	5.07 (0.24)	13.46 (0.38)	13.21 (0.46)	30.10 (1.08)
gam	26.32 (1.39)	24.60 (0.76)	1.46 (0.17)	13.54 (0.38)	17.38 (0.66)	29.27 (1.02)
bi	55.22 (1.00)	34.21 (1.00)	15.47 (0.48)	16.68 (0.41)	22.10 (0.84)	32.19 (1.00)
bi3	20.02 (0.71)	19.51 (0.49)	-10.54 (0.06)	13.87 (0.34)	14.05 (0.43)	30.73 (0.98)
dep	20.36 (0.73)	20.02 (0.50)	-6.25 (0.08)	15.33 (0.38)	14.41 (0.44)	30.99 (0.99)

Table S2.3 (cont'd).

gz3	24.62 (0.10)	21.36 (0.55)	15.62 (1.22)	13.95 (0.34)	16.28 (0.52)	30.56 (0.97)
gzm	30.15 (1.64)	29.25 (0.10)	-4.45 (0.09)	16.05 (0.39)	22.08 (0.84)	35.20 (0.10)
sA	21.35 (0.79)	22.06 (0.57)	21.11 (0.77)	15.05 (0.37)	15.51 (0.48)	33.32 (1.15)
sB	20.04 (0.71)	20.49 (0.52)	2.72 (0.17)	15.54 (0.38)	14.04 (0.43)	31.61 (1.03)

[a] Indicators included: *Bacteroides thetatiaotaomicron*, BT; enterococci, ENT; and *Escherichia coli*, EC.

[b] Conditions included liquid suspension, LS; or attached to a solid matrix, SM.

[c] The models evaluated were: first-order exponential decay (ep), one parameter logistic (lg1), two parameter logistic (lg2), exponential damped (epd), two-stage (jm1), log-logistic (jm2), Gompertz (gz), Weibull (wb), log-normal (ln), Gamma (gam), biphasic exponential decay with preset breakpoint at 3 days (bi), biphasic exponential decay (bi3), double exponential decay (dep), Gompertz 3-parameter (gz3), Gompertz-Makeham (gzm), Sigmoid-A (sA), and Sigmoid-B (sB). Bi was included in the R programming platform, but its BIC value wasn't compared to the other models because its breakpoint was pre-determined.

CHAPTER 3. COMPARISON OF THE LONG TERM PERSISTENCE OF ENTERIC BACTERIA ATTACHED TO A SOLID MATRIX AND IN LIQUID SUSPENSION

3.1 Introduction

Fecal pollution in recreational and other environmental waters is an ongoing concern for public health. In the United States, environmental waters that consistently do not meet water quality standards are assigned to total maximum daily loads (TMDL) programs. These programs identify, and measure fecal indicator bacteria (FIB) loading from point and non-point sources, and then create remediation plans in order to restore the environmental to meet water quality standards.

The development of TMDL programs for FIB is dependent on the many factors that affect their concentrations in recreational waters. One such factor is the survival of FIB in an aquatic environment. Previous research has determined that culturable populations of FIB, *Escherichia coli* (EC) and enterococci (ENT), were persistent in the aquatic ecosystem, and can even form reservoirs in sediments (Haller, Poté, et al., 2009). These reservoirs can impede the progress of remediation efforts.

Quantitative polymerase chain reaction (qPCR) methods are now used to measure concentrations of FIB independent of metabolic state and allow for source tracking. Monitoring and modeling of the long term persistence of genetic markers can help interpret long term pollution problems and the ensuing remediation efforts. Culturable populations of EC and ENT were shown to have $T_{90} > 60$ days in microcosms containing sediment covered with freshwater spiked with sewage effluent and stored at 10°C (Pote et al., 2009). Another study determined that molecular markers, ENT *23S rDNA* (Entero1), EC *23S rDNA* (EPA-EC23S) and general *Bacteroidales 16S rDNA* (GenBac3), in swine amended soil stored at 10°C had $T_{90} = 62$, 10, and 52 days, respectively (Rogers et al., 2011). However, there are few studies that have reported the persistence of FIB in various storage conditions on longer time scales.

Previously, we reported that the persistence of cellular equivalents of genetic markers from EC-uidA, ENT-23, and human specific *Bacteroides thetataiotaomicron alpha-mannanase* (BT-am) via qPCR measurements from sewage spiked river water stored at 4°C for up to 28 days was significantly affected by attachment to a membrane, time in storage, and type of indicator (**Chapter 2**). We also determined that the biphasic decay model predicted that the T_{90} of BT-am stored attached on a membrane filter (T_{90} = 27 days) was twice as large as the predicted value in liquid suspension (T_{90} = 13 days) at 4°C. The above study sparked the interest into the further investigation of the persistence of FIB on a longer time scale. Therefore, the purpose of this investigation was to:

a) Describe the long term persistence (up to 366 days) of three enteric molecular markers, *Bacteroides thetaiotaomicron 1,6 alpha-mannanase*, enterococci *23S rDNA*, and *E.coli uidA*, stored in liquid suspension or attached to a solid matrix, at a low temperature (4°C).
b) Compare the types of models and their parameters to data from previous short term persistence analyses.

3.2 Methods

The methods are described in detail in **Chapter 2.2**. Below is a brief an overview of the methods.

3.2.1 Sample preparation and storage

Water from the Red Cedar River was collected on May 1, 2013 and autoclaved. The river water was spiked with 10% (vol/vol) raw sewage from the East Lansing Wastewater Treatment. Attached samples (SM) were prepared by filtering 100ml of the spiked river water, and

producing 39 separately seeded Nucleopore Track Etch polycarbonate membrane filters (0.45 μm pore size, 47 mm diameter, Whatman Inc., Piscataway, NJ). Additionally, 4.2 L of 10% (vol/vol) sewage spiked river water was divided into 14 liquid suspension samples (LS). All samples were stored in the dark at 4°C.

3.2.2 Sample processing and DNA extraction

Triplicate samples from SM, and one LS sample bottle were removed on days 0, 31, 61, 93, 128, 155, 187, 219, 248, 279, 306, 337, and 366 (SM only) days. Then, LS samples were membrane filtered in 100 ml increments onto 3 separate membrane filters. DNA extraction from all samples was performed using the EPA DNA Crude Extraction Method (USEPA, 2012b). The concentrations of the total eluted DNA (ng/μl) was recorded, and the samples were stored at -80°C. The EPA DNA Crude Extraction Method (EPA-DNA) was chosen because it was determined in **Chapter 2** that the concentrations of total eluted DNA were statistically larger than two commercial methods, and had the best detection limit.

3.2.3 qPCR quantification of three indicators

Molecular markers from *Bacteroides thetataiotaomicron alpha mannanase* (BT-am), *Escherichia coli uidA* (EC-uidA), and *Enterococcus* spp. *23S rDNA* (ENT-23) were chosen because they were previously used to measure general and human specific (BT-am only) pollution (Frahm & Obst, 2003; Srinivasan et al., 2011; Yamahara et al., 2012). The primers, probes, amplified sequence length, and qPCR protocols for the markers are described in **Table 2.1**. Preparation of the qPCR reagents and the standard curves were completed as described in **Chapter 2.2.3**. For all standard curves, the R^2 was greater than 0.96.

Table 3.1: The qPCR reaction efficiency, CT values of the lowest detected dilutions (and representative copies/5 µl), and detection limits of EC-uidA, ENT-23, and BT-am [a].

Genetic marker (Species)	**Efficiency**	**Average lowest detected dilution's CT value (representative copies/5 µl)**	**Detection limits (and indicator persistence as measured by Log N/N_0)** [b]
BT-am (BT)	97%	36.13 (82)	1.34×10^3 copies/100 ml (Log N/N_0 = -1.71)
ENT-23 (ENT)	100%	34.62 (19.7)	6.90×10^3 copies/100 ml (Log N/N_0 = -3.77)
EC-uidA (EC)	102%	38.60 (1.87×10^3)	6.51×10^5 copies/100 ml (Log N/N_0 = -2.69)

[a] The genetic markers EC-uidA, ENT-23 and BT-am represent *Escherichia coli* (EC), enterococci (ENT), and *Bacteroides thetataiotaomicron* (BT), respectively.
[b] DNA extracts from EPA-DNA were diluted 5x prior to qPCR, the detection limits of BT, EC, and ENT were transformed to ≤ 2.68 x 10^2, ≤ 1.30 x 10^5, and ≤ 1.38 x 10^3 CE/ 100 ml-water sample.

3.2.4 Persistence modeling and statistical analyses

The statistical and modeling analyses considered all data points. Any time points that were represented by three non-detect replicates were evaluated at the detection limit in the persistence modeling and statistical analyses, as advised by the USEPA (USEPA, 1991). Further description of the number of non-detects in the datasets are discussed in the Results Section. As described in **Chapter 2.2.4**, a model fitting tool in R (R Development Core Team, 2013) was used for data analysis to choose one models out of 17 models. The full list of models are listed in **Table S2.1**. Best fit was analyzed with the Bayesian Information Criterion (BIC). The BIC value takes into account the number of parameters in the model while evaluating the model's fit to the data. The model with the smallest BIC value within each dataset was chosen. However, if the smallest BIC value was < 2 of bi3, then bi3 was chosen. The following models were chosen as best fits for the persistence data: biphasic exponential decay, bi3 (Carret, Flandrois, & Lobry, 1991); and log-logistic model, jm2 (Juneja, Marks, Mohr, et al., 2003). Descriptions of the parameters of best fit

models are outlined in **Table 2.3**. The parameters of the models were used to calculate the T_{90}, and T_{99}, the time required for 1 and 2 $\log_{10}$ reductions of the indicator concentration**,** respectively. The predicted T_{90} (or T_{99}), the time needed for 1 $\log_{10}$ (or 2 $\log_{10}$) reduction was also calculated by substituting -1.0 (or -2.0) = Log_{10} (N/N_0), and solving for t in each equation. The standard errors of the T_{90} and T_{99} values were not provided in the modeling program and were not calculated.

A student's T-test was performed in order to determine if the initial and final concentrations of cellular equivalents (CE) per 100 ml-water sample of BT, ENT, and EC on a SM were statistically different. This statistical analysis was evaluated with SPSS 22.0 (SPSS, Inc., Chicago, IL).

Multiple linear regression analyses was performed in order to determine how the fractional persistence was affected by time, storage condition and indicator species in the following datasets: a) the fractional persistence of replicates from all indicators, and b) fractional persistence specific to the indicator. The analyses were performed with SPSS 22.0 (SPSS, Inc., Chicago, IL) with the following equation:

$$\text{Log}\left(N_t/N_0\right) = b_S \times S + b_C \times C + b_I \times I + b_o.$$

with N/N_0 = fractional persistence; b_0 = log (N/N_0) intercept; b with a letter subscript represented the coefficients of the following independent variables: S = time in storage (0 - 366 days); C = storage condition (LS or SM); and I = indicator (BT, EC, and ENT). The dependent variable, fractional persistence, described the indicator's concentration (N, CE/ 100 ml-water sample) at t days in relation to its initial concentration (N_0). The class variables transformed to numerical designations starting at 1 for the class variable closes to the beginning of the alphabet, and increasing by one integer for each class within the variable. The independent variables that were

not significantly associated to fractional persistence were not removed from regression equation in order to fully explain the associations of the variables to the fractional persistence.

3.3 Results

3.3.1 Summary of molecular marker persistence

In total, 72 samples were analyzed by qPCR for each indicator after storage for up to 333, and 366 days in LS, and SM, respectively. At t = 187, and 337 days, there were instances of inhibition (defined as technical duplicates from qPCR differing by > 1 log) from samples that measured EC-uida on a SM, and ENT-23 in LS, respectively. Overall, 55, 24, and 0% of the samples were below the detection limit for BT-am, EC-uida, and ENT-23, respectively. At $t \geq 61$ days, all samples that measured BT in LS were non-detects, except for one sample at t = 219 days (**Figure 3.1A**). The average concentration for BT at t = 219 days was above the detection limit, but was within one standard error of the detection limit (**Figure 3.1A**). The standard error bars for ENT in LS and all indicators on a SM showed that variability of the measurements of the indicator concentration increased with time in storage (**Figure 3.1A-B**). There was an observable increase in the observed concentration of BT, and EC (but not ENT) in the SM samples after 64, and 279 days, respectively (**Figure 3.1B**).

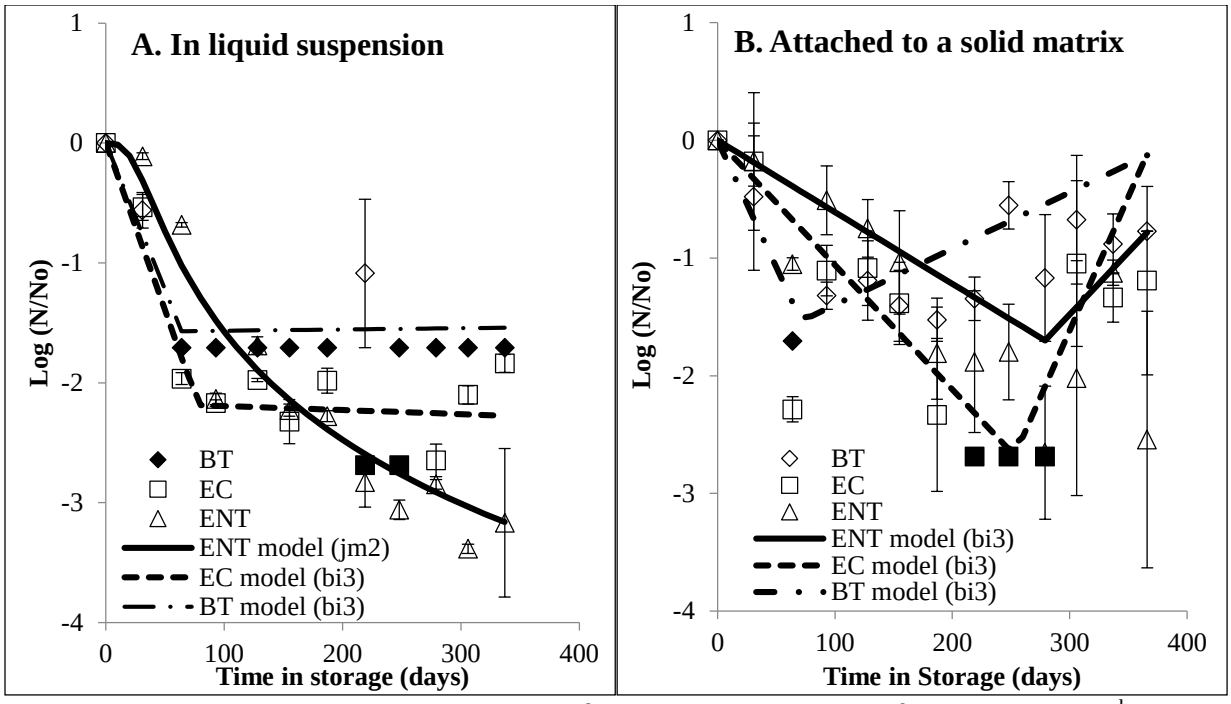

Figure 3.1A-B: The observed and predicted fractional persistence [a] of three indicators [b] stored for up to 366 days at 4°C **A)** in suspension (LS); or **B)** attached to a solid matrix (SM).
[a] Fractional persistence was measured as Log (N/N_0), where N is concentration of the indicator after t days in storage normalized to cell equivalents (CE) per 100 ml-water sample N_0 is the initial indicator concentration.
[b] The diamond (◊), circle (○), and square (□) symbols represent the observed fractional persistence of *Bacteroides thetataiotaomicron* (BT), enterococci (ENT), and *Escherichia coli* (EC), respectively. The lines represent the persistence models for each indicator in the storage conditions. The evaluated persistence models represented were biphasic exponential decay (bi3), and log-logistic model (jm2). Filled in data points illustrate the data that were below the detection limit, and represent 10 the indicator specific detection limit. The error bars represent one standard error.

3.3.2 Comparison of models estimating indicator persistence

Table 3.2 outlines the models' parameters for each indicator in the LS and SM storage conditions along with the BIC values, and the T_{90} and T_{99} values predicted from the persistence models. The BIC values of all of the best fit models analyzed in this study are in **Table S3.1**. A non-linear model, log-logistic (jm2), was fit to ENT in LS, while a linear model, biphasic exponential decay (bi3) was fit to the remainder of the persistence datasets, ENT on a SM, and BT and EC in both conditions. The slopes (decay rates) of the models in **Figure 3.1A-B**

indicated that their rates of decay were not constant. Model selection and shape of equations measuring BT and EC in both conditions were affected by non-detect samples. The models also calculated increases in the fractional persistence of BT, EC and ENT on a SM after 71, 253, and 279 days, respectively, in storage **(Figure 3.1B)**. At t = 366 days, the models predicted that the fractional persistence of BT and EC on a SM (log N/N_0 = -0.126, and -0.116, respectively) increased to close to the initial concentration **(Figure 3.1B)**. A Student's T-test analyzed the concentrations (CE/100 ml-water sample) of ENT, EC, and BT on a SM between the initial and final (t = 366 days) time points (n = 6 for each indicator), and determined that the final concentration of ENT was significantly less than its initial concentration (p = 0.006), while the final and initial concentrations of BT and EC were not significantly different (p = 0.73, and 0.71, respectively).

Table 3.2: Descriptions of the parameters, BIC values, predicted T_{90} and T_{99} values (days) from the models that evaluated the persistence of cell equivalents three indicators [a] in two storage conditions [b] at 4°C for up to 366 days.

Indicator	Storage condition	Model [c]	BIC value	Predicted T_{90} (days)	Predicted T_{99} (days)	Model parameters
BT	SM	bi3	41.78	46.36, 183.34 [d]	N/A[e]	k_1 = 0.05; k_2 = 0.06; bpt [f] = 71.23
	LS	bi3 *	41.24	40.76	N/A[e]	k_1 = 0.06; k_2 = 0.06; bpt = 64.00
EC	SM	bi3	57.18	94.46, 327.05 [d]	188.91	k_1 = 0.02; k_2 = 0.08; bpt = 253.06
	LS	bi3 *	34.37	35.80	71.59	k_1 = 0.06; k_2 = 0.06; bpt = 78.25
ENT	SM	bi3	35.05	164.03, 345.24[d]	N/A[e]	k_1 = 0.01; k_2 = 0.04; bpt = 279.00
	LS	jm2	34.90	62.93	138.94	k_1 = -10.34; k_2 = 3.03

*Denotes if the BIC value of bi3 was ± 2 of the smallest reported BIC value of the available models.

[a] Indicators included: *Bacteroides thetatiaotaomicron*, BT; enterococci, ENT; and *Escherichia coli*, EC.

[b] Conditions included liquid suspension, LS; or attached to a solid matrix, SM.

[c] The persistence models were biphasic exponential decay (bi3) and log-logistic decay (jm2).

[d] The models predicted an increase in indicator concentration that allowed for a second T_{90} value.

[e] The persistence model did not predict a 99% decrease in indicator concentration during the experiment duration.

[f] bpt is the break point value, x, in the bi3 equation in **Table 2.2**.

3.3.3 Comparison of predicted T_{90} and T_{99} values

The T_{90} and T_{99} values, time in days that was needed in order to reduce the indicator population by 90, and 99%, respectively, were calculated from the persistence models (**Table 3.2)**. The equations of the best fit models are in **Table S3.2**. The relative order of persistence in LS based on the T_{90} values was: EC < BT < ENT, while the relative order on a SM was: BT < EC < ENT. A T_{99} value for BT in LS was not calculated because the detection limit was larger

than the concentration needed to convey > 99% reduction of the initial BT concentration **(Figure 3.1A)**. The T_{99} values for ENT and BT on a SM were not calculated because the predicted fractional persistence in each dataset was not reduced to < 1% of the original concentration during the experiment duration **(Figure 3.1B)**. In **Figure 3.1B,** the persistence models of the indicators, BT, EC, and ENT, on SM predicted that their concentrations decreased to < 10% of the initial concentrations after 46.4, 94.5, and 164.0 days, respectively (**Table 3.2)**. After decreasing to < 10% of their initial concentrations, the predicted concentrations of BT, EC, and ENT on a SM increased to > 10% of the original concentration, creating two time points where the concentration of the indicators was 10% of the initial concentration **(Figure 3.1B)**. The second T_{90} values for BT, EC, and ENT on a SM were: 183.3, 327.1, and 345.2 days, respectively (**Table 3.2)**. Additionally, EC on a SM had two predicted T_{99} values, 188.9, and 283.0 days (**Table 3.2)**.

3.3.4 Comparison of genetic marker persistence at t = 31 days in the persistence models and observed data in both storage conditions of our study and Chapter 2.3.3

A comparison of the observed and predicted N/N_0 at t = 31 and 28 days for ENT, EC and BT in both storage conditions was investigated in order to compare the datasets in this experiment with a previous short term study (**Chapter 2.3.3**), respectively (**Table 3.3)**. In our study, the largest observed (and predicted) $N_{31}/N_0 = 0.78$ was from ENT stored in LS ($N_{31}/N_0 = 0.65$; ENT on a SM) while the smallest observed $N_{31}/N_0 = 0.26$ was from BT in LS ($N_{31}/N_0 = 0.14$; EC on LS), respectively. Also in our study, there was < 10% error, and > 50% error between the predicted and observed N_{31}/N_0 in ENT on a SM (predicted and observed $N_{31}/N_0 =$

0.65, and 0.67, respectively), and EC in LS (predicted and observed N_{31}/N_0 = 0.14 and 0.29, respectively), respectively (**Table 3.3**).

The observed N/N_0 from **Chapter 2.3.3** and this study at t = 28 and 31 days, respectively, were compared. There was < 50% difference of the following indicators: ENT on a SM and LS, and EC on a SM. The remaining indicators, EC in LS, and BT on a SM and LS, experienced > 50% difference. The predicted N/N_0 from **Chapter 2.3.3** and our study at t = 28 and 31 days, respectively, was compared **(Table 3.3)**, and there was > 50% error for all indicators except ENT on a SM, which experienced < 50% error.

Table 3.3: Comparison of the fractional persistence of cellular equivalents, *Escherichia coli* (EC), enterococci (ENT), and *Bacteroides thetataiotaomicron* (BT), quantified in our study and **Chapter 2.3.3** at N_{31}/N_0 and N_{28}/N_0, respectively.

Indicator	**Storage condition**	**Observed N_x/N_0 [a]**		**Predicted N_y/N_0 [a]**	
		Our study x = 31 days	**Chapter 2.3.3 x = 28 days**	**Our study y = 31 days**	**Chapter 2.3.3 y = 28 days**
ENT	SM	0.67	0.27	0.65	0.27
	LS	0.78	0.18	0.49	0.19
EC	SM	0.66	0.01	0.47	0.17
	LS	0.29	0.05	0.14	0.05
BT	SM	0.33	0.09	0.21	0.08
	LS	0.26	0.003	0.17	0.003

[a] N_0 is the initial concentration of the indicator normalized to cell equivalents (CE) per 100 ml-water sample, and N_{28} and N_{31} were the indicator concentrations after 28 and 31 days in storage (CE/100 ml-water sample), respectively. The samples were stored in suspension (LS) or attached to a solid matrix (SM).

3.3.5 Linear regression analysis

Results of linear regression analyses of all samples (n = 225) in our study, and the indicator specific data sets, ENT, EC, and BT (n = 72) including the correlation coefficients of the independent variables, significance of correlation coefficient, and their corresponding R^2 values are outlined in **Tables 3.4 and 3.5**. The linear regression analysis of all of the samples had R^2 =

0.343, which was larger than the R^2 value for ENT and BT specific datasets (R^2 = 0.244, and 0.152, respectively). The largest R^2 was 0.609, and was from the ENT specific dataset. All of the independent variables including, time in storage ($p < 0.01$), indicator species ($p = 0.001$, dataset of all samples only), and storage condition ($p < 0.05$) significantly affected fractional persistence of all of the linear regression analyses (**Table 3.4**). The analyses also determined the following relative order of indicator persistence: BT < EC < ENT.

Table 3.4: Correlation coefficient and p-values of multiple linear regression analyses that evaluated the association of the fractional persistence of the indicators [a] to: storage time (days), storage condition [b], and indicator species [c].

	Independent variables' correlation coefficient (p-value)			
Data set (n = sample size)	**R^2**	**Storage time (S)**	**Storage attachment (C)**	**Indicator (I)**
All data (n = 216)	0.343	-0.005 ($p < 0.001$)	0.543 ($p < 0.001$)	**-0.217 ($p = 0.001$)**
BT (n = 72)	0.152	-0.002 ($p = 0.008$)	0.382 ($p = 0.014$)	**N/A**
EC (n = 72)	0.244	-0.004 ($p < 0.001$)	0.431 ($p = 0.033$)	**N/A**
ENT (n = 72)	**0.609**	**-0.008 ($p < 0.001$)**	**0.818 ($p < 0.001$)**	**N/A**

[a] The evaluated data sets included: all data, BT only, EC only, and ENT only.
[b] Conditions included liquid suspension, LS; or attached to a solid matrix, SM.
[c] Indicators included: *Bacteroides thetatiaotaomicron*, BT; enterococci, ENT; and *Escherichia coli*, EC.

Table 3.5: A list of the coefficients of the linear regression equation [a], that evaluated the correlations of the independent variables to fractional persistence [b].

	Coefficients of the independent variables (and their standard errors) of the linear regression equations			
Data set (sample size)	**Storage time- *S***	**Storage attachment- C**	**Indicator- I**	**Y intercept- b_0**
All data (n = 216)	-0.005 (< 0.001)	0.543 (0.109)	-0.217 (0.066)	-1.125 (0.232)
BT (n = 72)	-0.002 (0.001)	0.382 (0.152)	N/A	-1.482 (0.265)
EC (n = 72)	-0.004 (0.001)	0.431 (0.198)	N/A	-1.664 (0.346)
ENT (n = 72)	-0.008 (0.001)	0.818 (0.176)	N/A	-1.531 (0.306)

[a] $Log_{10}\left({}^{N}/_{N_0}\right) = b_S \times S + b_E \times E + b_C \times C + \mathrm{b_T} \times T + \mathrm{b_I} \times I + b_o$

[b] $Log_{10}\left({}^{N}/_{N_0}\right)$

3.4 Discussion

Our study is one of the first to investigate and compare the long term persistence (up to 366 days) of three genetic markers, enterococci *23S rRNA* (ENT-23), *E. coli uidA* (EC-uidA), and *B. thetataiotaomicron alpha-mannanase* (BT-am) in an attached state and in liquid suspension at 4℃. Our study is an extension of **Chapter 2**, which measured and modeled the short term persistence (up to 28 days) of the above markers in various temperatures. During our study, 31 and 23% of measurements BT on a SM, and EC on a SM were below the detection limit, respectively, while EC in LS and BT in LS produced 21 and 72%, respectively, of their samples that were below their respective detection limits **(Figure 3.1A-B)**. The indicators' detection limits were substituted for these time points. It is acknowledged that the best-fit models of BT in LS (and on a SM) and EC in LS (and on a SM) were affected by non-detects at t ≥ 64 (t = 64), and t =219 and 248 (t = 219, 248, and 279) days in storage, respectively.

Two best fit models were chosen to represent the persistence patterns of the indicators in two storage conditions, liquid suspension and attachment to a solid matrix. The biphasic

exponential decay model was fit to all datasets except one, ENT on a SM, which was fit to log-logistic model (jm2). The selection of bi3 to fit a persistence pattern for BT and EC was not dependent on storage condition. Also, the persistence pattern of the three indicators in LS was described with bi3. The overwhelming selection of bi3 as the best fit model for five of the six conditions may indicate that the persistence of genetic markers independent on their source organisms removed from water samples has varying decay rates that is best modeled with a biphasic exponential decay model. However, model selection was influenced by samples that were below the detection limit. This is especially true for BT in LS. There were only three time points where ≥ 1 replicate was above the detection limit. Selection of this dataset's best fit model, bi3, was evaluated by replacing the time points that were below detection with the detection limit. Therefore, the model and its parameters may not accurately depict the persistence pattern of the dataset.

Also, there were two time points during the storage of the SM samples, where the concentration of the three indicators was at 10% of the original concentration of the indicators. These values were reported as T_{90}. The first value represented a reduction to 10% of the original concentration of the genetic marker, and the second value represented regrowth of the markers to 10% of the original population. Reporting both of these values is important because the data demonstrates that genetic marker from FIB in water samples can be measured for months. Therefore, these measurements may cause regulatory consequences for TMDL remediation efforts.

During our experiment, the persistence decay rates of the models were not constant **(Figure 3.1A-B)**. This is consistent with all of the 4°C persistence models in **Chapter 2.3.3**, except ENT

in LS which was modeled with the linear exponential decay model. The variability in the decay seen in these studies may indicate that rate of marker decay may be affected by time in storage.

The T_{90} values from our study were similar with previous studies that measured genetic markers from *E.coli*, enterococci and *Bacteroidales* spp. in water samples. For example, in the 4°C SM condition, our study and **Chapter 2.3.3** determined that BT was the least persistent indicator. Marti et al. (2011) also determined that general *Bacteroidales 16S rDNA*, Allbac, and pig associated *Bacteroidales 16S rDNA*, Pig-1-Bac, and Pig-2-Bac, experienced $T_{90} > 43$ days when stored in 4°C microaerophilic microcosms of freshwater spiked with pig manure, which was consistent with BT in LS ($T_{90} = 40.76$ days), and SM ($T_{90} = 46.36$ days) in our study.

There were also disagreements between the T_{90} values of the indicators in our study compared to previous studies. Marti et al. (2011) determined that pig associated *Bacteroidales 16S rDNA* marker, Pig-1-Bac, stored at 4°C in aerobic and microaerophilic microcosms of diluted pig manure spiked to river water had $T_{90} = 22$, and 10.3 days, respectively, which was smaller than in BT in LS and SM ($T_{90} = 40.76$, and 46.36 days, respectively) in our study. Based on T_{90} values in our study, EC in LS was the least persistent indicator. However, the least persistent indicator in **Chapter 2.3.3** was BT in LS. When comparing the observed N/N_0 of EC and BT in LS at t = 31 days in our study (0.29 and 0.26, respectively, **Table 3.3**), it can be verified that EC in LS in our study persisted longer than BT in LS. This coupled with the discrepancy between the T_{90} values of BT in LS and SM at 4°C from **Chapter 2.3.3** ($T_{90} = 27$, and 9.6 days, respectively) and in our study ($T_{90} = 40.76$, and 46.36 days, respectively) indicate that experiment duration can affect the persistence of these genetic markers.

During our experiment, the concentration of ENT, EC, and BT on a SM increased, which was consistent with a previous study that showed that there was an increase in the concentrations

of culturable enterococci, and general and pig-specific *Bateroidales* genetic markers in pig manure spiked river water stored at 4°C in microaerophilic conditions (Marti et al., 2011). Also, the concentration of Allbac increased over two weeks in microcosms consisting of 28% moisture sand spiked with raw sewage (Eichmiller, Borchert, Sadowsky, & Hicks, 2014). The above findings may indicate that the clumping of cells attached to a matrix may have created anoxic or microaerophilic micro-environments, which may have decreased the DNA decay of BT, or even supported its growth in our study. Additionally, there was not a statistical difference between the initial and final concentrations of EC and BT on a SM, which was consistent with a previous investigation that determined that culturable EC can grow in mescocosms of sewage spiked sediment stored at 25°C (Byappanahalli, Roll, & Fujioka, 2012). These results indicate that attachment to solid particles may offer a mechanism to enhance the persistence of enteric bacteria in the environment.

The significance of the variables of time, indicator species, and storage condition to the fractional persistence of the indicators in our study was compared to **Chapter 2.3.3**. Both studies showed that storage time significantly affected indicator persistence in the 4°C treatments ($p = 0.001$, **Chapter 2.3.3**; and $p < 0.001$, our study). However, our study determined that attachment to a solid matrix significantly affected persistence.

The results in our study indicated that low temperatures and attachment to a solid matrix can increase the persistence of genetic markers of *E.coli, B.thetaiotaomicron*, and enterococci for up to 34 days before a 90% decrease in the concentration.

APPENDIX

Table S3.1: BIC values and the standard deviations of the 17 best-fit models that analyzed the persistence of genetic markers from three indicators [a] that were stored in two conditions [b] at 4°C for up to 366 days.

	BIC values (and standard deviations)					
Model [c]	**BT**		**ENT**		**EC**	
	LS	**SM**	**LS**	**SM**	**LS**	**SM**
ep	50.80 (1.63)	57.08 (1.78)	43.80 (1.22)	42.82 (1.03)	53.59 (1.83)	66.06 (2.52)
lg1	52.75 (1.77)	58.03 (1.85)	46.69 (1.38)	44.88 (1.12)	56.02 (2.03)	67.17 (2.63)
lg2	51.79 (1.53)	67.46 (1.00)	46.60 (1.24)	44.63 (1.00)	55.37 (1.78)	68.13 (2.47)
epd	172.95 (1.00	95.62 (1.00)	35.92 (0.79)	113.85 (1.00)	296.34 (1.00)	217.74 (1.00)
jm1	42.57 (1.05)	49.91 (1.00)	42.23 (1.03)	42.02 (0.91)	41.53 (1.00)	64.53 (2.15)
jm2	42.19 (1.03)	58.63 (1.35)	34.90 (0.76)	40.91 (0.87)	38.25 (0.87)	64.12 (2.12)
gz	40.57 (0.96)	51.77 (1.00)	36.52 (0.81)	40.35 (0.85)	34.11 (0.75)	63.16 (2.04)
wb	42.36 (1.03)	51.91 (1.32)	39.94 (0.94)	41.52 (0.88)	42.29 (0.93)	64.65 (2.34)
ln	42.28 (1.03)	51.78 (1.32)	37.62 (0.85)	41.16 (0.88)	39.1 (0.91)	64.23 (2.13)
gam	42.70 (1.05)	51.82 (1.32)	41.52 (1.00)	41.93 (0.90)	41.33 (0.99)	64.85 (2.18)
bi	38.98 (0.89)	39.22 (1.33)	39.47 (0.93)	41.65 (0.89)	32.12 (0.68)	62.87 (1.75)
bi3	41.24 (0.89)	41.78 (0.81)	37.94 (0.78)	35.05 (0.63)	34.37 (0.67)	57.18 (1.47)
dep	41.99 (0.92)	49.32 (1.16)	37.19 (0.75)	45.65 (0.10)	49.60 (0.10)	67.44 (2.18)
gz3	40.85 (0.88)	55.73 (0.10)	36.83 (0.74)	45.40 (0.96)	32.41 (0.62)	63.83 (1.90)
gzm	55.77 (1.63)	62.21 (1.78)	37.32 (0.75)	47.95 (1.03)	34.20 (0.66)	71.19 (2.52)
sA	43.78 (0.99)	50.14 (1.12)	55.42 (5.15)	68.86 (2.30)	79.25 (4.34)	81.64 (3.77)
sB	43.46 (0.10)	55.84 (0.10)	39.46 (0.10)	45.81 (0.10)	34.93 (0.10)	84.02 (0.10)

[a] The indicators were: *Bacteroides thetataiotaomicron* (BT), enterococci (ENT), and *Escherichia coli* (EC).

[b] The storage conditions were: liquid suspension (LS) and attached to a solid matrix (SM).

[c] The models evaluated were: first-order exponential decay (ep), one parameter logistic (lg1), two parameter logistic (lg2), exponential damped (epd), two-stage (jm1), log-logistic (jm2), Gompertz (gz), Weibull (wb), log-normal (ln), Gamma (gam), biphasic exponential decay with preset breakpoint at 3 days (bi), biphasic exponential decay (bi3), double exponential decay (dep), Gompertz 3-parameter (gz3), Gompertz-Makeham (gzm), Sigmoid-A (sA), and Sigmoid-B (sB). Bi was included in the R programming platform, but its BIC value wasn't compared to the other models because its breakpoint was pre-determined.

Table S3.2: The best fit equations that were chosen to represent the persistence patterns of threes indicators [a] stored at three temperatures, and two storage conditions [b].

Indicator	Storage condition	Model [c]	Equation [d]
BT	SM	bi3	**for 0 ≤ t < 1709.5:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-0.05*t})$ **for t ≥ 1709.5:** $Log(^{N}/_{N_0}) = Log(e^{-0.05*t+0.06*(t-1709.5)})$
	LS	bi3*	**for 0 ≤ t < 1536:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-0.06*t})$ **for t ≥ 1536:** $Log(^{N}/_{N_0}) = Log(e^{-0.06\ *t+\ 0.06\ *(t-\ 1536)})$
EC	SM	bi3	**for 0 ≤ t < 6073.4:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-0.02*t})$ **for t ≥ 6073.4:** $Log(^{N}/_{N_0}) = Log(e^{-0.02*t+0.08*(t-6073.4)})$
	LS	bi3*	**for 0 ≤ t < 1878:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-0.06*t})$ **for t ≥ 1878:** $Log(^{N}/_{N_0}) = Log(e^{-0.06\ *t+\ 0.06\ *(t-\ 1878)})$
ENT	SM	bi3	**for 0 ≤ t < 6696:** $Log\ (^{N}/_{N_0}) = Log\ (e^{-0.01*t})$ **for t ≥ 6696:** $Log(^{N}/_{N_0}) = Log(e^{-0.01*t+\ 0.04*(t-6696)})$
	LS	jm2	$Log(^{\mathrm{N}}/_{\mathrm{N}_0}) = -Log(1 + e^{\ (-10.34+3.03*ln\ (t))})$

[a] The indicators included: *Bacteroides thetatiaotaomicron*, BT; enterococci, ENT; and *Escherichia coli*, EC.
[b] The storage conditions included: liquid suspension, LS; or attached to a solid matrix, SM.
[c] The models' abbreviations: first-order exponential decay (ep), biphasic exponential decay (bi3), and log-logistic (jm2).
[d] Time, t, is measured in hours.
*Denotes if the BIC value of bi3 was ± 2 of the smallest reported BIC value of the available models.

CHAPTER 4. DNA YIELDS, AND CONCENTRATIONS OF ESCHERICHIA COLI UIDA AND ENTEROCOCCI 23S rDNA IN FRESHWATER SEDIMENTS: A COMPARISON OF DNA EXTRACTION METHODS

4.1 Introduction

Freshwater sediments provide a long term reservoir for culturable fecal indicator bacteria (FIB), enterococci (ENT) and *Escherchia coli* (EC, Thevenon et al., 2012). These sediments can influence the pollution inputs into recreational waters (Pote et al., 2009). Culture independent methods like quantitative polymerase chain reaction (qPCR) methods were developed by the United State Environmental Protection Agency (USEPA) in order to address the impacts of sewage in environmental waters. These methods can monitor ENT in beaches, and microbial source tracking with human specific *Bacteroides thetataiotaomicron* (USEPA, 2010b, 2012b).

However, obtaining precise and accurate measurements of pollutions from sediment has remained a challenge.

Improving DNA extraction efficiency is essential for characterizing pollution levels in sediments. Many of the current DNA extraction methods have variable and distinct yields as well as detection limits for sediments (Xiong, Xie, Wang, & Niu, 2014). Inconsistent DNA yields can occur from incomplete cell lysis, or losses during the purification step (Kirk et al., 2004; Sharma et al., 2007). The concentration of organic matter and clay density of sediments can also affect DNA yields (Lloyd, Macgregor, & Teske, 2010) by adsorbing DNA (Hoshino & Matsumoto, 2007). DNA extraction efficiency also affects downstream applications. Methods that produce small DNA fragments, do not completely lyse cells, or elute DNA bound to inhibitors can reduce the qPCR amplification of specified genetic markers (Bürgmann, Pesaro, Widmer, & Zeyer, 2001; Inceoglu et al., 2010; Sharma et al., 2007).

Few studies have investigated how DNA extraction protocols may affect FIB measurements in sediments. Such research would give public health officials a better understanding of the accumulated pollution in aquatic ecosystems, and provide a better analysis of changes in water

quality. Therefore, the purpose of this study was to compare the DNA yields and qPCR measurements of spiked *E.coli uidA* (EC-uidA) and enterococci *23S rDNA* (ENT-23) using six modifications of three DNA extraction methods from surface sediments in the Lake St. Clair watershed. *E.coli* and enterococci were chosen because they represent gram-positive and gram-negative FIB, respectively. Additionally, EC-uidA is specific to *E.coli*, and has been used previously to measure *E.coli* in water samples (Srinivasan et al., 2011). ENT-23 was chosen because it was recommended by the USEPA to monitor pollution in recreational waters (USEPA, 2012b).

The specific objectives of this study were to:

a. Compare the DNA yields, and qPCR measurements of *E.coli uidA,* and enterococci *23S rDNA* spiked into sediments that were extracted by six modifications of three DNA extraction protocols.
b. Identify the DNA extraction protocol and modification that produced the largest DNA yield, and qPCR measurements of *E.coli uidA,* and enterococci *23S rDNA* spiked into sediments from the Lake St. Clair watershed.

4.2 Methods

An overview of the experiment design is illustrated in **Figure 4.1**. Each heading in **Figure 4.1** represents a sub-section described below.

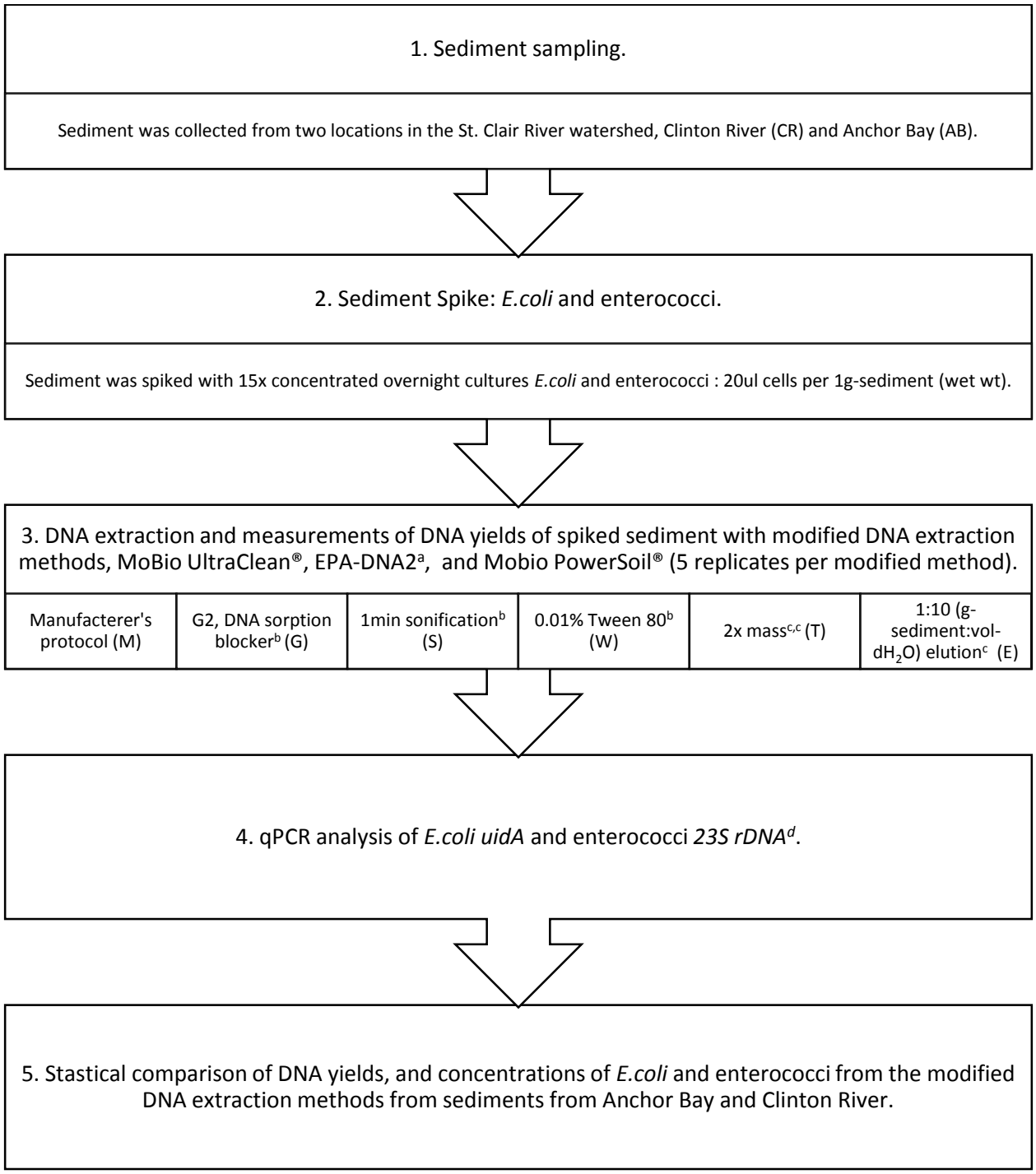

Figure 4.1: Overview of experimental design.

[a] This method is based on EPA Method 1611 for water samples.

[b] Completed at bead beating step.

[c] Completed as an initial step.

[d] Only applicable for modified methods of MoBio UltraClean® and EPA-DNA2.

4.2.1 Sediment sampling

Lake St. Clair is a small lake in the North American Great Lakes (max depth = 6.4 m, surface area = 1114 km^2). It connects Lake Huron to Lake Erie. Surface sediment was removed from Anchor Bay in Northwest Lake St. Clair (AB, 42˚62.175'N, 82˚74.935'W), and the mouth of the Clinton River (CR, 42˚59.494'N, 82˚79.241'W) on August 21, 2012. The average percent dry weight of the surface sediments collected from AB and CR were 62 and 40%, respectively. The two sites were chosen because of their historical differences in anthropogenic pollution. The Clinton River was named an USEPA Area of Concern in 1986 because of excess nutrient loading, fecal pollution, and habitat loss (Esman, 2007). The Anchor Bay watershed has historically been rural with recent increases in population (Baustian et al., 2014). The sediments from AB (and CR) had the following carbon, nitrogen, and phosphorus concentrations: 80.2 (34.8), 3.2 (1.6), and 279.6 ug/g-dry wt (437.2 ug/g-dry wt), respectively. The sediment samples were stored in a cooler on dry ice during transport, and then at -80˚C.

2.1 Sediment spike with enterococci and *E.coli.*

The sediments were thawed at 4°C. Once thawed, the sediments had a slurry consistency. Overnight cultures of *E.coli* ATCC strain 15597 and *Enterococcus faecalis* ATCC strain 19433 were mixed together after two washings with sterile dH_20, and concentrated 15x. Then, 20 µl of the solution was spiked into 1 g (wet wt) of surface sediments from AB and CR. The sediments were briefly vortexed to initiate mixing and stored overnight in the dark at 4°C to facilitate adhesion to sediment particles. In order to address the background levels of ENT and EC, samples of un-seeded sediments from AB and CR (negative controls) were also stored overnight at 4°C. Positive controls (20 µl of the spiking solution) were stored overnight at 4°C.

4.2.2 DNA extraction of spiked sediment with modified methods of MoBio UltraClean®, EPA-DNA2, and Mobio PowerSoil®.

All DNA extraction methods in our study included a bead beating step, and are listed as follows: MoBio PowerSoil® (MB-PS, MoBio Laboratories, Inc., Carlsbad, CA), Mobio UltraClean® (MB-UC, MoBio Laboratories, Inc., Carlsbad, CA), and a method (EPA-DNA2) based on USEPA Method 1611 (USEPA, 2012b). MB-PS and MB-UC required 0.25 g (wet mass) of sediment, and the manufacturers' protocols were followed. MB-UC was chosen because it was shown to achieve DNA yields similar to laborious home-made DNA extraction methods (Luna, Dell'Anno, & Danovaro, 2006), and it had a low detection limit for *F.tularensis* in soils (Whitehouse & Hottel, 2007). MB-PS was chosen because downstream applications showed increased species richness in agricultural and clay soils (Inceoglu et al., 2010). EPA-DNA2 was developed for this study. Procedures for EPA-DNA2 are outlined as follows: 1 g (wet mass) of sediment was added to a 2 ml centrifuge tube and centrifuged at 10,000x g for 30 s to remove excess liquid. Then, 0.3 g of sterilized and acid washed glass beads with diameter 212-300 µm (Sigma-Aldrich Corp., St. Louis, MO), and 500 µl of AE buffer (Qiagen, Inc., Valencia, CA) were added. The sediment was homogenized in Mini-BeadBeater 8 (Biospec Products, Inc., Bartlesville, OK) for 50 s. The sediment was centrifuged for 1 min at 12,000x g, and the supernatant was recovered. An additional 200 µl of AE buffer was added to the sediment, and it was homogenized for 10 s. The sediment was centrifuged again for 1min at 12,000x g. The supernatant was combined with the previously recovered supernatant. The combined supernatant was centrifuged for 5 min at 12,000x g. The supernatant was recovered and contained the eluted DNA.

The following modifications (and their abbreviations) were applied to each DNA extraction method: 1) manufacturer's protocol, no modification (**M**); 2) addition of 0.45 g of DNA sorption blocker reagent, G2 (GEUS, Copenhagen, Denmark) (**G**); 3) sonification (FS220H, Fisher Scientific, Waltham, MS) of a microcentrifuge tube containing the sediment for 1 min in dH_2O at room temperature (**S**); 4) addition of 0.01% Tween 80 to bead beating matrix (**W**); 5) 2x mass of sediment (**T**, only applied to MB-UC and EPA-DNA2); and 6) 1:10 elution of 1g-sediment (wet mass) per 10 ml sterile dH_2O (Boehm et al., 2009), and membrane filtered with a polycarbonate filter (**E**, 0.45 μm pore size, 47 mm diameter, Whatman Inc., Piscataway, NJ). Modifications #2-4 were completed before the bead beating step, while modifications #5-6 were the initial steps of the DNA extraction methods. Within each modified DNA extraction method, there were five replicates of spiked sediment from AB and CR. Three replicates of negative controls from AB and CR sediments were evaluated using the manufacturer's protocols of each DNA extraction method. Three replicates of the positive controls, the spiking solutions, were evaluated with the manufacturer's protocols of each DNA extraction method. Regardless of the modification, the elution volumes of EPA-DNA2, MB-UC, and MB-PS were: 700, 50, and 100 μl, respectively. All of the modifications, except G2, have been used in previous culturable bacterial enumeration studies to remove cells from various matrices (Boehm et al., 2009; Downey, Da Silva, Olson, Filliben, & Morrow, 2012; Natvig, Ingham, Ingham, Cooperband, & Roper, 2002; Thevenon et al., 2012). G2 is a DNA sorption blocker reagent with glass beads that is manufactured by the Geological Survey of Denmark and Greenland (GEUS). G2 was analyzed in our study because DNA yields from low biomass sediments increased by > 1000x when G2 was added to MoBio PowerLyzer PowerSoil® (MoBio Laboratories, Inc., Carlsbad, CA) and Griffith's protocol (Jacobsen, Nielsen, & Bælum, 2012). The DNA yield of each replicate of our study was

measured with a UV spectrophotometer at OD_{260}, and transformed to ng /g-dry wt. The eluates were stored at -80°C.

The average theoretical DNA yields of the positive controls, 20 µl of the spiking solution, produced from the manufacturer's protocols of EPA-DNA2, MB-UC and MB-PS were normalized to ng /g-dry wt and represented a spiked sample of sediment from AB (and CR), and were 6.48×10^4 (1.02×10^5), 1.20×10^3 (1.88×10^3), and 5.09×10^2 ng/g-dry wt (7.98×10^2 ng/g-dry wt), respectively. The average DNA yields measured from of the negative controls, unseeded sediments, of the manufacturer's protocols of EPA-DNA2, MB-UC, and MB-PS from AB (and CR) were 6.63×10^4 (3.06×10^5), 1.58×10^4 (3.68×10^4), and 1.17×10^4 ng/g-dry wt (2.2×10^4 ng/g-dry wt), respectively.

4.2.3 qPCR analysis of *E.coli uidA* and enterococci *23S rDNA*.

Measurements of the spiked EC and ENT in the replicates of each modified DNA extraction method were measured via genetic markers from enterococci *23S rDNA* (ENT-23) and *E.coli uidA* (EC-uidA) with qPCR using the Roche LightCycler® 480 Instrument (Roche Applied Science, Indianapolis, IN). The primer and probe sequences and qPCR protocols are described in **Table 2.1**. Genomic DNA of overnight cultures of *E.coli* ATCC strain 15597 and *Enterococcus faecalis* ATCC strain 19433 was extracted using Qiamp DNA Mini Kit® (Qiagen Inc, Valencia, CA).

The standard curves of ENT-23 and EC-uidA calculated their respective indicator concentrations. The genomic DNA was serially diluted in a 1:10 dilution series with at least six steps. The average efficiencies, r^2 values, and CT value of the lowest detected dilution step of the standard curves for ENT-23 (and EC-uidA) were: 95% (96%), 0.99 (0.95), 35.07 (37.15),

respectively. A new standard curve was made after every 4 qPCR runs. The theoretical detection limits of the markers were specific to the sediments' water content, and the DNA extraction method (**Table 4.1)**. One modification, 2x mass (T), affected the detection limits of MB-UC and EPA-DNA2 (**Table 4.1)**.

Table 4.1: Calculated detection limits of the qPCR amplified cellular equivalents of *E.coli* and enterococci extracted with the modified DNA extraction methods [a] in 20 µl qPCR reaction volumes from sediments from the Clinton River (CR) and Anchor Bay (AB).

Kit [a]	**Sample mass (g-wet wt)** [b]	**Indicator** [c]	**Detection limits** [d]	
			Clinton River (CE/g-dry wt)	**Anchor Bay (CE/g-dry wt)**
MB-PS	0.25	ENT	$\leq 1.43 \times 10^2$	$\leq 2.25 \times 10^2$
		EC	$\leq 5.61 \times 10^3$	$\leq 8.79 \times 10^3$
MB-UC	0.25	ENT	$\leq 7.17 \times 10^1$	$\leq 1.12 \times 10^2$
	0.5		$\leq 3.58 \times 10^1$	$\leq 5.62 \times 10^1$
	0.25	EC	$\leq 2.80 \times 10^3$	$\leq 4.40 \times 10^3$
	0.5		$\leq 1.40 \times 10^3$	$\leq 2.20 \times 10^3$
EPA-DNA2	1	ENT	$\leq 2.51 \times 10^2$	$\leq 3.93 \times 10^2$
	2		$\leq 1.26 \times 10^2$	$\leq 1.97 \times 10^2$
	1	EC	$\leq 9.81 \times 10^3$	$\leq 1.54 \times 10^4$
	2		$\leq 4.91 \times 10^3$	$\leq 7.70 \times 10^3$

[a] The DNA extraction methods were: MoBio PowerSoil® (MB-PC), MoBioUltraClean® (MB-UC), and an EPA Method 1611 based method (EPA-DNA2).
[b]The modification, 2x mass (T), affected the detection limits of MB-UC and EPA-DNA2.
[c] The indicator organisms were: enterococci (ENT) and *E.coli* (EC).
[d] The detection limits were transformed into cell equivalents (CE) per g-dry wt.

One amplified copy of EC-uidA represented one CE of EC (Srinivasan et al., 2011), while four amplified copies of ENT-23 represented one CE of ENT (USEPA, 2012b). The concentrations of ENT-23 and EC-uidA from the analytical duplicates were averaged, and transformed to CE/g- dry wt. The average ENT concentrations of the positive controls, spiking solutions, for AB (and CR) sediment produced from the manufacturer's protocols of EPA-DNA2, and MB-UC were transformed to CE/g-dry wt, and were 2.74×10^8 (1.75×10^8), and 1.07

x 10^7 CE/g-dry wt (6.81 x 10^6 CE/g-dry wt), respectively. The average concentrations of EC calculated from the positive controls, spiking solutions, for AB (and CR) sediment were measured from the manufacturer's protocols of EPA-DNA2, and MB-UC, and were 1.45 x 10^9 (9.27 x 10^8), and 7.64 x 10^8 CE/1 g-dry wt (4.87 x 10^8 CE/1 g-dry wt), respectively. The average ENT concentrations of the unseeded sediment measured with the manufacturer's protocols of EPA-DNA2, and MB-UC from AB (and CR) were ≤ 3.93 x 10^2- the detection limit, (≤ 2.51 x 10^2- the detection limit), and 1.46 x 10^5 CE/g-dry wt (2.76 x 10^4 CE/g-dry wt), respectively. The average EC concentrations of the unseeded sediments measured from the manufacturer's protocols of EPA-DNA2, and MB-UC from AB (and CR) were 4.18 x 10^6 (1.24 x 10^7), and 1.15 x 10^7 CE/g-dry wt (7.24 x 10^4 CE/g-dry wt), respectively.

4.2.4 Kruskal-Wallis statistical analyses of DNA yields and concentrations of *E.coli* and enterococci produced from the modified DNA extraction methods from AB and CR sediments

In each sediment location, three non-parametric ANOVA and Tukey's HSD tests, Kruskal-Wallis tests, were performed with SPSS 22.0 (SPSS, Inc., Chicago, IL) in order to determine if the modified DNA extraction methods affected 1) the DNA yields; 2) EC concentrations; and 3) ENT concentrations. Within each location, the statistical analysis first ranked the DNA yields, EC concentrations, and ENT concentrations of each replicate from the modified DNA extraction methods. The rankings were averaged per each modified DNA extraction method. Then, the ranked means were evaluated for significant differences ($p < 0.05$) using a pairwise comparison from the Kruskal-Wallis test. The modified MB-PS methods were excluded from the statistical evaluation of the qPCR amplified ENT and EC concentrations due to low DNA yields, with

further explanation in the **Results 4.3.1**. The Kruskal-Wallis test was chosen for all analyses because the ANOVA assumption of equal variance wasn't met in the sample distributions.

4.2.5 Comparison of the DNA yields and FIB concentrations produced from the modified DNA extraction methods to their respective standard method

Within each sediment location, the average DNA yields, and ENT and EC concentrations produced from the modifications of EPA-DNA2, MB-UC, and MB-PS (DNA yields only) were compared to their manufacturers' protocols with the following ratio, Y_X / Y_M, where Y_M was the DNA yield, ENT or EC concentration produced from the manufacturer's protocol of a DNA extraction method, and Y_X was the average DNA yield, or ENT and EC concentration produced from *x* modification of a DNA extraction method. The subscripts of the modifications were: *W* was 0.01% Tween 80, *T* was 2x mass; *S* was sonication; *E* was 1:10 elution; and *G* was G2 DNA sorption blocker.

4.3 Results

4.3.1 Site specific comparison of the DNA yield produced by the modified DNA extraction methods

The average DNA yields from the spiked sediments from AB (and CR) produced across all of the modified methods including the manufacturers' protocols of EPA-DNA2, MB-UC, and MB-PS were: 7.79 x 10^5 (9.97 x 10^5), 3.37 x 10^4 (1.39 x 10^4), and 6.48 x 10^3 ng/g-dry wt (8.74 x 10^3 ng/g-dry wt), respectively. EPA-DNA2 + G produced the largest average DNA yields from CR and AB sediments, and were 5.22 x 10^6 and 4.36 x 10^6 ng/g-dry wt, respectively (**Figure 4.2A-B**).

Of the three DNA extraction methods, EPA-DNA2 had the largest average DNA yields within each modification (**Figure 4.2A-B)**. The modified MB-PS methods had the lowest average DNA yields, the exceptions were MB-PS +S in CR, and MB-PS +G in AB and CR sediments, which had yields larger than the respective modifications of MB-UC (**Figure 4.2A-B)**.

When comparing the modifications, the average DNA yield from AB sediment using EPA-DNA2 + G was significantly larger than MB-UC +E, +S and MB-PS +E, +S ($p < 0.05$). For CR sediment, the average DNA yield from EPA-DNA2 + G was also significantly larger than MB-PS + E, +S, +W, and MB-UC +S, +W ($p < 0.05$).

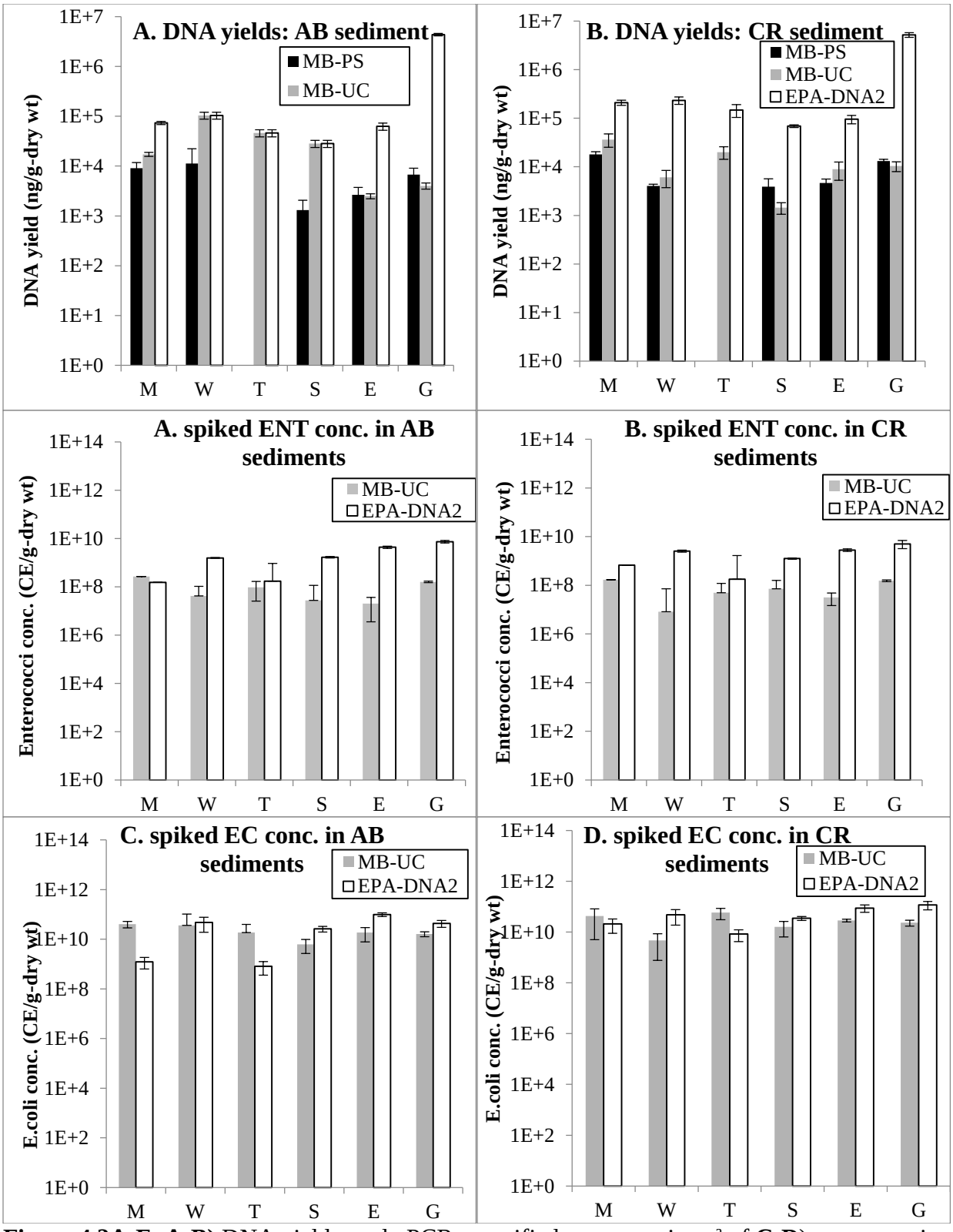

Figure 4.2A-F: A-B) DNA yields, and qPCR quantified concentrations [a] of **C-D**) enterococci (ENT), and **E-F**) *Escherichia coli* (EC) spiked into surface sediment from Anchor Bay (AB) and the Clinton River (CR), and extracted by modifications [b] of three DNA extraction methods [c].

Figure 4.2 (cont'd).
[a] The DNA yields were reported in ng/g-dry wt, and the qPCR amplified concentrations of *E. coli* and enterococci were normalized to cell equivalents (CE) per g-dry wt.
[b] The modifications were: manufacturer's protocol (M); 0.01% Tween 80 (W); 2x mass (T); sonication (S); 1:10 elution (E); and G2 DNA sorption blocker (G).
[c] The DNA extraction methods were: MB-PS (MoBio PowerSoil®), MB-UC (MoBio UltraClean®), and EPA-DNA2 (based on USEPA Method 1611). There were five replicates per each modified DNA extraction method. The error bars represent one standard error.

4.3.2 Site specific comparison of the qPCR amplified concentrations of enterococci and *E.coli* produced by the modified DNA extraction methods.

DNA extracts from the modified MB-PS methods were not used to evaluate the concentrations of EC and ENT in sediments from AB and CR, because of its comparatively low DNA yields (**Figure 4.2A-B**).

The average ENT concentrations from AB (and CR) sediments across all of the modified methods, including the manufacturers' protocols, of EPA-DNA2 and MB-UC were: 2.57×10^9 (2.09×10^9), and 1.03×10^8 CE/g-dry wt (8.12×10^7 CE/g-dry wt), respectively. Within each modification, EPA-DNA2 provided the largest average ENT concentrations from CR and AB sediments, except MB-UC +M (2.71×10^8 CE/g-dry wt) which was slightly larger than EPA-DNA2+M (1.52×10^8 CE/g-dry wt) in CR (**Figure 4.2C-D**). Using EPA-DNA2 +G, the largest average ENT concentrations in CR and AB sediments were measured to be 5.04×10^9 and 7.46×10^9 CE/g-dry wt, respectively (**Figure 4.2C-D**), and were significantly larger than MB-UC+W, +E, +T, and +S ($p < 0.05$).

The average EC yields from AB (and CR) sediments across all of the modifications of EPA-DNA2 and MB-UC methods, including the manufacturers' protocols, were 3.64×10^{10} (5.30×10^{10}), and 2.26×10^{10} CE/g-dry wt (2.1×10^{10} CE/g-dry wt), respectively. Again, the largest average concentration of EC from CR and AB sediments were measured from EPA-DNA2 +G

(1.17 x 10^{11} CE/g-dry wt), and EPA-DNA2 +E (9.89 x 10^{10} CE/g-dry wt), respectively (**Figure 4.2E-F)**. The average EC concentration in AB sediment extracted with EPA-DNA2 +E was significantly larger than EPA-DNA2 +M, +T ($p < 0.05$). In CR, the concentration of EC extracted with EPA-DNA2 +G was significantly larger than EPA-DNA2 +T, and MB-UC +W and +S ($p < 0.05$).

4.3.3 Comparisons of DNA extraction efficiency of the modified DNA extraction methods to their respective manufacturer's protocols

The ratio, Y_X / Y_M, compared the DNA yields, and ENT and EC concentrations produced from the modifications of EPA-DNA2, MB-UC, and MB-PS (DNA yields only) to their manufacturers' protocols, M, in sediments from CR and AB. **(Table 4.2)**. Therefore, $Y_x / Y_M > 1.0$ indicated that the modification, *x*, produced a larger DNA yield or FIB concentration than the respective manufacturer's protocol. Overall, no improvement was seen in the modified variations of MB-PS and MB-UC **(Table 4.2)**. Specifically, the DNA yields and FIB concentrations measured in CR sediments were reduced dramatically after the modifications were applied to MB-PS (DNA yields only) and MB-UC **(Table 4.2)**. However, 0.01% Tween 80, 1:10 elution and G2 improved the extraction efficiency of the standard method of EPA-DNA2 from AB and CR sediments **(Table 4.2)**.

Table 4.2: Within each sediment location, Anchor Bay and Clinton River, a ratio compared the average DNA yields, and concentrations of enterococci and *E.coli* produced from modifications of DNA extraction methods to their respective manufacturer's protocol.

DNA extraction Method [a]	Site [b]	Output [c]	Ratio: Y_X/Y_M [d]				
			W	E	T	G	S
MB-PS	AB	DNA yield	1.25	0.29	-	0.75	0.15
	CR		0.22	0.26	-	0.73	0.07
MB-UC	AB	DNA yield	6.03	0.14	2.67	0.23	1.64
	CR		0.17	0.24	0.55	0.28	0.04
	AB	ENT conc.	0.15	0.07	0.35	0.60	0.10
	CR		0.05	0.18	0.28	0.90	0.42
	AB	EC conc.	0.89	0.46	0.46	0.40	0.15
	CR		0.11	0.66	1.35	0.54	0.38
EPA-DNA2	AB	DNA yield	1.41	0.85	0.63	59.18	0.38
	CR		1.11	0.45	0.70	24.83	0.33
	AB	ENT conc.	3.79	4.14	0.27	7.47	1.87
	CR		3.79	4.14	0.27	7.47	1.87
	AB	EC conc.	2.28	4.24	0.40	5.63	1.69
	CR		2.28	4.24	0.40	5.63	1.69

[a] The DNA extraction methods were: MB-PS (MoBio PowerSoil®), MB-UC (MoBio UltraClean®), and EPA-DNA2 (based on USEPA Method 1611).
[b] The sites were Anchor Bay (AB) and Clinton River (CR).
[c] The outputs were: DNA yields (transformed to ng/g-dry wt), enterococci concentration (ENT, transformed to CE/g-dry wt), and *E.coli* concentrations (EC, transformed to CE/g-dry wt).
[d] Y_X was the DNA yield or FIB concentration of the modification, *x*. The modifications were: 0.01% Tween 80 (W), 2x mass (T), sonication (S), 1:10 elution (E), and G2 DNA sorption blocker (G). Y_M was the DNA yield or FIB concentration of the manufacturer's protocol.

4.4 Discussion

The ability to improve DNA extraction efficiency and amplify FIB markers such as ENT-23 and EC-uidA from sediments is an ongoing pursuit. Our study examined two sediment locations, the mouth of the Clinton River, and Anchor Bay in Northwest Lake St. Clair. Sediments from AB had twice the concentration of carbon and nitrogen compared to CR, while CR had twice the concentration of phosphorus than measured in AB. Previous research has determined that DNA

extraction efficiency decreased in soils with large organic content (Zhou, Bruns, & Tiedje, 1996). However, within each sediment location of our study, modifications of the DNA extraction methods overall resulted in minimal differences based on location in extraction efficiency of the DNA yields, and FIB concentrations.

Our results suggested that two of the investigated DNA extraction methods, MB-UC and EPA-DNA2, overcame some of the challenges impeding the extraction of DNA in sediments with varying organic content. The manufacturer's protocol of MB-UC performed better than its modifications, which indicated that optimization was not generally needed. Within EPA-DNA2, the modifications, 1:10 elution, sonication, 0.01% Tween 80 and G2, resulted in larger ENT and EC concentrations when compared to its standard method for AB and CR sediments **(Table 4.2)**. A previous comparison of eighteen methods to measure culturable ENT and EC in sands also determined that significant differences ($p < 0.05$) in the measurements were mainly restricted to methods that used blending versus shaking (Boehm et al., 2009). This suggests that the variations in sampling protocols account for minimal variation of ENT and EC measurements in sands and sediments.

The number of steps of a DNA extraction method may have some impact on its efficiency. There were only three steps in the standard method for EPA-DNA2, while MB-UC and MB-PS had five and six steps, respectively. It has also been shown that DNA purification with a silica column in the manufacturers' protocols of MB-UC and MB-PS decreased DNA yields in sediments by 9 - 55% when compared to the same methods that purified DNA with an acrylamide gel (Lloyd et al., 2010). Preferential binding of humic acids and other organic compounds to a silica column was presumed to be one of the mechanisms that decreased DNA

extraction efficiency (Zhou et al., 1996), which may have accounted for the decrease in DNA extraction efficiency in MB-UC and MB-PS compared to EPA-DNA2 in our experiment.

Additionally, in both unseeded sediments, the manufacturer's protocol of MB-UC had > 2 log increase of the naturally occurring ENT concentration when compared to the standard method of EPA-DNA2. These results are different from the seeded samples, and may indicate that the indigenous populations of ENT have a distinct extraction efficiency compared to the spiked concentrations used in this study. The differential extraction efficiency may be due to increased resistance to disrupt the cell wall with bead beating, as was observed in a previous study of viable but not culturable *Enterococcus faecalis* in microcosms of sterilized freshwater stored at 4°C for 16 days (Signoretto, del Mar Lleo, Tafi, & Canepari, 2000).

In our study, a novel DNA sorption blocker reagent, G2, produced larger DNA yields, and FIB concentrations from AB and CR sediments (**Figure 4.2A-F).** G2 contains a proprietary blocking reagent and glass beads. It is produced by The Geological Survey of Denmark and Greenland (GEUS). It was previously shown to help significantly increase ($p < 0.05$) qPCR measurements of *rpoB* from clay subsoil with low biomass (Jacobsen et al., 2012). MB-UC used with G2 was shown to increase the DNA extraction efficiency of *Dehalococcoides* spp. in clay till by 3 logs (Bælum et al., 2014). As previous research also suggested, G2 may act as an adsorption-site competitor like spiked salmon sperm DNA, which increased DNA yields extracted from soils (Paulin & Nicolaisen, 2013).

The time and money needed to extract indicator DNA from each of the modified methods was also compared. DNA extraction using MB-UC and MB-PS can be completed in 30 min, while EPA-DNA2 took ~10 min. The material costs for the manufacturer's protocol of MB-UC, MB-PS and EPA-DNA2 were 3, 5, and 2 $USD, respectively. The additional costs for the

materials for the modifications was < 1 $USD, except G2, which added an additional 7 $USD. The additional time required for the modifications were < 10 min. Thus, EPA-DNA3 with G2 was an easy, and time efficient method to extract FIB concentrations from sediments.

CHAPTER 5. HISTORICAL ASSOCIATIONS OF FECAL INDICATOR CONCENTRATIONS TO ANTHROPOGENIC ACTIVITIES AND CLIMATE IN FREHSWATER SEDIMENTS

5.1 Introduction

The United States has a fragmented history of water quality standards. Culturable total coliforms were first regulated in drinking water in 1914, and were used to measure ambient water quality until the 1950s(Wolf, 1972). By the 1960s, fecal coliforms were used to monitor wastewater discharges, and then to guide the safety of recreational waters (Dufour, 2001). Since 1986, culturable *Escherichia coli* (EC) and/or enterococci (ENT) have been the indicators of choice for monitoring recreational waters (USEPA, 2003). Recently, quantitative polymerase chain reaction (qPCR) measurements of ENT *23S rDNA* and *Bacteroidales 16S rDNA* were recommended by the USEPA to monitor water quality at beaches (USEPA, 2010b, 2012b).

Current methods primarily measure fecal indicator bacteria (FIB) in the water column. However, qPCR measurements of cell equivalents (CE) and culturable concentrations of ENT and EC revealed higher concentrations in sediments and benthic sand, respectively (Eichmiller, Hicks, & Sadowsky, 2013; Wheeler Alm et al., 2003). There was a ~5-log increase of CE of ENT in the top 11 cm of sediment and sand cores obtained from Lake Superior when compared to culturable forming units (CFUs) (Eichmiller et al., 2013). It appears that culturable ENT and EC stabilized (< 1-log reduction) in sediment microcosms stored at 10°C for 60 days, indicating that sediments have the potential to be long term reservoirs of FIB (Pote et al., 2009), and their resuspension can negatively impact water quality (D L Craig et al., 2004).

Scientific questions regarding water quality trends over large time scales and their associations to climate change and human interactions have remained unanswered. An approach which measures FIB in sediment cores could be used to evaluate water quality changes over longer timescales. For example, a previous study observed viable concentrations of EC and ENT in sediments deposited up to 245 ± 45 years ago, and their observed concentrations increased

when eutrophic conditions were observed in Switzerland (Thevenon et al., 2012). Therefore, the purpose of our study was to evaluate the relationship between the concentrations of enterococci *23S rDNA* and *E.coli uidA* in sediment cores from two sub-watershed of a lake system to climatic and anthropogenic variables over fairly long time scales. Two locations representing different sub-watersheds in the Lake St. Clair watershed were chosen. Anchor Bay, Northwestern Lake St. Clair represented historical agricultural lands that have transitioned to developed areas, while the mouth of the Clinton River in Western Lake St. Clair represented an increase in developed lands (J. A. Fry et al., 2011). Sediment cores were taken from both locations, and assessed for changes in sedimentary concentrations of total nitrogen, total phosphorus and total carbon, as well as FIB. The watersheds' historical anthropogenic activities were also evaluated, and included human population in Clinton River and Lake St. Clair watersheds. Finally, historical climate variables were also obtained for inclusion in the analysis, and included river discharge, and air temperature.

5.2 Methods

5.2.1 Field site description, sample collection and processing

The Clinton River and Anchor Bay sub-watersheds within the Lake St. Clair watershed, and are 1968 and 443 km^2, respectively. The sites, Anchor Bay (AB, 42˚62.175'N, 82˚74.935'W) and Clinton River (CR, 42˚59.494'N, 82˚79.241'W**, Figure 5.1**), were chosen because they have distinct historical records of environmental perturbation (Healy, Chambers, Rachol, & Jodoin, 2008). Also, these sites were reported to be sediment accumulation zones, and thus are useful sites for the reconstruction of historical changes (International Joint Commission & Upper Great Lakes Connecting Channels Study, 1988). At each site, a pontoon boat with a Pneumatic

Vibracore Core Sampler with 14,000 vibrations per min (Great Lakes Environmental Center, Traverse City, MI) removed five sediment cores using sterile acetate butyrate tubes with an inside diameter of 9.5 cm. Surface sediment was removed with a petite ponar sampler. Surface sediment and the cores were stored vertically on dry ice during transport, and subsequently stored vertically at -80°C. One frozen sediment core from each site was aseptically cut into 2 cm vertical sections (core length: AB = 86 cm and CR = 58 cm), and stored at -80°C. There were a total of 43 and 29 sediment sections from the AB and CR cores, respectively.

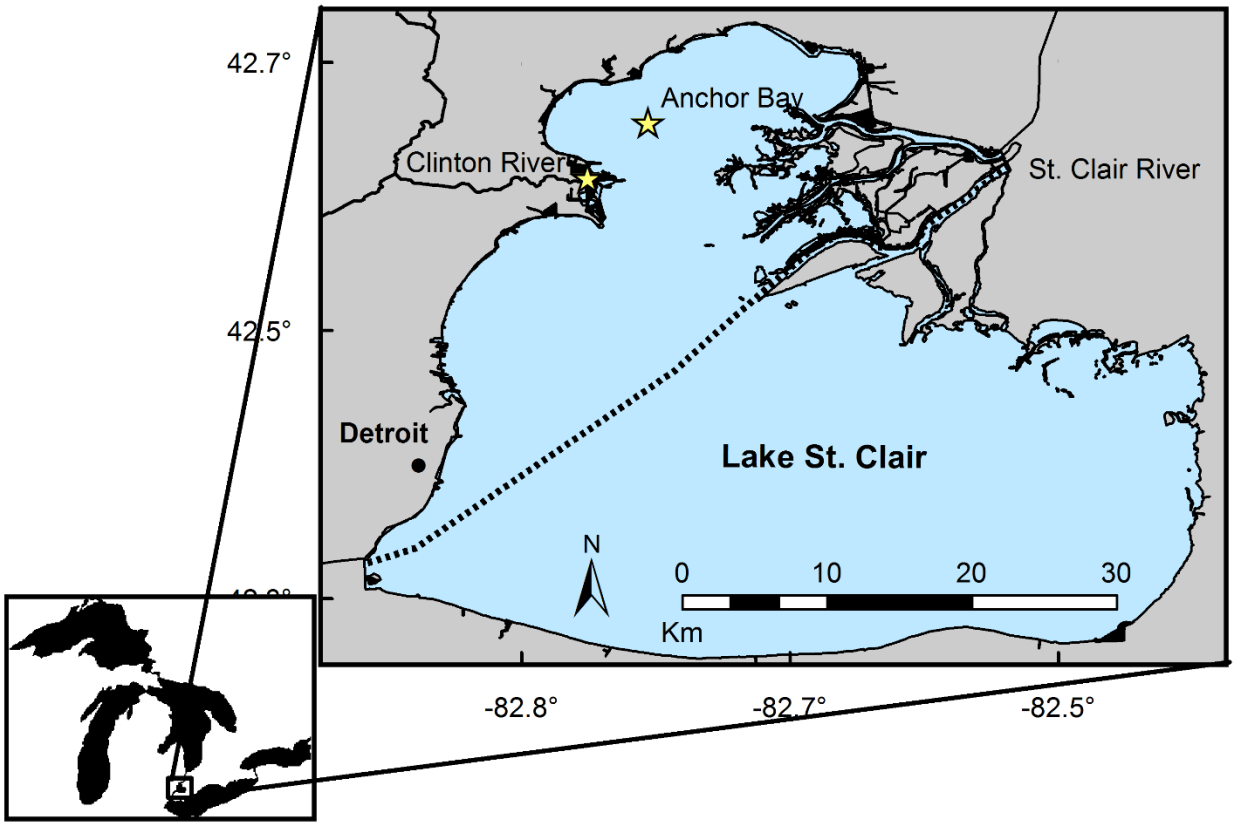

Figure 5.1: Map of the Lake St. Clair basin, and the sediment core sites labeled with stars: Anchor Bay (AB), and the Clinton River (CR).

5.2.2 DNA Extraction and qPCR measurements of enterococci *23S rDNA* and *E.coli uidA*

The AB and CR sediment sections were thawed at 4˚C overnight and homogenized with EPA-DNA2 with G2 (Geological Survey of Denmark and Greenland, GEUS, Coppenhagen, Denmark). In triplicate, the total DNA of 1 g (wet weight) of each sediment section was extracted using the method EPA-DNA2 +G2, outlined in **Chapter 4.2.2.** Enterococci *23S rRNA* (ENT-23) and *E.coli uidA* (EC-uidA) were measured using qPCR. The primer probe sequences, qPCR protocols and reaction matrices are outlined in **Table 2.1**. The genome copy number of EC-uidA was one, while the estimated copy number of ENT-23 was an average of four copies per *Enterococcus faecalis* cell (Srinivasan et al., 2011; USEPA, 2012b). The concentrations of ENT-23 and EC-uidA were transformed to cell equivalents (CE) per g-dry wt. The EC-uidA and ENT-23 concentrations in the DNA extracts were calculated from their respective qPCR standard curves. The ENT-23 and EC-uidA standard curves were produced from genomic DNA extracted from overnight cultures of *Enterococcus faecalis* ATCC strain 19433 and *E.coli* ATCC strain 15597, respectively, using Qiamp DNA Mini Kit (Qiagen Inc, Valencia, CA). The genomic DNA was serially diluted (1:10) to create at least 6 dilution steps for a standard curve. A new standard curve was made after every four qPCR runs. The average efficiency, r^2 value, and threshold cycle value of the lowest quantified concentration in the standard curve, and its representative copies/rxn of ENT-23 (and EC-uidA) were: 102% (99%), 0.98 (0.99), 34.44 (36.05), and 19.7 copies/rxn (1860 copies/rxn), respectively.

5.2.3 Sediment chronostratigraphy

Approximately, ~30 g (wet weight) of each sediment section was dried at 90˚C for 48 hrs. The percent water content in each sediment section was determined, and used to calculate the porosity and commutative mass depths (Jweda & Baskaran, 2011). From the measured vertical

profiles of Cs-137 and excess Pb-210 activity, the approximate years of deposition were calculated as previously outlined (Jweda & Baskaran, 2011). The activities of total ^{210}Pb, ^{226}Ra, and ^{137}Cs were measured using high-resolution gamma-ray spectrometer, and the details are given in Jweda and Baskaran (2011). Details on the excess ^{210}Pb ($^{210}Pb_{xs}$) and ^{137}Cs-based chronologies are discussed in Baustian et al. (in prep.). From the peak ^{137}Cs activity corresponding to 1963, the chronology of the sedimentary layers were established. There was an overall agreement between $^{210}Pb_{xs}$ and ^{137}Cs based chronologies.

5.2.4 Nutrient measurements

Percent total C (% tC) and total N (% tN) were measured with modifications of a previously described method (B. Fry, 2007). Briefly, the samples were acidified in a 10% 1 M HCl solution overnight at 50°C to dissolve carbonates. The samples were added to a three series system: EuroVector EA3000 CHNS elemental analyzer (EuroVector S.p.A, Milan, Italy), a GC+ O_2 column scrubber system, and an IsoPrime IRMS stable isotope ratio mass spectrometer (Elementar Americas, Inc., Mt. Laurel, New Jersey). The first combustion furnace consisted of Cr_2O_3, CoO, and quartz chips. It remained at 1060°C to allow for CO_2 SO_2, H_2O, and N_2 combustion products to form. The H_2O was scrubbed out in a glass water trap. The samples passed to a second furnace at 650°C that consisted of Cu and quartz chips. The combustion gases passed to a GC column in a 10 cm long glass oxygen trap. Mass spectrometry measured N_2 and CO_2 sequentially from peak heights of 28, and 44 g/mol, respectively, in the GC column (3 m long) at 50˚C. Alanine standards (A7627-1G, Sigma Aldrich, St. Louis, MO) of varying masses were also prepared. Linear regression analysis of the standards was calculated using Microsoft Excel (Microsoft Corp., Redwood, Washington, USA) in order to accurately calculate the N and

C concentrations in the sediment samples. Concentrations were calculated as % total N and % total C based on the mass of the dried sediment.

Concentrations of total P (tP) were measured with previously outlined methods (American Public Health Association, 1998; Andersen, 1976). Briefly, the sediment (0.15 - 0.2 g) was ashed at 550°C for 2 hr in a muffle furnace. The cooled sediment residues were washed with 15 ml 1 M HCl, and boiled for 15 min. The samples were diluted with dH_2O to 100 ml. The solutions were analyzed for PO_4^{-3}–P using a previously described perchloric acid method. Briefly, 50 ml of the sample was added to a flask with one drop of phenolphthalein. If the sample turned red, then enough drops of 5 M H_2HO_4 were added to dilute the color. A reaction matrix of the following solutions was made: 0.1 M $C_6H_8O_6$, 5M H_2HO_4, 0.005M $K(SbO)C_4H_4O_6 \times ½ H_2O$ and 0.043 M $(NH_4)_6 Mo_7O_{24} \times 4H2O$. Of this reaction matrix, 8ml was added to the samples and mixed. The samples rested for 10 – 30 min and the absorbance was measured at 880 nm using Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific Inc, Waltman, MA). A calibration standard of serially diluted phosphate solutions was analyzed with Microsoft Excel (Microsoft Corp., Redwood, Washington, USA), and used to calculate the samples' concentration of tP.

5.2.5 Measurements of the anthropogenic and climate data

The average monthly temperature (°C) was measured at Selfridge Air National Guard Base (42°36.498'N, 82°49.098'W) during 1937 – 2012 (Midwestern Regional Climate Center, 2014). The base represented the air temperatures of Anchor Bay and Clinton River because of its close proximity to the two sites. The monthly average discharge (m^3/sec) from St. Clair River (1900 - 2012) was previously published (Hunter & Croley, T.E., 1993), with data continuously added on

http://www.glerl.noaa.gov/data/arc/hydro/mnth-hydro.html. The St. Clair River was chosen to represent Anchor Bay because it contributes the majority of the water discharged into Anchor Bay. The average daily discharge (m^3/sec) of the Clinton River was measured during 1935 - 2012 from the USGS weather station **04165500** (42°35.75'N, 82°54.53'W) upstream of CR(USGS, 2014). These measurements were averaged over a calendar year.

The data concerning the human population in the communities of the Clinton River and Anchor Bay watersheds during 1900 - 2010 were gathered from the U.S. Census Bureau (SEMCOG, 2002). Communities in the watersheds were identified with having ≥ 50% of their area within the watershed boundaries. A linear regression was performed on the census data gathered from the Anchor Bay and Clinton River watersheds during 1900 – 2010, in order to estimate the annual population in the respective watersheds, and the linear regression equations were:

$Ln(P_{AB}) = -38.86 + 0.03 * Y$;

$Ln(P_{CR}) = -53.39 + 0.03 * Y$,

respectively, where P_x was the estimated population in the Clinton River or Anchor Bay watershed, and Y was the year.

Each core section represented multiple calendar years. Therefore, the average air temperature, river discharge from the St. Clair River and the Clinton River, and estimated population data points were calculated for each section of the core within the range of years represented in each core section in order to develop a dataset representative of the time frames represented in the cores.

5.2.6 Multiple linear regression analyses

Multiple linear regression analyses evaluated two datasets: ENT concentrations, C_{ENT}, deposited during c.1932 - 2012 in both cores; and EC concentrations, C_{EC}, deposited during c.1932 - 2012 in the AB core and during c.1951 - 2012 in the CR core, and their associations to the following independent variables: EC (when C_{ENT} was the dependent variable) or ENT (when C_{EC} was the dependent variable) concentrations, C_{EC} or C_{ENT}, respectively; calendar year, *Y*; sedimentary nutrient (P, N, and, C) concentrations (ug/g-dry wt), *H*, *N*, and *A*, respectively; watershed population, *P*; river discharge, *D*; air temperature, *T*; and site, *S*. The ENT and EC linear regression equations were:

$$\ln(C_{ENT}) = \mathrm{b}_{\mathrm{C-EC}} * \ln(C_{EC}) + b_P * P + b_Y * Y + b_H * H + b_N * N + b_A * A + b_T * T + b_D * D + b_S * S + b_0;$$

$$\ln(C_{EC}) = \mathrm{b}_{\mathrm{C-ENT}} * \ln(C_{ENT}) + b_P * P + b_Y * Y + b_H * H + b_N * N + b_A * A + b_T * T + b_D * D + b_S * S + b_0,$$

respectively. Also, b_0 was the y-intercept, and *b* with a letter subscript represented the correlation coefficient of each independent variable. In the EC dataset, there was a lag of three sediment core sections in the discharge rate, which translated to ~6 years. The class variable, site, had two outputs, AB and CR, which were transformed the numbers to 1 and 2, respectively. Independent variables that were non-significant were not removed from the regression model.

5.3 Results

5.3.1 Sedimentation rate and sediment chronostratigraphy

It appears that there was little vertical mixing of sediment in the Cs-137 profile (**Figure 5.2**). The mass accumulation rates (and linear sedimentation rates) in the AB and CR cores were 0.35

g/cm^2 (0.39 cm/yr), and 0.42 g/cm^2 (0.67 cm/yr) respectively. Therefore, the AB and CR cores represented approximately 255 and 117 years, respectively. Uniform sedimentation rate over the whole core was assumed over this time period which cannot be validated by other methods.

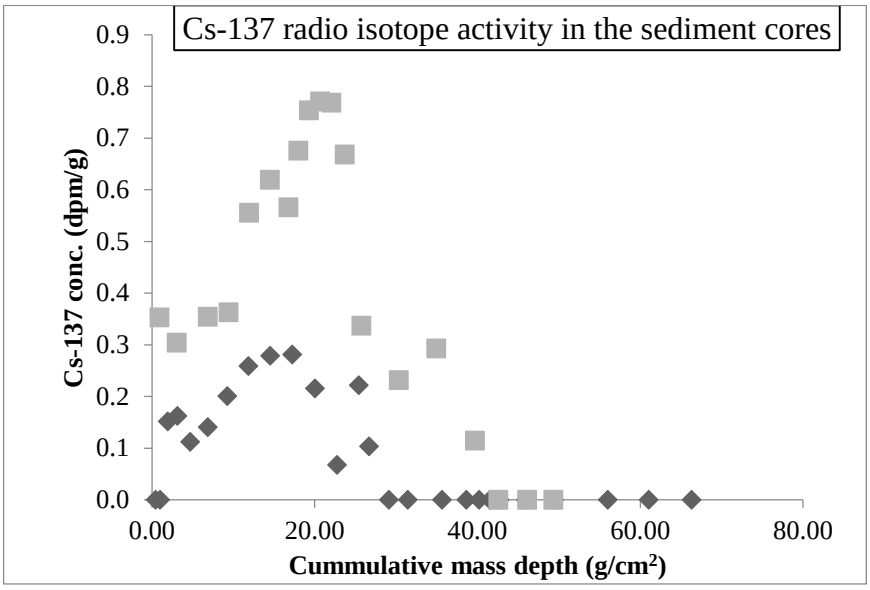

Figure 5.2: Cs-137 radio isotope activity profile (dpm/g-dry wt) of the sediment cores from Anchor Bay (AB, ♦) and the Clinton River (CR, ■) measured against the cumulative mass depth of the cores (g-dry wt/cm2).

5.3.2 Climatic measurements: air temperature and discharge rates

The average annual air temperatures were averaged to represent the time interval estimated in each sediment section, and ranged from 8.4° - 10.27°, and 7.9° -10.2° C for AB and CR, respectively **(Figures 5.3A and 5.4A,** respectively**)**. From 1932 to 1951 and from 1978 to 2012, the air temperature increased, while it decreased during 1951 to 1978 **(Figure 5.3A and 5.4A)**.

The annual average river discharge representative of AB and CR was averaged within the time intervals estimated in each sediment section. The range of discharge in St. Clair River (4.52 x10^3 - 5.84 x10^3 m^3/s) was larger than the Clinton River (0.65 x 10^1 - 3.23 x 10^1 m^3/s, **Figures 5.3B and 5.4B,** respectively**)**. The discharge in the Clinton River increased, then decreased, and

then increased again from c.1934 – c.1945; c.1945 – c.1963; and c.1963 – 2012, respectively (**Figure 5.4B)**. The discharge in St. Clair River decreased c.1971 - 2012 (**Figure 5.3B).**

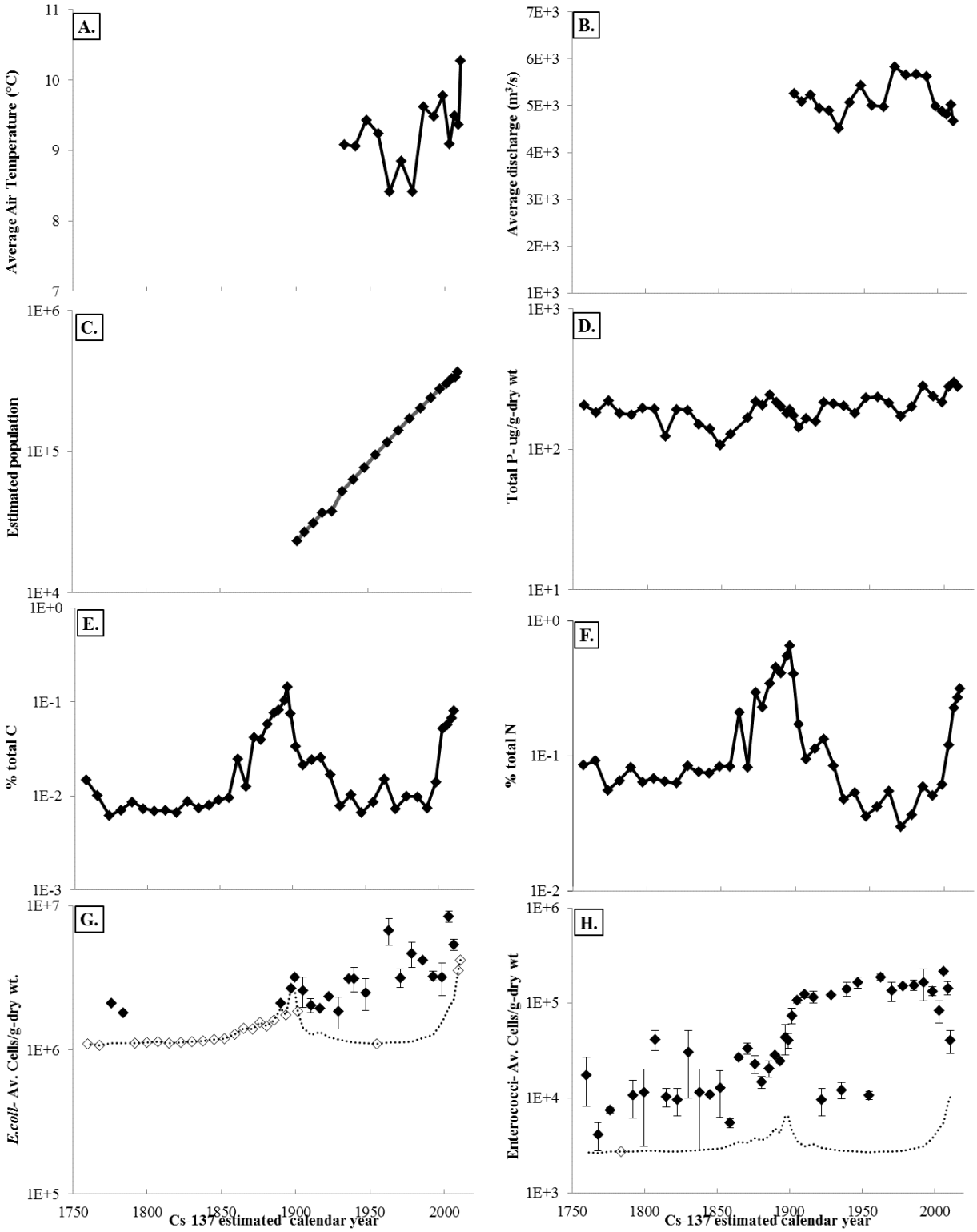

Figure 5.3A-H: Indictors of climatic variables [a] **(A-B)**, population in the watershed [b] **(C)**, sedimentary nutrient concentrations [c] **(D-F)**, and fecal indicator concentrations [d] **(G-H)** in the Anchor Bay watershed.

[a] Air temperature at Selfridge Air National Guard Base, Mt. Clemens, MI (1937 - 2012), and B) discharge (m^3/s) in the St. Clair River (1900 - 2012). The annual average air temperature, and

Figure 5.3 (cont'd).
discharge were averaged within the time interval estimated in each sediment section of the Anchor Bay core.
[b] Population in the Anchor Bay watershed was estimated from census data (1900 - 2010). The human population in the watershed was averaged within the time interval estimated in each sediment section of the Anchor Bay core.
[c] Sedimentary nutrient concentrations in the Anchor Bay core (c.1760 - 2012) were described with: D) total P (ug/g-dry wt), E) % total C, and F) % total N.
[d] Fecal indicator concentrations in the Anchor Bay core were measured via G) *Escherichia coli* (EC), and H) Enterococci (ENT) concentrations (cell equivalents/ g-dry wt, c.1760 - 2012). The lines represent the ENT and EC detection limits in the Anchor Bay core sections. Data points that are not filled indicate samples that were below the detection limit. The error bars represent one standard error. **Table S5.1** gives a further description of *E.coli* and enterococci concentrations in the cores, respectively.

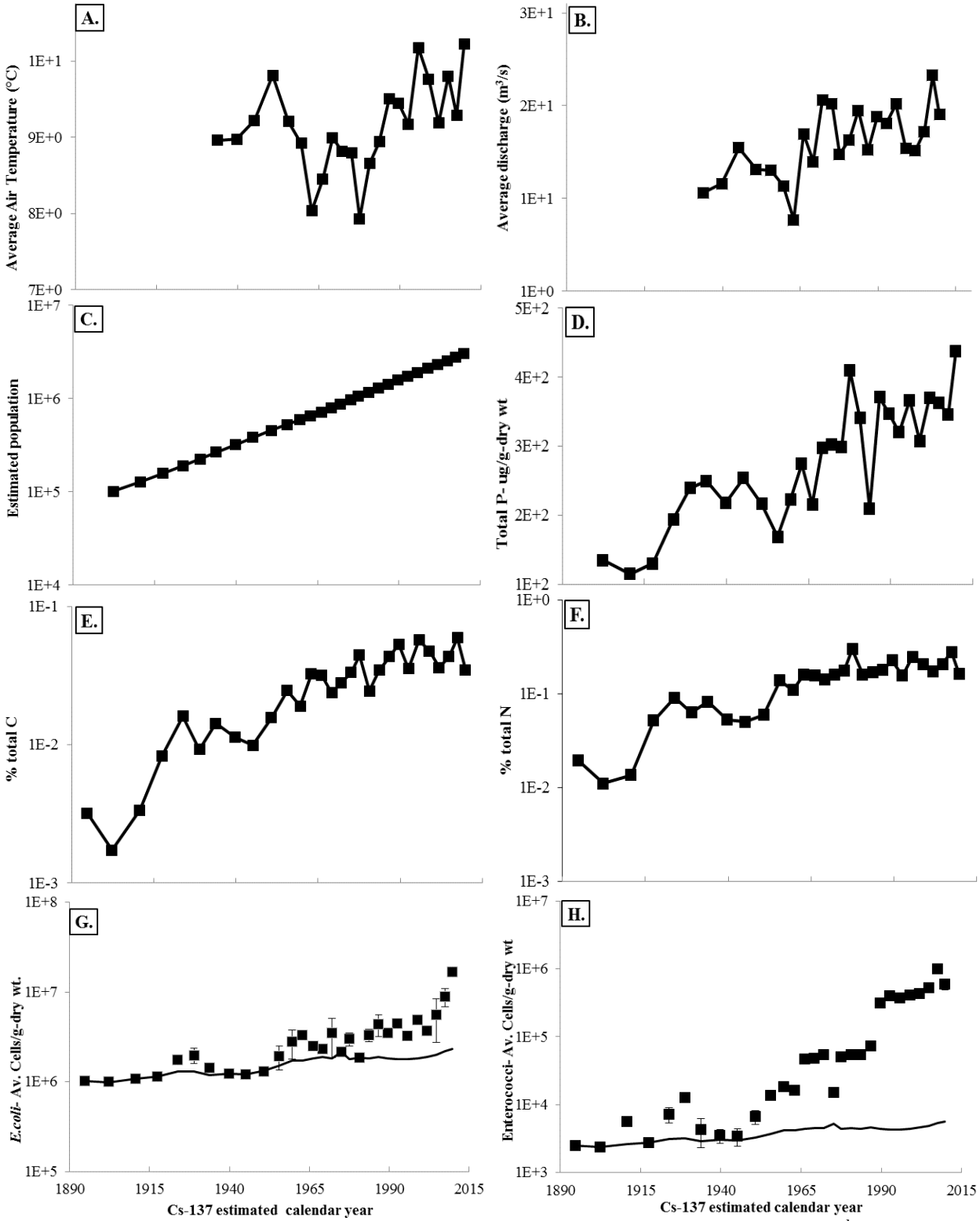

Figure 5.4A-H: Indictors of climatic variables [a] **(A-B)**, population in the watershed [b] **(C)**, sedimentary nutrient concentrations [c] **(D-F)**, and fecal indicator concentrations [d] **(G-H)** in the Clinton River watershed.

Figure 5.4 (cont'd).
[a] Air temperature at Selfridge Air National Guard Base, Mt. Clemens, MI (1937 - 2012), and B) discharge (m^3/s) in the Clinton River (1935 - 2012). The annual average air temperature, and discharge were averaged within the time interval estimated in each sediment section of the Clinton River core.
[b] Population in the Clinton River watershed was estimated from census data (1900 - 2010). The human population in the watershed was averaged within the time interval estimated in each sediment section of the Clinton River core.
[c] Sedimentary nutrient concentrations in the Clinton River core (c.1895 - 2012) were described with: D) total P (ug/g-dry wt), E) % total C, and F) % total N.
[d] Fecal indicator concentrations were measured via **G)** *Escherichia coli* (EC), and **H)** enterococci (ENT) concentrations (cell equivalents/ g-dry wt, c.1895 - 2012). The lines represent the ENT and EC detection limits in the Clinton River core sections. Data points that are not filled indicate samples that were below the detection limit. The error bars represent one standard error. **Table S5.2** gives a further description of *E.coli* and enterococci concentrations in the cores, respectively.

5.3.3 Anthropogenic attributes: estimated census population and nutrient loading in the cores.

The estimated human populations in the Anchor Bay and Clinton River watersheds (1900 - 2010) were averaged to represent the time intervals in each sediment section. In 1900 (and 2010), the population density per km^2 in AB and CR watersheds was: 52.76 and 51.32 people per km^2, respectively (1.84×10^3 and 1.55×10^3 people per km^2, respectively). The Clinton River watershed had a larger total population compared to the Anchor Bay watershed **(Figures 5.3C and 5.4C)**, while the estimated growth rates in both watersheds were similar. Since 1900, the estimated populations in both watersheds have increased by ~1-log **(Figures 5.3C and 5.4C)**.

The nutrient concentrations from the CR core steadily increased towards present day (**Figures 5.4D-F**). The tP concentrations in the AB and CR cores were similar until c.1966 (~ 1.5×10^2 ug/g-dry wt; **Figures 5.3D and 5.4D,** respectively), and thereafter doubled in CR core (**Figure 5.4D**). The range of the % tC in the AB core was larger (6.2×10^{-1} – 140×10^{-1} % tC) compared to the CR core (9.3×10^{-1} – 59.3×10^{-1} % tC, **Figures 5.3E and 5.4E,** respectively),

while the range of the % tN in the AB core was smaller ($3.0 \times 10^{-2} – 66 \times 10^{-2}$ % tN) compared to the CR core ($5.0 \times 10^{-2} – 30 \times 10^{-2}$ % tN, **Figures 5.3F and 5.4F**). Starting in c.1856, the % tC and % tN in the AB core began to increase > 1-log until they reached their largest values in c.1896 (140×10^{-1} % tC, and 6.6×10^{-1} % tN, respectively), and then began to decrease by > 1-log until the concentrations returned to the pre-1856 values at c.1932 (**Figures 5.3E-F**). The % tC (and % tN) was lower in the CR core than in the AB core during two intervals: pre-1925 (pre-1918), and post-2007 (post-2009; **Figures 5.4E-F**). The concentrations of sedimentary nutrients measured in AB and CR are detailed in **Tables 5.1 and 5.2**, respectively.

5.3.4 Fecal indicator concentrations in sediment cores

ENT-23 and EC-uidA concentrations were determined in each section of the AB and CR cores (129 and 87 samples in total, respectively). In the AB (and CR) sediment sections, 9% (13%) and 72% (41%) of the samples were below the detection limits of ENT-23 and EC-uidA, respectively, and those ENT and EC concentrations were reported at their detection limits (**Figures 5.3G-H and 5.4G-H**). The oldest detectable ENT (and EC) concentrations in the AB and CR cores were deposited in c.1760 (c.1776), and c.1911 (c.1924), respectively (**Figures 5.3G-H and 5.4G-H**). The EC concentrations in the CR and AB cores increased towards present day in both sites, and ranged from 0.14×10^7 to 1.69×10^7 CE/g-dry wt, and 1.8×10^6 to 8.5×10^6 CE/g-dry wt, respectively (**Figures 5.3G and 5.4G**). The ENT concentrations were at steady-state in the AB core during the following time intervals: c.1760 - c.1860 (~0.01×10^6 CE/g-dry wt), and c.1910 – c.2003 (~1.0×10^6 CE/g-dry wt, **Figure 5.3H**). ENT concentrations in CR increased towards present day, and ranged from ~0.03×10^5 to 9.9×10^5 CE/g-dry wt

(**Figure 5.4H)**. The EC and ENT concentrations in the AB and CR cores are further detailed in **Tables 5.1 and 5.2,** respectively.

5.3.5 Linear regression analyses

Multiple linear regression analyses evaluated ENT concentrations (deposited during c.1932 - 2012 in both cores), and EC concentrations (deposited during c.1932 - 2012 in the AB core and during c.1951 - 2012 in the CR core), and their associations to the following independent variables: year, site, EC or ENT concentration in the core sections (opposite of the dependent variable), river discharge, air temperature, sedimentary nutrient concentrations (% tC, % tN, and tP), and estimated population in the watersheds. The R^2 values (and sample size) for the ENT and EC datasets were 0.86 (n = 36), and 0.72 (n = 33), respectively **(Table 5.1)**. Also, the coefficients of the linear regression equation and their standard errors are listed in **Table 5.2**. The ENT concentrations in both cores were significantly correlated to the discharge in the Clinton River and the St. Clair River (p = 0.046), and the estimated population in the Clinton River and Anchor Bay watersheds (p = 0.003, **Table 5.1)**. The EC concentrations in both cores were significantly associated with tN and tC concentrations (p = 0.038, and 0.029, respectively), estimated population in the Clinton River and Anchor Bay watersheds (p = 0.023), and air temperature (p = 0.018, **Table 5.1**). ENT and EC concentrations were not significantly correlated to each other **(Table 5.1),** nor were the FIB concentrations significantly different between the sites **(Table 5.1)**.

Table 5.1: Linear regressions evaluated the correlation strengths (and p-values) of climatic and anthropogenic variables [a] to 1) enterococci concentrations (ENT) [b,c], C_{ENT}; and 2) *E.coli* concentrations (EC) [b,d].

		Correlation strength of the independent variables (p-value)								
Data set (sample size)	**R^2**	**P**	**Y**	**H**	**N**	**A**	**T**	**D**	**S**	**C**
C_{ENT} (n = 37)	0.860	0.570 (p = 0.003)	0.779 (p = 0.483)	0.471 (p = 0.306)	0.421 (p = 0.145)	0.483 (p = 0.492)	0.358 (p = 0.804)	0.168 (p = 0.046)	-0.161 (p = 0.175)	0.791[e] (p = 0.079)
C_{EC} (n = 33)	0.724	0.478 (p = 0.023)	0.613 (p = 0.498)	0.430 (p = 0.289)	0.269 (p = 0.038)	0.408 (p = 0.029)	0.214 (p = 0.018)	0.068 [g] (p = 0.058)	-0.046 (p = 0.117)	0.715 [f] (p = 0.230)

[a] *Escherichia coli* concentrations (when C_{ENT} was the dependent variable), C_{EC} or enterococci concentrations (when C_{EC} was the dependent variable), C_{ENT}; calendar year, Y; nutrient (total P, % N, and % C) concentrations in the cores, H, N, and A, respectively; population in the Anchor Bay and Clinton River watersheds, P; St. Clair River and Clinton River discharge, D; air temperature, T; and site, S.

[b] Enterococci (C_{ENT}), and *E.coli* (C_{EC}) concentrations were normalized to cell equivalents per g dry wt.

[c] Linear regression analysis evaluated core sections deposited during c.1932-2012 in both cores.

[d] Linear regression analysis evaluated core sections deposited during c.1932-2012 and during 1951-2012 in the AB and CR core, respectively, C_{EC}.

[e] Measured the coefficient and significance of the independent variable, C_{EC}.

[f] Measured the coefficient and significance of the independent variable, C_{ENT}.

[g] There was a ~6 year lag in the discharge rates in the *E.coli* dataset.

Table 5.2: The coefficients and their standard errors of the climatic and anthropogenic variables in linear regression equations [a] that evaluated their associations to: 1) enterococci concentrations (ENT) [b,c], C_{ENT}; and 2) *E.coli* concentrations (EC) [b,d], C_{EC}.

	Coefficient values (b_n, where n is the coefficient) of the independent variables (standard error of the coefficients)									
Data set[a] (sample size)	**P**	**Y**	**H**	**N**	**A**	**T**	**D**	**S**	**C**	**b_0**
C_{ENT} (n = 37)	0.000 (0.000)	0.008 (0.011)	-0.003 (0.003)	0.822 (0.547)	-0.016 (0.023)	0.077 (0.306)	0.001 (0.001)	3.977 (2.850)	0.682 (0.373)	-25.252 (20.173)
C_{EC} (n = 33)	0.000 (0.000)	-0.004 (0.006)	0.002 (0.002)	-0.589 (0.267)	0.024 (0.010)	-0.349 (0.137)	0.001 [e] (0.000)	2.309 (1.417)	0.116 (0.088)	19.466 (10.509)

[a] The equations were:

$\ln(C_{ENT}) = b_{C-EC} * \ln(C_{EC}) + b_P * P + b_Y * Y + b_H * H + b_N * N + b_A * A + b_T * T + b_D * D + b_S * S + b_0$;

$\ln(C_{EC}) = b_{C-ENT} * \ln(C_{ENT}) + b_P * P + b_Y * Y + b_H * H + b_N * N + b_A * A + b_T * T + b_D * D + b_S * S + b_0$.

Their variables: *Escherichia coli* concentrations (when C_{ENT} was the dependent variable), C_{EC} or enterococci concentrations (when C_{EC} was the dependent variable), C_{ENT}; calendar year, Y; nutrient (P,N, and, C) concentrations in the cores, H, N, and A, respectively; watershed population in Anchor Bay and Clinton River, P; St. Clair River and Clinton River discharge, D; air temperature, T; and site, S.

[b] Enterococci (C_{ENT}), and *E.coli* (C_{EC}) concentrations were normalized to cell equivalents per g dry wt.

[c] in core sections deposited during 1932-2012 in both cores

[d] are deposited in core sections during 1932-2012 in the AB core and during 1951-2012 in the CR core

[e] There was a ~6 year lag in the discharge rates in the *E.coli* dataset.

5.4 Discussion

Previous investigations have measured chemical contaminants, and nutrients in sediment cores in order to reconstruct large scale assessments of historical land management practices (Furl & Meredith, 2011; Kaushal & Binford, 1999). Our study is one of the first studies to evaluate the associations of anthropogenic attributes, and climate variables to FIB concentrations in sediment cores over ~100 to 250 years, contrasting two locations in the Lake St. Clair watershed near the mouth of the Clinton River (CR), and Anchor Bay (AB) in Northwestern Lake St. Clair. The Lake St. Clair watershed is important to the Great Lakes basin as a key connector between the Upper and Lower Great Lakes, as well as home to > 4 million people, and is a drinking water source for metropolitan Detroit and southwestern Ontario (Baustian et al., 2014).

Summaries of the anthropogenic, climatic, nutrient loading and FIB data in both locations is illustrated in **Figures 5.5A-B**. Analysis of the AB core determined that the bottom most sections (c.1760 – c.1800) were deposited when the watershed was a trading outpost and a Native American settlement. During this time, nutrient and FIB measurements were at steady state (**Figures 5.3D-H** and **5.4A**). Starting in 1766, small scale logging of the area began and the timber was rafted on the St. Clair River to Detroit (Crampton, 1921). Wide scale logging, timber processing, and transport began after the Swampland Act of 1850 (Unknown, 1883), which allowed settlers to obtain wetlands at no cost if they were drained and developed (Fishbeck, Thompson, Carr & Huber, 2007). Subsequently, the Anchor Bay watershed was deforested by the early 20th Century (Crampton, 1921). The effects of deforestation, destruction of wetlands, and the booming logging industry were seen in the increasing nutrient and enterococci concentrations until around the start of the 20th Century (**Figures 5.3D-F and 5.5A**). Also, the

C:N ratio before the Swamplands Act of 1850 was ~11:1 (Baustian et al., in prep.), indicating that the tC and tN were mainly from aquatic sources. After the policy change, the C:N ratio increased to 21.85 (Baustian et al., in prep.), which indicated that the tC and tN were from terrestrial sources (Meyers & Ishiwatari, 1993). Similarly, a previous study also observed increases in organic matter and C:N values measured in sediment cores from the Great Lakes that coincided with deforestation (Meyers, 2007). The formation of the second steady state concentration of ENT at 1-log larger than the pre-deforestation levels, and the return to the pre-deforestation levels of % tC, and % tN in the AB core began in c.1896 (**Figures 5.3E-G and 5.5A)**. This demonstrated that activities in the watershed formed a new "equilibrium", which could have been the result of forest re-growth or widespread agricultural practices that buffered nutrient loading and wastewater/fecal pollution inputs into Anchor Bay. Similarly, the nutrients concentrations in a sediment core from Lake Ontario decreased following massive deforestation events (Hodell & Schelske, 1998), and could be the result of re-forestation activities that buffered runoff. Increases in EC and tP concentrations in the AB core starting c.1890 and c.1849 (**Figures 5.3D, 5.3G, and 5.5A**), respectively, could be related to the increase in population of the watershed, which may be related to wastewater production. The sedimentary % tN and % tC returned to a cyclical steady state during c.1932 – c.1992, which occurred during the wide scale development of Michigan's shorelands (Fishbeck, Thompson, Carr & Huber, 2007) (**Figures 5.3E-F, and 5.5A**). Such development did not allow for natural buffers to retain run off from nonpoint inputs of wastewater and fertilizers, which may have accounted for the cyclic nature of the nutrient loading. In 1972, The National Pollutant Discharge Elimination System (NDPES) began regulating P loading in the Great Lakes (Harrington-Hughes, 1978), and during this period, its concentration was in a cyclical steady state (**Figures 5.3D and 5.5A**), which can be

partially attributed to erosion in the riverbed caused by dredging and increased air temperatures that lead to higher evaporation rates (Associated Press, 2007) (**Figures 5.3B and 5.5A**). The ENT and EC concentrations began to decrease in 2003, which could be the result of programs initiated in 2002 in St. Clair and Macomb counties to identify and fix failing septic systems (St. Clair County Health Department, 2009) (**Figures 5.3G-H; and 5.5A**).

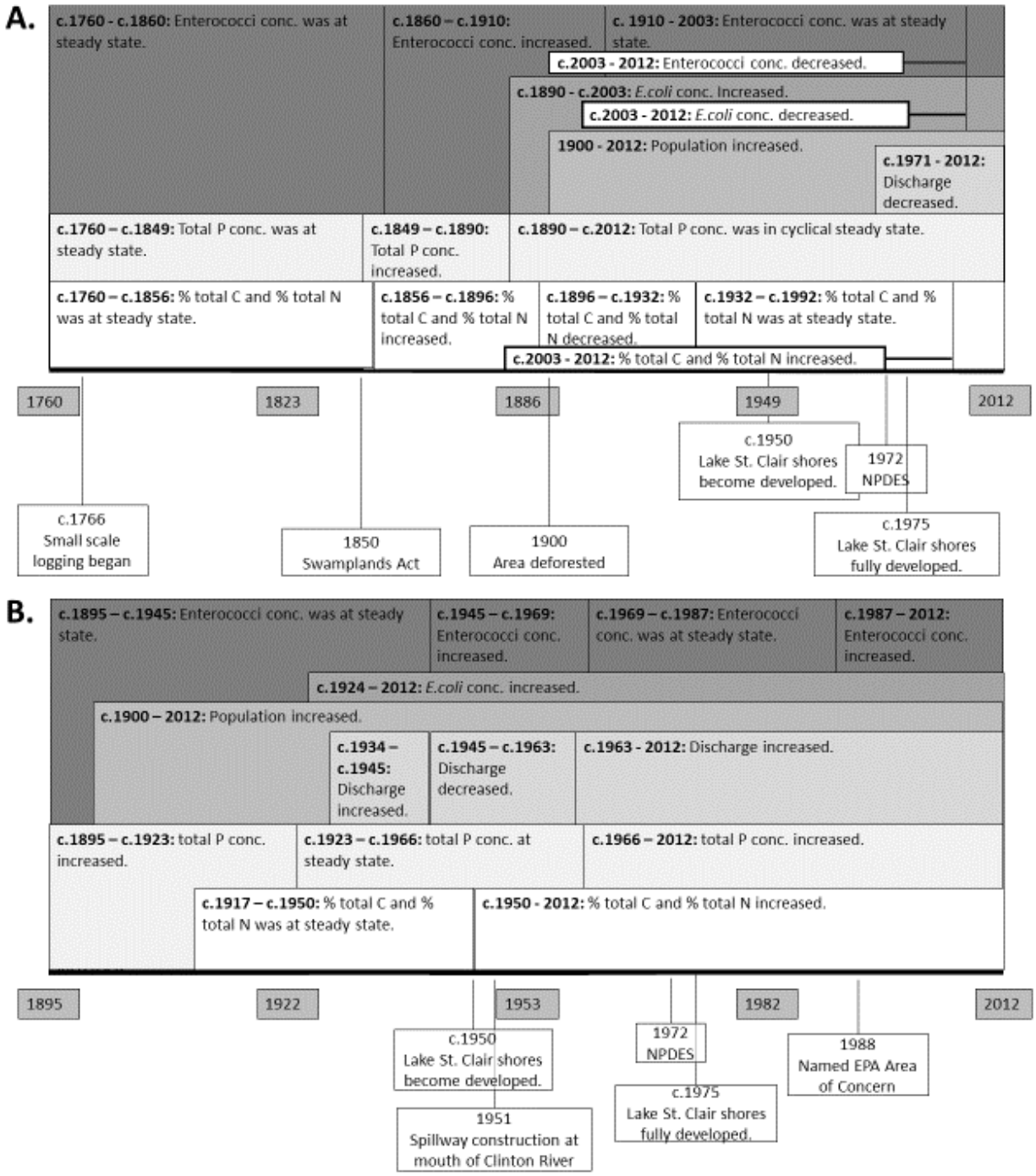

Figure 5.5A-B: Illustrations of the patterns of discharge of the St. Clair River **(A)** and the Clinton River **(B)**, anthropogenic variables [b], sedimentary nutrient concentrations [c], and fecal indicator concentrations [d] from the Anchor Bay [e] **(A)** and Clinton River [e] **(B)** watersheds.
[a] The NDPES is the National discharge pollutant elimination system.
[b] Anthropogenic variables included human population in watershed, and policy changes.
[c] Sedimentary nutrient measurements included: total phosphorus, % total C, and % total N.
[d] The measurements included: *Escherichia coli* and enterococci cell equivalents.

Figure 5.5 (cont'd).
[e] The Anchor Bay and Clinton River sediment cores spanned the years: c.1760 - 2012 and c.1895 - 2012, respectively.

The large sedimentation rate in the CR core could be due to higher sediment supply (per unit volume of water discharge) from the Clinton River compared to St. Clair River. Overall, the river discharge, and FIB and nutrient concentrations in the CR core have increased since c.1895 (and since c.1934 for river discharge only, **Figures 5.4B, and 5.4D-H**). The increase of nutrient and FIB concentrations in the Clinton River were previously attributed to failing septic systems, storm water, and runoff(Environmental Conuslting and Technology, 2007). The sedimentary tP profile suggest that its loading began to increase in c.1966 **(Figures 5.4D and 5.5B)**. However, the rate of tC and tN loading decreased in c.1962 (**Figures 5.4E-F)**, and ENT concentrations stabilized during c.1969 – c.1987 (**Figure 5.4H, and 5.5B)**, perhaps due to the elimination of raw sewage inputs and regulation of point source pollution instituted by National Pollutant Discharge Elimination System in 1972 (Michigan Department of Environmental Quality, 1988). The discharge at the mouth of the Clinton River decreased in 1951 (**Figures 5.4B, and 5.5B)** as a result of the construction of a spillway to prevent flooding (Michigan Department of Environmental Quality, 1988), which didn't curb the loading of FIB or nutrients into the core (**Figures 5.3D-H, and 5.5B**). The Clinton River was first listed as an EPA Area of Concern in 1988, and a remedial action plan was drafted in order to remediate the watershed. Revitalization efforts of the Clinton River may have decreased the C:N ratio c.1987 (Baustian et al., in prep.). However, delisting of the Clinton River has yet to be accomplished, and is witnessed in the increasing FIB and nutrient loading in the core since c.1987 **(Figures 5.4D-H, and 5.5B).**

Our study is one of the first to measure genetic markers from ENT and EC in sediments that were deposited in the Lake St. Clair watershed > 200 yrs ago. Previously, culturable ENT and

EC were enumerated from 60 cm beneath the surface sediment in Lake Geneva, Switzerland (Thevenon et al., 2012). Their and our results further suggest that sediments are long term reservoirs for FIB. The sensitivity of EC-uidA resulted in incomplete profiles of the EC concentrations in the cores. However, when detected, the EC concentrations were larger than ENT **(Figures 5.3G, and 5.4G)**, which was echoed in measurements of culturable ENT and EC from sediment cores obtained from Lake Geneva (Thevenon et al., 2012).

Our study evaluated the statistical associations of anthropogenic (human population in watershed, and nutrient concentration), and climate (river discharge, and air temperature) variables to FIB concentrations in both cores. The ENT and EC concentrations in both cores were significantly correlated to population ($p = 0.003$, and 0.023, respectively). A previous study also reported that urbanized areas of a marine estuary experienced larger fecal coliform concentrations (Kelsey, Porter, Scott, Neet, & White, 2004). EC concentrations were significantly associated to % tC and % tN ($p = 0.029$, and 0.038, respectively) in our study. Similarly, culturable EC concentrations in sediments increased with organic matter and N concentrations (Haller, Amedegnato, et al., 2009). The air temperature was negatively associated with EC concentrations in the cores ($p = 0.018$). EC *23S rDNA* demonstrated longer persistence in beef manure amended soil stored at 10°C than at 25°C (Rogers et al., 2011). ENT concentrations were significantly correlated to river discharge in our study ($p = 0.046$), suggesting that ENT attachment to suspended particles facilitates their movement to the benthos (Jeng, England, & Bradford, 2005). Therefore, increased discharge partially made up of wastewater effluent, discharges from combined sewer overflow events, and excessive non-point runoff from flooding could increase ENT concentrations in surface sediments. The lack of

correlation between ENT and EC concentrations could be due to the type of pollution inputs and their distinct decay rates.

Our data provided evidence that paleolimnological investigations can determine associations between climate, anthropogenic attributes, regulation, and water quality over long time scales. Sediment core studies can be tools to analyze historical fecal pollution as markers of anthropogenic transformation of the area, watershed management and shape future practices.

APPENDIX

Table S5.1: Concentrations of *Escherichia coli*, enterococci, total phosphorus, % total nitrogen and % total carbon measured in the Anchor Bay sediment core.

Depth (cm)	**Year deposited** [a]	***E.coli* conc. (CE/g-dry wt)** [b]	**Enterococci conc. (CE/g-dry wt)** [b]	**Total Phosphorus conc. (ug/g-dry wt)**	**% total Nitrogen**	**% total Carbon**
2	2011	$\leq 4.20 \times 10^{6}$ [c]	4.01×10^{4}	-	3.16×10^{-1}	8.02×10^{0}
4	2009	$\leq 3.58 \times 10^{6}$ [c]	1.43×10^{5}	2.80×10^{2}	2.69×10^{-1}	6.71×10^{0}
6	2006	5.38×10^{6}	2.14×10^{5}	3.01×10^{2}	2.26×10^{-1}	5.67×10^{0}
8	2003	8.46×10^{6}	8.35×10^{4}	2.82×10^{2}	1.21×10^{-1}	5.23×10^{0}
10	1999	3.20×10^{6}	1.33×10^{5}	2.16×10^{2}	6.18×10^{-2}	1.40×10^{0}
12	1992	3.26×10^{6}	1.65×10^{5}	2.40×10^{2}	5.09×10^{-2}	7.49×10^{-1}
14	1986	4.23×10^{6}	1.53×10^{5}	2.85×10^{2}	5.96×10^{-2}	9.83×10^{-1}
16	1978	4.66×10^{6}	1.49×10^{5}	2.01×10^{2}	3.69×10^{-2}	9.96×10^{-1}
18	1971	3.17×10^{6}	1.34×10^{5}	1.73×10^{2}	2.99×10^{-2}	7.34×10^{-1}
20	1963	6.76×10^{6}	1.86×10^{5}	2.15×10^{2}	5.50×10^{-2}	1.53×10^{0}
22	1955	$\leq 1.11 \times 10^{6}$ [c]	1.06×10^{4}	2.38×10^{2}	4.23×10^{-2}	8.68×10^{-1}
24	1947	2.51×10^{6}	1.65×10^{5}	2.33×10^{2}	3.59×10^{-2}	6.64×10^{-1}
26	1940	3.14×10^{6}	1.40×10^{5}	1.80×10^{2}	5.39×10^{-2}	1.03×10^{0}
28	1932	3.12×10^{6}	1.22×10^{4}	2.05×10^{2}	4.79×10^{-2}	7.82×10^{-1}
30	1926	1.86×10^{6}	1.21×10^{5}	2.12×10^{2}	8.48×10^{-2}	1.68×10^{0}
32	1919	2.36×10^{6}	9.56×10^{3}	2.16×10^{2}	1.34×10^{-1}	2.56×10^{0}
34	1913	1.95×10^{6}	1.16×10^{5}	1.59×10^{2}	1.14×10^{-1}	2.41×10^{0}
36	1907	2.04×10^{6}	1.22×10^{5}	1.66×10^{2}	9.50×10^{-2}	2.15×10^{0}
38	1902	2.59×10^{6}	1.07×10^{5}	1.43×10^{2}	1.71×10^{-1}	3.36×10^{0}
40	1898	$\leq 1.87 \times 10^{6}$ [c]	7.37×10^{4}	1.75×10^{2}	4.08×10^{-1}	7.44×10^{0}
42	1896	3.21×10^{6}	4.03×10^{4}	1.93×10^{2}	6.57×10^{-1}	1.44×10^{-1}
44	1894	$\leq 2.68 \times 10^{6}$ [c]	4.35×10^{4}	1.81×10^{2}	5.53×10^{-1}	1.04×10^{-1}
46	1890	$\leq 1.75 \times 10^{6}$ [c]	2.44×10^{4}	2.02×10^{2}	4.13×10^{-1}	8.15×10^{0}
48	1887	2.13×10^{6}	2.82×10^{4}	2.17×10^{2}	4.53×10^{-1}	7.60×10^{0}
50	1883	$\leq 1.61 \times 10^{6}$ [c]	2.04×10^{4}	2.44×10^{2}	3.43×10^{-1}	5.85×10^{0}
52	1878	$\leq 1.47 \times 10^{6}$ [c]	1.46×10^{4}	2.06×10^{2}	2.31×10^{-1}	3.94×10^{0}
54	1873	$\leq 1.56 \times 10^{6}$ [c]	2.29×10^{4}	2.19×10^{2}	2.95×10^{-1}	4.19×10^{0}
56	1868	$\leq 1.39 \times 10^{6}$ [c]	3.30×10^{4}	1.68×10^{2}	8.29×10^{-2}	1.27×10^{0}

Table S5.1 (cont'd).

58	1862	$\leq 1.42 \times 10^{6}$ [c]	2.66×10^{4}	1.28×10^{2}	2.09×10^{-1}	2.48×10^{0}
60	1856	$\leq 1.28 \times 10^{6}$ [c]	5.45×10^{3}	1.07×10^{2}	8.37×10^{2}	9.62×10^{-1}
62	1849	$\leq 1.19 \times 10^{6}$ [c]	1.28×10^{4}	1.40×10^{2}	8.36×10^{-2}	9.08×10^{-1}
64	1842	$\leq 1.18 \times 10^{6}$ [c]	1.09×10^{4}	1.51×10^{2}	7.47×10^{-2}	8.01×10^{-1}
66	1835	$\leq 1.16 \times 10^{6}$ [c]	1.14×10^{4}	1.91×10^{2}	7.63×10^{-2}	7.49×10^{-1}
68	1827	$\leq 1.14 \times 10^{6}$ [c]	3.05×10^{4}	1.93×10^{2}	8.42×10^{-2}	8.79×10^{-1}
70	1820	$\leq 1.13 \times 10^{6}$ [c]	9.51×10^{3}	1.24×10^{2}	6.31×10^{-2}	6.70×10^{-1}
72	1812	$\leq 1.12 \times 10^{6}$ [c]	1.03×10^{4}	1.96×10^{2}	6.47×10^{-2}	7.01×10^{-1}
74	1805	$\leq 1.14 \times 10^{6}$ [c]	4.12×10^{4}	1.98×10^{2}	6.86×10^{-2}	6.97×10^{-1}
76	1797	$\leq 1.13 \times 10^{6}$ [c]	1.15×10^{4}	1.77×10^{2}	6.43×10^{-2}	7.33×10^{-1}
78	1789	$\leq 1.12 \times 10^{6}$ [c]	1.07×10^{4}	1.81×10^{2}	8.25×10^{-2}	8.65×10^{-1}
80	1781	1.81×10^{6}	$\leq 2.70 \times 10^{3}$ [c]	2.21×10^{2}	6.62×10^{-2}	7.07×10^{-1}
82	1774	2.12×10^{6}	7.39×10^{3}	1.83×10^{2}	5.57×10^{-2}	6.21×10^{-1}
84	1765	$\leq 1.08 \times 10^{6}$ [c]	4.11×10^{3}	2.06×10^{2}	9.22×10^{-2}	1.01×10^{0}
86	1760	$\leq 1.10 \times 10^{6}$ [c]	1.74×10^{4}	2.80×10^{2}	8.550×10^{-2}	$1.50*10^{0}$

[a] Year of deposition was estimated from the Cs-137 radioactivity in each sediment section.

[b] The enterococci and *E.coli* concentrations were normalized to cell equivalents (CE) per gram-dry wt.

[c] The measurement was below the detection limit, and reported value is the detection limit specific to the sediment section.

Table S5.2: Concentrations of *Escherichia coli*, enterococci, total phosphorus, % total nitrogen and % total carbon measured from the Clinton River sediment core.

Depth (cm)	**Year deposited** [a]	***E.coli* conc. (CE/g-dry wt)** [b]	**Enterococci conc. (CE/g-dry wt)** [b]	**Total Phosphorus conc. (ug/g-dry wt)**	**% total Nitrogen**	**% total Carbon**
2	2010	16.9×10^{6}	5.93×10^{5}	4.37×10^{2}	1.64×10^{-1}	3.48×10^{0}
4	2007	8.87×10^{6}	9.94×10^{5}	3.45×10^{2}	2.77×10^{-1}	5.93×10^{0}
6	2005	5.56×10^{6}	5.20×10^{5}	3.63×10^{2}	2.07×10^{-1}	4.34×10^{0}
8	2002	3.72×10^{6}	4.36×10^{5}	3.70×10^{2}	1.71×10^{-1}	3.61×10^{0}
10	1999	4.87×10^{6}	4.08×10^{5}	3.08×10^{2}	2.07×10^{-1}	4.77×10^{0}
12	1996	3.23×10^{6}	3.75×10^{5}	3.67×10^{2}	2.47×10^{-1}	5.71×10^{0}
14	1993	4.49×10^{6}	3.97×10^{5}	3.20×10^{2}	1.55×10^{-1}	3.53×10^{0}
16	1990	3.50×10^{6}	3.10×10^{5}	3.47×10^{2}	2.25×10^{-1}	5.34×10^{0}
18	1987	4.36×10^{6}	7.39×10^{4}	3.71×10^{2}	1.78×10^{-1}	4.33×10^{0}
20	1984	3.30×10^{6}	5.45×10^{4}	2.09×10^{2}	1.68×10^{-1}	3.45×10^{0}
22	1981	$\leq 1.84 \times 10^{6}$ [c]	5.48×10^{4}	3.41×10^{2}	1.60×10^{-1}	2.45×10^{0}
24	1978	3.01×10^{6}	5.02×10^{4}	4.10×10^{2}	2.99×10^{-1}	4.44×10^{0}
26	1975	$\leq 2.13 \times 10^{6}$ [c]	1.50×10^{4}	2.99×10^{2}	1.76×10^{-1}	3.33×10^{0}
28	1972	3.48×10^{6}	5.40×10^{4}	3.02×10^{2}	1.59×10^{-1}	2.81×10^{0}
30	1969	2.33×10^{6}	4.81×10^{4}	2.97×10^{2}	1.41×10^{-1}	2.37×10^{0}
32	1966	2.50×10^{6}	4.74×10^{4}	2.16×10^{2}	1.58×10^{-1}	3.15×10^{0}
34	1963	3.29×10^{6}	1.61×10^{4}	2.75×10^{2}	1.60×10^{-1}	3.24×10^{0}
36	1960	2.78×10^{6}	1.85×10^{4}	2.22×10^{2}	1.09×10^{-1}	1.91×10^{0}
38	1956	1.92×10^{6}	1.37×10^{4}	1.69×10^{2}	1.39×10^{-1}	2.46×10^{0}
40	1951	1.31×10^{6}	6.63×10^{3}	2.16×10^{2}	5.93×10^{-2}	1.56×10^{0}
42	1945	$\leq 1.21 \times 10^{6}$ [c]	3.41×10^{3}	2.54×10^{2}	5.04×10^{-2}	9.93×10^{-1}
44	1940	$\leq 1.24 \times 10^{6}$ [c]	3.48×10^{3}	2.17×10^{2}	5.26×10^{-2}	1.13×10^{0}
46	1934	1.42×10^{6}	4.29×10^{3}	2.50×10^{2}	8.22×10^{-2}	1.43×10^{0}
48	1929	1.97×10^{6}	1.28×10^{4}	2.39×10^{2}	6.32×10^{-2}	9.23×10^{-1}
50	1924	1.73×10^{6}	7.13×10^{3}	1.94×10^{2}	9.02×10^{-2}	1.61×10^{0}
52	1917	$\leq 1.13 \times 10^{6}$ [c]	$\leq 2.75 \times 10^{3}$ [c]	1.30×10^{2}	5.18×10^{-2}	8.24×10^{-1}

Table S5.2 (cont'd).

54	1911	$\leq 1.09 \times 10^{6}$ [c]	5.69×10^{3}	1.15×10^{2}	1.36×10^{-2}	3.35×10^{-1}
56	1902	1.01×10^{6}	$\leq 2.38 \times 10^{3}$ [c]	1.35×10^{2}	1.11×10^{-2}	1.72×10^{-1}
58	1895	$\leq 1.02 \times 10^{6}$ [c]	$\leq 2.49 \times 10^{3}$ [c]	4.37×10^{2}	1.96×10^{-2}	$3.19*10^{-1}$

[a] Year of deposition was estimated from the Cs-137 radioactivity in each sediment section.

[b] The enterococci and *E.coli* concentrations were normalized to cell equivalents (CE) per gram-dry wt.

[c] The measurement was below the detection limit, and reported value is the detection limit specific to the sediment section.

CHAPTER 6. CONCLUSIONS

6.1 Goals of the research and summary of the results

The goal of this dissertation was to investigate the factors that were associated with concentrations of persistent fecal indicators measured from water quality samples in various storage schemes and from sediment cores from the Lake St. Clair watershed. The first research project investigated the short term and long term persistence of qPCR measurements of three fecal indicators in water samples stored in various conditions. The second study compared the concentrations of total eluted DNA and qPCR measurements of two fecal indicators extracted with modifications to three DNA extraction methods. The last study investigated the associations of anthropogenic attributes and climate variables to qPCR measurements of persistent fecal indicators measured with the optimal DNA extraction method identified in the second experiment.

In the first research project, two bench scale studies investigated how indicator species, storage condition, time, and temperature (short term study only) were associated with the short term and long term persistence of naturally occurring *Escherichia coli*, *Bacteroides thetataiotaomicron*, and enterococci in water samples stored up to 28 and 366 days, respectively. Raw sewage (10% vol/vol) was spiked into autoclaved river water and stored in two conditions, containers in liquid form (liquid suspension), and membrane filters that were membrane filtered in 100 ml increments at initial sampling (solid matrix). The short term and long term studies included 4˚C storage temperatures maintained with ice packs and refrigeration, respectively, while the short term study also included 27˚ and 37˚C storage temperatures. The persistence of the indicators was evaluated from qPCR measurements of *Escherichia coli uidA*, *Bacteroides thetataiotaomicron alpha-mannanase*, and enterococci *23S rDNA* that were transformed into

concentrations of cellular equivalents. The persistence patterns of the indicators were mathematically evaluated with sixteen linear and non-linear best fit models. Of those models, five linear and nonlinear models were selected. The most popular model was biphasic exponential decay, representing 75% of the treatments in the long term and short term studies. From the best fit models, the general order of least to most persistent indicators were: *B.thetataiotaomicron* < *E.coli* < enterococci. Indicator persistence significantly increased when attached to a solid matrix compared to storage in liquid suspension. Increased storage time was also associated with the shortened persistence of indicators. Persistence was significantly affected by indicator species. In the short term study, the time needed for 90% reduction of the initial concentrations of the indicators, T_{90}, was estimated from their best fit models, and ranged from 1.0 day (*B.thetataiotaomicron* in liquid suspension at 37˚C) to > 28 days (enterococci and *E.coli* attached to a solid matrix at all temperatures). In the long term study, the best fit models predicted T_{90} values that ranged from 35.8 days for *E.coli* in liquid suspension to 164.0 days for enterococci attached to a solid matrix. The T_{90} values of the indicators of the long term study were longer than the short term study, and may be a result of storage cooling strategies i.e. storage on ice vs. refrigeration, and/or storage duration.

Comparisons of concentrations of total eluted DNA, and cellular equivalents of qPCR measurements of *E.coli uidA* and enterococci *23S rDNA* in surface sediment from Lake St. Clair and the Clinton River spiked with *E.coli* and *Enterococcus faecalis* were evaluated with six modifications of three DNA extraction methods, MoBio UltraClean®, MoBio PowerSoil®, and EPA-DNA2 (based on USEPA Method 1611). The six modifications included: manufacturer's protocol; a DNA sorption blocker, G2; sonication; 1:10 elution step; double recommended

sample mass; and addition of 0.01% Tween 80. Overall, there were few significant differences in total eluted DNA, and cellular equivalent concentrations of *E.coli* and enterococci extracted using the modified DNA extraction methods from the two locations. Modifications to the MoBio methods decreased concentrations of total eluted DNA and the indicators (MoBio UltraClean only), while modifications to EPA-DNA2 generally resulted in larger concentrations of total eluted DNA and the indicators. The optimal method was determined to be EPA-DNA2 modified with G2, and was used in the investigation below.

Sediment cores were collected from the mouth of the Clinton River and from Anchor Bay, northwestern Lake St. Clair, in order to investigate how anthropogenic attributes (human population in the watershed, and vertical concentrations of total P, total C, and total N) and climate variables (air temperature and river discharge) were associated to vertical concentrations of cellular equivalents of *E.coli uidA* and enterococci *23S rDNA.* Radio-isotope activity of Cs-137 and Pb-210 determined that the Clinton River (CR) and Anchor Bay (AB) cores represented the years, 1757 - 2012 and 1895 - 2012, respectively. There were steady state concentrations of enterococci in the AB core at ~0.1 x 10^5 and ~2 x 10^5 cell equivalents (CE) per g-dry wt during 1757 - 1878, and 1902 - 2010, respectively. The temporal change of enterococci concentrations in the AB core trended with increased sedimentary nutrient loading. Enterococci concentrations in the CR core increased steadily with time, and ranged ~0.03 x 10^5 - 9.94 x 10^5 CE/g-dry wt. The *E.coli* concentrations in the CR and AB cores increased with time, and ranged 0.14 x 10^7 – 1.69 x 10^7 CE/g-dry wt, and 0.18 x 10^7 – 0.85 x 10^7 CE/g-dry wt, respectively. Statistical analyses of the core sections of the AB and CR during 1932 - 2012 (and 1951 - 2012 for *E.coli* concentrations measured in the CR core) determined that concentrations of enterococci were

significantly associated with human population in the watershed, and river discharge, while *E.coli* concentrations were significantly associated with human population in the watershed, air temperature, and sedimentary concentrations of total nitrogen and total carbon. The historical record of the St. Clair watershed illustrated that changes in sedimentary nutrient loading, and fecal indicator concentrations occurred after watershed policy enactments and watershed management practices like the Swampland Act of 1860, the Clean Water Act of 1972, naming the Clinton River an EPA Area of Concern, and development of the shore lands surrounding the mouth of the Clinton River and Lake St. Clair.

6.2 Correlations of the results of the long term persistence study to persistent concentrations of fecal indicators in sediment cores

One of the objectives of the bench scale persistence studies was to determine if the persistence of fecal indicators was significantly different in the two storage conditions, liquid suspension and attached to a solid matrix. The solid matrix not only represented a novel storage scheme for water quality samples, it was also a representative of the possible attachment of fecal indicators to sediment particles in the environment. The results of the long term study determined that attachment to a surface such a membrane filter was associated with the extended persistence of three fecal indicators. The results supported previous research that determined that attachment to sediment particles could be one of the mechanisms that allows for extended persistence of fecal indicators in sediments. However, one of the limitations of the long term study is the lack of three dimensional interaction between the indicators and the abiotic and biotic factors in the environment, which is possible in sediments.

Another objective of the long term study was to measure the persistence of fecal indicators over a large time scale. The results in this dissertation indicated that the persistence of fecal indicators attached to a solid matrix supported the existence of highly persistent populations of fecal indicators in sediments at a low temperature (4˚C). The temperature in vertical sections of the sediments of Lake St. Clair is unknown. However, the storage temperature was chosen because 4˚C was considered to be the lowest temperature of the benthic portion of Lake St. Clair during the winter months.

6.3 Implications of the results of this dissertation and recommendations to water quality monitoring

The Clean Water Act ensures that all navigable waters are safe for swimming and fishing. The biological safety of recreational waters is monitored with culturable concentrations of general fecal indicators, *E.coli* and enterococci. Such monitoring has reduced the risk of adverse health outcomes of swimmers. However, there is still much progress to be made to ensure that standardized water quality monitoring methods can accurately and precisely estimate the health risk associated with swimming in recreational waters. Research has recently indicated that qPCR measurements of enterococci *23S rDNA* were better associated with the risk of waterborne illness incidence than its culturable concentrations. The results in of this dissertation demonstrated that enterococci *23S rDNA* and *E.coli uidA* can persist for extended periods of time in various environmental conditions. These persistent populations may not accurately predict the presence or concentration of waterborne pathogens. Also, previous research has suggested that qPCR measurements of human associated fecal indicators may better reflect the risk of waterborne illness incidence. The observations in this dissertation indicated that concentrations

of a human associated indicator, *B.thetataiotaomicron*, reflected recent pollution inputs. The use of both general and human associated indicators may give better representation of recent and persistent fecal pollution. **Therefore, it is recommended that qPCR measurements of human associated pollution indicator, *B.thetataiotaomicron,* and qPCR measurements of general pollution indicators, *E.coli* and enterococci, should be used in conjunction to better evaluate the risk of waterborne illness incidence in recreational waters.**

The ability of water quality monitoring to accurately measure fecal indicator concentrations is dependent on the validity of its methods. The standardized transportation scheme for water quality monitoring was optimized for culturable measurements of fecal indicators. Briefly, water samples are stored in closed containers in liquid suspension. Samples can spend up to 6 hrs in transport at 4˚C (on ice) before analysis in the laboratory. The transportation time limit constrains the number of samples and sites that can be sampled. The results from this dissertation illustrated that qPCR measurements of three naturally occurring fecal indicators can spend at least 9 days on ice and at least 35 days in refrigeration in liquid suspension before 90% removal of the initial concentrations. Additionally, ice can be a limiting factor for water sample storage in low resource settings. However, this dissertation estimated that the time needed for 90% removal of initial concentrations of the three indicators in liquid suspension in storage temperatures, 27º and 37ºC, does not occur until after at least 1 day in storage. **Therefore, it is recommended that water samples in liquid suspension can be stored for up to 1 day in storage temperatures > 4˚C, and water samples in liquid suspension can be stored at 4˚C (on ice) for up to 9 days before 90% removal of concentrations of *E.coli*, enterococci, and *B.thetataiotaomicron* measured with qPCR. It is also recommended that water samples can be stored long term if**

refrigerated at 4˚C for up to 35 days before 90% removal of the initial concentrations of *E.coli*, enterococci, and *B.thetataiotaomicron* measured with qPCR.

The validity of the standard methods to access the biological safety of recreational waters is also dependent on the storage matrix of water samples. The USEPA standardized methods mandate that water samples are stored in liquid suspension in a closed container before enumeration of culturable fecal indicators. Storage in liquid suspension can limit sampling efficiency because the samples occupy more space and mass. This dissertation compared the persistence of qPCR measurements of naturally occurring *E.coli uidA*, enterococci *23S rDNA*, and *B.thetataiotaomicron alpha-mannanase* in water samples stored in liquid suspension and attached to a solid matrix (membrane filter). The results from this dissertation predicted that the indicators persisted up to 2.8x longer when attached to a solid matrix than in liquid suspension when stored at 4ºC for up to 28 days and 366 days. At elevated storage temperatures, i.e. 27º and 37ºC, the persistence of the indicators in water samples was between 3.2x - 10x longer when attached to a solid matrix for up to 28 days. **Therefore, it is recommended that water samples be filtered at initial sampling in order to increase the persistence of indicators measured with qPCR, while decreasing the space and mass of water samples during transportation to a laboratory.**

6.4 Implications of results to watershed management and recommendations for management actions

Standardized water quality monitoring methods do not take into account the potential fecal pollution in the sediments of recreational waters. Previous research has observed that fecal

indicators and fecal pathogens are highly concentrated in the sediments, which indicate that sediments can be reservoirs of fecal pollution. The results of this dissertation have illustrated that concentrations of fecal indicators, *E.coli* and enterococci, and sedimentary nutrients like total nitrogen, total carbon, and total phosphorus, can be measured in sediments deposited > 200 years ago. Thus, vertical measurements of nutrients and fecal indicators in sediment cores can be used to measure historical impacts specific to the watershed, including population, land use, and policy enactments. Such research would allow for better identification of the strengths and deficiencies regarding watershed management and facilitate the determination of sustainable management practices. **Therefore, it is recommended that the historical water quality of the Great Lakes Region should be surveyed via sediment core students in order to better understand how changes in water chemistry and quality are associated to previous management practices and policies. Such studies will help guide future watershed management practices and policies.**

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