Title
Evaluation and Performance of Rapid Methods for Identifying and Tracking Sources of Fecal Pollution in Coastal Watersheds

Author
Thulsiraj, Vanessa

Publication Date
2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Evaluation and Performance of Rapid Methods for Identifying and Tracking Sources of Fecal Pollution in Coastal Watersheds

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Civil Engineering

by

Vanessa Thulsiraj

2014

ABSTRACT OF THE DISSERTATION

Application of Rapid Methods for Identifying and
Tracking Sources of Fecal Pollution in Coastal Watersheds

by

Vanessa Thulsiraj
Doctor of Philosophy in Civil Engineering
University of California, Los Angeles, 2014
Professor Jennifer A. Jay, Chair

Fecal contamination of coastal waters is known to degrade the environment and poses a health risk to recreational beach users. Fecal indicator bacteria (FIB) are used around the world to assess water quality and characterize fecal contamination. Elevated levels of FIB have been linked to health risks in epidemiological studies. However, some limitations exist with this indicator. FIB cannot be used to identify the specific sources as they originate from both human and animal sources. FIB may also persist and regrow in the environment.

In order to effectively remediate the cause of pollution and characterize the hazards at chronically impaired beaches it is necessary to measure indicators that can provide information about the sources of the general fecal pollution. Tracking pollution sources at impaired beaches is critical to ensuring the health of coastal watersheds and reducing the incidence of swimming related illness. Molecular methods have gained popularity to identify and detect sources of fecal

contamination using host-associated markers. The work presented here addresses areas warranting further research in the state of the science of water quality monitoring. In Chapter 2, we demonstrate that host-associated markers exhibit similar limits of detection in different water types and are robust in environmental field applications. Additionally, we provide a cost-benefit analysis and provide water quality managers with information supporting the inclusion of molecular methods in current monitoring practices.

This body of work also presents novel methods for rapid and viability-based detection of recent fecal contamination with propidium monoazide (PMA-qPCR) and a field portable method covalently-linked IMS/ATP technique (Cov-IMS/ATP). In Chapters 3 and 4, we present results on optimization and specificity of the Cov-IMS/ATP. We evaluated the performance of Cov-IMS/ATP at three different watersheds for rapid quantification of enterococci, and show this method to be a robust tool in assessing water quality at complex sites. This work also addresses drawbacks of traditional qPCR to quantify viable fecal contamination. We validate the PMA-qPCR method and demonstrate its performance in detecting recent fecal contamination in environmental waters. Use of these methods demonstrates a new framework that can enhance current microbial source tracking studies and water quality monitoring.

The dissertation of Vanessa Thulsiraj is approved.

Richard F. Ambrose

Michael K. Stenstrom

Shaily Mahendra

Jennifer A Jay, Committee Chair

University of California, Los Angeles

2014

Dedication Page

To my family, friends and better half for all the love and support throughout the years, I could never have achieved this goal without you all by my side.

Table of Contents

List of Tables.

List of Figures.

I. Acknowledgements

I would like to acknowledge my advisor, Professor Jenny Jay, for providing me with guidance throughout the years. Her never ending support, kindness and encouragement has helped shape me into the researcher I am today. She has always led by example, and inspires me to be more environmentally aware of my behavior and choices. Her confidence and belief in me has enriched my experience at UCLA.

I would also like to thank my committee members Drs Ambrose, Stenstrom, and Mahendra. Their guidance and expertise has helped me greatly throughout the years. Thank you for being available for questions, and taking the time to provide suggestions throughout the years. I would also like to acknowledge Donna Ferguson for sharing her scientific knowledge on enterococcus speciation and the Southern California Coastal Water Research Project for sharing equipment, specifically their Promega Glo-Max. I would also like to acknowledge the Orange County Sanitation District for providing primary influent.

This work has been supported in part by a number of grants including the NSF GK12 Fellowship, the UC MEXUS Dissertation Grant, a Clean Beach Initiative Grant from the California State Water Resources Control Board, Los Angeles County funding for the Topanga County Project and the UCLA Dissertation Year award.

Two chapters of the dissertation are in review. Chapter 2 of this dissertation is a version of the manuscript currently submitted to the Journal of Environmental Management with the following coauthors: Timothy E. Riedel, Amity G. Zimmer-Faust, Tania Madi, Kaitlyn T. Hanley, Darcy L. Ebentier, Blythe Layton, Muruleedhara Byappanahalli, Meredith Raith, Alexandria B. Boehm, John F. Griffith, Patricia A. Holden, Orin C. Shanks, Stephen B. Weisberg, Jennifer A. Jay (PI). Chapter 3 of this dissertation is a modified form of the

manuscript submitted to the Journal of Applied and Environmental Microbiology with the following coauthors: Amity G. Zimmer-Faust, Donna Ferguson and Jennifer A. Jay (PI). In addition, portions of Chapter 5 have been adapted from the "Topanga Source ID Study Annual Report" with the following coauthors: Rosi Dagit, Jenna Krug, Krista Adamek, Jennifer A. Jay, Timothy Riedel, Amity Zimmer-Faust, Steve Braeband, David Tufto and Richard Sherman.

I would also like to acknowledge all my current and former lab mates their help and friendship: Amity Zimmer-Faust, Dr. Timothy Riedel, Tiffany Lin, Dr. Kathryn Mika, Saeed Hafeznezami, Dr. Christine Lee, Raven LoGiurato, Ariel Flores, Uriel Cobian, Robert Torres, Helen Sanchez, and Cristina Echeverria.

Thank you to my family for believing in me and supporting me throughout this process. Their unconditional love and unwavering faith helped me to realize this dream. My incredible parents, Raj and Ana Thulsiraj, inspire me every day to work hard and to be a better version of myself. A special thanks to both my wonderful sisters Vijaya Thulsiraj and Usha Bhushan for their help with grammatical edits of this dissertation. And my dearest thanks to my partner and better half, Alex Eugene Orosco. Thank you for picking me up when I fall, for always lending an ear to listen when I needed to talk, for being my constant. Thank you for walking this path with me. You have always been my biggest fan and I love you.

VITA

2010 M.S. Civil Engineering, University of California, Los Angeles. Thesis title: Characterization of microbial contamination within the Santa Monica Canyon watershed

2007 B.S. Environmental Engineering, University of California, Irvine

AWARDS

2012 NASA DEVELOP Fellow

2011 National Science Foundation GK-12 Fellow

2010 Mildred Mathias Award for best UC MEXUS Dissertation Research Grant Proposal in the Natural Sciences, University of California

2009 Outstanding Master of Science in Civil Engineering, Department of Civil and Environmental Engineering, University of California, Los Angeles

PUBLICATIONS

Mika, K. B., T. Y. Lin, M. Ferreira, J. Lacson, C. M. Lee, C. Lin, K. O'Byrne, W. Sandoval, **V. Thulsiraj**, J. A. Jay. Incorporating service learning in traditionally lecture-based environmental engineering courses through researching bacterial contamination at a local beach. Global Journal of Engineering Education. 2012, 14(2), p. 155 – 162.

Riedel, T. E., A. G. Zimmer-Faust, **V. Thulsiraj**, T. Madi, K. T. Hanley, D. L. Ebentier, B. Layton, M. Byappanahalli, M. Raith, A. B. Boehm, J. F. Griffith, P. A. Holden, O. C. Shanks, S. B. Weisberg, J. A. Jay. Detection limits and cost comparisons of human- and gull-associated conventional and quantitative PCR assays in artificial and environmental waters, *submitted to Journal of Environmental Management*.

Mika, K., D. W. Ginsburg, C. M. Lee, **V. Thulsiraj**, J. A. Jay. Fecal indicator bacteria levels do not correspond with incidence of human-associated HF183 *Bacteroides* 16S rRNA genetic marker in two urban Southern California watersheds, *submitted to Water, Air, & Soil Pollution.*

Zimmer-Faust, A. G., **V. Thulsiraj**, D. Ferguson, J. A. Jay. Specificity and performance of the Covalently Linked Immunomagnetic Separation/ Adenosine Triphosphate (Cov-IMS/ATP)

method for rapid assessment of coastal water quality, *submitted to Applied and Environmental Microbiology*.

TEACHING EXPERIENCE

Fall 2008, 2009, 2012	Teaching Assistant for CEE 153 – Introduction to Environmental Engineering and Science Taught two discussion sections each week, held office hours, lectured in absence of professor, helped design midterm and final exams, and proctored exams. Posted assignments, homework solutions and handouts on the course web site. Selected as Reader for CEE 153 (Fall 2007) and graded homework assignments.
Fall 2010 to Spring 2011	NSF GK12 Fellowship Served as "Scientist in Residence" helping teach and improve science curriculum in physics, astronomy and earth sciences. Assisted two different high school teachers in their classrooms, twice a week for an academic year. Designed and implemented lesson plans (grade levels range between 9th – 12th grade), developed hands-on curriculum and class demonstrations for students.
Spring 2010	Teaching Assistant for CEE 110 – Introduction to Probabilities and Statistics Posted assignments, homework solutions and handouts on the course web site. Printed and distributed handouts, collected and distributed homework assignments to readers, graded late assignments, answered emails regarding coursework and maintained the master grade sheet.

Chapter 1: Introduction to the evaluation and performance of rapid methods for identifying and tracking sources of fecal pollution in coastal watersheds

1. Introduction

Water quality and microbial contamination in marine recreational beaches is an economic and public health concern. It is estimated that 150 to 400 million visits are made to coastal marine beaches in California annually. These beaches act as a recreational resource and generate revenue from local swimmers and tourism, providing significant economic benefits of billions of dollars (Given et al., 2006). However, impaired beaches are a potential public health risk to visitors that come into contact with the water (Pruss 1998; Wade et al., 2006). To protect the health of swimmers and prevent exposure to contaminated waters, California State Legislature passed and implemented Assembly Bill AB411 in 1997. AB411 enforces monitoring of recreational bathing waters for fecal indicator bacteria during the dry season (April through October).

Use of FIB for water quality monitoring is based on previous studies that have shown a dose response relationship between gastrointestinal illness and elevated levels of FIB (Cabelli et al., 1982). State statute AB411 mandates that beaches be monitored for fecal indicator bacteria (FIB); however, current monitoring methods use a culture-based procedure to measure FIB. These methods take up to 24 hours to obtain a result. Due to the lag time between testing and public notification, swimmers may still be exposed to contaminated waters under this system (Kim and Grant 2004).

Despite the evidence supporting the use of fecal indicator bacteria to assess water quality, some studies question the predictive accuracy of FIB to proxy for sewage contamination, due to

non-human sources and observed regrowth in sediment (Hardina and Fujioka 1991; Byappanahalliet al., 2003; Yamahara et al., 2007). As sources of FIB are not exclusively human, it is difficult to determine if human fecal pollution is present in a watershed when using this indictor to assess contamination. In addition, fecal indicator bacteria may survive at different rates than certain pathogens in the environment and a weak relationship between FIB and pathogens has been reported in some cases (Boehm et al., 2003; Noble and Fuhrman 2001; Jiang and Chu 2004; McQuaig et al., 2006).

Microbial source tracking (MST) refers to a variety of methods used to investigate and identify sources of fecal pollution to water bodies based on the association of particular microorganisms with a specific host. Many MST methods depend on identification of sources through detection of unique molecular DNA sequences of the host-associated fecal microorganisms (Harwood et al., 2013). MST is an actively growing and important area of research, as information on host-associated sources of fecal contamination can be the key to successful remediation efforts (Stoeckel et al., 2007; Stewart et al., 2013). Significant research has been directed toward the development of conventional and quantitative PCR methods associated with human, bovine, pig, horse, and dog, among others (Boehm et al., 2013; Sinigalliano et al; 2013; Raith et al., 2013; Schriewer et al; 2013). Studies have shown that many of these markers are sensitive and specific in artificial water (Layton et al., 2013, Ebentier et al., 2013). However, limits of detection (LOD) of the end-point and quantitative PCR assays in different water types need to be established, and the performance of the markers needs further validation in environmental waters across a regional scale.

Another emerging MST approach is use of an immunomagnetic separation method for

targeting and enumerating fecal associated microorganisms and pathogens. The immunomagnetic separation/adenosine triphosphate (IMS/ATP) technique has been proven as a successful alternative approach to culture-based methods for enumerating FIB in less than one hour (Lee and Deininger 2004: Bushon et al., 2009; Lee et al., 2010). An in-field, adaptive and rapid method such as IMS/ATP can serve as a tool to determine source locations of contamination that need immediate remediation efforts. It has an advantage over culture and molecular methods as being field portable; IMS/ATP can be used to hone in on locations of contamination. However, specificity of the IMS/ATP method, along with an equivalent water quality threshold for FIB with this method has not been established.

The following chapters focus on these water quality issues, with the purpose of addressing gaps in current research and to improve future microbial source tracking investigations. These studies help to serve as a tool for watershed managers and policy makers. To determine whether water quality monitoring laboratories should upgrade to qPCR technology, we compared the limits of detection of three human and two gull-associated molecular markers in artificial freshwater, environmental marine water and environmental creek water. We tested assays in three water matrices to determine if assays are robust across water types, which had not been done in a previous comprehensive multi-laboratory study (Boehm et al., 2013). We also compared the performance and costs per reaction for the human and gull end-point assays against their corresponding quantitative PCR assays. This provided information that had not been previously determined on whether performance improvements in limits of detection justify upgrading from PCR to qPCR processing capabilities.

In Chapter 3, we investigated the performance of another MST method, the Cov-

IMS/ATP technique for rapidly assessing water quality in recreational waters. Although this method has been shown to rapidly measure *Escherichia coli* and enterococci in marine and freshwaters, we applied Cov-IMS/ATP to several beaches in Southern and Baja California to determine if the method could adequately determine water quality across a wider regional scale. In addition, previous work by the Jay Lab showed problems applying the Cov-IMS/ATP method at Doheny State Beach; this work looked to further optimize the method to successfully utilize the Cov-IMS/ATP technique at this site. We also examine the specificity of the method for several *Enterococcus* species. Determining performance and specificity is necessary for implementation of Cov-IMS/ATP in future monitoring efforts.

In Chapter 4 we compare two viability based methods, the Cov-IMS/ATP method against PMA-qPCR assay to determine recent fecal contamination events in environmental waters. A viability-based PMA-qPCR assay has been used to measure the live portions of pure cultures and sewage, but has not been incorporated in microbial source tracking studies, nor has it been utilized to assess adequate sewage treatment in a region with frequent faulty infrastructure. Finally, in Chapter 5 we conduct a long-term microbial source tracking study in Topanga Creek Watershed utilizing two human- and three animal-associated assays. Typically MST studies are conducted in short periods over a few months. We explore the seasonality of molecular markers at Topanga State Beach in a 21 month MST investigation. The work presented in these chapters can advance monitoring efforts to rapidly assess water quality and may better allow for identification of several problem areas requiring remediation efforts. The sampling methods and approach presented in these chapters provide an improved framework for future MST studies.

2. References

Boehm, A.B., Fuhrman, J.A., Mrse, R.D., Grant, S.B., 2003. A tiered approach for the identification of a human fecal pollution source at a recreational beach: Case study at Avalon Bay, Catalina Island, California. Environmental Science and Technology 37, 673-680.

Boehm, A.B., Van de Werfhorst, L.C., Griffith, J.F., Holden, P.A., Jay, J.A., Shanks, O.C., Wang, D., Weisberg, S.B., 2013. Performance of forty-one microbial source tracking methods: a twenty-seven lab evaluation study. Water Research 47 (18), 6812-6828.

Bushon R.N., Brady A.M., Likirdopulos C.A., Cireddu J.V. 2009. Rapid detection of Escherichia coli and enterococci in recreational water using immunomagnetic separation⁄adenosine triphosphate technique. J Appl Microbiol. **106**: 432–441.

Byappanahalli, M.N., Shively, D.A., Nevers, M.B., Sadowsky, M.J., Whitman, R.L., 2003. Growth and survival of Escherichia coli and enterococci populations in the macro-alga Cladophora.

Cabelli, V., Dufour, A., McCabe, L., Levin, M., 1982. Swimming-associated gastroenteritis and water quality. American Journal of Epidemiology 115(4), 606-616.

Ebentier, D.L., Hanley, K.T., Cao, Y., Badgley, B.D., Boehm, A.B., Ervin, J.S., Goodwin, K.D., Gourmelon, M., Griffith, J.F., Holden, P.A., Kelty, C.A., Lozach, S., McGee, C., Peed, L.A., Raith, M., Ryu, H., Sadowsky, M.J., Scott, E.A., Santo Domingo, J., Schriewer, A., Sinigalliano, C.D., Shanks, O.C., Van De Werfhorst, L.C., Wang, D., Wuertz, S., Jay, J.A., 2013. Evaluation of the repeatability and reproducibility of a suite of qPCR-based microbial source tracking methods. Water Research 47(18), 6839-6848.

Given, S., Pendleton, L. and Boehm, A. (2006) Regional public health cost estimates of contaminated coastal waters: a case study of gastroenteritis at southern California beaches. Environmental Science & Technology, 40, 4851-4858.

Hardina, C.M., Fujioka, R.S., 1991. Soil: the environmental source of Escherichia coli and enterococci in Hawaii's streams. Environmental Toxicology and Water Quality: An International Journal 6, 185-195.

Harwood, V.J., Staley, C., Badgley, B.D., Borges, K., Korajkic, A., 2013. Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. FEMS Microbiology Reviews, 1-40. doi:10.1111/1574-6976.12031

Jiang, S.C., Chu, W., 2004. PCR detection of pathogenic viruses in southern California urban rivers. Journal of Applied Microbiology 97 (1), 17-28.

Kim, J. H. and Grant, S.B. (2004) Public mis-notification of coastal water quality: A probabilistic evaluation of posting errors at Huntington Beach, California. Environmental Science & Technology, 38, 2497-2504.

Layton, B., Cao, Y., Ebentier, D.L., Hanley, K.T., Ballest , E., Brandao, J., Byappanahalli, M.N., Converse, R.R., Farnleitner, A.H., Gentry-Shields, J. Gidley, M.L., Gourmelon, M., Lee, C.S., Lee, J., Lozach, S., Madi, T., Meijer,W.G., Noble, R.T., Peed, L., Reischer, G.H., Rodrigues, R., Rose, J.B., Schriewer, A., Sinigalliano, C., Srinivasan, S., Stewart, J.R., Van De Werfhorst, L.C., Wang, D., Whitman, R., Wuertz, S., Jay, J., Holden, P.A., Boehm, A.B., Shanks, O.C., Griffith, J.F., 2013. Performance of human fecal anaerobe-associated PCR-based assays in a multi-laboratory method evaluation study. Water Res. 47(18), 6897-6908.

Lee J.Y. and Deininger R.A. 2004. Detection of E-coli in beach water within 1 hour using immunomagnetic separation and ATP bioluminescence. Luminescence. 19: 31–36.

Lee, C.M., J.G., G., W., K. and J.A., J., 2010. Covalently linked immunomagnetic separation/adenosine triphosphate technique (Cov-IMS/ATP) enables rapid, in-field detection and quantification of *Escherichia coli* and *Enterococcus* spp. in freshwater and marine environments. Journal of Applied Microbiology 109, 324-333.

McQuaig, S.M., Scott, T.M., Harwood, V.J., Farrah, S.R., Lukasik, J.O., 2006. Detection of human-derived fecal pollution in environmental waters by use of a PCR-based human polyomavirus assay. Applied and Environmental Microbiology 72 (12), 7567-7574.

Noble, R.T., Fuhrman, J.A., 2001. Enteroviruses detected by reverse transcriptase polymerase chain reaction from the coastal waters of Santa Monica Bay, California: low correlation to bacterial indicator levels. Hydrobiologia 460, 175-184.

Pruss, A., 1998. Review of epidemiological studies on health effects from exposure to recreational water. International Journal of Epidemiology 27 (1), 1-9.

Raith, M.R., Kelty, C.A., Griffith, J.F., Schriewer, A., Wuertz, S., Mieszkin, S., Gourmelon, M., Reischer, G.H., Farnleitner, A.H., Ervin, J.S., Holden, P.A., Ebentier, D.L., Jay, J.A., Wang, D., Boehm, A.B., Gim Aw, T., Rose, J.B., Balleste, E., Meijer, W.G., Sivaganesan, M., Shanks, O.C., 2013. Comparison of PCR and quantitative real-time PCR methods for the characterization of ruminant and cattle fecal pollution sources. Water Res 47 (18), 6921-6928.

Schriewer, A., Goodwin, K.D., Sinigalliano, C.D., Cox, A.M., Wanless, D., Bartkowiak, J. Ebentier, D.L., Hanley, K.T., Ervin, J., Deering, L.A., Shanks, O.C., Peed, L.A., Meijer, W.G., Griffith, J.F., SantoDomingo, J., Jay, J.A., Holden, P.A. Wuertz,S., 2012. Performance evaluation of canine-associated Bacteroidales assays in a multi-laboratory comparison study. Water Research 47(18) 6909-6920.

Sinigalliano, C.D., Ervin, J., Van De Werfhorst, L.C., Badgley, B., Balleste, E., Bartkowiak, J., Boehm, A.B., Byappanahalli, M., Goodwin, K.D., Gourmelon, M., Griffith, J., Holden, P.A.,

Jay, J.A., Layton, B.A., Lee, C., Lee, J., Meijer, W.G., Noble, R.T., Raith, M., Ryu, H., Sadowsky, M.J., Schriewer, A., Wang, D., Wanless, D., Whitman, R., Wuertz, S., Santo Domingo, J.W., 2013. Multi-Laboratory Evaluations of the Performance of Catellicoccus marimammalium PCR Assays Developed to Target Gull Fecal Sources. Water Research, 47(18) 6883-6896.

Stewart, J.R., Boehm, A.B., Eric A. Dubinsky, E.A., Fong, T., Goodwin, K.D., Griffith J.F., Noble, R.T., Shanks, O.C., Vijayavel, K. Weisberg, S.B., 2010. Recommendations following a multi-laboratory comparison of microbial source tracking methods. Water Research, In Press.

Stoeckel, D.M., Harwood, V.J., 2007. Performance, design, and analysis in microbial source tracking studies. Applied and Environmental Microbiology 73 (8), 2405-2415.

Wade, T.J., Calderon, R.L., Sams, E., Beach, M., Brenner, K.P., Williams, A.H., Dufour, A.P., 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environmental Health Perspectives 114, 24-28.

Yamahara, K.M., Layton, B.A., Santoro, A.E., Boehm, A.B., 2007. Beach sands along the California coast are diffuse sources of fecal bacteria to coastal waters. Environmental Science & Technology 41 (13), 4515-4521.

Chapter 2: Detection limits and cost comparisons of human- and gull-associated conventional and quantitative PCR assays in artificial and environmental waters

Abstract: Modern techniques for tracking fecal pollution in environmental waters allow for the identification of the original fecal host with DNA-based methods. To help water quality managers decide whether to employ conventional end-point polymerase chain reaction (PCR) or quantitative PCR (qPCR), this study compared the limit of detection (LOD) in several matrices and costs associated with the human-associated *Bacteroidales* HF183 Endpoint, HF183 Taqman, HF183 SYBR assays and gull-associated Catellicoccus marimammalium Gull2 Endpoint and Gull2 Taqman assays. LODs were determined by logistic modeling of results on dilutions of DNA from extractions of sewage or gull feces spiked in three water types (artificial freshwater, environmental creek water, and environmental marine water). The HF183 Endpoint LOD was 70 times higher than the HF183 Taqman and HF183 SYBR LODs. The instrument and annual costs of HF183 Endpoint were the lowest, and the per reaction cost was 62% of HF183 Taqman and 1.8 times higher than HF183 SYBR. The low reaction cost of SYBR offsets the high instrument cost in 5 years or less if 9,600 or more reactions are run per year. There was no significant difference between the Gull2 Endpoint and Gull2 Taqman LODs, but the Gull2 Endpoint assay had a lower instrument, annual, and per reaction cost than Gull2 Taqman. Upgrading to qPCR involves greater startup and annual costs, but this increase may be justified for human-associated assays with lower detection limits and a reduced cost per sample.

1. Introduction

Recreational water quality monitoring often involves measuring fecal indicator bacteria (FIB) via culture-based enumeration methods by either membrane filtration (U.S. Environmental Protection Agency, 2006, 2010) or defined substrate technology (IDEXX, Westbrook, ME) (American Public Health Association et al., 2005). There are two drawbacks to these methods. First, the required incubation time of 18-24 hours minimum precludes a same-day judgment of water quality, and rapid coastal water quality fluctuations (Boehm, 2007) can lead to misclassifications (Kim and Grant, 2004). Second, FIB can derive from multiple host sources in the environment (Byappanahalli and Ishii, 2011; Ferguson and Signoretto, 2011), which culture-based methods cannot distinguish.

Culture-independent, molecular-based water quality monitoring methods for detection and quantification of host-associated fecal bacterial DNA can address the inherent drawbacks associated with culture-based methods. Polymerase chain reaction (PCR) and the more complex quantitative polymerase chain reaction (qPCR) do not require overnight incubations and both can use host-associated primers to identify the original host of fecal pollution in environmental water samples. While thermal cyclers for conventional PCR are relatively inexpensive and readily available in most molecular biology capable laboratories, thermal cyclers for qPCR can be much more expensive.

A comparison of the detection capabilities and costs of PCR and qPCR methods can help inform decisions regarding whether an environmental laboratory should invest in new instrumentation. Numerous performance comparisons of PCR and qPCR have been conducted previously in the medical field; for example, although PCR and qPCR methods for *Chlamydia pneumonia* were shown to have the same detection limit (Mygind et al., 2002), qPCR was shown to be somewhat more sensitive for *Pneumocystis jiroveci* (Flori et al., 2004) and *Leishmania*

(Carson et al., 2010), greater than one order of magnitude more sensitive for rhinovirus in cultured cells (Dagher et al., 2004), and two orders of magnitude more sensitive for capripoxyvirus present in clinical specimens from sheep and goats (Balamurugan et al., 2009).

These clinical assay results indicate that the sensitivity difference between PCR and qPCR is assay specific, but qPCR is typically more sensitive than PCR. The reasons for qPCR's greater sensitivity have been well described (Ginzinger, 2002; Smith and Osborn, 2009). Briefly, during PCR amplification, the target sequence is amplified exponentially under ideal conditions. However, due to potential amplification interference, as well as reactant and reagent limitations, the target sequence ceases to amplify exponentially and a plateau phase follows. Accurate quantification of the starting target quantity requires extrapolation from the exponential phase of amplification. With end point PCR gel, visualization may occur after the exponential phase, making quantitative assumptions unreliable (Polz and Cavanaugh, 1998). This problem is alleviated with qPCR because the amplification progress is monitored in real-time, ensuring that only the exponential phase portion of the amplification is used in calculations. Additionally, qPCR amplification products can be shortened to lengths not easily visualized on a gel and time is saved by obviating the need to visualize the product with gel electrophoresis.

For this study, we focused on human- and gull-associated assays, which are relevant to beach related management decisions since such sources of FIB are of high priority and corrective action can be taken to address them. The particular assays chosen have been evaluated for specificity and sensitivity in artificial freshwater by the Source Identification Protocol Project (SIPP, Boehm et al., 2013). The SIPP study compared several MST assays among 27 different laboratories for assay performance including sensitivity, specificity, and repeatability of each method, with the goal of generating standard operating procedures (SOPs) for technology

transfer. A complete description of the SIPP study can be found in Boehm et al. (2013). For quantitative human assays, both HF183 Taqman and BacH were the best performing in terms of both sensitivity and specificity (Boehm et al., 2013; Layton et al., 2013); furthermore, an analysis of repeatability showed both intra- and inter-laboratory variability to be low (Ebentier et al., 2013). For the gull assays, Gull2 SYBR and LeeSeaGull performed with high sensitivity and specificity (Boehm et al., 2013; Sinigalliano et al., 2013) in the conditions tested. The sensitivity of the assays in various environmental waters, a current gap in our knowledge, was addressed in this study.

In this work, we: 1) determined the limits-of-detection (LODs) for three human-associated assays (HF183 Endpoint PCR, HF183 Taqman qPCR, and HF183 SYBR qPCR) and two gull-associated assays (Gull2 Endpoint PCR and Gull2 Taqman qPCR)(Table 2-1); 2) tested these assays for changes in LOD using artificial freshwater, environmental coastal water, and environmental creek water; 3) detailed the cost differences between the assays; and 4) discussed whether performance differences justify differences in cost.

2. Methodology

2.1 Method Background

For this study, we used the standardized protocols (reagents and standards) that were tested in the method evaluation phase of SIPP. The SIPP SOPs used in this study were HF183 Taqman, HF183 SYBR, HF183 Endpoint, Gull2 Taqman, Gull2 Endpoint, DNA extraction SOP, and a gel visualization SOP. Brief SOP details follow and full descriptions are provided in Supplementary Materials.

2.2 Dilutions

2.2.1 Diluent Water Types

Either sewage or gull feces were spiked into three water types: artificial freshwater (AW, distilled water with 0.3 mM $MgCl_2$, 0.6 mM $CaCl_2$, and 1.4 mM $NaHCO_3$), environmental freshwater collected from a creek (CW), or environmental marine water (MW) collected from a coastal site (Table 2-2). Environmental water samples were collected within and at the discharge of a 47 km^2 watershed drained by Topanga Creek in the Santa Monica Mountains in Southern California. Over 70% of the watershed is undeveloped public, open space (GeoPentech, 2006). Approximately 3 liter grab samples of CW and MW were collected on two occasions in the morning in polypropylene plastic bottles (prewashed with 10% HCl and rinsed three times with source water before collection) and immediately stored on ice for transport to the laboratory. CW was collected from actively flowing sections of the lower reach of the creek in Topanga State Park that did not indicate a significant presence of human fecal contamination during snapshot monitoring in 2011-2012 (data not shown). CW was collected at a site named Shale Falls for the sewage dilutions and at a site named Lower Brookside Drive (Dagit et al., 2013) for the gull feces dilutions. MW was collected from incoming waves in knee deep surf at Topanga State Beach approximately 100 meters up-current from the outlet of Topanga Creek at a location that was free from a significant presence of human or gull fecal contamination during spot monitoring in 2011-2012 (data not shown). Physical and chemical parameters of CW and MW collected as the source water for the gull spiked samples were measured *in situ* using a Hydrolab Quanta Multiparameter Sonde (Hach Hydromet, Loveland CO) (Table 2-2).

2.2.2 Sewage Dilutions

Primary influent sewage was obtained from the Orange County Sanitation District (Fountain Valley, CA) and transported on ice to the laboratory on the same morning that environmental water samples were collected. Three 1:20 dilutions of the sewage were generated

using AW, CW, or MW as the water matrix. These dilutions were made by first spiking 20 ml of each matrix with 20 ml of sewage and then transferring 5 ml from these tubes into another 50 ml Falcon centrifuge tube preloaded with 45ml of matching matrix type. The 1:20 dilutions in each matrix were mixed on a rotating platform for one hour at 24°C. After incubation, *Enterococcus* spp. (ENT) concentrations were measured for each dilution (Table 2-3) using Enterolert with Quanti-Tray/2000 (IDEXX, Westbrook ME). Then 10 ml volumes of each 1:20 dilution were filtered onto Isopore 0.4µm HTTP membrane polycarbonate filters (EMD Millipore, Billerica MA) depositing a final volume of 0.5 ml sewage onto the filters. Each filter was placed into a separate 2 ml polypropylene screw cap tube (Sarstedt Inc., Newton NC) preloaded with 0. 3 g 212-300 µm (50-70 U.S. sieve) acid washed glass beads (Sigma-Aldrich, St. Louis MO). Tubes containing filters were immediately archived at -80°C until DNA extraction. For clarity, these filters will be referred to in this study as 0.5S-AW, 0.5S-CW, and 0.5S-MW (Table 2-3). For the purposes of negative controls, filters were created by filtering 10 ml of AW, CW, or MW without prior spiking with sewage. These controls will be referred to as AW_NS, CW_NS, and MW_NS.

2.2.3 Gull Feces Dilutions

Moist gull feces were collected at Mariners Point in Mission Bay San Diego, CA (32° 45' 50" latitude, -117° 14' 47" longitude) in a location that is known for a large congregation of gulls typically devoid of other birds such as pigeons. Twenty-five to 30 individual droppings were collected from beach sand with a sterile spatula into a single sterile 50 ml centrifuge tube, transported on ice to the laboratory, stored overnight at 4°C, and processed the following day. AW was added to 16.7 g of feces until reaching a final volume of 50ml. Ten ml of this slurry was transferred to 40 ml of AW, CW, or MW for a 1:5 dilution. These 1:5 dilutions were further

diluted to 1:10 three times by adding 5 ml to 45 ml of matrix in a 50 ml falcon tube, resulting in final dilutions of 1:5000. The 1:5000 dilutions were mixed on a rotating platform for one hour at 24°C. After mixing, ENT concentrations were measured for each dilution (Table 2-3) using Enterolert with Quanti-Tray/2000. Then 10 ml volumes of each 1:5000 dilution were filtered onto Isopore 0.4µm HTTP membrane polycarbonate filters (EMD Millipore, Billerica MA) depositing a final mass of 0.67 mg onto the filters. Each filter was placed into a separate 2 ml polypropylene screw cap tube (Sarstedt Inc., Newton NC) preloaded with 0. 3 g 212-300 µm (50-70 U.S. sieve) acid washed glass beads (Sigma-Aldrich, St. Louis MO). Tubes containing filters were immediately archived at -80°C until DNA extraction. For clarity, these filters will be referred to in this study as 0.67G-AW, 0.67G-CW, and 0.67G-MW (Table 2-3).

2.3 DNA Extraction

DNA extraction of the 0.5S-AW, 0.5S-CW, 0.5S-MW, AW_NS, CW_NS, and MW_NS filters (Sections 2.2.2) and the 0.67G-AW, 0.67G-CW, and 0.67G-MW filters (Sections 2.2.3) were completed with the DNA-EZ ST1 Extraction Kit according to the manufacture's protocol (GeneRite, North Brunswick NJ) (see Section S1 in Appendix A for SOP details). The extracted DNA was eluted into 100 µl of elution buffer and DNA concentration (Table 2-3) was determined using UV absorption with a Nanodrop 2000C (Thermo Scientific, Waltham MA).

2.4 PCRs and qPCRs

Working dilutions of the 0.5S-AW, 0.5S-CW, 0.5S-MW, 0.67G-AW, 0.67G-CW, and 0.67G-MW extractions (Section 2.3, Table 3) spanning up to 6 orders of magnitude were made using molecular biology grade water (MO BIO Laboratories, Inc., Carlsbad CA). HF183 Endpoint (Bernhard and Field, 2000) (Table S1, S2), HF183 Taqman (Haugland et al., 2010) (Table S3, S4), and HF183 SYBR (Seurinck et al., 2005) (Table S5, S6) SOPs were followed as

written in Sections S2 and inoculated with 2 µl of template from the extractions and their working dilutions. Gull2 Endpoint (Lu et al., 2008) (Table S7, S8), and Gull2 Taqman (Shibata et al., 2010) (Table S9, S10) SOPs were followed as written in Section S3 and inoculated with 2 µl of template from the 0.67G-AW, 0.67G-CW, or 0.67G-MW extractions and working dilutions of these extractions. All supplementary information (SI) in this chapter is provided in Appendix A, including Tables S1 –S10 referenced above.

qPCR reactions were amplified for 50 cycles in an Applied Biosystems StepOnePlus and instrument performance was verified with a TaqMan RNase P Fast 96-Well Instrument Verification Plate (Life Technologies, Carlsbad, CA). PCR reactions were cycled 35 rounds in an Applied Biosystems GeneAmp PCR System 9700. Two µl of each PCR products were visualized and scored by eye (see Section S4 for SOP details) utilizing a FlashGel DNA System (Lonza, Allendale NJ). A less subjective digital analysis of the gel visualization was attempted with ImageJ computer software (Schneider et al., 2012), but the detection algorithm did not detect bands that were visually apparent (data not shown). This failure of the detection algorithm may be an artifact of the resolution (640 by 480 8-bit pixels) of the images taken by the camera in the FlashGel DNA System.

2.5 Statistical Analysis

Statistical analyses were done using Stata version 12.1 (StataCorp, 2011). For human-associated assays, an initial linear regression was run to analyze performance differences of SYBR versus Taqman. To adjust for performance differences between water type, water was included in the model as a factor variable and an interaction term was included between feces/sewage concentration and water type to allow for differences in slope.

Separate analyses were also completed to compare assay performance of SYBR and Taqman among the three water types (AW, CW, and MW) for human- and gull-associated assays. Water type and feces/sewage concentrations were regressed on C_q values. Firth's logistic regression analyses (Firth, 1993) were performed to quantify the relationship between probability of detects and analyte (i.e. feces or sewage concentration). The firthlogit function uses a penalized likelihood estimation method to deal successfully with problems of separation (Heinze and Schemper, 2002). Likelihood ratio tests were completed by comparing a full model that allowed the slope to vary by water type to a condensed model that allowed for a common slope across water types for each assay. Predicted probabilities of detection were computed for varying levels of sewage and feces concentration and were applied as in Burns and Valdivia (2007) to determine generalized LODs for each assay type.

2.6 LOD Determinations

Many assumptions typically applied to the structure of the data distributions used for LOD analysis (Currie, 1995, 1968) fail for qPCR data . This is because qPCR data may not always be normally distributed, is not homoscedastic and negative controls do not result in a zero value (Burns and Valdivia, 2007). Using the methods described in Burns and Valdivia, 2007, we constructed logistic models to determine LODs at a Currie detection decision (i.e. detection frequency) of 95%. Concentrations above the LOD are predicted by the logistic model to have a false negative rate lower than 5%. Confidence intervals (95%) are reported for each LOD. For PCR assays, samples were scored as detected when a reaction was visualized by eye as a band on an electrophoresis gel (Section 2.4) after 35 cycles. For qPCR, samples were scored as detected when an amplification signal greater than a fluorescence threshold of 0.03 (ΔRn) was detected within 40 thermal cycles. A PCR maximum cycle number of 35 and qPCR of 40 were

determined as optimal by the SIPP study (Boehm et al., 2013). Any SYBR qPCR reactions with a melting temperature deviating greater than 0.8°C from the expected value known for plasmid controls were scored as non-detects.

For this study, six replicate qPCR reactions of each dilution were analyzed, with the exception of HF183 SYBR MW, which had an instrument failure that resulted in one less replicate at each dilution. For PCR assays, three replicate reactions of each dilution were analyzed, with the exception of HF183 Endpoint CW, which was analyzed with six replicate reactions. No difference was found between using three or six replicates for end-point analysis, therefore all other samples were analyzed with three replicates to conserve supplies and costs.

2.7 Cost Analysis

HF183 Taqman qPCR, HF183 SYBR and HF183 Endpoint conventional PCR assays were used to model cost differences between PCR and qPCR. The PCR and qPCR thermal cycler prices used in the analysis are both quoted from Applied Biosystems to provide some level of consistency; however, prices vary considerably among different manufacturers. Annual maintenance costs are based on the instrument manufacturer's recommended procedures and frequencies. Consumables used in the cost analysis are, when possible, matched to the exact catalog numbers of those used in this study. The cost analysis does not include service contracts, many materials that are consistent between PCR and qPCR (such as pipettes and microcentrifuge tubes), technician training, or infrastructure requirements (such as dedicated rooms, refrigeration equipment, and hoods). No educational or other discounts were factored into the prices used for the cost analysis and all prices are in 2012 United States dollars and taken directly from the manufacturer.

The PCR TaKaRa Ex Taq DNA Polymerase RR001B Kit (Clontech, Mountain View CA) contains 200 µl of 1000 U TaKaRa Ex Taq DNA Polymerase, 4000 µl of 10X Ex Taq Buffer, 4000 µl of 25 mM $MgCl_2$ solution and 3200 µl of dNTP mixture (2.5 mM each dNTP). For each PCR reaction, 0.1 µl, 2.5 µl, 1.5 µl and 2 µl of Ex Taq Polymerase, buffer, $MgCl_2$ and dNTP mixture were required, respectively. The total number of reactions possible with one kit was calculated to be 1600 (Table S11 and S14).

qPCR primers, probes and plasmids (used for standard curves and positive controls) were purchased from Integrated DNA technologies (Coralville, IA). A 5-point plasmid standard curve, measured in triplicate, was used for each 96-well reaction plate in the qPCR assay in addition to three no-template negative controls. For PCR, a single concentration standard run in triplicate and three no-template negative controls were assumed. This limited the wells available for samples to 78 reactions per qPCR plate and 90 reactions per PCR plate. As such, the "Units/Sample" for the optical adhesive films and 96-well reaction plates are listed as 1/78 for qPCR (Tables S12, S13, S15) and 1/90 for PCR (Tables S11, S14). A plasmid containing sequences of the marker is used as a positive control for making standards. This plasmid is estimated to cost $240.90 per 2 µg. The cost of the plasmid per sample amounted to such an insignificant cost that it is reported as $0.00 due to the very small amount of plasmid (3×10^{-7} ng) required to generate a 5-point dilution series that begins at 1×10^5 target copies (Tables S12, S13, S15).

3. Results

3.2 Human-Associated Assays

3.2.1 HF183 Endpoint PCR

Extractions in each water type (Section 2.3) were serially diluted and the HF183 Endpoint PCR assay was tested for its ability to detect sewage at concentrations spanning from 0.5 ml sewage/10 ml water to the equivalent of 1.4×10^{-7} sewage/10 ml water. In AW, CW and MW, a <100% detection rate was observed at and below a 5.0×10^{-3} ml sewage/10 ml, 2.5×10^{-2} ml sewage/10 ml and 5.0×10^{-3} ml sewage/10 ml concentration, respectively (Figure 2-1A, B, C). Two replicates of the AW_NS, CW_NS, and MW_NS (Section 2.2.2) non-spiked negative control extractions were analyzed and none produced visible bands via gel visualization. One AW, three CW and two MW no-template-controls (NTC) were also run and did not produce visible bands.

To predict a 95% detection frequency LOD and associated confidence intervals (CI) a Firth's logistic model was used. The model was run on combined data from all water types. We feel this is justified based on the results from the Taqman and SYBR data which show no statistically significant (Taqman P=0.4092, SYBR P=0.441) influence of water type on assay LOD (Section 3.2.2, 3.2.3). The logistic model for HF183 Endpoint is plotted against sewage concentrations (Figure 2-1A, B, C, black lines) and predicts an LOD of 4.5×10^{-2} ml sewage/10 ml water (95% CI = 1.8×10^{-2} - 1.6×10^{-1}, Wald $Chi^2 = 143.7$, P=0.000).

3.2.2 HF183 Taqman qPCR

Extractions in each water type (Section 2.3) were serially diluted and the HF183 Taqman qPCR assay was tested for its ability to detect sewage at concentrations spanning from 0.5 ml sewage/10 ml water to the equivalent of 1.4×10^{-7} sewage/10 ml water. To determine LODs, the C_q results (Figure 2-1G, H, I, grey points) were analyzed in a binary fashion. In AW, CW, and MW, a <100% detection rate was observed at and below a 5.0×10^{-5}, 5.0×10^{-4}, and 2.5×10^{-4} ml sewage/10 ml concentration, respectively (Figure 2-1D, E, F, grey points). Six replicates of the

AW_NS, CW_NS, and MW_NS (Section 2.2.2) non-spiked negative control extractions did not amplify by 40 cycles. Six CW and MW no-template-controls (NTCs) were also run with none amplifying by 40 cycles. Although, one AW NTC had a C_q of 38.6 and the other five did not amplify by 40 cycles.

Neither the main effects term for water type ($F_{2, 73}$=0.92, P=0.4092) nor the interaction term between water type and sewage ($F_{2,73}$=0.01, P=0.99) were significant, indicating no significant performance difference between the three water types. A Firth logistic model was created with the combined HF183 Taqman AW, CW, and MW binary data. This combined model (Figure 2-1D, E, F, grey lines, note: the model shown on each panel is identical and replicated for comparison purposes) predicts the HF183 Taqman LOD at a concentration of 6.4 x 10^{-4} ml sewage/10 ml water (CI 3.6 x 10^{-4} - 1.4 x 10^{-3}, Wald Chi^2 =17.11, P<0.001.

3.2.3 HF183 SYBR qPCR

The HF183 SYBR qPCR assay was tested with the same dilutions used for the Taqman assay (Section 3.2.2). HF183 SYBR performed erratically in this study. Out of eight experimental runs, utilizing multiple batches of dye and enzymes, only three runs, one in each matrix type, were considered acceptable for data analysis. The five failed runs all showed poor amplification of positive controls. We were unable to determine the source of failure, and every SYBR run had a complementing Taqman run on the same working dilutions that successfully amplified. To determine LODs, the C_q results of the successful runs (Figure 2-1G, H, I, black points) were analyzed in a binary fashion. The MW SYBR qPCR run experienced an instrument malfunction (drifting baseline) in one column of samples, resulting in only five replicates. In AW, CW, and MW, a <100% detection rate was observed at and below a 5.0 x 10^{-5}, 5.0 x 10^{-4}, and 2.5 x 10^{-4} ml sewage/10 ml concentration, respectively (Figure 2-1D, E, F, black lines).

Three replicates of the AW_NS, CW_NS, and MW_NS (Section 2.2.2) non-spiked negative control extractions were analyzed. AW_NS had a C_q of 31.7, 32.3, and 32.2 with the last replicate melting temperature 5.6 degrees off from expected. CW_NS had C_qs of 39.3, 38.2, and 38.7. MW_NS had a C_q of 37.7, ND, and ND. AW NTCs had C_qs of 32.5, ND, and 34.9. CW and MW NTCs did not amplify by 40 cycles.

For the HF183 SYBR assay, there was no significant difference of the slope of C_q for sewage between water types ($F_{2,71}$=0.83, P=0.441). However, the main effect was significant indicating a difference in average C_q value (after adjusting for sewage concentration) for the SYBR AW reactions when compared to the SYBR CW reactions (β=2.47, t=2.58, p=0.012). A Firth logistic model was created with the combined HF183 SYBR AW, CW, and MW binary data. This combined model (Figure 2-1D, E, F, black lines, note: the model shown on each panel is identical and replicated for comparison purposes) predicts an LOD of 6.5 x 10^{-4} ml sewage/10 ml (CI 3.5 x 10^{-4} - 1.7 x 10^{-3}, Wald Chi^2 =13.47, P=0.0002).

The lower two concentrations (5 x 10^{-7} and 5 x 10^{-6} ml sewage/10 ml water) of the AW data and the 5 x 10^{-6} ml sewage/10 ml water MW sample have a detection frequency greater than zero, in spite of concentrations lower than the LOD. For AW, these detections along with those in non-spiked and NTC reactions suggest that the HF183 SYBR AW assay run had a low level of sewage contamination throughout the reactions. To explore the impact of these possible false positives on the model output, we reran the HF183 SYBR combined water model with these putative false positives changed to a 0% detection rate. This new (false positive removed) model predicts an LOD of 5.3 x 10^{-4} ml sewage/10 ml water (CI 3.0 x 10^{-4} - 1.1 x 10^{-3}, Wald Chi^2 =19.0, P=0.0).

3.3 *Gull-Associated Assays*

3.3.1 Gull2 Endpoint PCR

Extractions in each water type (Section 2.3) were serially diluted and the Gull2 Endpoint PCR assay was tested for its ability to detect feces at concentrations spanning from 0.67 mg feces/10 ml water to the equivalent of 6.7×10^{-10} g feces/10 ml water. In AW, CW and MW, a <100% detection rate was observed at and below a 3.3×10^{-6}, 6.7×10^{-6}, and 6.7×10^{-6} mg feces/10 ml water, respectively (Figure 2-2A, B, C). One reaction each of the AW_NS, CW_NS, and MW_NS (Section 2.2.2) non-spiked negative control extractions did not produce visible bands via gel visualization. Two AW, CW, and MW no-template-controls (NTC) were also run and did not produce visible bands.

A Firth logistic model (Figure 2-2A, B, C, black lines) of the combined end point data was used to predict an LOD for Gull2 Endpoint at 9.8×10^{-6} mg feces/10 ml water (CI 4.3×10^{-6} - 3.1×10^{-5}, Wald Chi^2 =143.7, P=0.000).

3.3.2 Gull2 Taqman qPCR

The Gull2 Taqman qPCR assay was tested with the same dilutions used for the Endpoint assay (Section 3.3.1). When the C_q results (Figure 2-2G, H, I) are analyzed in a binary fashion AW, CW, and MW have a <100% detection rate at and below a 3.4×10^{-6}, 6.7×10^{-6}, and 3.4×10^{-6} mg feces/10 ml water, respectively (Figure 2-2D, E, F). Three replicates of the AW_NS, CW_NS, and MW_NS (Section 2.2.2) non-spiked negative control extractions did not amplify by 40 cycles. Three AW, CW, and MW no-template-controls (NTC) were also run with none amplifying by 40 cycles.

When water type and gull feces concentrations were regressed on C_q values neither the interaction of water type and feces ($F_{2, 66}$=0.20, P=0.82), nor the water type main effects terms ($F_{2,66}$=0.68, P=0.50) were significant for feces concentration versus C_q value, indicating no

significant performance difference between the three water types. To determine the LOD, a Firth logistic model was created with the combined Gull2 Taqman AW, CW, and MW binary data. This combined model (Figure 2-2D, E, F, grey lines, note: the model shown on each panel is identical and replicated for comparison purposes) predicts an LOD for Gull2 Taqman at 5.5×10^{-6} mg feces/10 ml water (CI 3.4×10^{-6} - 9.9×10^{-6}, Wald $Chi^2 = 28.84$, P=0.000).

3.4 Assays Cost Analyses

To assist managers in deciding which assays are best for their needs, the costs associated with setting up and running a qPCR versus a conventional PCR system were estimated (Table 2-4). The instrument costs, annual maintenance costs, and assay consumable costs were compiled for HF183 Endpoint PCR (Table S11), HF183 SYBR qPCR (Table S12), HF183 Taqman qPCR (Table S13), Gull2 Endpoint PCR (Table S14), and Gull2 Taqman qPCR (Table S15). The PCR instrument cost (Table S11 and S14) is 51% of the qPCR instrument cost (Table S12, S13, S15) because the more complex qPCR optical thermocycler is more expensive. PCR instrumentation requires a temperature validation every two years requiring a $6,097 temperature verification kit which is included in the instrument cost. Alternatively, temperature validation may be included with a service contract if one is purchased (service contract costs are not included in this study). Excluding a Temperature Verification Kit reduces the PCR startup cost to 30% of the qPCR startup cost. qPCR instrumentation requires a calibration and performance verification test every 18 months resulting in a qPCR annual maintenance cost of $716 (Table S12, S13, S15). PCR has no annual costs associated with it (Table S11 and S14).

For both HF183 and Gull2 assays the Endpoint PCR cost per reaction is approximately 63% lower than the Taqman qPCR cost per reaction (Table 2-4). While the Endpoint reactions have a $0.43 per reaction cost associated with FlashGel visualization that qPCR assays do not

have (Table S11 and S14), the higher costs of the Taqman polymerase, primers, and probes (Table S13 and S15) more than offsets the visualization costs leading to Taqman assays being the most expensive option. The SYBR assay cost per reaction is 56% lower than the Endpoint PCR assay (Table 2-4) as the primers and polymerase are approximately the same cost for both assays; however, the SYBR assay does not require a FlashGel visualization step (Table S11 and S12).

4. Discussion

4.1 Detection Results

To help managers determine the detection improvements possible with upgrading to qPCR technology, we considered host-associated assays relevant to beach water quality assessment that utilize both PCR and qPCR technologies and have a shared DNA target. Previous medically-related comparisons of PCR versus qPCR found a range of detection improvement from zero to two orders of magnitude (Balamurugan et al., 2009; Carson et al., 2010; Dagher et al., 2004; Flori et al., 2004; Mygind et al., 2002). Similarly, with the HF183 and Gull2 assays tested in this study we found the improvement of qPCR LODs over PCR to be assay specific. The LODs for HF183 are 4.5×10^{-2} (CIs $1.8 – 16 \times 10^{-2}$), 6.4×10^{-4} (CIs $3.6 – 14 \times 10^{-4}$), and 6.5×10^{-4} (CIs $3.5 – 17 \times 10^{-4}$) ml sewage/10 ml for Endpoint, Taqman, and SYBR, respectively. HF183 Endpoint has an LOD 70 times higher than the HF183 qPCR assays in a statistically significant way. This suggests a significant improvement in the ability of HF183 Taqman or SYBR over Endpoint to detect contamination of sewage in water samples.

The AW HF183 SYBR experimental run may have had low levels of contamination leading to a false positive rate that was as high as 50% (Figure 2-1D). The effect of these false positives on the Firth logistic LOD model utilized throughout this study was tested by changing

the putative false positive results to non-detects and rerunning the model (Section 3.2.3). The new model predicts a HF183 SYBR LOD of 5.3×10^{-4} (CIs 3.0 - 11×10^{-4}) ml sewage/10 ml water which is 18% lower than when false positives are included in the model and not significant given the CIs of the LODs. This suggests that using a Firth logistic model approach to predict LODs is a robust technique not overly sensitive to low levels of false positives. In a management context, preventing contamination during sample processing is critical to ensure an accurate and reliable result, nonetheless contamination problems may sometimes occur. Here, the predicted LODs were similar even when low levels of contamination were detected. Therefore, the logistic modeling approach presented here lends itself to other MST studies where low levels of false positives may occur during sample processing.

The LODs for Gull2 Endpoint and Gull2 Taqman are 9.8×10^{-6} (CIs $4.3 – 31 \times 10^{-6}$) and 5.5×10^{-6} (CIs $3.4 – 9.9 \times 10^{-6}$) ml sewage/10 ml, respectively. In contrast to the human assays, the Gull2 results do not suggest a significant improvement in the ability of Gull2 Taqman to detect gull feces in water samples compared to Gull2 Endpoint. The performance difference between the human- versus the gull-associated assays could be due to the reduction in qPCR product size with the HF183 Taqman and HF183 SYBR optimization (Bernhard and Field, 2000; Haugland et al., 2010; Seurinck et al., 2005) that was not done for the gull assays (Lu et al., 2008; Shibata et al., 2010). It should also be noted that the FlashGel DNA System used for the end point assays in this study has a proprietary DNA stain reported to be more sensitive than ethidium bromide (Riley and White, 2008). Therefore, labs using ethidium bromide visualization may experience an even greater difference between PCR and qPCR LODs than this study determined.

All five assays considered in this study performed similarly in artificial, creek, and marine waters. Staley et al. (2012) tested HF183 Taqman against sewage spikes in 5 different water types (tannic, river, lake, estuarine, and marine) on two dates and reported an average process LOD dilution of 10^{-5} with a standard deviation of one order of magnitude. While this level of variation is greater than that reported in this study, both reports suggest that recently developed PCR and qPCR environmental assays are not greatly affected by diverse water matrix types.

4.2 Cost Comparison Results

For the human assays, the 70 times improvement of detection of qPCR over PCR (Section 4.1) comes at an increased instrument cost of $14,766 and $716 greater annual maintenance costs. HF183 Taqman costs 62% more than Endpoint per reaction while HF183 SYBR costs 56% less per reaction. This lower cost may be negated by the much higher failure rate of the SYBR reactions. In our experiments, we experienced a failure rate as high as 60% with the HF183 SYBR assay. We do not feel that this failure level is typical in our lab but we were unable to determine the cause. If this high failure rate were to persist, then 2.5 times the number of SYBR reactions would need to be run to cover the same number of samples as Endpoint or Taqman. This would increase the equivalent per reaction cost for SYBR to $1.30. This equivalent per reaction cost is now greater than Endpoint but still lower than Taqman. Although similar failure rates were not reported in other SIPP labs, one of the four laboratories that tested the HF183 SYBR also experienced problems with the assay. They reported a specificity of only 28%, while the remaining three reported specificity of the assay >85% (Boehm et al., 2013), conveying that this assay shows reduced performance at times.

If the failure rate of HF183 SYBR is not considered, then the higher startup and annual costs of the HF183 SYBR versus HF183 Endpoint will eventually be offset by the lower cost per reaction (Table 2-4). The time it will take for HF183 SYBR to become less expensive than HF183 Endpoint is dependent on the number of reactions run per year. For example, if a laboratory is analyzing 9600 reactions per year (100 96-well plates), then the two assays will reach an equivalent "break-even" cumulative cost by 5 years. If 48,000 reactions (500 96-well plates) are analyzed per year, then the break-even point is reached in less than one year; however, if only 4800 reactions (50 96-well plates) are analyzed per year, then a break-even point is not reached until eight years (Figure 2-3). When management is deciding if the performance increase of qPCR assays over end-point assays justifies the startup investment in new equipment, it is important to consider how many reactions the laboratory facility anticipates analyzing per year.

For the Gull2 assay there was no significant difference in the LODs for the Taqman qPCR and the Endpoint PCR assays. The primers and amplicon sizes of the two assays are equivalent, suggesting that optimization is possible (such as shortening the amplicon) for the qPCR assay, which may lead to a lower LOD. A SYBR based version of the Gull2 assay showed better sensitivity and specificity in the SIPP study (Boehm et al., 2013; Sinigalliano et al., 2013) but this assay also uses the same primers as Endpoint and Taqman and therefore may have a similar LOD. Like the HF183 assays, the Taqman assay is 1.6 times more expensive than the Endpoint option. Thus, there is no break-even point for the Gull2 assays tested in this study. Since the SYBR version of Gull2 uses the same primers as Gull2 Endpoint it can be assumed to have a similar reduction in the per reaction costs as HF183. Further investigation into the performance of the possibly less expensive SYBR qPCR version of Gull2 is warranted.

4.3 Other Factors for Consideration

In addition to differences in LOD and cost, there are several other factors that may influence the decision to invest in either an end-point or qPCR technology. First, qPCR requires the preparation, storage, and handling of reference DNA standard materials. Previous studies have shown that these materials are critical for the successful estimation of DNA target concentrations and that improper use can lead to erroneous results (Shanks et al., 2012). Second, it is well documented that the amplification of DNA targets isolated from environmental samples can partially or completely inhibit PCR-based methods (Wilson, 1997). While strategies are available to detect inhibition in end-point PCR methods (Shanks et al., 2006), these methods can only detect the presence or absence of complete amplification inhibition. In contrast, there are many strategies available to characterize the presence of amplification inhibition (even partial) with qPCR technologies (King et al., 2009). Because environmental water samples can harbor many types of substances that only partially inhibit amplification, the use of qPCR inhibition screening strategies may result in a lower incidence of false negatives and higher confidence in results. From a management perspective, false negatives can be detrimental as they convey beaches to be clean when in actuality they are contaminated. This could prevent needed remediation efforts to target a certain source based on absence of marker detection. Third, substances that can co-extract with the DNA target from environmental samples can not only lead to partial or complete inhibition, but may also interfere with DNA target isolation (Stoeckel et al., 2009). Just like inhibition, end-point PCR strategies are available, but they can only identify the complete failure of the DNA isolation step (for example see Rossen et al., 1992), whereas qPCR methods offer more refined strategies that can detect more subtle changes in the efficiency of DNA recovery needed for accurate quantification (Fredricks et al., 2005). Fourth,

DNA amplification-based technologies can be severely confounded by the presence of contaminating DNA molecules. It is important to consider that the lower the LOD is for a given method, the easier it is to potentially contaminate experiments with extremely low concentrations of extraneous DNA. Therefore, if watershed managers decide to invest in qPCR technology, investment in highly trained technicians and laboratory personnel are also required to maintain quality control in sample processing. While both end-point and qPCR methods are very similar in terms of mixing reagents, qPCR methods require additional steps where a high level of proficiency is required for successful application. For qPCR, small deviations in protocols can lead to large differences in results (Shanks et al., 2012).

5. Conclusion

As DNA amplification-based technology use becomes more widespread for water quality monitoring applications, it will be necessary for sample processing laboratories to carefully consider and weigh all of these issues before deciding to invest in end-point PCR or qPCR technology. In this study, there was not a systematic difference in the LOD between end-point and qPCR assays evaluated. For the human-associated HF183 assays, the Taqman and SYBR LODs are 70 times lower than the Endpoint LOD. In contrast, for the gull-associated Gull2 assays, the Taqman LOD is not statistically distinguishable from the Endpoint LOD. The results for HF183 and Gull2 were not affected by artificial, creek, or marine water matrices, indicating the assays evaluated can be robust across waters tested in this study. However, these assays were only compared in water matrices from one watershed. Future work could also incorporate artificial seawater, and compare assay performance and LODs in creek and marine waters collected from other watersheds to further support the findings in this study across a wider regional scale.

The instrument and annual maintenance costs of qPCR methods are more expensive than those for end-point PCR. SYBR based assays can be cheaper per reaction than end-point assays, while Taqman based assays are more expensive. If enough reactions are run per year the increased startup costs of SYBR qPCR can be offset by the reduced reaction costs. The significant LOD improvement of HF183 qPCR assays compared to Endpoint may help justify the greater costs for human-associated qPCR methods, while the similar performance of gull-associated assays do not justify adapting Taqman qPCR technology (a SYBR version may be preferred but was not tested in this study). Again it is important for water monitoring processing laboratories to examine the host-associated assay that is relevant to their watershed when deciding whether to invest in upgrading the laboratory's current technology. If for example, human contamination is a possible fecal source impairing recreational water quality, improvements in inhibition screening, lower rates of false positives, and increased sensitivity with lower LODs suggest qPCR technology may better inform needed remediation efforts and may justify the added cost. In addition, although performance in LODs were similar for the gull-associated Enpoint and Taqman assays, likely detection of gull at beaches with larger bird populations makes Enpoint less useful. Instead, quantitative analysis of the magnitude of gull marker present at beaches may be more informative for management purposes. Work presented in this chapter can help inform future decisions in financing and guiding sampling proposals which incorporate Endpoint and qPCR assays for microbial source tracking investigations in coastal watersheds. Future work should focus on testing a wider range of sources than just the two (human and gull) selected here, and provide a corresponding cost breakdown.

Figures

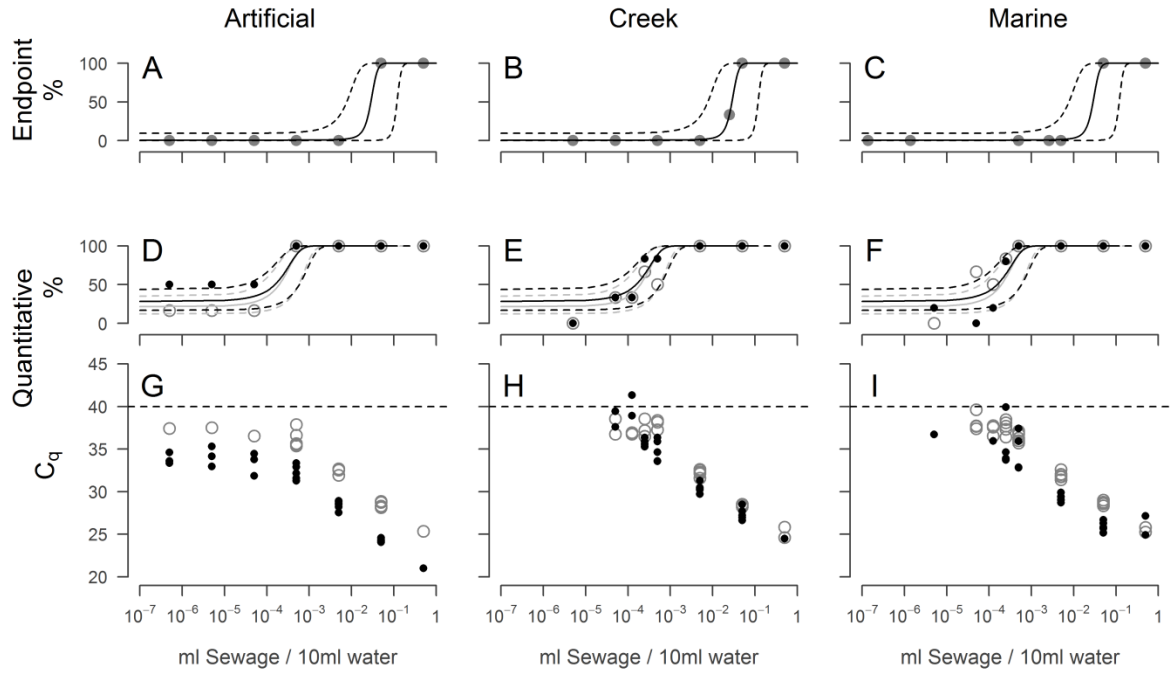


Figure 2-1. Detection of the HF183 gene target in dilutions of extracted artificial, creek, or marine water spiked with sewage. Target was detected with either the PCR Endpoint assay, the Taqman qPCR assay, or the SYBR qPCR assay. A, B, C) The percentage of replicate reactions detected via gel visualization are indicated for Endpoint (grey filled circles). A logistic model of combined Endpoint data is shown (black line) on all three panels with corresponding CIs (dashed lines). D, E, F) The percentage of replicate reactions amplifying by 40 cycles are indicated for Taqman (open gray circles) and SYBR (black circles). Logistic models of Taqman (grey line) and SYBR (black line) are shown with corresponding CIs (dashed lines). G, H, I) Quantification cycle (Cq) is shown for Taqman (open grey circles) and SYBR (black circles).

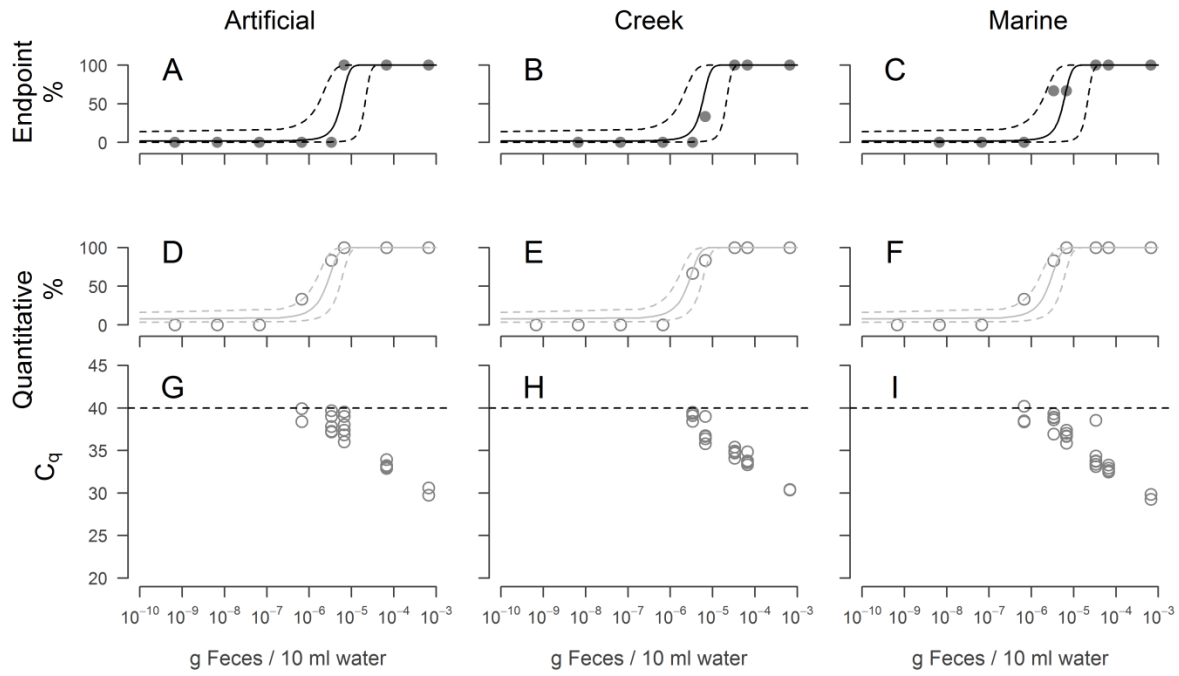


Figure 2-2. Detection of the Gull2 gene target in dilutions of extracted artificial, creek, or marine water spiked with gull feces. Target was detected with either a PCR Endpoint assay or a Taqman qPCR assay. A, B, C) The percentage of replicate reactions detected via gel visualization are indicated for Endpoint (grey filled circles). A logistic model of combined Endpoint data is shown (black line) on all three panels with corresponding CIs (dashed lines). D, E, F) The percentage of replicate reactions amplifying by 40 cycles are indicated for Taqman (open circles). A logistic model of combined Taqman data is shown (grey line) on all three panels with corresponding CIs (dashed lines). G, H, I) Quantification cycle (Cq) is shown for Taqman (open grey circles).

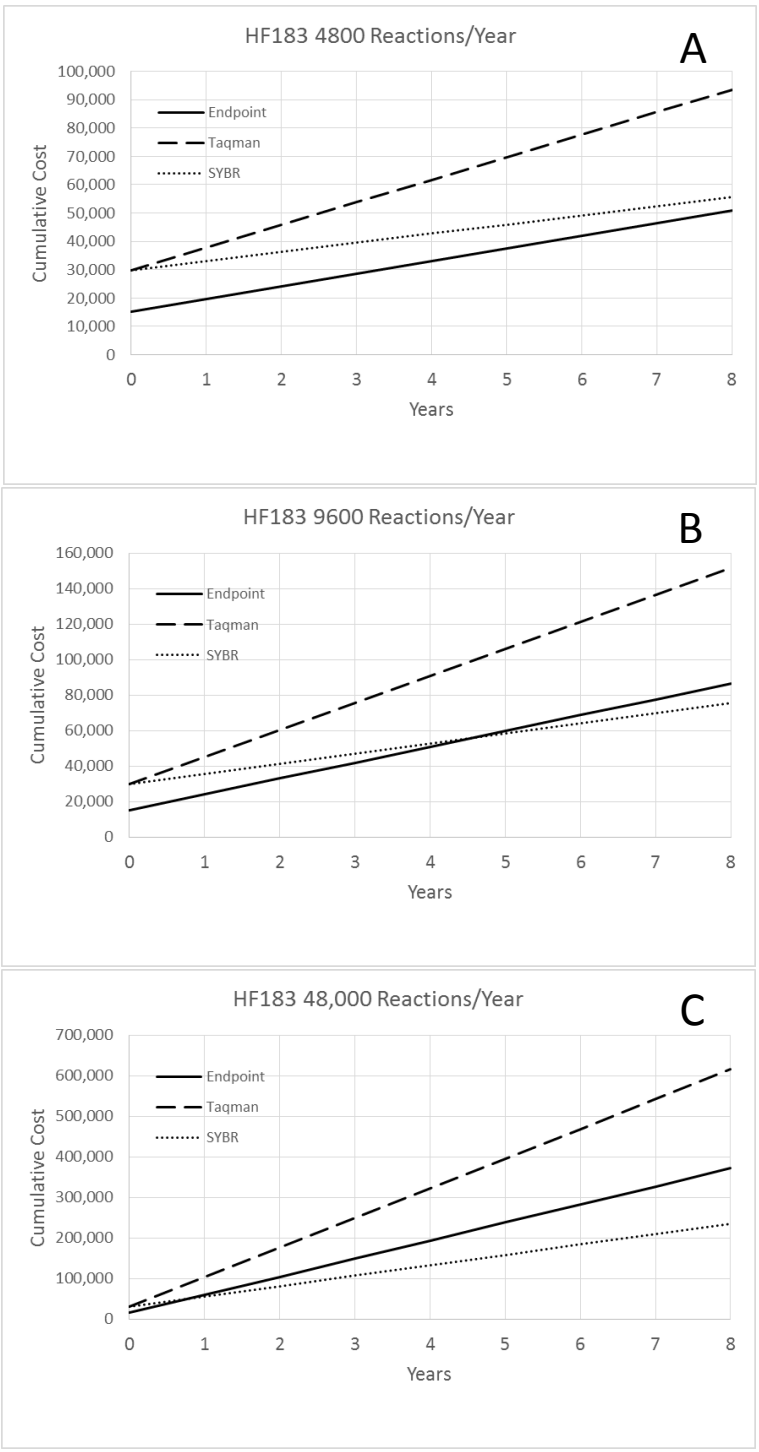


Figure 2-3. Cumulative cost comparison of HF183 Endpoint PCR (solid line), Taqman qPCR (dashed line), and SYBR qPCR assays (dotted line). The cumulative cost for each assay is estimated for A) 4800 (50 96-well plates), B) 9600 (100 96-well plates), or C) 48,000 (500 96-well plates) reactions and includes the startup, annual, and reaction costs.

Tables

Table 2-1. PCR and qPCR assays used in this study.

Name	Type	Target	Forward Primer / Reverse Primer	Probe/Dye	Amplicon Size (bp)	Reference
HF183 Endpoint	PCR	*Bacteroides* 16S	ATCATGAGTTCA CATGTCCG/ CAATCGGAG TTCTTCGTG	N/A	525	Bernhard and Field, 2000
HF183 Taqman	qPCR	*Bacteroides* 16S	ATCATGAGTTCA CATGTCCG / CGTAGGAGTTTG GACCGTGT	FAM-CTGAGAGGAAG GTCCCCCACA TTGGA-TAMRA	167	Haugland et al., 2010
HF183 SYBR	qPCR	*Bacteroides* 16S	ATCATGAGTTCA CATGTCCG / TACCCCGCCTAC TATCTAATG	SYBR Green	82	Seurinck et al., 2005
Gull2 Endpoint	PCR	*Catellicoccus marimammaliu m*	TGCATCGACCTA AAGTTTTGAG/ GTCAAAGAGCGA GCAGTTACTA	N/A	412	Lu et al., 2008
Gull2 Taqman	qPCR	*Catellicoccus marimammaliu m*	TGCATCGACCTA AAGTTTTGAG/ GTCAAAGAGCGA GCAGTTACTA	FAM-CTGAGAGGGTG ATCGGCCACAT TGGGACT-BHQ1	412	Shibata et al., 2010

Table 2-2. Water Types. Environmental water was collected during dry weather for same day use in the sewage spiking event and again at the same sites two weeks later for the gull feces spiking event. Chemical and physical CW and MW values were measured in situ during the 7/17/2012 collection event.

Water	Spike	Location lat./long.	Date, Time	ENT MPN/100ml	Temp. °C	Sp. Cond. ms/cm	DO mg/L	pH	Salinity PSU	ORP mV	Turb. NTU
AW			7/1/12	<10	24	1.4	7.33	8.15	0.69	159	2.1
CW	Sewage	34° 3' 26" / -118° 35' 1"	7/2/12, 9:25a	41	N/D	N/D	N/D	N/D	N/D	N/D	N/D
MW	Sewage	34° 2' 16" / -118° 35' 5"	7/2/12, 10:10a	10	N/D	N/D	N/D	N/D	N/D	N/D	N/D
CW	Feces	34° 3' 26" / -118° 35' 1"	7/17/12, 6:20a	10	17.7	1.5	9.06	8.08	0.75	158	4.6
MW	Feces	34° 2' 16" / -118° 35' 5"	7/17/12, 6:50a	<10	19.56	52	7.28	7.66	33.9	160	N/D

N/D = not determined.

Table 2-3. Characteristics of extractions. Sewage and Gull feces refer to the amount that was filtered. Water Type indicates whether artificial (AW), creek (CW), or marine (MW) water was used as the matrix. Enterococcus spp. (ENT) values reported are the concentration prior to filtration. The DNA value is the concentration (as determined via absorbance at 260 nm) of the extracted eluent.

Filter	Sewage (ml/ 10ml)	Gull Feces (mg/10 ml)	Water Type	ENT (MPN/100 ml)	DNA (ng/µl)
0.5S-AW	0.5		AW	15500	4
0.5S-CW	0.5		CW	29100	7
0.5S-MW	0.5		MW	21400	8
0.67G-AW		0.67	AW	199000	1
0.67G-CW		0.67	CW	242000	2
0.67G-MW		0.67	MW	242000	3

Table 2-4. Cost comparison between PCR/qPCR assays.

Assay Name	PCR/qPCR	Cost/Reaction (US$)	Annual Cost (US$)	Startup Cost (US$)
HF183	Endpoint	0.93	0	15,100
	SYBR	0.52	716	29,900
	Taqman	1.51	716	29,900
Gull2	Endpoint	1.08	0	15,100
	Taqman	1.70	716	29,900

6. Appendix A

Supplementary information for Chapter 2 is provided below.

S1 DNA Extraction SOP details

Utilizing the DNA-EZ ST2 Extraction Kit (Generite, Kendall Park, NJ):

1) 500µL of lysis buffer was added to each filter tube.
2) Tubes were agitated in a Mini-Beadbeater-8 (BioSpec, Bartlesville, OK) for 2 minutes on the maximum setting.
3) Tubes were centrifuged 12,000 RCF for 1min in a Microfuge 18 Microcentrifuge (Beckman Coulter, Inc., Brea CA).
4) The maximum volume of supernatant was pipetted out of the bead beating tube, and added to a new 1.7 ml prelubricated microcentrifuge tube (Costar, Corning NY).
5) Tubes were centrifuged at 12,000 RCF for 1 minute.
6) 350 µl of supernatant was pipetted and added to 1000 µl of binding buffer in a new, 1.7 ml microcentrifuge tube. Samples were pipette mixed and gently vortexed.
7) 675 µl of the DNA/binding buffer mixture (from step 6) was added to a spin-column with a collection tube and centrifuged for 1 minute at 10,000 RCF (flow through was discarded). This step was repeated.
8) The spin-column was placed in a new collection tube.
9) 500 µl of EZ wash buffer was added to the spin-column and then centrifuged for 1 minute at 10,000 RCF (flow through was discarded). This step was repeated.
10) To remove any residual ethanol in the wash buffer, the spin-column was placed in a new collection tube and centrifuged at 10,000 RCF for 1 minute.
11) The spin-column was placed into a 1.7 ml microcentrifuge tube. 50 µl of elution buffer (warmed to 60 deg C) was added and incubated for 1 minute before centrifuging for 1 minute at 10,000 RCF. This step was repeated once.
12) This final 100 µl elution buffer/DNA solution was vortexed and aliquoted into microcentrifuge tubes and stored in -20°C freezer until analysis.

S2 Human-Associated SOP Details

S2.1 HF183 Endpoint (Bernhard and Field, 2000)

Table S1: Reagent specifics.

Reagent	Reaction Conc.	Per Reaction Vol (µl)
TaKaRa ExTaq PCR Buffer (no $MgCl_2$)	1X	2.5
$MgCl_2$	2 mM	2.0
dNTPs	200 µM each	2.0
Forward primer	0.2 µM	0.5
Reverse primer	0.2 µM	0.5
Bovine Serum Albumen	0.075%	0.25
TaKaRa ExTaq DNA Polymerase	0.025 U/µl	0.125
Ultrapure H2O		15.125
Template	varies	2

Table S2: Temperature cycling specifics.

Time	Temp °C	No. Cycles
2 min	94	1
30 sec	94	35
45 sec	63	
45 sec	72	
7 min	72	1

S2.2 HF183 Taqman (Haugland et al., 2010)

Table S3: Reagent specifics.

Reagent	Reaction Conc.	Per Reaction Vol (µl)
ABI Master Mix	1X	12.5
Bovine Serum Albumen	0.005 mg/ml	2.5
Primer/Probe Mix	0.093 µM probe 1.2 µM Rev primer 1.2 µM Fwd primer	3.5
Ultrapure H2O		4.5
Template	varies	2

Table S4: Temperature cycling specifics.

Time	Temp °C	No. Cycles
2 min	50	1
10 min	95	
15 sec	95	50
1 min	60	

S2.3 HF183 SYBR (Seurinck et al., 2005)

Table S5: Reagent specifics.

Reagent	Reaction Conc.	Per Reaction Vol (µl)
Ultrapure H2O		17.5
Forward primer	0.25 µM	0.06
Reverse primer	0.25 µM	0.06
dNTPs	200 µM each	1.0
$MgCl_2$	2 mM	1.0
PCR 10X buffer	1X	2.5
Hot GoldStar DNA Polymerase	2.5 U	0.13
SYBRGreen I		0.75
Template	varies	2

Table S6: Temperature cycling specifics.

Time	Temp °C	No. Cycles
2 min	50	1
10 min	95	
30 sec	95	50*
1 min	53	
1 min	60	

* followed by a melt curve of 60°C up to 94.8°C at a rate of 0.4°C per 10 sec.

S3 Gull-Associated SOP Details

S3.1 Gull2 Endpoint (Lu et al., 2008)

Table S7: Reagent specifics.

Reagent	Reaction Conc.	Per Reaction Vol (µl)
TaKaRa ExTaq PCR Buffer (no $MgCl_2$)	1X	2.5
$MgCl_2$	1.5 mM	1.5
dNTPs	200 µM each	2.0
Forward primer	0.8 µM	2.0
Reverse primer	0.8 µM	2.0
TaKaRa ExTaq DNA Polymerase	0.5 U	0.1
Ultrapure H2O		12.9
Template	varies	2

Table S8: Temperature cycling specifics.

Time	Temp °C	No. Cycles
3 min	95	1
30 sec	95	35
30 sec	64	
1 min	72	
10 min	72	1

S3.2 Gull2 Taqman (Shibata et al., 2010)

Table S9: Reagent specifics.

Reagent	Reaction Conc.	Per Reaction Vol (µl)
Qiagen Quantitect MMX	1X	12.5
Forward Primer	0.9 µM	2.25
Reverse Primer	0.9 µM	2.25
Probe	0.3 µM	0.75
Ultrapure H2O		5.25
Template	varies	2

Table S10: Temperature cycling specifics.

Time	Temp °C	No. Cycles
15 min	95	
15 sec	95	50
1 min	62	

S4 DNA Visualization SOP details

Utilizing the FlashGel DNA System (Lonza, Allendale NJ):

1) The white seals on the FlashGel DNA Cassette (2.2%, 16+1 well, double tier) were removed.
2) The cassette was rinsed with dI water and any excess liquid was removed from the gel cassette by tilting the cassette and blotting. Wells were avoided.
3) The cassette was inserted into the FlashGel Dock.
4) Two µl of PCR product was mixed with 2 µL of FlashGel Loading Dye (5X concentrate) and loaded into cassette wells.
5) To verify product size, 4 µl of FlashGel DNA Marker (100bp - 4kb) were used in two wells.
6) The PowerPac 300 (Bio-Rad Hercules CA) power supply was set to 275V and the gel was run for ~4 minutes.
7) Bitmap digital pictures were archived with the FlashGel Camera at ~1 minute intervals.

S5 Detailed Costs Associated With PCR and qPCR

Table S11: HF183 Endpoint PCR equipment, annual, and consumable costs for a 25 µl reaction. Unit refers to the unit of measure while the amount is the typical number of units purchased. The cost is reported in US dollars and classified as consumable (cost/sample), annual, or capital.

	Unit	Amount	Cost/ Amount	Cost/ Unit	Units/ Reaction	Cost/ Reaction	Annual Cost	Capital Cost
ABI Veriti 96-Well Fast Thermal Cycler	Instrument	1	7995.00	7995.00				7995
Temperature Verification Kit	Kit	1	6097.00					6097
Forward Primer	µM	100	11.00	0.11	0.2	0.022		
Reverse Primer	µM	100	9.90	0.10	0.2	0.020		
Nuclease-free Water	mL	1000	33.18	0.03	0.015	0.0005		
TaKaRa Ex Taq DNA Polymerase Kit	Reaction	1600	590.00	0.37	1	0.369		
Optical Films	Plate	100	206.00	2.06	1/90	0.023		
96 Well Reaction Plates	Plate	10	31.00	3.10	1/90	0.034		
Flash Gel Camera	Instrument	1	606.00	606.00				606.00
FlashGel Dock System	Instrument	1	436.00	436.00				436.00
FlashGel Loading Dye	mL	5	140.00	28.00	0.002	0.056		
FlashGel Marker	mL	0.5	125.00	250.00	0.004/32	0.031		
FlashGel DNA Cassette	Cassette	9	107.00	11.89	1/32	0.372		
Total						0.93	0	15134

Table S12: HF183 SYBR qPCR equipment, annual, and consumable costs for a 25 µl reaction. Unit refers to the unit of measure while the amount is the typical number of units purchased. The cost is classified as consumable (cost/sample), annual, or capital.

	Unit	Amount	Cost/ Amount	Cost/ Unit	Units/ Sample	Cost/ Sample	Annual Cost	Capital Cost
StepOnePlus PCR System	Instrument	1	29900.00					29900.00
Spectral Calibration Kit	Plate	1	508.00	508.00			338.66	
RNase P Verification Plate	Plate	1	566.00	566.00			377.33	
Forward Primer	µM	100	11.00	0.11	0.2500	0.028		
Reverse Primer	µM	100	11.00	0.11	0.2500	0.028		
Nuclease-free Water	mL	1000	33.18	0.03	0.0175	0.001		
qPCR Core Kit for SYBR Green 1 10X buffer, Passive Reference dNTP/dUTP mix HotGoldStar DNA Polymerase SYBR Green I MgCl2	25 µL Reaction	5000	1970.00	0.39	1	0.394		
Optical Films	Plate	100	206.00	2.06	1/78	0.026		
96 Well Reaction Plates	Plate	10	31.00	3.10	1/78	0.040		
Plasmid Standard Curve	µg	2	240.90	120.45	0.0000	0.000		
Total						0.52	715.99	29,900

Table S13: HF183 Taqman qPCR equipment, annual, and consumable costs for a 25 µl reaction. Unit refers to the unit of measure while the amount is the typical number of units purchased. The cost is classified as consumable (cost/sample), annual, or capital.

	Unit	Amount	Cost/ Amount	Cost/ Unit	Units/ Reaction	Cost/ Reaction	Annual Cost	One time cost
StepOnePlus PCR System	Instrument	1	29900.00					29900.00
Spectral Calibration Kit	Plate	1	508.00	508.00			338.66	
RNase P Verification Plate	Plate	1	566.00	566.00			377.33	
Forward Primer	µM	100	11.00	0.11	1.1600	0.128		
Reverse Primer	µM	100	11.00	0.11	1.1600	0.128		
Probe 5'6-FAM/3'TAMRA	µM	100	195.00	1.95	0.0930	0.181		
Nuclease-free Water	mL	1000	33.18	0.03	0.0045	0.000		
ABI Universal Master Mix Kit 2X Buffer, Passive Reference dNTP/dUTP mix AmpliTaq Gold DNA Polymerase AmpErase UNG	50 µL Reaction	2000	3974.00	1.99	0.5	0.994		
Bovine Serum Albumen	mg/mL	50	93.00	1.86	0.0050	0.009		
Optical Films	Plate	100	206.00	2.06	1/78	0.026		
96 Well Reaction Plates	Plate	10	31.00	3.10	1/78	0.040		
Plasmid Standard Curve	µg	2	240.90	120.45	0.0000	0.000		
Total						1.51	715.99	29,900

Table S14: Gull2 Endpoint PCR equipment, annual, and consumable costs for a 25 µl reaction. Unit refers to the unit of measure while the amount is the typical number of units purchased. The cost is classified as consumable (cost/sample), annual, or capital.

	Unit	Amount	Cost/ Amount	Cost/ Unit	Units/ Reaction	$ Cost/ Reaction	Annual Cost	Capital Cost
ABI Veriti 96-Well Fast Thermal Cycler	Instrument	1	7995	7995				7995
Temperature Verification Kit	Kit	1	6097	6097				6097
Forward Primer	µM	100	12.10	0.12	0.8	0.096		
Reverse Primer	µM	100	12.10	0.12	0.8	0.096		
Nuclease-free Water	ml	1000	33.18	0.03	0.015	0.0005		
TaKaRa Ex Taq DNA Polymerase	Reaction	1600	590.00	0.37	1	0.37		
96 Well Reaction Plates	Plate	10	31.00	3.10	1/90	0.034		
Optical Films	Plate	100	206.00	2.06	1/90	0.023		
Flash Gel Camera	Instrument	1	606.00	606.00				606.00
FlashGel Dock System	Instrument	1	436.00	436.00				436.00
FlashGel Loading Dye	ml	5	140.00	28.00	0.002	0.056		
FlashGel Marker	ml	0.5	125.00	250.00	0.004/32	0.031		
FlashGel DNA Cassette	Cassette	9	107.00	11.89	1/32	0.372		
Total						1.08	0.00	15134

Table S15: Gull2 Taqman qPCR equipment, annual, and consumable costs for a 25 µl reaction. Unit refers to the unit of measure while the amount is the typical number of units purchased. The cost is classified as consumable (cost/sample), annual, or capital.

	Unit	Amount	Cost/ Amount	Cost/ Unit	Units/ Sample	Cost/ Sample	Annual Cost	One time cost
StepOnePlus PCR System	Instrument	1	29900.00					29900.00
Spectral Calibration Kit	Plate	1	508.00	508.00			338.66	
RNase P Verification Plate	Plate	1	566.00	566.00			377.33	
Forward Primer	µM	100	11.00	0.11	0.9000	0.099		
Reverse Primer	µM	100	11.00	0.11	0.9000	0.099		
Probe 5'6-FAM/3'BHQ1	µM	100	195.00	1.95	0.3000	0.585		
Nuclease-free Water	mL	1000	33.18	0.03	0.0035	0.000		
QuantiTect Probe PCR Kit	50 µL Reaction	1000	1710.00	1.71	0.5000	0.855		
HotStarTaq DNA Polymerase								
QuantiTect Probe PCR Buffer								
dNTP/dUTP mix								
ROX dye								
Optical Films	Plate	100	206.00	2.06	1/78	0.026		
96 Well Reaction Plates	Plate	10	31.00	3.10	1/78	0.040		
Plasmid Standard Curve	µg	2	240.90	120.45	0.0000	0.000		
Total						1.70	715.99	29,900

7. References

American Public Health Association, Eaton, A.D., American Water Works Association., Water Environment Federation., 2005. Standard methods for the examination of water and wastewater, 9000. APHA-AWWA-WEF, Washington, D.C.

Balamurugan, V., Jayappa, K.D., Hosamani, M., Bhanuprakash, V., Venkatesan, G., Singh, R.K., 2009. Comparative Efficacy of Conventional and TaqMan Polymerase Chain Reaction Assays in the Detection of Capripoxviruses from Clinical Samples. Journal of Veterinary Diagnostic Investigation 21, 225–231.

Bernhard, A.E., Field, K.G., 2000. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. Applied and Environmental Microbiology 66, 1587–1594.

Boehm, A.B., 2007. Enterococci Concentrations in Diverse Coastal Environments Exhibit Extreme Variability. Environ. Sci. Technol. 41, 8227–8232.

Boehm, A.B., Van De Werfhorst, L.C., Griffith, J.F., Holden, P.A., Jay, J.A., Shanks, O.C., Wang, D., Weisberg, S.B., 2013. Performance of Forty-Three Microbial Source Tracking Methods: A Twenty-Seven Lab Evaluation Study. Water Research In Press.

Burns, M., Valdivia, H., 2007. Modelling the limit of detection in real-time quantitative PCR. European Food Research and Technology 226, 1513–1524.

Byappanahalli, M.N., Ishii, S., 2011. Environmental sources of fecal bacteria., in: Sadowsky, M.J., Whitman, R.L. (Eds.), The Fecal Bacteria. ASM Press, pp. 93–110.

Carson, C., Quinnell, R.J., Holden, J., Garcez, L.M., Deborggraeve, S., Courtenay, O., 2010. Comparison of Leishmania OligoC-TesT PCR with Conventional and Real-Time PCR for Diagnosis of Canine Leishmania Infection. Journal of Clinical Microbiology 48, 3325–3330.

Currie, L.A., 1968. Limits for qualitative detection and quantitative determination. Application to radiochemistry. Anal. Chem. 40, 586–593.

Currie, L.A., 1995. Nomenclature in evaluation of analytical methods including detection and quantification capabilities (IUPAC Recommendations 1995). Pure and Applied Chemistry 67, 1699–1723.

Dagher, H., Donninger, H., Hutchinson, P., Ghildyal, R., Bardin, P., 2004. Rhinovirus detection: comparison of real-time and conventional PCR. Journal of Virological Methods 117, 113–121.

Dagit, R., Krug, J., Adamek, K., Jay, J.A., Riedel, T.E., Zimmer-Faust, A., Thulsiraj, V., Braband, S., Tufto, D., Sherman, R., 2013. Topanga Source ID Study Annual Report Dec 2012- October 2013.

Ebentier, D.L., Hanley, K.T., Cao, Y., Badgley, B.D., Boehm, A.B., Ervin, J.S., Goodwin, K.D., Gourmelon, M., Griffith, J.F., Holden, P.A., Kelty, C.A., Lozach, S., McGee, C., Peed, L.A., Raith, M., Ryu, H., Sadowsky, M.J., Scott, E.A., Santo Domingo, J., Schriewer, A., Sinigalliano, C.D., Shanks, O.C., Van De Werfhorst, L.C., Wang, D., Wuertz, S., Jay, J.A., 2013. Evaluation of the repeatability and reproducibility of a suite of qPCR-based microbial source tracking methods. Water Research In Press.

Ferguson, D., Signoretto, C., 2011. Environmental Persistence and Naturalization of Fecal Indicator Organisms, in: Hagedorn, C., Blanch, A.R., Harwood, V.J. (Eds.), Microbial Source Tracking: Methods, Applications, and Case Studies. Springer New York, New York, NY, pp. 379–397.

Firth, D., 1993. Bias Reduction of Maximum Likelihood Estimates. Biometrika 80, 27.

Flori, P., Bellete, B., Durand, F., Raberin, H., Cazorla, C., Hafid, J., Lucht, F., Sung, R.T.M., 2004. Comparison between real-time PCR, conventional PCR and different staining techniques for diagnosing Pneumocystis jiroveci pneumonia from bronchoalveolar lavage specimens. Journal of medical microbiology 53, 603–607.

Fredricks, D.N., Smith, C., Meier, A., 2005. Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J. Clin. Microbiol. 43, 5122–5128.

GeoPentech, 2006. Hydrogeologic Study, Lower Topanga Creek Watershed, Los Angeles County. California. Resource Conservation District of the Santa Monica Mountains.

Ginzinger, D.G., 2002. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. Experimental Hematology 30, 503–512.

Haugland, R.A., Varma, M., Sivaganesan, M., Kelty, C., Peed, L., Shanks, O.C., 2010. Evaluation of genetic markers from the 16S rRNA gene V2 region for use in quantitative detection of selected Bacteroidales species and human fecal waste by qPCR. Syst. Appl. Microbiol. 33, 348–357.

Heinze, G., Schemper, M., 2002. A solution to the problem of separation in logistic regression. Stat Med 21, 2409–2419.

Kim, J.H., Grant, S.B., 2004. Public Mis-Notification of Coastal Water Quality: A Probabilistic Evaluation of Posting Errors at Huntington Beach, California. Environ. Sci. Technol. 38, 2497–2504.

King, C., Debruyne, R., Kuch, M., Schwarz, C., Poinar, H., 2009. A quantitative approach to detect and overcome PCR inhibition in ancient DNA extracts. BioTechniques 47, 941–949.

Layton, B., Cao, Y., Ebentier, D.L., Hanley, K.T., Van De Werfhorst, L.C., Wang, D., Madi, T., Whitman, R.L., Byappanahalli, M.N., Balleste, E., Meijer, W., Schriewer, A., Wuertz, S., Converse, R., Noble, R.T., Srinivasan, S., Rose, J., Soo Lee, C., Lee, J., Sheilds, J., Stewart, J., Reischer, G., Farnleitner, A., Sinigalliano, C.D., Rodrigues, R., Lozach, S., Gourmelon, M., Peed, L., Shanks, O.C., Jay, J.A., Holden, P.A., Boehm, A.B., Griffith, J.F., 2013. Performance of human fecal-associated PCR based assays: An international source identification method evaluation. Water Research In Press.

Lu, J., Domingo, J.W.S., Lamendella, R., Edge, T., Hill, S., 2008. Phylogenetic Diversity and Molecular Detection of Bacteria in Gull Feces. Appl. Environ. Microbiol. 74, 3969–3976.

Mygind, T., Birkelund, S., Birkebaek, N., Østergaard, L., Jensen, J., Christiansen, G., 2002. Determination of PCR efficiency in chelex-100 purified clinical samples and comparison of real-time quantitative PCR and conventional PCR for detection of Chlamydia pneumoniae. BMC microbiology 2, 17.

Polz, M.F., Cavanaugh, C.M., 1998. Bias in Template-to-Product Ratios in Multitemplate PCR. Appl Environ Microbiol 64, 3724–3730.

Riley, M., White, H., 2008. FlashGel System: Results in 5 Minutes. Resource Notes, Lonza Rockland, Inc. 3.

Rossen, L., Nørskov, P., Holmstrøm, K., Rasmussen, O.F., 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int. J. Food Microbiol. 17, 37–45.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9, 671–675.

Seurinck, S., Defoirdt, T., Verstraete, W., Siciliano, S.D., 2005. Detection and quantification of the human-specific HF183 Bacteroides 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater. Environ. Microbiol. 7, 249–259.

Shanks, O.C., Nietch, C., Simonich, M., Younger, M., Reynolds, D., Field, K.G., 2006. Basin-wide analysis of the dynamics of fecal contamination and fecal source identification in Tillamook Bay, Oregon. Appl. Environ. Microbiol. 72, 5537–5546.

Shanks, O.C., Sivaganesan, M., Peed, L., Kelty, C.A., Blackwood, A.D., Greene, M.R., Noble, R.T., Bushon, R.N., Stelzer, E.A., Kinzelman, J., Anan'eva, T., Sinigalliano, C., Wanless, D., Griffith, J., Cao, Y., Weisberg, S., Harwood, V.J., Staley, C., Oshima, K.H., Varma, M., Haugland, R.A., 2012. Interlaboratory Comparison of Real-Time PCR Protocols for Quantification of General Fecal Indicator Bacteria. Environ. Sci. Technol. 46, 945–953.

Shibata, T., Solo-Gabriele, H.M., Sinigalliano, C.D., Gidley, M.L., Plano, L.R.W., Fleisher, J.M., Wang, J.D., Elmir, S.M., He, G., Wright, M.E., Abdelzaher, A.M., Ortega, C., Wanless, D., Garza, A.C., Kish, J., Scott, T., Hollenbeck, J., Backer, L.C., Fleming, L.E., 2010. Evaluation of Conventional and Alternative Monitoring Methods for a Recreational Marine Beach with Nonpoint Source of Fecal Contamination. Environ. Sci. Technol. 44, 8175–8181.

Sinigalliano, C.D., Ervin, J.S., Van De Werfhorst, L.C., Wang, D., Wanless, D., Bartkowiak, J., Layton, B., Raith, M., Schriewer, A., Badgley, B.D., Lee, C., Goodwin, K.D., Lee, J., Boehm, A.B., Noble, R.T., Holden, P.A., Jay, J.A., Wuertz, S., Byappanahalli, M.N., Whitman, R.L., Sadowsky, M.J., Meijer, W., Balleste, E., Gourmelon, M., Griffith, J.F., Ryu, H., Domingo, J.W.S., 2013. Multi-laboratory assessment on the performance of PCR assays targeting Catellicoccus marimammalium for microbial source tracking of coastal birds. Water Research In Press.

Smith, C.J., Osborn, A.M., 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiology Ecology 67, 6–20.

Staley, C., Gordon, K.V., Schoen, M.E., Harwood, V.J., 2012. Performance of two quantitative PCR methods for microbial source tracking of human sewage and implications for

microbial risk assessment in recreational waters. Appl. Environ. Microbiol. 78, 7317–7326.

StataCorp, 2011. Stata Statistical Software. StataCorp LP, College Station, TX.

Stoeckel, D.M., Stelzer, E.A., Dick, L.K., 2009. Evaluation of two spike-and-recovery controls for assessment of extraction efficiency in microbial source tracking studies. Water Research 43, 4820–4827.

U.S. Environmental Protection Agency, 2006. Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-Beta-D-Glucoside Agar (mEI) (No. EPA-821-R06-009).

U.S. Environmental Protection Agency, O. of W., 2010. Method 1103.1: Escherichia coli (E. coli) in Water by Membrane Filtration Using membrane-Thermotolerant Escherichia coli Agar (mTEC) (No. EPA-821-R-10-002).

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63, 3741–3751.

Chapter 3. Optimization of the Cov-IMS/ATP method for rapid assessment of coastal water quality

Abstract: The performance and specificity of the Covalently-linked Immunomagnetic Separation/ Adenosine triphosphate (Cov-IMS/ATP) method for enterococci was evaluated in recreational waters. Cov-IMS/ATP performance was compared with standard methods: defined substrate technology (Enterolert, IDEXX Laboratories), membrane filtration (EPA Method 1600), and an Enterococcus-specific qPCR assay (EPA Method A). We extend previous studies by 1) analyzing the stability of the relationship between the Cov-IMS/ATP method and culture-based methods at different field sites, 2) evaluating specificity of the assay for seven ATCC *Enterococcus* species, 3) identifying cross-reacting organisms binding the antibody-bead complexes with 16S rRNA gene sequencing and evaluating specificity of the assay to five non-enterococci species, and 4) conducting preliminary tests of preabsorption as a means of improving the assay. Cov-IMS/ATP was found to perform consistently and with strong agreement rates (based on exceedance/compliance with regulatory limits) of between 89% and 100% when compared to the culture-based Enterolert method at a variety of sites with complex inputs. The specificity of Cov-IMS/ATP is robust to different strains of *Enterococcus* spp. However, there is potential for non-target bacterium to bind the antibody which may be reduced by purification of the IgG serum with preabsorption at problematic sites. The findings of this study help to validate the Cov-IMS/ATP method, suggesting a predictable empirical relationship between the Cov-IMS/ATP method and traditional culture-based methods, which will allow for more widespread application of this rapid and field portable method for microbial source tracking and coastal water quality assessment.

1. Introduction

Pollution from diverse and multiple sources, as well as extreme variability in enterococci concentrations, have historically confounded efforts to improve coastal and freshwater water quality (1-3). The development of rapid detection assays, which enumerate microbial contaminants in as quickly as one hour, has progressed significantly in recent years (4-6). Such assays may be more protective of human health and increasingly beneficial for regulatory reporting when compared to traditional methods (7). Traditional reporting has relied on culture-based methods (*e.g.*, defined substrate technology, membrane filtration), which can take between 24-48 hours to yield results, making these methods less effective for microbial source tracking and assessment of short duration beach contamination events (5, 8). The method explored in this study, Covalently-linked Immunomagnetic Separation/ Adenosine Triphosphate (Cov-IMS/ATP), is field portable and the quickest of the current rapid methods being explored for coastal water quality assessment; environmental enterococci concentrations can be enumerated in marine and freshwaters within one hour of sample collection (9). Furthermore, the Cov-IMS/ATP method measures ATP of viable bacteria only, potentially allowing for better comparison with traditional culture-based technologies than nucleic-acid based technologies. Cov-IMS/ATP also has reasonable startup costs and is user-friendly, eliminating the need for highly experienced technicians.

Immunomagnetic separation has been used in the past for isolation and measuring of *Giardia* (10, 11) and *Cryptosporidium parvum* (12). IMS for isolation in combination with DAPI for enumeration of *Giardia* and C. *parvum* in drinking water is U.S. EPA approved. More recently, IMS/ATP has been used to analyze recreational water quality. Lee and Deininger first applied the IMS/ATP assay to measure *E. coli* in recreational freshwater in 2004 (13) which was optimized by Bushon to measure *Enterococcus* in wastewater recreational water in 2004 (14).

Bushon adopted this protocol to quantify *Enterococcus* and *E.coli* wastewater (15). The selective magnetic bead–antibody complex applied in these studies relied on hydrophobic interactions between the antibody and the magnetic bead as the primary attachment mechanism for isolation of target organisms from environmental samples. Lee et al. (2009) further optimized the use of the IMS/ATP assay to quantify *E. coli* and *Enteroccous* in marine waters with the development of the Cov-IMS/ATP assay. The Cov-IMS/ATP assay relies on a more robust covalently-linked antibody-bead complex that cannot be destabilized as easily as the original adsorption-based complex (9). These earlier studies suggest that the *Enterococcus* IMS/ATP method can be useful for coastal water quality assessment and source tracking in fresh and marine waters; however, Cov-IMS/ATP has only been validated at a few sites and application potential as well as limitations have not been expressly evaluated. Further, Bushon et al. (2009) found that the IMS/ATP assay was site specific, requiring a different calibration curve when comparing the assay to culture-based methods at different sites (15).

Differential specificity has been reported to lead to intrinsic differences in enumeration by mEI (Difco, Becton, Dickinson and Company, San Jose, CA) and Enterolert (IDEXX) media (16) and potentially may influence site-specific performance of the Cov-IMS/ATP assay. A similarly constructed IgG *Enterococcus* antibody showed potential for cross-reactivity (17), and the specificity of this polyclonal *Enterococcus* antibody has not been examined (18).

In this study, performance of the Cov-IMS/ATP assay was evaluated for *Enterococcus* and compared to traditional culture-based methods and an *Enterococcus*-specific qPCR assay at several complex, diverse environmental sites through development of calibration curves with both ambient concentrations of enterococci and with wastewater-spiked ambient water. Further, specificity of the Cov-IMS/ATP was tested against different strains of *Enterococcus* spp. In

addition, potential cross-reacting organisms were identified with 16S rRNA gene sequencing and ability of the assay to detect non-enterococci species was evaluated. In the final stage of the study, an additional preabsorption step was tested for potential to decrease non-specific binding of the antibody-bead complex. These results help validate the Cov-IMS/ATP method and improve successful application of the assay to coastal water quality assessment and source tracking.

2. Materials and Methods

Cov-IMS/ATP Methodology

Enterococcus spp. (cat #B65173R, Meridian Life Sciences) polyclonal antibodies and Dynabead particles (Invitrogen, Carlsbad, CA) were used to generate antibody-bead complexes. Dynabead particles (M-280; Invitrogen, Carlsbad, CA) are uniform, superparamagnetic, polystyrene beads functionalized with sulfonyl ester groups permitting covalent binding to immunoglobins. *Enterococcoccus spp.* antibodieswere applied for isolation of target organisms.

Samples were processed according to the Cov-IMS/ATP method developed by Lee et al. 2009 with several modifications. Briefly, 200 µL of Dynabeads were washed in borate buffer in phosphate-buffered saline (PBS) (10% borate buffer w/v in PBS, pH 9.5) and separated for 1 minute from solution using a magnet. After two washes, the clean Dynabeads were added to 40 µL of IgG solution creating an anti-ent biosorbent. This antibody-bead complex was incubated at 37°C for 16-24 hours. Following incubation, the anti-ent biosorbent mixture was washed with and stored in bovine serum albumin (0.1% BSA w/v in PBS) buffer at continuous rotation at 4°C for up to two weeks.

Culture-based methods

Measurements made by Cov-IMS/ATP were compared with counts of *Enterococcus* determined by two standard methods, USEPA Method 1600 and defined substrate technology (Enterolert, IDEXX). For the EPA membrane filtration Method 1600 (MF) (19), water samples were filtered on a GN-6 mixed cellulose-gridded membrane filter with a standard platform manifold (in triplicate) and incubated on mEI agar (BD, Franklin Lakes, NJ, USA) for 24 hours at 41°C. Presumptive enterococci colonies were then enumerated as colony forming units (CFU). Detection of *Enterococcus* with Enterolert was performed according to the manufacturer's instructions (IDEXX Laboratories, Canada Corp., Toronto, Ontario, Canada). Positive identification of presumptive enterococci was determined by samples presenting fluorescence under UV light (365 nm) and quantified in units of most probable number (MPN).

Quantitative PCR

Cell densities of *Enterococcus* were also determined by quantitative PCR (qPCR) using USEPA Method A (15). For the measurement of *Enterococcus* gene copies mL^{-1}, recorded volumes of sample water were filtered through 47-mm, 0.4-µm pore size HTTP polycarbonate filters (EMD Millipore, Billerica MA) in duplicate. Each filter was placed in an individual two mL polypropylene screw cap tube, containing 0.3g, 212 – 300 µm (50 – 70 U.S. sieve) acid washed glass beads (Sigma-Aldrich, St. Louis MO) and stored at -80°C until DNA extraction. Filter blanks, consisting of 50 mL of PBS passed through the polycarbonate filter, were also generated with each set of samples processed. DNA was extracted as described below and run in parallel with calibration standards. Calibration standards were prepared using *Enterococcus faecalis* ATCC 29212 and analyses were performed according to USEPA Method 1A (20). Briefly, 2 µL of DNA template was added to 12.5 µL 1x ABI Universal Mater Mix, 2.5 µL of 2 mg mL^{-1} BSA, 3.5 µL of primer/probe working solution and 4.5 µL molecular grade RNase free

water for a final reaction volume of 25 µL and cycled at 50°C for two min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for two min. Primers and probe sequences used are detailed in Table 3-1. Samples and calibration standards were run in triplicate. In addition, negative controls were run on every plate and filter and extraction blanks were included to ensure contamination of samples did not occur during either the filtration or extraction processes. All four methods were run in parallel in order to calibrate Cov-IMS/ATP measurements of relative light units (RLU) to standard units of CFUs, MPNs, and gene copies per 100 mL.

DNA Extraction

DNA was recovered from water samples and calibration standards were constructed according to manufacturer's guidelines of the DNA-EZ ST1 Extraction Kit (GeneRite, North Brunswick NJ). Extracted DNA was eluted into 100 µl of elution buffer and aliquots were stored at -20°C until analysis with qPCR. DNA concentration was determined using UV absorption with a Nanodrop 2000C (Thermo Scientific, Waltham MA). A preloaded tube (containing only 0.3g of glass beads) was extracted in the same manner as a sample and used to assess any possible contamination during the extraction process (extraction blank). Additionally, extraction efficiency was evaluated by spiking a preloaded tube with 1µL of sewage extracted DNA, known to amplify at a specific cycle threshold value (Cq). If amplification varied from the expected Cq value, a new set of archived sample filters would undergo the DNA extraction process; however, this never occurred in the study.

Bacteria attached to the bound portion of the bead-antibody complex were also identified by sequencing- DNA was extracted and recovered according to the method in Shanks et al. 2012 (21). Universal primers were used to amplify partial 16S rRNA genes by PCR (Table 3-1). The MoBio 12500-50 UltraClean PCR Clean-Up kit was used according to manufacturer's guidelines

for DNA purification. Further processing and sequencing of the 16S gene was performed at UCLA Genotyping and Sequencing Core (GenoSeq, Los Angeles, CA) with the Biosystems 3730 Capillary DNA Analyzer, using capillary technology. Sequences were realigned with CLUSTALW (SDSC WorkBench 3.2) and blasted against the NCBI nucleotide database (NCBI-BLAST).

Field Sampling

Grab samples were collected by immersing a 2 L polypropylene bottle at the water surface for creek and lagoon samples and at ankle depth for marine samples. Bottles were acid washed with 10% HCL and pre-rinsed three times with sample water prior to sample collection. Approximately 500 mL of sample was filter concentrated and analyzed using the Cov-IMS⁄ATP assay. Samples were filtered through a 0.45 µm filter (SA1J792H5; Millipore), and bacteria were resuspended by vortexing the filter for 1 min in 10 mL of PBS (1x pH 7.2). One mL of the resuspended solution was added to the anti-ent biosorbent, incubated on a rotating mixer for 40 min at ambient temperature and processed according to methods described previously. The remaining sample was analyzed with Enterolert, membrane filtration, and qPCR for enterococci at each site, respectively.

Water samples were collected on multiple field days between August 2012 – June 2013 from Topanga State Beach, California; Doheny State Beach, California; and Tijuana, Mexico, (for full site descriptions see Supplemental Information Table S1 in Appendix B) for environmental calibration curves. At Topanga State Beach, three samples were collected along an approximately half mile transect that ran perpendicular to the Topanga lagoon outlet. In addition, two samples were collected within the Topanga lagoon. Potential sources of FIB include significant bird populations, local septic systems, dogs, lagoon input, algae and kelp.

At Doheny State Beach five sites were sampled along a 2.5 mile ocean transect. Two sites were sampled upcoast of the San Juan Creek outlet. The outlet of the Harbor creek was also sampled as was one down coast site. Potential sources of FIB include transients (homeless individuals) as well as faulty sewage infrastructure and significant bird populations.

Samples were also collected from eight sites in Tijuana, Baja California, Mexico, approximately 17 miles south of the US-Mexico border. Four marine water samples were collected along a five mile along-shore ocean transect. In addition, samples were collected from four freshwater inputs that discharge directly into the ocean including Real del Mar Creek, San Antonio de los Buenos (SADB) Creek and two storm drains (SADB drain and Isla drain). SADB Wastewater treatment plant discharges approximately 25 MGD of secondary treated and chlorinated sewage into the SADB Creek, making up the majority of the creeks flow (22). Sources of fecal pollution to ocean water include raw sewage from inadequate or lack of treatment, dogs, and gulls. In addition, improperly disposed animal fecal waste from horses and/or dairy cattle farms could potentially contribute to impaired water quality in this region. Fecal contamination within the watershed may be transported to the ocean via creeks and storm water runoff.

Samples were collected for sewage-spiked calibration curves at Topanga State Beach (34º2’19.67”, -118º34’56.21”), Topanga Lagoon (34º2’19.85”, -118º34’58.42”), Topanga Creek (34º3’50.48”, -118º35’13.95”), Doheny State Beach (33º27’40.40”, -117º40’54.45”) and Santa Monica Beach (34º0’12.41”, -118º29’29.57”).

Cov-IMS/ATP Specificity: *Enterococcus spp.*

Specificity of the Cov-IMS/ATP assay was tested with seven ATCC strains representative of *Enterococcus* species found in environmental waters: *Enterococcus hirae*

(ATCC 8043), *Enterococcus mundtii* (ATCC 43186), *Enterococcus durans* (ATCC 6056), *Enterococcus faecium* (ATCC 35667), *E..faecalis* (ATCC 29212), *Enterococcus casselflavus* (ATCC 12755), and *Enterococcus gallinarum* (ATCC 70025) (Difco, Becton, Dickinson and Company, San Jose, CA). Each ATCC strain described was grown to semi-logarithmic phase and adjusted to a concentration of 10^8 cells mL^{-1} PBS (1x, pH 7.4) using optical density (OD 595), before being serially diluted ten-fold in phosphate-buffered saline (1x, pH 7.4) to three to five different concentrations ranging between approximately 10 cells mL^{-1} and 1000 cells mL^{-1}. Standard curve measurements were taken in parallel by the mEI, Enterolert, qPCR, and the Cov-IMS/ATP assays as described above.

Cov-IMS/ATP Specificity: Non-Target Bacteria

Samples from each field site were evaluated for potential binding to non-enterococcal bacterium. After the bead-antibody complex had been incubated with environmental sample for 40 minutes, a subset of the bound antibody-bead complex was plated on both nutrient agar (pH 7.2, Becton Dickinson and Company) and/or tryptic soy agar (pH 7.2, Becton Dickinson and Company) for 24 hours at 37°C. Following incubation, approximately 50 isolates were randomly selected and purified. A subset of these isolates was then further purified and sequenced using 16S rRNA primers described above.

The IgG serum was further tested for cross-reactivity with four non-target bacterial species previously identified by our laboratory in high prevalence from Doheny and Topanga beach water by 16S rRNA sequencing. *E. faecalis* was grown to semi-logarithmic phase and adjusted to a concentration of 10^8 cells mL^{-1} PBS (1x, pH 7.4) using optical density (OD 595), before being serially diluted to a starting concentration of 100 cells mL^{-1} PBS (1x pH 7.4). A standard curve generated with *E. faecalis* was compared to a standard curve generated with *E.*

faecalis spiked with *Staphylococcus aureus, Enterobacter cloacae, Exiguobacterium sp.,* or *Bacillus cereus* to test for possible cross-reactivity of the non-target bacteria with the IgG serum.

Preabsorption process for purified antibody-bead complexes.

The antiserum was purified by preabsorption with mixtures of *S. aureus, E. cloacae,* and *B. cereus*, bacterial species likely found in marine waters and isolated with high prevalence from the bound-bead population. Cell cultures used for preabsorption were initially grown for 24 hours at 37 °C. Bacterial densities were adjusted to 10^8 cells mL^{-1} using optical density (595 nm). One mL of each bacterial strain was centrifuged at 12,000 x g at room temperature for ten min. The pellet was washed and re-suspended three times in one mL of 0.1% Tween in PBS (1x pH 7.4). 50 µL of the cell pellet was then used to inoculate and preabsorb the antibodies (500 µL) according to Saraswat (1994) (23). Following a 40 min incubation period, the cell-antibody solution was centrifuged at 12,000 x g for 10 min at room temperature. Supernatant was then retrieved and the concentration of the purified antibody serum was measured using the ELISA IgG Rabbit assay (Immunology Consultants Laboratory, Portland, OR). The Cov-IMS/ATP assay was run in parallel using purified antibody serum and non-preabsorbed antibodies to compare potential performance improvements.

Statistical Analyses

Statistical analyses were performed in STATA 12.1 (STATA Corp LP, College Station, Texas). Linear regression models were applied to estimate MPN per 100 mL as a function of RLU per 100 mL and were computed using $\log_{10}$-transformed data. Pearson's correlation coefficients were calculated to further examine the linear relationship between methods.

3. Results

Water samples (sewage-spiked and environmental) were measured using the Cov-IMS/ATP, Enterolert, and EPA Method A (ENT1A) qPCR assays. Luminescence, reported by the Cov-IMS/ATP method as RLU per 100 mL, was plotted against MPN per 100 mL, per the Enterolert method. Linear regression was used to model the association between the Enterolert and Cov-IMS/ATP methods based on the USEPA enterococci single standard of 104 MPN per 100 mL as well as an equivalent single sample threshold for the Cov-IMS/ATP method. Table 3-2 presents the results of linear regression models applied for sewage-spiked and environmental data. Confidence intervals (CIs) were applied to the exceedance threshold predicted for the Cov-IMS/ATP measurements by the linear regression equation. Values falling within the 95% CI were excluded from agreement rate calculations. Frequency of observations falling within these bands were calculated and reported as percentage of observations within uncertainty region (Table 3-2, Column Unc.). Agreement rate was calculated based on the percentage of data points where the Cov-IMS/ATP and Enterolert methods-agreed in indicating samples exceeding the 104 MPN per 100 mL threshold.

Sewage-Spiked Calibration Curves

Raw influent samples from the Orange County Sanitation District were diluted and spiked into ambient waters and analyzed with the Cov-IMS/ATP and Enterolert methods at Doheny State Beach, Dockweiler Beach, Santa Monica Beach, Topanga Creek, Topanga State Beach, and Topanga Lagoon. Average *Enterococcus* concentrations as measured by Enterolert and Cov-IMS/ATP correlated well both among the six sites ($R>0.90$, $P<0.05$) and between the six sites when data were combined ($R=0.90$, $P<0.05$) (Table 3-2). When data were combined between all sites, between all marine sites, and between all sites within the Topanga watershed,

Pearson's correlation coefficients remained high ($R>0.80$, $P<0.05$) as did the agreement rates (AR> 85%) when results were examined in comparison to the USEPA recreational water enterococci single standard of 104 MPN per 100 mL (Figure 3-1).

Due to the low number of points per site, a mixed effects model allowing for a random slope and random intercept for each site was applied to further quantify potential site specific effects on the relationship between Enterolert and Cov-IMS/ATP measurements. There was no evidence of a site effect; although the sample size was fairly small, there was no indication of a dramatic sample size effect. The variance associated with site effects on slope and intercept were negligible when compared to the variance due to randomness in the model: σ^2 =0.00 and 0.001 versus 0.13, respectively (see supplementary information Table S2). When data were combined between sites with a common input (raw influent) a site specific calibration curve does not appear necessary or provide a particular advantage.

Environmental Calibration Curves

The three sites reported represent various challenging inputs, both freshwater and marine, thus the combined calibration curve presented is robust to a variety of complex inputs evaluated in coastal water quality for source tracking. For Doheny State Beach, Topanga State Beach, and Tijuana, Mexico samples, calibration curves were created using ambient source water over an approximately six month period. Luminescence, reported by the IMS/ATP method as RLU per 100 mL, was plotted against MPN per 100 mL, as reported for the Enterolert method (Figure 3-2). Significant correlations and linear relationships between the Cov-IMS/ATP and Enterolert results ($R > 0.75$, $P <0.05$) were observed at each site.

Due to differences in the relationship between MPN and RLU measurements between sites for environmental calibration curves, an additional linear regression model was run to allow

for differences between the three sites (see supplementary information Table S3). There was not a significant difference between Mexico and Doheny Beach ($F_{1,63}$= 0.66, P=0.42) or between Doheny Beach and Topanga State Beach profiles (t=-0.61, p=0.542), only between the Topanga and Mexico profiles (t=0.22, p=0.030), which resulted in a significant improvement in fit when the model was allowed to account for differences in site as well as profile by site (relationship between MPN and RLU at each site) ($F_{1, 65}$ =5.215, P=0.026).

Although the linear relationship between Enterolert and Cov-IMS/ATP results differed among the three sites, with coefficient of the slope varying between 0.25 at Topanga State Beach and 0.58 at Tijuana, Mexico (Table 3-2), a combined environmental calibration curve was applied effectively demonstrating strong agreement with Enterolert (AR= 94%) for the enterococci single sample limit and a strong correlation between the two methods (R=0.83, P<0.05) (Figure 3-2, Panel D).

Best performance at certain sites may require site-specific calibration curves, however a combined calibration curve was applied effectively and with strong predictability. Further, site-specific calibration curves improves predictability of Cov-IMS/ATP measurements for the Topanga site only and did not offer an improvement over the combined calibration curve for either the Doheny or Tijuana sites. Here, the improvement was small (the AR was increased from 84% to 89%) when applying the site-specific calibration curve to Topanga site measurements. The measurements falling within the region of uncertainty did increase from 5% to 32% signaling a larger region of uncertainty for certain measurements when applying the more generalized combined calibration curve to environmental data for the Topanga site (Table 3-2).

Site specific calibration curves may still be utilized for best performance at certain sites, as indicated by the improved performance at Topanga State Beach when a site-specific

calibration curve was applied. However, overall performance of the combined calibration curve was strong (R =0.83, AR=91%) and comparable to performance if individual site-specific calibration curves were applied to each site.

Luminescence, reported by the IMS/ATP method as RLU per 100 mL, was also plotted against copies per 100 mL, as reported for the Method A qPCR assay. The linear relationship between Enterolert and Cov-IMS/ATP results differed among the three sites, with coefficient of the slope varying between 0.31 at Tijuana, 0.40 at Mexico and 1.06 at Doheny State Beach (Table 3-3). For Topanga State Beach samples, there was a linear relationship between qPCR and Cov-IMS/ATP measurements (R=0.68). Similarly, at Doheny State Beach qPCR and Cov-IMS/ATP measurements were correlated (R=0.71). Measurements at the Tijuana site both when comparing Cov-IMS/ATP and qPCR (R=0.37) measurements and when comparing the Enterolert and QPCR assay (R=0.30) measurements were not as predictable.

Model Variability

Model variance was analyzed on smoothed data for the combined environmental curves and sewage-spiked calibration curves. Sum of squared error (SSE), as defined as the sum of squared differences between actual and predicted values, was calculated for binned data, such as from between 0.5 to 1 MPN, adapted from method applied in Whitman et al. 2010. Estimated sum of squared error was plotted against cell concentration (MPN/100 mL) (see supplemental information Figure S1). For the sewage-spiked samples, the range of SSE was small overall, ranging between 0.5 and 2.3, resulting in more consistent and smaller overall error. For the environmental model, the predictions made were the most robust and least affected by model error at lower and mid-range concentrations and especially around the enterococci

exceedance threshold of 104 MPN/100 mL. At higher concentrations (between 3 and 6 log MPN) there was a trend of consistent, increased error.

Limit of Detection

Blank samples were processed by analyzing PBS in a similar manner to that of the environmental samples. The limit of detection for the Cov-IMS/ATP method was computed by multiplying the SD of the blank sample results by a factor of three and then adding the mean (14). The limit of detection was calculated to be 6190 RLU/ 100 mL based on 15 blank samples.

Speciation of Enterococci at study sites

Site water was further characterized at Doheny State Beach, Topanga State Beach, and Imperial Beach through examination of enterococci species assemblages. Enterococci isolates were found to include the following six species: *E. faecalis, E. faecium, E. gallinarum, E. casseliflavus, E. mundtii*, and *E. hirae,* as well as additional unidentified enterococci and unidentified non-enterococci individuals. Enterococci isolates examined in this study from Doheny State Beach and Imperial Beach were previously collected and identified as part of a comprehensive comparison of *Enterococcus* species diversity (12) but have not been analyzed previously by site. Out of 65 isolates examined at Doheny Beach, *E. faecalis* and *E. faecium* were the most prevalent species obtained, occurring at frequencies of 42% and 22% out of 65 isolates examined. The remaining isolates were characterized as follows: *E. casseliflavus* (8%), *E. gallinarum* (11%), and either unidentified enterococci (6%) or non-enterococci (8%).

At Imperial Beach, isolates were examined from a mixture of the Tijuana River mouth and Imperial Beach Water. Water samples were collected approximately ten miles from the Tijuana site sampled in this study, due to difficulty associated with transporting bacterial isolates across border lines from samples collected in Mexico. Out of 60 isolates isolated from either

Enterolert broth media or mEI agar, 43% were identified as non-*Enterococcus*. *E. faecium* and *E. casseliflavus* were the most pre-dominant enterococci species, representing 18% and 17% of isolates examined, respectively. The remaining isolates were characterized as follows: *E. hirae* (7%), *E. faecalis* (8%), *E. mundtii* (2%), and unidentified enterococci (5%). In addition, in this study, 20 isolates were isolated from mEI agar at Topanga State Beach and Lagoon, and 100% of these isolates were identified as *E. faecalis*. Isolates were identified according to methods described in Ferguson et al. 2013 (16).

Cov-IMS/ATP Assay Specificity

Specificity of the Cov-IMS/ATP assay was verified by testing seven common enterococci species found in marine waters. Of the enterococci species tested, average concentrations as measured by Enterolert and Cov-IMS/ATP correlated well for six out of the seven species (Table 3-3). *E. gallinarum* measurements made by Cov-IMS/ATP correlated poorly with measurements made by the Enterolert assay. Measurements for all seven species were well correlated for the Cov-IMS/ATP and MF assays (Table 3-3). Cov-IMS/ATP exhibited differential sensitivity to the seven enterococci species, when compared to the traditional methods (MF and Enterolert) (Figure 3-3). The Cov-IMS/ATP method may potentially be the most sensitive to *E. faecalis* and *E. faecium,* evidenced by the steeper slope of the linear regression relationship between the Cov-IMS/ATP assay and the Enterolert and MF assay measurements (Figure 3-3) and the least sensitive to *E. gallinarum*, *E. casseliflavus*, and *E. mundtii.* Average *Enterococcus* concentrations as measured by culture-based (Enterolert and MF) and qPCR (ENT1A) methods correlated well (R> 0.80) for all seven of the tested *Enterococcus spp*. (Table 3-4). Fourteen samples used in the specificity experiments were run in duplicate to quantify the variability of the Cov- IMS/ATP assay. On average, duplicates differed by 10040 RLU or 13%.

Non-enterococcal bacteria that were bound to the antibody-bead complex were identified. A subset of environmental isolates cultured from water samples incubated with the bead-antibody complex were purified and sequenced from the bound population. Water samples were collected on three separate days from both Doheny State Beach and Topanga State Beach. A rarefaction curve indicated that even if considerably more isolates were sequenced, similar species richness would still be observed (see Supplemental Information Figure S2). There were some similarities in species identified at the two sites, although the frequency of species differed (Table 3-5). *Bacillus spp*. (29%), *Exiguobacterium spp*. (19%), and *Enterobacter spp*. (15%) were the most common non-enterococcal bacteria isolated. Other species identified at both sites included *Staphylococcus spp*. (10%) and *Aeromonas spp*. (8%). For a full species list of isolates sequenced see supplemental information Table S4 .

Specificity of the antibody-bead complex was tested against four ATCC species of cross reactors identified in high frequency from 16S rRNA sequencing results: *B. cereus* (ATCC 14579), *A. calcoaceticus* (ATCC 23055), *E. cloacae* (ATCC 13047), and *S. saprophyticus* (ATCC 15305) as well as against *S. gallolyticus* (no ATCC number provided). Sensitivity of the Cov-IMS/ATP assay was decreased, evident by a substantial reduction on slope of the linear relationship between Enterolert and the Cov-IMS/ATP method, when several of these species were present, individually or in combination, in samples spiked with different concentrations of *E. faecalis*. *S. gallolyticu*s (P=0.07), *E. clocae* (P=0.03), and *S. saprophyticus* (P=0.10) all significantly affected measurements made by the Cov-IMS/ATP method. Further, combinations of various cross reactors including a mixture of *E. clocae* and *B. cereus* (P=0.00) and a mixture of *E. clocae, B. cereus,* and *S. saprophyticus* (P=0.01) were also found to significantly affect Cov-IMS/ATP measurements (Table 3-6).

Preabsorption

In an effort to improve the specificity of the assay, *Enterococcus* IgG serum was preabsorbed with *S. aureus*, *E. cloacae,* and *B. subtilis.* These species were all isolated in high frequency from the bound portion of the antibody-bead complex at both Topanga State Beach and Doheny State Beach. Moreover, *S. aureus* and *E. cloacae* were found to significantly affect Cov-IMS/ATP measurements. An ELISA assay (Immunology Consultants Laboratory, Portland, OR) was used before and after the preabsorption test to quantify the portion of antibody remaining in suspension. On average (out of five trials), concentration was decreased from 1mg mL^{-1} to 0.85 mg/ml signaling a 15 % loss of antibodies to preabsorption. The ratio of antibody to magnetic bead was adjusted for this loss. Preabsorbed antibodies were applied in laboratory experiments. Preabsorption may increase sensitivity of the assay (subset of trial results included in supplemental information Figure S3). Antibody-bead complexes that had been preabsorbed and were spiked with a starting concentration between 10^4 and 10^5 cells/100 mL of *S. aureus*, and *B. subtilis* bound less of the interfering species. Measurements were on average reduced by 60% for *S. aureus* and 40% for *B. subtilis* when beads were purified with an additional preabsorption step. Preabsorption did not appear to improve potential cross reactivity from *E. cloacae*.

4. Discussion

Standard enterococci detection methods for coastal water quality are culture-based and require a lengthy incubation period. A rapid, portable method can be an important facet to a multi-tiered approach to source tracking (24) which has previously relied on culture- and nucleic-acid based methods (25). Molecular methods based on qPCR can be highly sensitive and

have less expensive per sample costs, but require experienced technicians, expensive startup costs, and measure a genetic end-point that does not distinguish between viable and non-viable organisms. In comparison, the Cov-IMS/ATP method requires minimal equipment, is affordable (per sample cost is comparable to culture methods) and simple to perform. The Cov-IMS/ATP method also has the ability to filter higher volumes through the HAWG filter used when compared to the HTTP filter required for qPCR, which is advantageous for analyzing turbid water samples including runoff. Moreover, Cov-IMS/ATP quantifies viable bacteria only making it more comparable to culture-based methods. Cov-IMS/ATP quantifies ATP in target organisms, making measurements in relative light units, while culture-based methods directly measure metabolic activity, providing measurements in either most probable number or colony forming units. Therefore, it is important to have a comprehensive understanding of the empirical relationship between culture-based and Cov-IMS/ATP measurements.

In this study, a strong, positive association was observed between the measurements produced by the Enterolert and Cov-IMS/ATP assays in different water samples and with different environmental source inputs of enterococci. Less variability was noted in the Cov-IMS/ATP estimates among samples with mid-range concentrations, between 100 and 300 MPN/100 mL. Further, when the two methods were compared across water types, either under ambient conditions or sewage-spiked conditions, relationships were consistent and provided a strong linear relationship and strong agreement when measurements were analyzed based on a single sample limit of 104 MPN enterococci per 100 mL. Although there was evidence of some site heterogeneity in proportion of Enterolert to Cov-IMS/ATP measurements between sites, especially when comparing Topanga State Beach to Tijuana and Doheny sites, an overall relationship was developed with success for both ambient and sewage-spiked waters.

Site heterogeneity and method variability may result in a region of uncertainty for the Cov-IMS/ATP assay. Therefore, values falling in this region may require verification from another method. Even so, these results suggest that the relationship between Enterolert and Cov-IMS/ATP measurements may reasonably predict and help differentiate sites with high and low *Enterococcus* levels from sites that may need further verification.

QPCR measurements were also compared with both Enterolert and Cov-IMS/ATP measurements. There was a positive trend between both Cov-IMS/ATP and Enterolert compared to qPCR measurements at Topanga State Beach and Doheny State Beach. However, at the Tijuana sampling sites the relationship between qPCR and the other methods (Cov-IMS/ATP and Enterolert) was poor. The weaker linearity at this site could be due to environmental factors such as point-sources of FIB to the Tijuana sites. Sources of fecal pollution have been found to be an important part of describing the relationship between culture and qPCR results and can result in decreased correlation between the two methods at certain sites (26). At the discharge point of the SAB wastewater treatment plant, high levels of ENT were measured by qPCR; yet low to non-detectable levels were observed with culture-based methods. Elevated signal of ENT at this site may be the result of qPCR amplifying DNA from both live and dead cells of *Enterococcus;* contributing to the decoupling of qPCR and culture-based methods. However, Cov-IMS/ATP measurements still correlated well with Enterolert measurements at the Tijuana sites suggesting that this rapid and viability-based method may be useful for detection of complex and recent inputs of FIB.

To explain discrepancies between sites, differential sensitivity to various *Enterococcus* species and potential cross-reactive binding of the antibody-bead complex was evaluated using the seven most common *Enterococcus* species in marine water (27, 28) and five species of

potential non-target bacteria. Previous studies indicate that there is potential for Enterolert and EPA Method 1600 to differ in species selectivity between sites (16). To our knowledge, this is the first report on the comparison of these culture-based methods to Cov-IMS/ATP using pure cultures of varied *Enterococcus* species. The antibody used in the Cov-IMS/ATP is polyclonal in nature and has not been absorbed on other *Enterococcus* species besides *E. faecium* (18). Caruso et al. 2008 analyzed specificity of a similar *Enterococcus* IgG serum for application to a fluorescent antibody technique, finding the serum to be effective at labeling *E. faecium* species only (17). However, the IgG serum as utilized in the Cov-IMS/ATP assay appears to have a more robust specificity profile to *Enterococcus spp*. A linear relationship was observed for all enterococci species tested when measurements were compared between the Cov-IMS/ATP and MF methods and for all but one of the enterococci species when measurements were compared between the Cov-IMS/ATP and Enterolert methods. Variations in assay sensitivity were observed for the different *Enterococcus spp*. and could contribute to differences between Cov-IMS/ATP and the culture-based measurements at certain sites where *Enterococcus* species other than *E. faecium* are dominant. Depending on the site and source input, enterococcal communities can differ drastically (29, 30) as was found between the three sites (Doheny, Topanga and Tijuana) tested in this study.

Potential for cross-reactivity of the antiserum to non-specific antigens was also assessed using several common species (*A. calcoaceticus, E. cloacae, S. saprophyticus* and *B. subtilis)* isolated in high frequency from the antibody-bead complex. *E. cloacae*, *S. saprophyticus*, and *S. gallolyticus* were found to significantly affect Cov-IMS/ATP measurements.

A purification procedure, based on preabsorption of the polyclonal antibody with non-enterococcal bacteria, shows promise to increase specificity of the assay. Without prior serum

purification, the addition of certain non-enterococci species can result in decreased correlation between traditional methods and the Cov-IMS/ATP assay. However, further research is needed to develop more target-specific antibodies and to better optimize preabsorption of the antibody to reduce cross reactivity.

5. Conclusions

This study extends upon previous efforts by sampling a variety of location types during both the wet and dry season, and a range of bacterial concentrations. Previous reports have documented poorly correlated measurements between IMS/ATP and traditional methods in primary influent obtained from the Orange County Sanitation District (OCSD) (15), as well as at Doheny State Beach (Jay Lab, unpublished data). In this study, Cov-IMS/ATP and traditional methods correlated well in both OCSD sewage-spiked ambient waters, OCSD sewage-spiked directly into PBS, and environmental waters (Topanga State Beach, CA, Doheny State Beach, CA and Tijuana, Mexico).

Standard enterococci detection methods for coastal water quality are culture-based and require a lengthy incubation period. The Cov-IMS/ATP method presented rapidly measures viable enterococci, providing a useful field tool for microbial source tracking and assessing coastal water quality. This study helps to validate the Cov-IMS/ATP method as well as identify mechanisms, such as non-specific binding to antibody-coated beads and differential sensitivity of the method for different enterococci species, which can lead to site differences in measurements made by the Cov-IMS/ATP assay. Further, in this study the Cov-IMS/ATP assay illustrates robust measurements and a predictable relationship between enterococci measurements made by the Cov-IMS/ATP and Enterolert methods. Application of a reliabel consistent relationship between Cov-IMS/ATP and culture-based methods would substantially increase ease and

efficiency of application of the Cov-IMS/ATP method for source tracking of coastal water quality.

Acknowledgments

We thank Uriel Cobian, Tim Riedel, Sofi Peterson, Robert Torres, and Chris Carandang for field and laboratory support as well as SCCWRP for additional assistance and materials.

This work was supported by Los Angeles County and the UC Mexus Program as well as by the California State Water Resources Control Board which funded the Source Identification Protocol Project.

Figure 3 - 1. Logarithmic MPN versus RLU for sewage-spiked calibration curves generated with ambient water at A) all sites (data combined between Doheny State Beach, Topanga watershed sites, Santa Monica, and Dockweiler), B) all marine sites, and C) sites within Topanga watershed (Topanga creek, lagoon, and ocean). Solid line: linear mean trend between MPN and RLU; grey dashed line: 95% CI of exceedance threshold predicted by Cov-IMS/ATP; black dashed lines: exceedance threshold at 104 MPN/100 mls for MPN and predicted exceedance threshold for RLU/100 mls.

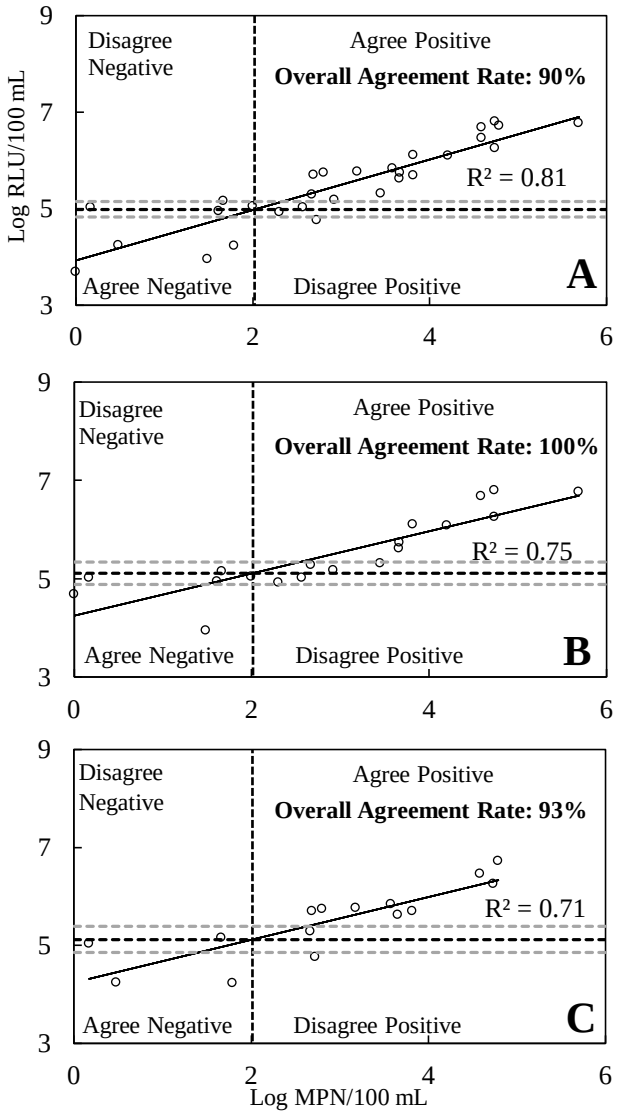


Figure 3- 2. Logarithmic MPN versus RLU for environmental calibration curves generated with ambient water at A) Topanga State Beach sites, B) Doheny State Beach sites, C) Tijuana, Mexico sites, and D) all sites combined (Doheny, Topanga, and Tijuana). Solid line: linear mean trend between MPN and RLU; grey dashed line: 95% CI of exceedance threshold predicted by Cov-IMS/ATP; black dashed lines: exceedance threshold at 104 MPN/100 mL for MPN and predicted exceedance threshold for RLU/100 mL.

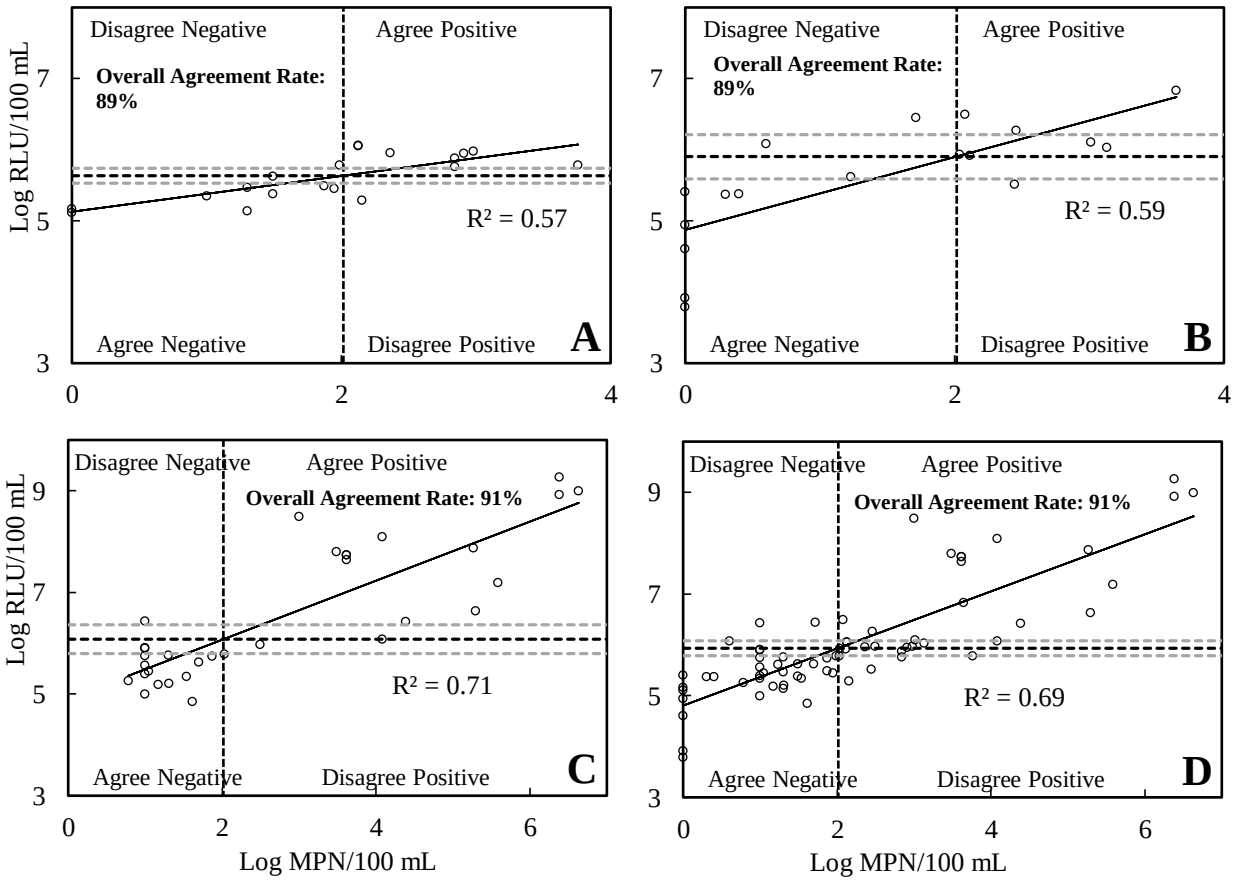


Figure 3- 3. Linear regression results for Enterococcus spp. specificity experiments. Logrithmic cell concentration versus RLU for A) *E. faecalis*, B) *E. faecium*, C) *E. gallinarum*, D) *E. hirae*, E) *E. durans*, F) *E. mundtii*, G) *E. casseliflavus*. Dashed line: relationship between RLU and cell concentration for *E. faecium* plotted for comparison; solid lines: linear mean trend between cell concentration and RLU/100 mL. Hollow circles = cell concentration as measured by membrane filtration (CFU/100 mL); black circles = cell concentration as measured by Enterolert (MPN/100 mL).

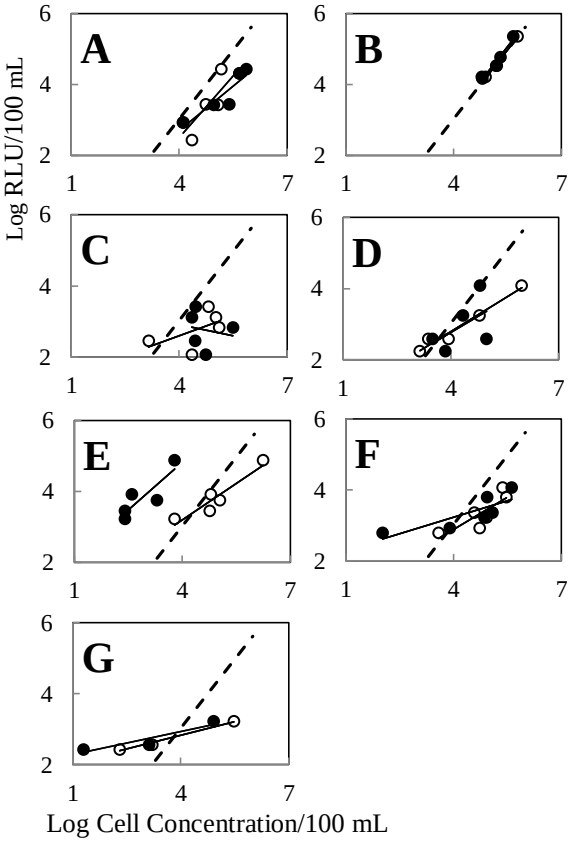


Table 3- 1. Specific primers used in this study for 16s rRNA sequencing and Ent 1A qPCR assays.

PCR and qPCR primers/probes used in this study					
Assay			Primer/Probe Sequences		Reference
Name	Type	Target	For Primer/ Rev Primer	Probe/Dye	
EPA Method A (ENT 1A)	qPCR	Enterococcus	AGAAATTCCAAACGAACTTG/ CAGTGCTCTACCTCCATCATT	FAM-TGGTTCTCTCCGAAATAG CTTTAGGGCTA-TAMRA	Ludwig and Schleifer, 2000
Sanger Seq. (16S r RNA)	PCR	Total Bacteria	AGAGTTTGATCCTGGCTCAG/ ACGGCTACCTTGTTACGACTT	N/A	

Table 3- 2. Results of linear regression models for sewage-spiked and environmental calibration curves. Relationship between MPN and RLU described by slope (ß) of the model. Agreement rates calculated based on frequency that Cov-IMS/ATP and Enterolert methods agreed on *Enterococcus* concentrations exceeding 104 MPN/100 mls threshold. Uncertainty (Unc.) calculated as percentage of measurements falling within 95% CI of predicted *Enterococcus* threshold for Cov-IMS/ATP method.

Treatment	Culture Results vs. IMS-ATP Results Model					Agreement Rate			
		Slope (ß)		Model		Site-Specific		Combined	
	Site (n)	ß	95% CI	R^2	R	AR	Unc.	AR	Unc.
Sewage-spiked	Dockweiler	0.80	[0.66, 0.94]	0.99	0.99				
	Doheny	0.48	[0.23, 0.73]	0.93	0.96				
	Santa Monica	0.40	[0.04, 0.76]	0.81	0.90				
	Topanga Creek	0.59	[0.17, 1.01]	0.87	0.93				
	Topanga Beach	0.26	[.05, 0.46]	0.92	0.96				
	Topanga Lagoon	0.39	[0.15, 0.63]	0.90	0.95				
	All Sites (29)	0.52	[0.42, 0.62]	0.81	0.90			89%	17%
	Marine (19)	0.43	[0.30, 0.56]	0.75	0.87	100%	47%	89%	16%
	Topanga (15)	0.44	[0.27, 0.60]	0.71	0.84	93%	20%	89%	5%
Ambient	Doheny (18)	0.51	[0.28, 0.74]	0.59	0.77	89%	33%	89%	22%
	Topanga (19)	0.25	[0.14, 0.36]	0.57	0.75	89%	5%	84%	32%
	Tijuana (32)	0.58	[0.44, 0.72]	0.71	0.84	91%	9%	94%	13%
	All Sites (69)	0.56	[0.47, 0.65]	0.69	0.83	90%	14%	91%	13%

Table 3 - 3. Results of linear regression models for qPCR measurements of *Enterococcus* concentrations of ambient water at Topanga State Beach, Doheny State Beach, and Tijuana, Mexico sites. Relationship between RLU and copy number as measured by Cov-IMS/ATP and Ent1A qPCR assay described as well as relationship between MPN (Enterolert) and copies (qPCR).

Environmental Calibration Curves QPCR Results								
	QPCR and Cov-IMS/ATP				QPCR and IDEXX			
Site	Slope (ß)		Model		Slope (ß)		Model	
	ß	95% CI	R^2	R	ß	95% CI	R^2	R
Topanga	0.4	[0.14, 0.66]	0.46	0.68	0.37	[0.06, 0.68]	0.37	0.61
Doheny	1.06	[0.42, 1.70]	0.50	0.71	0.33	[0.14, 0.53]	0.56	0.75
Tijuana	0.31	[0.02, 0.60]	0.14	0.37	0.25	[-0.04, 0.54]	0.09	0.30

Table 3- 4. Results of linear regressions models for *Enterococcus spp*. specificity experiments. *Enterococcus spp*. concentrations measured with MF (membrane filtration), IDEXX, Cov-IMS/ATP, and the Ent 1A qPCR assay. Separate regressions run comparing rapid methods to culture based as well as culture vs culture-based methods.

		Culture Results vs. Rapid Method (IMS-ATP/ QPCR) Results Model								Culture vs. Culture			
Treatment		IDEXX and Rapid Method				MF and Rapid Method				MF and IDEXX			
	Rapid	Slope (ß)		Model		Slope (ß)		Model		Slope (ß)		Model	
Ent sp.	Method	ß	95% CI	R^2	R	ß	95% CI	R^2	R	ß	95% CI	R^2	R
E. faecalis	IMS	0.84	[0.14, 1.54]	0.83	0.91	1.17	[0.20, 2.14]	0.74	0.86	0.99	[-.29, 2.27]	0.67	0.82
E. faecium	IMS	1.31	[0.86, 1.75]	0.97	0.98	1.30	[-0.32, 2.92]	0.99	0.99	0.96	[-0.35, 2.27]	0.99	0.99
E. gallinarum	IMS	-1.03	[-6.19, 4.12]	0.03	0.17	0.17	[-2.65, 2.99]	0.31	0.56	0.59	[-0.67, 1.85]	0.67	0.82
E. hirae	IMS	0.60	[-1.21, 2.42]	0.27	0.52	0.62	[0.42,0.82]	0.97	0.98	0.37	[-0.20, 0.95]	0.42	0.65
E. durans	IMS	0.80	[-0.88, 2.49]	0.68	0.82	0.38	[-0.08, 0.85]	0.70	0.84	0.81	[-0.44, 2.05]	0.80	0.89
E. mundtii	IMS	0.31	[0.00, 0.62]	0.66	0.81	0.61	[0.04, 1.17]	0.69	0.83	1.68	[0.45, 2.92]	0.78	0.88
E. casselfavis	IMS	0.22	[-0.86, 1.29]	0.87	0.93	0.26	[-0.13, 0.65]	0.99	0.99	0.22	[-0.86, 1.29]	0.87	0.93
E. faecalis	QPCR	0.79	[-0.33, 1.90]	0.63	0.79	1.14	[0.84, 1.44]	0.97	0.98				
E. faecium	QPCR	1.03	[0.06, 1.99]	0.73	0.85	1.04	[-0.07, 2.14]	0.99	0.99				
E. gallinarum	QPCR	1.13	[-0.45, 2.70]	0.99	0.99	0.68	[-0.19, 1.59]	0.92	0.96				
E. hirae	QPCR	0.76	[[-1.01, 2.53]	0.63	0.79	0.37	[-0.20, 0.95]	0.80	0.89				
E. durans	QPCR	0.49	[-0.26, 1.25]	0.80	0.89	0.41	[0.31, 0.51]	0.98	0.99				
E. mundtii	QPCR	0.40	[-0.02, 0.81]	0.64	0.80	0.89	[[0.45, 1.32]	0.89	0.94				
E. casselfavis	QPCR	0.45	[-0.72, 1.61]	0.96	0.98	0.50	[0.20, 0.81]	0.99	0.99				

Table 3 - 5. Bacteria isolated from bound antibody-bead complexes at either Topanga or Doheny State Beaches and sequenced with 16s rRNA sequencing.

	Site no. isolates from % (n):		%
Species	Doheny	Topanga	Total
Acinetobacter spp.	-	16% (8)	8%
Aeromonas spp.	4% (2)	12% (6)	8%
Bacillus spp.	49% (22)	12% (6)	29%
Delftia spp.	-	2% (1)	1%
Enterobacter spp.	24% (11)	6% (3)	15%
Exiguobacterium spp.	13% (6)	24% (12)	19%
Pantoea spp.	-	2% (1)	1%
Pseudomonas spp.	2% (1)	-	1%
Sphingopyxis spp.	4% (2)	-	2%
Sporosarcina spp.	-	4% (2)	2%
Staphylococcus spp.	2% (1)	18% (9)	10%
Vogesella spp.	-	6% (3)	3%
Total (n)	45	51	96

Table 3 - 6. Linear regression results testing potential effect of non-target bacteria on Cov-IMS/ATP measurements. EF= *E. faecalis* spiked standard curve; EF + CR = *E. faecalis* spiked with potential non-target bacterium. Presence of cross reactor included as indicator variable; significance of change in slope due to cross reactor included below.

Cov-IMS/ATP and IDEXX With Interference				
Treatment	Slope (ß)		Δ Slope	
Cross reactor (CR):	EF	EF + CR	t score	P-value
S. gallolyticus	0.72	0.06	2.84	0.07
B. cereus	0.66	0.78	1.87	0.158
A. calcoaceticus	0.72	0.64	-0.6	0.59
E. cloacae	1.42	0.59	3.91	0.03
S. saprophyticus	0.59	-0.03	2.09	0.13
EC + BC	1.21	0.44	9.38	0.00
EC +BC + AC	0.95	0.64	1.76	0.18
EC +BC +SS	0.80	0.17	5.25	0.01

6. Appendix B

Supplementary Table and Figures

Table S1. Coordinates of environmental sampling sites at Topanga State Beach, Doheny State Beach, and Tijuana field sites.

Site		Water Type	Coordinates	
Location	Sample Site ID		Latitude	Longitude
Tijuana, Mexico	Baja Malibu	Marine	32.41603	-117.096
	SADB Beach	Marine	32.43119	-117.1002
	SADB Storm drain	Fresh	32.43123	-117.0998
	SADB Creek	Fresh	32.44655	-117.1075
	SADB WWTP mixing zone	Marine	32.44633	-117.1075
	Real del Mar Creek	Fresh	32.4432	-117.1054
	Punta Bandera	Marine	32.46311	-117.117
	Isla Storm drain	Fresh	32.4273	-117.0987
Doheny State Beach, CA	San Juan Creek Outlet	Marine	33.46085	-117.6844
	Dana Point Creek	Brackish	33.46111	-117.689
	Dana Point Outlet	Marine	33.46167	-117.6894
	Beach Road	Marine	33.45401	-117.6675
	Cove Road	Marine	33.46045	-117.7085
Topanga State Beach, CA	Beach Upcoast	Marine	34.03785	-118.585
	Beach Outlet	Marine	34.0379	-118.5824
	Lifeguard Station	Marine	34.0383	-118.5817
	Topanga Lagoon	Fresh	34.03861	-118.5829
	Hwy 1 Bridge- lagoon	Fresh	34.03925	-118.5831

Table S2. Mixed effects model (random slope and intercept) results for combined sewage-spiked calibration curve (Wald Chi^2 123.79, P=0.00).

	Parameters		SE	95% CI	
	Predictor	Est.			
Model Coefficients	MPN	0.52	0.05	0.43	0.62
	ß	3.02	0.16	3.62	4.22
Random-Effects Parameters	sd (MPN)	0.00	0.00	0.00	0.07
	sd (ß)	0.25	0.34	0.00	6.8E+09
	sd (Residual)	0.36	0.05	0.27	0.48

Table S3. Multivariate regression model results for environmental calibration curve ($F_{5, 63}$=36, P=0.00, R^2=0.74). Cov-IMS/ATP measurements regressed on MPN measurements. Site included as a categorical covariate with Topanga as the referent site for analysis.

Parameters						
Predictor	Est.	SE	t	P>|t|	95% CI	
MPN	0.25	0.14	0.05	0.08	-0.03	0.53
Doheny	-0.23	0.38	0.16	0.54	-0.98	0.52
Tijuana	-0.23	0.35	0.00	0.51	-0.93	0.47
Doheny*MPN	0.23	0.18	0.34	0.21	-0.14	0.60
Tijuana*MPN	0.33	0.15	0.05	0.03	0.03	0.64

Table S4. Species sequenced from antibody-bead complex at Doheny State Beach and Topanga State Beach.

	Site no. isolates from:		
Species	Doheny	Topanga	Total
Acinetobacter sp.	-	6	6
Acinetobacter beijerinckii	-	1	1
Acinetobacter haemolyticus	-	1	1
Aeromonas hydrophila	1	-	1
Aeromonas popoffii	-	1	1
Aeromonas salmonicida	-	2	2
Aeromonas veronii	1	3	4
Bacillus cereus	15	1	16
Bacillus megaterium	2	4	6
Bacillus pumilus	4	-	4
Bacillus subtillus	1	-	1
Bacillus sp.	-	1	1
Delftia sp.	-	1	1
Enterobacter aerogenes	1	-	1
Enterobacter cloacae	6	1	7
Enterobacter sp.	4	1	5
Enterobacteriaceae bacterium	-	1	1
Exiguobacterium	-	3	3
Exiguobacterium antarcticum	5	2	7
Exiguobacterium sibiricum	1	7	8
Pantoea vagans	-	1	1
Pseudomonas stutzeri	1	-	1
Sphingopyxis alaskensis	2	-	2
Sporosarcina aquimarina	-	1	1
Sporosarcina newyorkensis	-	1	1
Staphylococcus aureus	-	1	1
Staphylococcus warneri	1	-	1
Staphylococcus saprophyticus	-	8	8
Vogesella sp.	-	3	3
Total	45	51	96

Figure S1. Sum of squared error calculated on smoothed data and plotted against cell concentration. Hollow circles indicate predicted error for sewage-spiked Cov-IMS/ATP measurements and filled-in circles indicate predicted error for environmental Cov-IMS/ATP measurements.

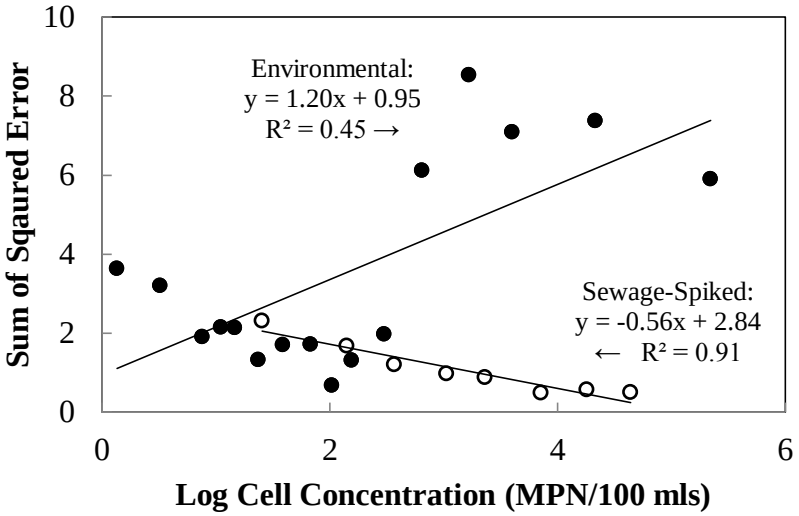


Figure S2. Rarefaction curve calculated for isolates sequenced from the bound portion of the antibody-bead complex at Doheny State Beach and Topanga State Beach. Mean species richness was calculated by random re-sampling of the number of species sequenced per sample in R Version 3.02.

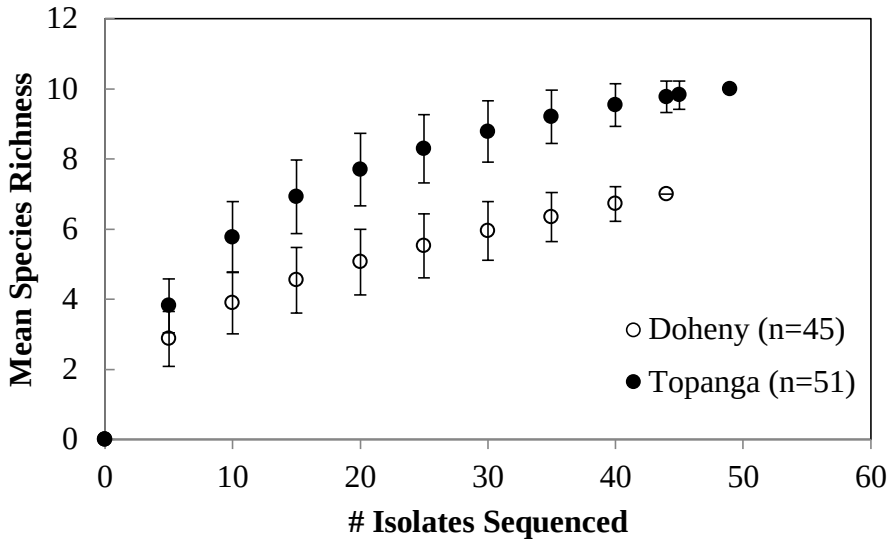
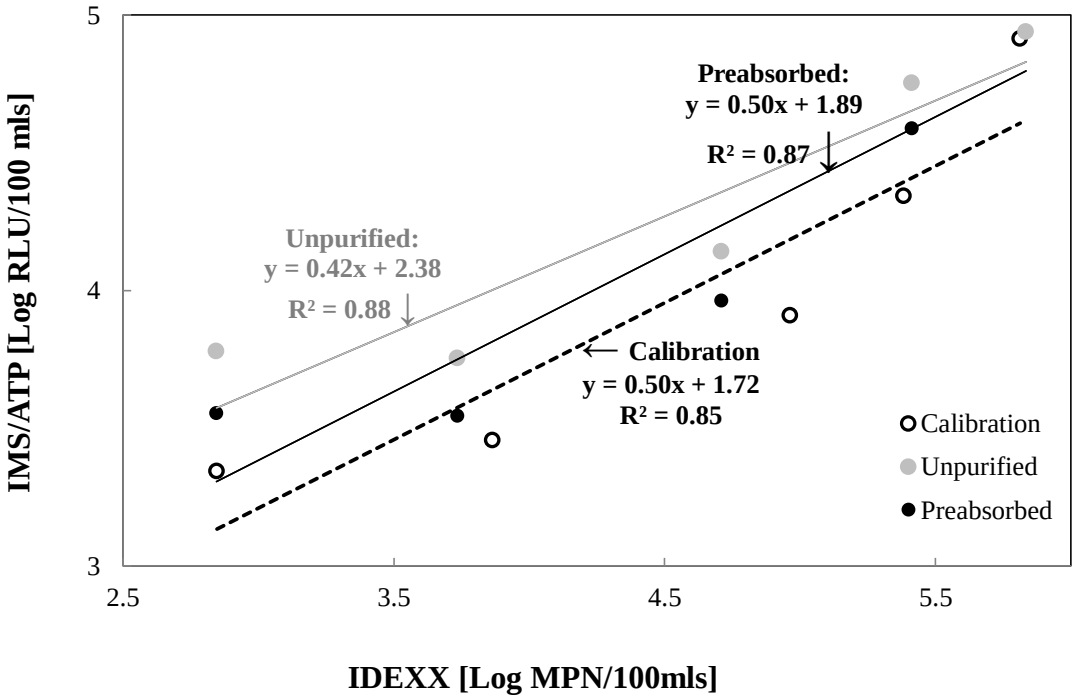


Figure S3. Subset of preabsorption experiment results. Calibration curve generated with different concentrations of *E. faecalis* spiked into PBS. Mixed model generated by spiking the *E. faecalis* calibration curve with *B. cereus* and *S. saprophyticus*. Cov-IMS/ATP measurements were taken with both the preabsorbed antibody-bead complexes and with unpurified antibody-bead complexes.



7. References

1. Kim J, Grant SB. 2004. Public Mis-Notification of Coastal Water Quality: A Probabilistic evaluation of Posting Errors at Huntington Beach, California. Env Sci Tech **38**: 2497- 2504.

2. Boehm AB. 2007. Enterococci concentration in diverse coastal environments exhibit extreme variability. *Environ Sci Technol* **41**, 8227–8232.

3. Boehm AB, Grant SB, Kim JH, Mowbray SL, McGee CD, Clark CD, Foley DM, Wellman DE. 2002. Decadal and shorter period variability of surf zone water quality at Huntington Beach, California. Environ Sci Technol. **36:** 3885–3892.

4. Noble RT, Griffith JF, Blackwood AD, Fuhrman JA, Gregory JB, Hernandez X, Liang XL, Bera AA, et al. 2006. Multitiered approach using quantitative PCR to track sources of fecal pollution affecting Santa Monica Bay, California. Appl Environ Microbiol**. 10:** 2598–2608.

5. Noble RT, Weisberg SB. 2005. A review of technologies being developed for rapid detection of bacteria in recreational waters. J. Water Health. **3**: 381–392.

6. Haugland RA, Siefring SC, Wymer LJ, Brenner KP, Dufour AP. 2005. Comparison of Enterococcus measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Res. **39**: 559–568.

7. Noble RT, Blackwood AD, Griffith JF, McGee CD, Weisberg SB. 2010. Comparison of rapid quantitative PCR-Based and conventional culture-based methods for enumeration of *Enterococcus* spp. and *Escherichia coli* in recreational waters. Appl Environ Microbiol. **76**: 7437-7443.

8. Boehm AB. 2009. A sea change ahead for recreational water quality criteria. J. Water Health **7**: 9 –20.

9. Lee CM, Griffith J F, Kaiser W, Jay JA. 2009. Covalently linked immunomagnetic separation⁄adenosine triphosphate technique (Cov-IMS⁄ATP) enables rapid, in-field detection and quantification of *Escherichia coli* and *Enterococcus* spp. in freshwater and marine environments. Appl Microbiol. **109:** 324-333.

10. USEPA. 2005. Method 1623: Cryptosporidium and Giardia in Water by Filtration/IMS/FA. EPA-815-R-05-002. Washington, DC: US Environmental Protection Agency: Office of Water Regulations and Standards, Criteria and Standards Division.

11. Hsu B, Huang C. 2007. IMS method performance analyses for Giardia in water using differing conditions. Environ Monit Assess. **131:** 129–134.

12. Rochelle PA, Leon RD, Johnson A, Stewart M.H, Wolfe RL. 1999. Evaluation of immunomagnetic separation for recovery of infectious Cryptosporidium parvum Oocysts from environmental samples. Appl Environ Microbiol**. 65:** 841–845.

13. Lee JY, Deininger RA. 2004. Detection of E-coli in beach water within 1 hour using immunomagnetic separation and ATP bioluminescence. Luminescence**. 19:** 31–36.

14. Bushon RN, Brady AM, Likirdopulos CA, Cireddu JV. 2009. Rapid detection of Escherichia coli and enterococci in recreational water using immunomagnetic separation⁄adenosine triphosphate technique. J Appl Microbiol. **106**: 432–441.

15. Bushon RN, Likirdopulos CA, Brady AMG. 2009. Comparison of immunomagnetic separation/adenosine triphosphate technique rapid method to traditional culture-based method for E. coli and enterococci enumeration in wastewater. Water Res. **43:** 4940–4946.

16. Ferguson DM, Griffith JF, McGEE CD, Weisberg SB, Hagedorn C. 2013. Comparison of Enterococcus species diversity in marine water and wastewater using Enterolert and EPA Method 1600. J Env and Public Health. Article ID 848049, 6 pgs.

17. Caruso G, Monticelli LS, Caruso P, Bergamasco A. 2008. Development of a fluorescent antibody method for the detection of Enterococcus faecium and its potential for coastal aquatic environmental monitoring. Marine Poll Bulletin**. 56:** 318-324.

18. Meridian Life Sciences. 2009. Specification Sheet, Catalog B65173R, Rabbit anti-Enterococcus species. Saco, ME: Meridian Life Science.

19. USEPA. 2006. Method 1600: Enterococci in Water by Membrane Filtration Using Membrane-Enterococcus Indoxyl-b-d-Glucoside Agar (mEI). EPA-821-R-06-009. Washington, DC: US Environmental Protection Agency: Office of Water Regulations and Standards, Criteria and Standards Division.

20. USEPA. 2010. Method A: Enterococci in water by TaqMan Quantitative Polumerase Chain reaction (qPCR) Assay. EPA-821-R-10-004. Washington, DC: US Environmental Protection Agency: Office of Water Regulations and Standards, Criteria and Standards Division.

21. Shanks OC, Sivaganesan M, Peed L, Kelty CA, Blackwood AD, Greene MR, Noble RT, Bushon RN, Stelzer EA, Kinzelman J, Ananeva T, Singalliano C, Wanless D, Griffith J, Cao Y, Weisberg S, Harwood VJ, Staley C, Oshima KH, Varma M, Haugland RA. 2012. Interlaboratory comparison of real-time PCR protocols for quantification of general fecal indicator bacteria. Environ Sci Technol. **46**: 945-953.

22. Border Environment Cooperation Commission, USEPA. 2009. Environmental assessment for the expansion of the water distribution and wastewater collection system for unserved areas in Tijuana, B.C.

23. Saraswat N, Alleman JE, Smith T J. 1994. Enzyme immunoassay detection of *Nitrosomonas europaea.* Appl Environ Microbiol. **60:** 1969-1973.

24. Field K, Samadpour M. 2007. Fecal source tracking, the indicator paradigm, and managing water quality. Water Res. **41**: 3517–3538.

25. Boehm AB, Van de Werfhorst LC, Griffith JF, Holden PA, Jay JA, Shanks OC, Wang D, Weisberg SB. 2013. Performance of forty-one microbial source tracking methods: A twenty-seven lab evaluation study. Water Res. **47**: 6812-6828.

26. Converse RR, Griffith JF, Noble RT, Haugland RA, Schiff KC, Weisberg SB. 2012. Corrrelation between Quanitative PCR and Culture-Based Methods measuring *Enterococcus* spp. over various temporal scales at three California beaches. Appl Environ Microbiol. 1237-1242.

27. Stern CS, Carvalho M, Teixeira LM. 1994. Characterization of enterococci isolated from human and nonhuman sources in Brazil. Diagn Microbiol Infect Dis. **20:** 61–67.

28. Pinto B, Pierotti R, Canale G, Reali D. 1999. Characterization of faecal streptococci as indicators of faecal pollution and distribution in the environment. Lett Appl Microbiol. **29:** 258–263.

29. Ferguson DM, Moore DF, Getrich MA, Zhowandal M H. 2005. Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California. Appl Microbiol. **99**: 598-608.

30. Maraccini PA, Ferguson DM, Boehm AB. 2012. Diurnal variation in Enterococcus species composition in polluted ocean water: A potential role for the enterococcal carotenoid in protection against photoactivation. Appl Environ Microbiol. **78:** 305-310.

Chapter 4. Use of viability-based methods for improved detection of recent fecal contamination in a coastal watershed near Tijuana, Mexico

Abstract: The San Antonio de los Buenos sub-drainage basin, located in Tijuana, Baja California, Mexico receives freshwater inputs from multiple creeks and storm drains. The SAB wastewater treatment plant treats and discharges 25 MGD of secondary treated waste to the SAB Creek, which outfalls into the ocean. SAB plume was previously reported to degrade water quality in the region, impacting beaches as far as the US-Mexico border. To evaluate the impact of the SAB outfall, samples were collected upcoast and downcoast of the discharge point. Presence and magnitude of six host-associated markers were used to identify possible sources impairing the watershed. This study evaluated the use of two novel viability-based methods for rapid detection and assessment of fecal contamination. Propidium monoazide (PMA), used to inhibit amplification of DNA from dead cells in qPCR, was used in a modified PMA-qPCR assay for the human-associated HF183 marker. In addition, the covalently-linnked, immunomagnetic separation/adenosine triphosphate (Cov-IMS/ATP) method was tested and compared with traditional culture-based methods for enumeration of enterococci. Results for the microbial source tracking study showed human and dog-associated markers were prevalent throughout the watershed, particularly in creek and storm drain sites sampled. PMA was found to successfully inhibit DNA resulting from dead cells in creek waters receiving a large volume of treated wastewater from the SAB treatment plant. In addition, PMA-qPCR samples were more comparable to two culture-based methods (IDEXX) and a rapid field portable Cov-IMS/ATP method than qPCR and shows promise for improved assessment of water quality in sewage impacted waters.

1. Introduction

Inadequate sewage collection and poor water treatment is known to adversely impact water quality and the coastal environment, and recreational waters polluted with wastewater can result in waterborne illness. Globally, an estimated 120 million cases of gastrointestinal and 50 million cases of severe respiratory illnesses are attributed to contact with sewage contaminated coastal waters (Shuval 2003). Regionally, residents living within the US-Mexico border are known to suffer disproportionately from respiratory illness and water-borne disease (Border 2012) including increased incidences of endemic diarrheal diseases and infectious hepatitis in comparison to non-border regions (Doyle and Bryan 2000). Tijuana Comisión Estatal de Servicios Públicos de Tijuana (CESPT), the local sanitation company, provides wastewater collection service for 87.1% of the region, and treats only 81% of wastewater generated in Tijuana (BECC 2009a). Residents who lack sewer collection rely on latrines, septic tanks and open ditches that contribute to potential groundwater and surface water contamination from untreated wastewater (BECC 2009b).

Although not necessarily pathogenic in nature, fecal indicator bacteria (FIB) such as *Escherichia coli* and enterococci are used as proxies for fecal contamination (Gerba 2000). These indicators are easy to enumerate and have been found to occur in high concentrations in wastewater. In addition, epidemiology studies have shown FIB to correlate with incidence of swimming-related illnesses (Colford et al., 2007; Wade et al 2006). Despite the widespread use of FIB to assess water quality, certain limitations exist. FIB can originate from both human and non-human sources. Detection of FIB does not provide information on the hosts of contamination which is critical for targeted remediation efforts, and current techniques are primarily culture-based, requiring a minimum of 18 hours for analysis.

The development of rapid detection assays, allowing enumeration of microbial contaminants as

quickly as one hour and enabling a more diverse suite of organisms to be studied, has progressed significantly in recent years. Among these explored technologies are quantitative polymerase chain reaction (Khan et al., 2007; McDaniels et al., 2005; Shanks et al., 2008; Siefring et al., 2008), fluorescent in situ hybridization (Lee and Deininger 2004; Field and Samadpour 2007), enzymatic methods (Scott et al., 2002), flow cytometry (Griffith et al., 2003) and immumomagnetic separation/ATP (IMS/ATP) quantification (Lee et al., 2010). IMS/ATP has been developed for rapid enumeration of *E. coli* and enterococci from fresh and marine waters. Of those mentioned, IMS/ATP has been found advantageous because it has been developed to rapidly detect FIB in less than one hour processing time, and is a viability-based field portable method.

The development of library-independent molecular based methods helped identify sources of contamination for alternative indicators such as *Bacteroidales*. Members of the order *Bacteroidales* are detected at high concentrations in feces and sewage, and various fecal hosts exhibit different genetic sequences, allowing distinction between human and non-human sources of fecal pollution (Griffith et al., 2003; Seurinck et al., 2005; Kildare et al., 2007). Quantitative Polymerase Chain Reaction (qPCR) assays also have been rigorously tested and applied for identification of different hosts such as humans, dogs, cattle, pigs, horses and gulls (Layton et al., 2006; Okabe et al., 2007; Shanks et al., 2008; Mieszkin et al., 2009; Schriewer et al., 2013; Sinigalliano et al., 2013). Boehm and colleagues (2013) conducted a methods comparison study to evaluate sensitivity and specificity of 41 different microbial source tracking (MST) methods across 27 laboratories and to establish standardized procedures for MST qPCR assays. Detection methods using qPCR have shown *Bacteroidales* to be a highly repeatable, specific and sensitive method of tracking human pollution back to its source (Ebdon et al., 2007; Gawler et al., 2007; Layton et al., 2013; Ebentier et al., 2013).

Current molecular assays using quantitative PCR do not discriminate between viable and non-viable cells as these methods amplify DNA of both live and dead cells as well as extracellular DNA (Bae and Wuertz 2009a). DNA of certain pathogens have been known to persist greater than three weeks, therefore, detection of human-associated markers by qPCR may not necessarily indicate recent contamination events (Nielsen et al., 2007; Bae and Wuertz 2009b). Detection of host-associated *Bacteroidales* marker from viable cells would better indicate recent human contamination as the microorganisms are strictly anaerobic and unlikely to survive for long periods in water. Propidium monoazide (PMA), a selective agent that penetrates and inhibits amplification of dead cells, has been used with qPCR to successfully discriminate between live and dead cells of pure cultures (*Bacteroides thetaiotaomicron*), and *Bacteroidales* in human feces and wastewater (Varma et al., 2009; Bae and Wuertz 2009a; Nocker et al., 2006). Yet the ability to measure fecal *Bacteroidales* with PMA in environmental samples or microbial source tracking studies has not been well documented (Bae and Wuertz, 2009a).

In this study, we investigated the presence and magnitude of FIB and host-associated markers for human, gull, dog and horse. We also tested two novel techniques, PMA-qPCR and an immuno-magnetic separation/Adenosine triphosphate method (Cov-IMS/ATP), for rapid assessment of target viable fecal bacteria in an urban sub-drainage basin in Baja California, Mexico. We evaluated the addition of a PMA treatment step to an existing qPCR assay to determine recent sewage contamination in environmental fresh and marine waters at the outflow of a wastewater treatment plant discharge point. This is the first study to our knowledge to utilize a PMA-qPCR assay for monitoring water quality at a discharge point. Samples with and without PMA treatments were also compared against traditional FIB enumerated with standard culture-based methods, and a second rapid viability-based method, Cov-IMS/ATP technique.

Performance and relationships between viability-based methods (culture-based and Cov-IMS/ATP) and the viability-based PMA-qPCR assay were also evaluated.

2. Methods

Field Site

Our study focused on the San Antonio de los Buenos (SAB) sub-drainage basin which is located in Tijuana, Baja California, Mexico approximately 17 miles south of the US-Mexico border (Figure 4-1). This region is subject to a Mediterranean climate, with typically low levels of rainfall in winter months (Nov – Apr) and drier summer months (May – Oct). Sewage collection infrastructure of nearby coastal residential communities varies drastically and includes locations with no infrastructure in place to centralized sewage collection and small scale onsite treatment facilities. Currently, approximately 25 million gallons per day (MGD) of sewage from Tijuana is treated at the San Antonio de los Buenos (SAB) wastewater treatment plant. Treated sewage is mixed with five MGD raw sewage, chlorinated, and discharged at the shoreline near Punta Bandera, Tijuana (BECC 2009b). Potential sources of fecal pollution within this region include raw sewage from inadequate or lack of water treatment, dogs, horses and cows, gulls and other wildlife. Feces from livestock serve as a diffuse source of FIB to the watershed. In addition, feral dogs reside in the area and beaches are animal-friendly, thus increasing the amount of possible fecal pollution.

Sample Collection

Samples were collected from eight sites within the SAB sub drainage basin between October 2011 and July 2013. Four marine sites were collected along an approximately 6 km along-shore transect along the Tijuana coastline (Figure 4-1). Marine samples were collected from residential beaches at Baja Malibu, San Antonio del Mar (SADM Beach), San Antonio de

los Buenos (SAB Beach) and Punta Bandera. In addition, samples were collected from four freshwater inputs that discharged directly into the ocean including Real del Mar Creek (RDM Creek), San Antonio de los Buenos Creek (SAB Creek) and two storm drains (SADB drain and Isla). SAB wastewater treatment plant discharged treated sewage into the SAB Creek, making up the majority of the creeks' flow. Water for bacterial and nutrient analyses were collected in 2L sterile polypropylene bottles at ankle-depth and stored on ice. Processing was done on site at a mobile laboratory in Tijuana, Mexico.

FIB Enumeration

Samples were processed for culture analyses according to standard methods. Samples were also processed with IDEXX (IDEXX Laboratories, Canada Corp., Toronto, Ontario, Canada) for enumeration of presumptive enterococci, *E.coli*, and total coliform. Samples were processed with Enterolert (to yield values of enterococci) and Colilert-18 (to yield values of *E.coli* and total coliform) according to manufacturer's instructions. Ten-fold and 100-fold dilutions of water samples were used as recommended by the manufacturer. Higher dilutions were periodically used for chronically contaminated sediments and sample locations (1000-fold and/or 10,000-fold dilutions). Subsets of samples were analyzed in parallel for enterococci by the membrane filtration method 1600 (USEPA 2006).

Sediment samples were also collected. Approximately 45g of sediment was resuspended in 60 mL of PBS (1x pH 7.2) and shaken by hand for two minutes. Supernatant was decanted after settling for one minute into a fresh, sterile bottle. This process was repeated for a total of two washes to obtain a final volume of 120 mL resuspension, which was then used for all FIB enumeration of sediment samples according to the methods above.

Sample Filtration and DNA Extraction

Each sample was filtered through a 47-mm, 0.4- µm pore size HTTP polycarbonate filters (Isopore Millipore, Billerica MA) in triplicate of varying volume from 15 to 500 mL on a standard platform manifold with sterile disposable filtration devices (Thermo Scientific, Logan, UT). Filters were stored in two mL polypropylene screw cap tub containing acid-washed glass beads (Sigma-Aldrich, St. Louis MO) at -80°C until DNA extraction. DNA was extracted using commercial kits. DNA was recovered from samples according to manufacturer's guidelines of the MoBio UltraClean fecal DNA extraction kit (Mobio Laboratories Inc, Carlsbad, CA), following manufactures' protocol with slight modifications. Sample filters were placed on the XX 8-Mini Bead Beater for 1.5 minutes in lieu of vortexing samples for ten minutes. On a subset of samples, the DNA was recovered according to manufacturer's guidelines of the DNA-EZ ST1 extraction kit (GeneRite, North Brunswick NJ) and ran for qPCR analyses in parallel of samples extracted with the MoBio UltraClean fecal DNA extraction kit for comparison.

MST analysis

Samples collected from 2010 to 2012 were analyzed using one human-associated marker. Detection of 16S rRNA gene markers for human-associated *Bacteroidales* were performed using the HF183 SYBR assay with DNA primers HF183F and HF183R (Bernhard and Field 2000a&b; Seurinck et al., 2005; Kildare et al., 2007). Each sample was measured in duplicate and "spiked" (i.e., positive controls) with 1-µl (~10^4 copies) *Bacteroidales* standard to estimate low rates of recovery and possible inhibition by contaminants in DNA extracts. In the case of interference, samples were diluted two-fold and reprocessed (Noble et al., 2006). qPCR assays were conducted on 25 µl reaction mixtures and with a StrataGene MX3000P thermocycler. In

addition, a subset of samples taken in 2012 and 2013 were processed for a suite of host-associated markers (Table 4-1).

Samples collected from 18 March 2012 to July 11 2013 were tested for the following assays: HF183 Taqman (Haugland et al. 2010), BacHum (Kildare et al. 2007), DogBact (Shibata et al. 2010), Gull2Taqman (Shibata et al. 2010), and ENT1A (US EPA Method A). qPCR analyses were carried out according to previously published and standardized protocols detailed in Boehm et al., 2013. qPCR assays were conducted on 25 µl reaction mixtures in triplicate in a Applied Biosystems StepOnePlus thermocycler. Cell concentrations were calculated according to pooled standard curve analysis based on the relative quantity of target DNA compared to that of a known quantity of target DNA (either genomic or plasmid). Equations used from pooled standard curves for each assay are listed in Appendix C. DNA concentration was determined using UV absorption with a Nanodrop 2000C (Thermo Scientific, Waltham MA).

PMA-qPCR for HF183 SYBR

Propidium monoazide (PMA) treatment was applied to a set of samples for analysis of viability of fecal bacteria detected using the HF183 SYBR human-associated marker. Each sample was filtered through a 47-mm, 0.4- µm pore size HTTP polycarbonate filters (Isopore Millipore, Billerica MA) in varying volume from 15 to 500 mL on a standard platform manifold with sterile disposable filtration devices (Thermo Scientific, Logan, UT). The filter was placed in 5 mL of PBS, and vortexed for one minute. One mL of resuspension was used for analysis of PMA treatment.

PMA was applied to samples as described in Nocker et al. 2007 and Bae and Weurtz et al 2009a with a few modifications. Breifly, a final concentration of 200 uM PMA (stock originally

dissolved in 20% dimethylsulfoxide) was added to 1 mL of sample resuspension and incubated in the dark for five minutes. The sample tubes were then placed in a container with ice aand agitated on an orbital mixer while exposed to a 700-W halogen lamp for ten minutes. Tubes were manually rotated approximately every minute to prevent excessive heating. Samples were then centrifuged at 10,000xg for five minutes at room temperature. The pellet was resuspended in one mL of AE buffer and filtered as described above through a 47-mm, 0.4- µm pore size HTTP polycarbonate filter (Isopore Millipore, Billerica MA). The filter was stored in a two mL polypropylene screw cap tub containing acid-washed glass beads (Sigma-Aldrich, St. Louis MO) at -80°C until DNA extraction.

Cov-IMS/ATP for ENT

A near-real time assay for *Enterococcus spp.* and *E. coli* based on immunomagnetic separation IMS/ATP quantification for use in freshwater and marine water sources within impaired watersheds has been developed and tested for assay performance (Lee et al. 2010). Samples in our study were processed in three main steps: (1) isolation of target organisms, during which samples were incubated on a rotating mixer with antibody-magnetic bead biosorbents; (2) lysing of target organisms, exposing specific ATP into solution; and (3) the addition of luciferin/luciferase, enzymes that catalyzed a light-emitting reaction while consuming ATP. This emission was quantified using a microluminometer (New Horizon Diagnostics, model 3560), and light intensity is correlated to cell concentration. Multiple samples were evaluated in less than one hour by this procedure (Lee et al 2010).

Also briefly, a solution of antibody-magnetic bead complexes was generated: 200 µl Dynabead particles (Invitrogen, Carlsbad, CA) were incubated with 50 µl *Enterococcoccus spp.*

antibodies (cat #B65173R, Meridian Life Sciences) on a rotating mixer for between 18-24 hours. Following incubation, the anti-ent biosorbent mixture was washed with and stored in bovine serum albumin (0.1% BSA w/v in PBS) buffer at continuous rotation at 4°C for up to two weeks.

Approximately 200-500 mL per sample was filter-concentrated and analyzed using the anti-ent biosorbent mixture. Samples were filtered 0.45-µm filter (SA1J792H5; Millipore) to capture bacteria. Filters were then resuspended in 10 mL PBS and bacteria was resuspended after one min of vortexing. One mL of resuspension was added to the anti-ent biosorbent, incubated on a rotating mixer for 40 minutes, and processed according to Lee et al. 2010.

Water Chemistry

Water samples were analyzed in the field for chemical water quality. Dissolved oxygen, salinity, specific conductivity, pH and water temperature were analyzed and recorded using a Hach Quanta Hydrolab multiparamter probe (Hach, Loveland, Colorado) deployed in knee-depth water. A subset of water samples collected were also analyzed for nitrate and ammonia (TNT 835 and TNT 813; Hach, Loveland, Colorado) using the Hach Spectrophotometer (model DR 280).

Statistical Analyses

Linear regression models were applied to estimate relationship between culture-based and IMS/ATP and qCPR measurements and were computed using $\log_{10}$-transformed data. Pearson's correlation coefficients were calculated to further examine the linear relationship between methods. Regression analyses were done in STATA 12.1 (STATA Corp LP, College Station, Texas). Box plots were generated in Matlab to assess the range and average FIB values at each site and were computed using $\log_{10}$-transformed data.

3. Results

HF183 SYBR Results

FIB in combination with the human-associated HF183 SYBR (HF183 SYBR) marker were initially used to assess presence and magnitude of human fecal pollution in the San Antonio de los Buenos sub-drainage basin for samples (n=41) collected between 2009 and 2011. The HF183 SYBR marker was detected in 71% of samples tested from the field site, with a median concentration (in ROQ) of 4.45×10^4 copies/100 mL (Table 4-2A). SAB Creek, SAB Beach and RDM Creek were impaired for the human-associated marker, with 100% detection of HF183 SYBR at creek sites and 83% detection at SAB Beach. Concentrations in SAB Creek water ranged on from $2.87 \times 10^3 – 9.15 \times 10^4$ copies/100 mL; SAB Beach had comparable levels ($2.28 \times 10^2 – 1.93 \times 10^5$ copies/100 mL). Although frequency of detection of the HF183 SYBR marker was high for these two sites, FIB values were variable and ranged from less than 10 MPN/100 mL to greater than 24,196 MPN/100 mL. RDM Creek was impaired with consistently high levels for both FIB and the human-associated marker. Median values for RDM were measured at 7.24×10^4 copies/100 mL for HF183 SYBR, and greater than 24,196 MPN/100mL for FIB.

In 2010, two storm drain sites (SADM and Isla drains) were also added to the study to investigate the impact of urban runoff from storm drains discharging to the ocean. All four samples collected from storm drains were positive for the HF183 marker with concentrations reaching as high as 7.30×10^6 copies/100 mL in runoff samples. HF183 SYBR was also detected in a 33 – 50% of samples analyzed for other sites (Baja Malibu, SADM Beach and Punta Bandera), with lowest frequency of detection at the furthest upcoast (Punta Bandera) and downcoast (Baja Malibu) sites.

Molecular Source Tracking Marker Results

Samples collected from 2012 – 2013 were analyzed for a suite of markers in an MST study. Combined data for presence and range of quantification of source-associated molecular markers is shown in Table 4-2 B-F. Human-associated markers were detected in 72% (HF183Taq) and 81% (BacHum) of samples collected from field sites. Creek sites (SAB Creek and RDM Creek) both had extremely high and consistent levels of BacHum and HF183Taq, ranging between 10^4 and 10^6 copies/100 mL. Storm drains were also impacted for human fecal contamination. HF183 and BacHum were detected in 83 – 100% of samples tested from storm drains (Table 4-2 B and C). The highest concentration and the highest median values (10^7 - 10^8 copies/100 mL) of human-associated markers were measured in runoff from the SADM storm drain. HF183 and BacHum were detected in 50 – 67% of samples collected from other sites (Baja Malibu, SADM Beach and Punta Bandera beaches). Human associated markers from beaches were typically measured at one to two orders of magnitude less than creek sites, and up to five orders of magnitude less than storm drain sites.

In addition to human-associated markers, three animal host-associated markers were used to assess if dog, gull and horse fecal waste were polluting the SAB sub-drainage basin. The dog-associated marker (DogBact) was detected in 66% of all samples analyzed from the field site. The SAB Creek and SAB Beach samples were positive for DogBact in 100% of the samples tested. Frequent detection of Dogbact (67-83%) was also observed in storm drain samples. The SAB Creek and storm drains (SADM and Isla) were the most impacted sites with highest concentrations of dog marker ranging from 10^4 – 10^6 copies/100 mL (Table 4-2E). One sample collected from the SADM drain had extremely high levels of DogBact that was two orders of magnitude greater (10^8 copies/100 mL) than any other sample tested.

Gull marker was not frequently detected in the SAB sub-drainage basin. Only 8.5% (4/47) samples were positive for the gull-associated marker. Gull marker was detected at Baja Malibu, SADM Beach and Punta Bandera beach sites and ranged between 2565 – 2787 copies/100 mL (Table 4-2F).

The horse-associated (HoF597) marker was also tested on all samples taken from 2012 – 2013. The HoF597 marker was positive for two of 47 samples tested, and was not found to be a significant source at this field site. Baja Malibu and SAB Creek each had one positive sample for this marker.

Spatial FIB results

Although a combination of freshwater and marine samples were collected, samples were analyzed using the more conservative US EPA marine recreational water quality single sample standard for Total coliforms (TC), *Escherichia coli* (EC) and *Enterococci* (ENT) bacteria, as freshwater inputs sampled discharged directly into the ocean and could potentially contribute to exceedances seen at marine sites. Sites were also analyzed for two Mexican water quality standards: the ENT water quality standard of 200 MPN/100mL and the TC daily limit for treated wastewater discharging to the ocean of 2000 MPN/100mL (BECC 2009b).

Fecal indicator bacteria exceeded US EPA water quality single-sample standards within the SAB sub-drainage basin in 42%, 45% and 44% of samples collected for TC, EC and ENT, respectively. A comparable fraction of samples (42%) also exceeded the Mexican water quality threshold for ENT (Cofepris 2012). FIB showed a strong relationship among indicators for this watershed, ENT and EC were well correlated (R^2 0.91). Forty-four percent of samples were in exceedance for both EC and ENT for all samples analyzed.

FIB results in marine and freshwater samples

FIB results in freshwater showed elevated levels at RDM Creek and in runoff samples from SADM and Isla storm drains. All three sites exceeded standards in 100% of samples tested (Table 4-3). FIB concentrations measured from creek and storm drain sites were two to four orders of magnitude higher than other sites (Figure 4-2). FIB levels for sites receiving direct inputs were generally higher than other marine sites (Baja Malibu and Punta Bandera). For example, FIB levels ranged from two to 35 times higher at SADM Beach and 12 to 270 times higher at SAB Beach in comparison to beaches free from inputs. Both SADM and SAB beaches had a greater frequency of exceedance for FIB than other beaches tested. Storm drains adjacent to the marine sites consistently showed elevated levels of FIB and likely contributed to exceedances in marine sites.

Variable levels of FIB were also observed for SAB Creek and Beach throughout the study, despite consistently high values for the human-associated markers. Twenty five to 27% of the time these sites were in exceedance of USEPA guidelines for coastal waters. Elevated FIB (>241960) for all three indicators were measured from SAB Creek on three different sampling days (Fig 4-6). During those events, TC concentrations were above the Mexican allowable daily limit for treated wastewater discharging to the ocean and were in exceedance of US EPA health standards. Concentrations at upcoast and downcoast sites remained two to three orders of magnitude lower, despite elevated levels as the SAB discharge point (Figure 4-6).

FIB results in sand samples

FIB levels in sand followed a similar trend to FIB levels measured from water. Levels were higher in sand collected near creek sites and SAB Beach. FIB enumerated from sand

collected near creek sites ranged from 0.17 – 1468 MPN/g dry weight for ENT, and sand samples from SAB beach reached concentrations of 651 MPN/g dry weight for ENT (Figure 4-2). Highest levels were measured from wet sand collected adjacent to SAB Creek, with values greater than 163,904 MPN/g dry weight for all three indicator bacteria on 19 February 2010. Low levels or non-detectable levels of FIB were measured in sand from all other sites. Median concentrations of FIB in sand for the study were 10.12, 11.41, and 0.65 MPN/g dry weight for TC, EC and ENT, respectively. Excluding creek impacted sites, FIB has been measured and shown to persist in beach sand at comparable levels (Lee et al., 2006). Creek sand had levels consistent with sewage impacted sand (Mika et al., 2010).

PMA-qPCR Results

Water samples were analyzed for the human-associated HF183 SYBR marker with and without PMA treatment. PMA-qPCR and qPCR samples (n=20) are plotted against FIB enumerated with culture-based methods (Figure 4-4). Inclusion of PMA treatment should result in amplification of DNA from live bacteria cells only, making PMA-qPCR a viability-based method. PMA-qPCR samples showed a stronger relationship with FIB than did qPCR. A significant linear relationship was seen for PMA treated samples for both EC and ENT (with an increased R^2 of 0.64 and 0.75) The relationship between qPCR and IDEXX measurements was close to a 1:1 relationship (indicated by grey dashed line) when samples were treated with PMA (Figure 4-4). The inclusion of a PMA treatment step to traditional analysis with qPCR can improve assessment of recent inputs of fecal contamination.

Concentrations of FIB in SAB Creek samples showed high variability possibly due to periodic failures of the SAB WWTP (which outfalls into the creek) to adequately treat sewage.

Despite dynamic changes in FIB concentrations, constantly high levels of the HF183 SYBR marker was measured with qPCR at SAB Creek and SAB Beach (Figure 4-5). However, samples treated with PMA showed a similar trend to FIB levels, in comparison to samples processed for qPCR only. PMA was able to capture dynamic changes in FIB. It is important to note that in this analysis samples falling below ROQ are estimated and non-detects were set to a value of 5 copies/100 mL. In addition, several samples exceeded the detection limit for FIB; therefore actual correlations may be stronger for PMA-qPCR and FIB than captured here (Figure 4-4).

Cov-IMS/ATP Results

Cov-IMS/ATP results correlated well with Enterolert measurements (R^2=0.75). However, the correlation was weak between Cov-IMS/ATP measurements and qPCR measurements. Cov-IMS/ATP measures viable bacteria only; thus, likely the decreased correlation between qPCR and Cov-IMS/ATP measurements was a result of non-viable bacteria in water samples at SAB Creek, which received a high volume of treated sewage from the SAB WWTP and at SAB Beach, located in the mixing zone and outlet of the creek (Figure 4-3). When points from SAB Creek and Beach sites were removed there was a significant linear relationship between Cov-IMS/ATP and qPCR measurements (R^2 increased from 0.31 to 0.59). A similar trend was seen when comparing qPCR ENT and IDEXX Enterolert measurements. When points from SAB Creek and Beach were removed, R^2 increased from 0.30 to 0.73, illustrating a significant linear relationship between IDEXX and qPCR measurements at the other sites.

4. Discussion

Elevated concentrations of FIB and exceedances in water quality standards, along with detection of several host-associated markers, have indicated widespread fecal contamination in

the San Antonio de los Buenos sub-drainage basin. Dog and human fecal contamination were important sources impairing the region with the highest frequency of detection of the four host-associated markers tested (gull, dog, horse and human). Creek sites (SAB Creek and RDM Creek) were identified as sources of contamination to the coastline with frequent presence of FIB, human and dog markers. Previous studies have also reported a spike in FIB values adjacent to the SAB Creek and have found that wastewater discharge from the SAB outfall was responsible for impacting water quality adjacent to Real del Mar (RDM) and San Antonio del Mar (SADM) beaches (Morales-Chavez 2000; Orozco Borbon et al., 2006). FIB levels measured in this study are comparable to those found in Orozco Borbon (2006), indicating a continued history of chronic pollution in the region. In addition to creek sites, this study found high levels of fecal contamination in freshwater storm drain inputs. Degraded water quality at SADM Beach may be the combined effect of SAB Creek discharge (outlet of the SAB WWTP) as well as in SADM storm drain discharges.

To investigate the extent of contamination associated with the SAB outfall, samples were collected at sites upcoast and downcoast of the discharge point. The SAB discharge plume has been estimated to travel northward approximately 56 times a year, even contributing to pollution sources at San Diego beaches on occasion (Terrill et al. 2009; Sung Yong et al., 2009). In addition, the SAB outfall may also contribute to downcoast contamination. Sassoubre and colleagues (2012) measured human enteric viruses, adenovirus and enterovirus, from the SAB Creek discharge point, and in marine waters collected from Baja Malibu (located 3.6km downcoast of the discharge point).

In this study, currents modeled with the Southern California Coastal Ocean Observing System (SCCOOS) were flowing northward (upcoast) during the time of sampling on 26 March

2013 (Appendix C), when markers and high levels of FIB were detected at the SAB outfall and both human-associated markers (HF183 Taqman and BacHum) were detected from Punta Bandera, located 2.05 km upcoast from the SAB discharge point. However, human-associated markers were not detected at downcoast sites, Baja Malibu and SADM beaches. The SAB discharge plume could potentially impact both upcoast and downcoast sites, depending on current flow, evidenced by corresponding presence of source markers and virus detection.

Due to widespread contamination associated with the SAB plume, it is critical to utilize effective monitoring methods for assessing water quality in the region. FIB and qPCR are routinely used in microbial source tracking studies to assess fecal contamination. Here, we found that HF183 SYBR qPCR measurements did not correlate with FIB measurements on days when water quality in creek and surfzone samples were impaired with treated sewage. Other studies have also reported a lack of correlation between FIB and human-associated source markers (Santoro and Boehm 2007; Litton et al., 2010; Flood et al., 2011). Litton et al. (2010) found that HF183 marker and enterococci measurements did not covary in a stream impacted with treated wastewater effluent. Similarly, in this study a poor relationship was seen between HF183 SYBR qPCR and FIB measurements at the SAB Creek and Beach ($R^2 = 0.13$). For the overall sub-drainage basin, an improved relationship was seen ($R^2 = 0.42$ for ENT). If SAB Creek and Beach sites were removed from overall analysis, the fit was improved further and a strong linear relationship was observed ($R^2 = 0.73$) when comparing qPCR and ENT measurements. As the human marker was consistently detected, HF183 SYBR qPCR was unable to distinguish between when the SAB wastewater treatment plant functioned properly versus when effluent had viable fecal waste from inadequate sewage treatment. Alternative rapid methods to qPCR are needed for assessing water quality in sampling locations receiving treated wastewater effluent.

Addition of a PMA treatment step to qPCR can improve the relationship of molecular source markers to FIB and the ability to detect recent sewage spills. In this work, samples with PMA treatment showed a similar trend to that of culture-based methods. Linear regression analysis showed a strong relationship between PMA-qPCR and FIB (R^2= 0.75). Low FIB levels (<10MPN/100mL) at the SAB discharge point coincided with low levels of the human-associated marker (HF183 SYBR <LOD) measured with PMA-qPCR on two different dates. In addition, a spike in FIB (>24916 MPN/100 mL) coincided with a spike for the human marker measured by PMA-qPCR (Figure 4-5). Samples analyzed with qPCR and with PMA treatment exhibited similar values (between 10^3 to 10^5 copies/100 mL) on Feb 19th, 2010, suggesting that viable cells were contributing a higher portion of the human-marker. Previous studies support the use of PMA-qPCR for detection of viable cells and wastewater effluent (Nocker et al., 2007, Bae and Wuertz 2009a; Sassoubre et al., 2012). Bae and Wuertz (2009a) measured human marker in influent and effluent samples from a wastewater treatment plant, effectively distinguishing viable fecal pollution. Additionally, Bae and Wuertz (2009a) reported PMA-qPCR concentrations for effluent at the sample Limit of Detection (LOD). Such results reflect our findings that PMA-qPCR better captures dynamic changes in concentrations relating to treatment of wastewater effluent. This is the first study to our knowledge to document the successful application of the HF183 SYBR PMA-qPCR assay for monitoring sewage treatment at a wastewater discharge point.

Other alternative rapid methods besides PMA-qPCR, may assist in detecting recent fecal contamination events. For example, in this study, a second alternative method, the covalently-linked immunomagnetic separation/adenosine triphosphate (Cov-IMS/ATP), was evaluated for performance in rapidly assessing water quality near point-sources of pollution. Cov-IMS/ATP

exhibited a strong linear relationship with IDEXX ($R^2 = 0.74$), when all sample locations were included in analysis.

The use of viability-based, rapid methods for detection of sewage contamination may greatly benefit regions with frequent infrastructure failure. SAB Creek, which is made up of mostly treated effluent from the SAB wastewater treatment plant, had FIB concentrations in exceedance of USEPA and Mexico water quality standards in three of 12 sampling events (25% of samples). Rapid detection of fecal contamination is critical for remediation efforts and to identify problems associated with sewage treatment at that facility. In addition, same day detection and notification of a sewage spill would help prevent exposure of swimmers and beach visitors to contaminated waters. The city of Tijuana collects and treats 81% of the sewage generated in the region, the remainder of which has the potential to enter and contaminate the environment (BECC 2009b). Work has been completed to improve collection and treatment with the addition of three new reclamation plants, aimed to reduce the overall burden on SAB wastewater treatment plant. Nonetheless, inadequate sewage treatment from the SAB plant was evident. Therefore, it is critical for appropriate monitoring methods to be used in assessing coastal water quality in waters impacted by wastewater effluent, especially in regions prone to infrastructure failures. PMA-qPCR and Cov-IMS/ATP both show promise in augmenting source tracking studies to accurately determine the presence and magnitude of human fecal contamination.

5. Conclusion

Although laboratory studies have shown samples treated with PMA can distinguish between live and dead *Bacteroidales* originating from sewage and human feces, use of PMA has been understudied in field investigations for assessing fecal contamination (Varma et al., 2009; Bae and Wuertz 2009a; Nocker et al., 2006). Therefore, it is important to understand the relationship

between qPCR samples treated with and without PMA, and their relationship to other viability-based methods. A viability-based, PMA-qPCR assay has been previously optimized for detection of the human-associated BacHum marker (Bae and Wuertz 2009a). In this study, we apply a modified PMA assay for detection of the human-associated HF183 SYBR marker to assess recent fecal contamination events in environmental waters. PMA was found to successfully inhibit DNA resulting from dead cells in creek waters receiving a large volume of treated wastewater from the SAB treatment plant. In addition, PMA-qPCR samples were more comparable to two viability-based methods, a standard culture-based method (IDEXX) and a rapid field portable IMS/ATP method, for quantification of waters impacted with sewage inputs.

PMA-qPCR may need to be optimized on a per assay basis for different molecular host markers. In this study, PMA-qPCR values were measured at times to be greater than samples without PMA-treatment. Possibly, this is a result of using an optimized BacHum PMA-qPCR assay for the HF183 SYBR marker. Although outside the scope of this work, future research is needed to evaluate the performance of PMA-qPCR for a suite of human-associated and animal host markers. In addition, further optimization may be required for use of the PMA-qPCR assay in molecular source tracking and environmental field studies. PMA-qPCR may have performed better at the SAB sub-drainage basin due to the high levels of contamination measured at this site. Future work to investigate the widespread use of PMA-qPCR will require testing the application of this viability-based method in less contaminated watersheds.

Although host-associated microbial source tracking markers can be helpful in identifying sources of contamination for targeted remediation efforts, detection of the human-associated marker with qPCR alone may not be appropriate for assessing sewage treatment and coastal water quality near wastewater discharge points. Instead, alternative viability-based methods such

as PMA-qPCR and Cov-IMS/ATP can assist monitoring efforts to detect recent fecal contamination events and sewage spills resulting from point-sources.

standards.

Figure 4 - 1. Map of Tijuana coastline depicting sampling locations and source inputs. Samples were collected up coast and down coast of the San Antonio de los Buenos (SAB) Creek outlet, which is made up of mostly treated effluent from the SAB wastewater treatment plant. Currents generally flow in a southward direction. Sampling sites are shown in green, yellow and red circles representing creeks, storm drains and surf zone samples from three community beaches. SAB Beach sample was taken at the mixing point where SAB creek discharges and mixes with marine waters. Satellite image provided by Google Maps (© Google 2013).

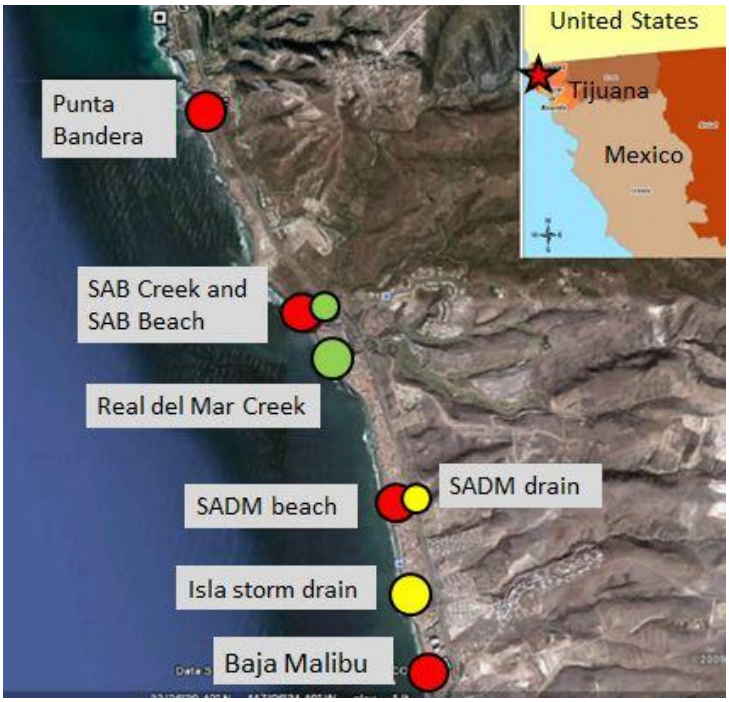


Figure 4 - 2. Box and whisker plot of *E. coli* and enterococci concentrations for the study period. Plots are shown for FIB results in sand (top panel) and in water (bottom panel) at each sampling site. FIB levels measured from water are reported in units of MPN per 100ml while FIB levels in sand are reported in units of MPN/ g dry weight. The box signifies the 25th, 50th and 75th percentiles and the whiskers depict the 10th and 90th percentiles.

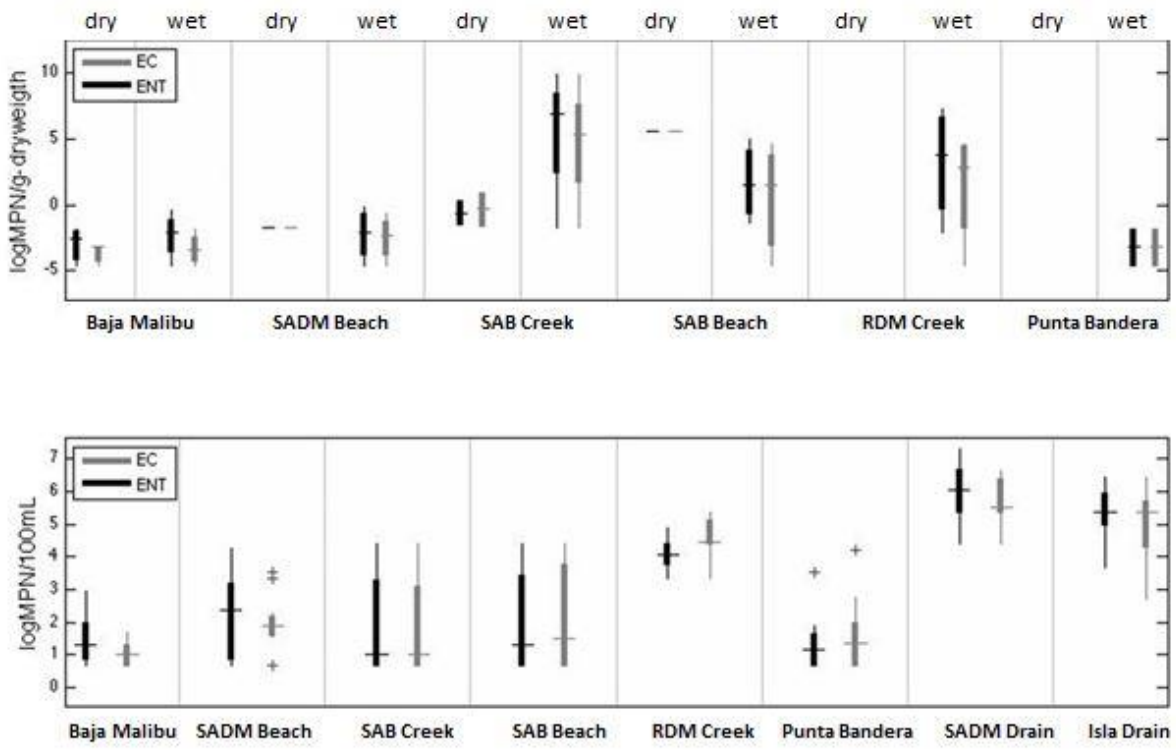


Figure 4 - 3. Results for qPCR and viability based methods (IDEXX and IMS/ATP for ENT). Enterococci concentrations measured by qPCR are plotted against enterococci enumerated with IDEXX and IMS/ATP. A weak relationship is observed between qPCR and viability-based methods for all sites (panel A).qPCR shows a stronger relationship with viability-based methods when SAB Creek and SAB Beach sites are omitted from analysis (panel B).

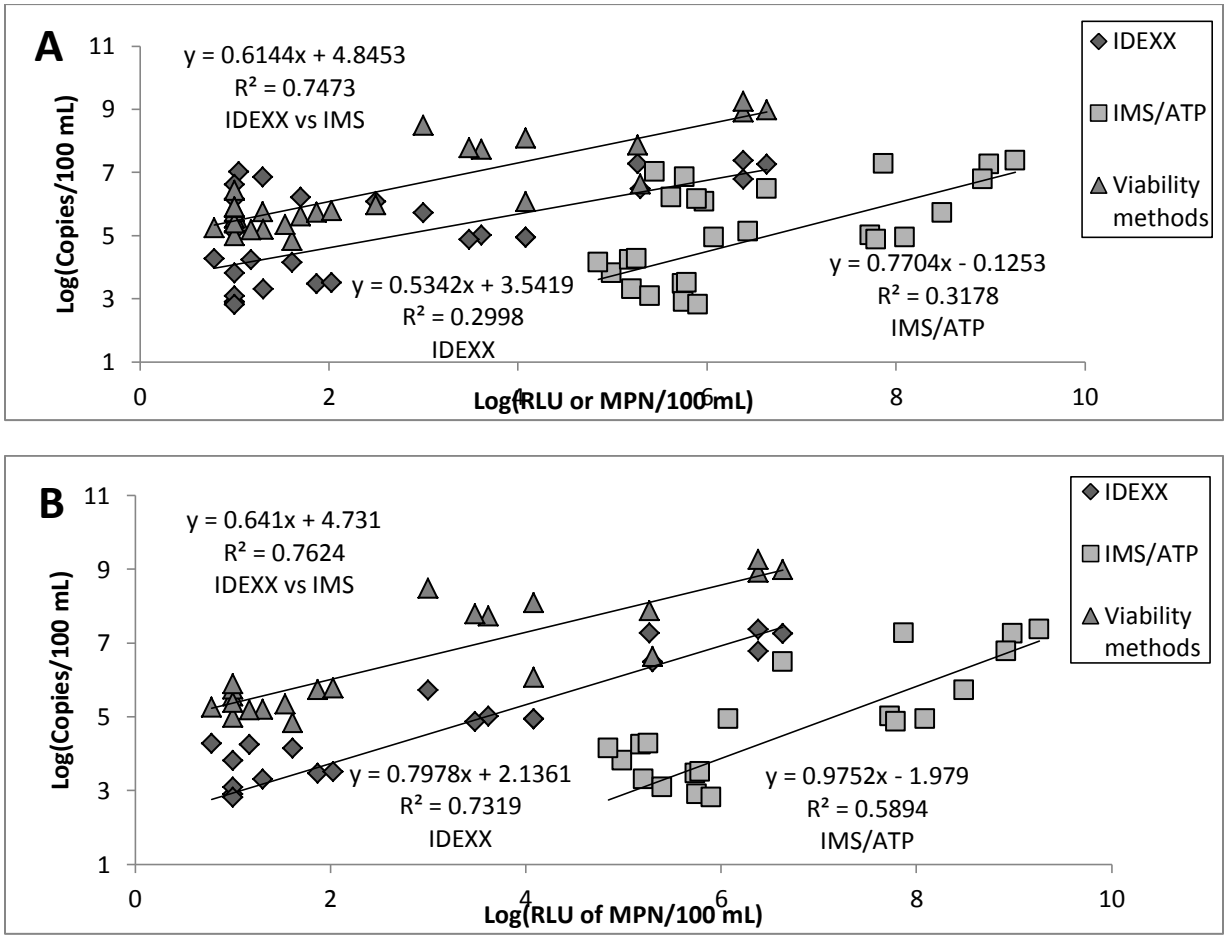
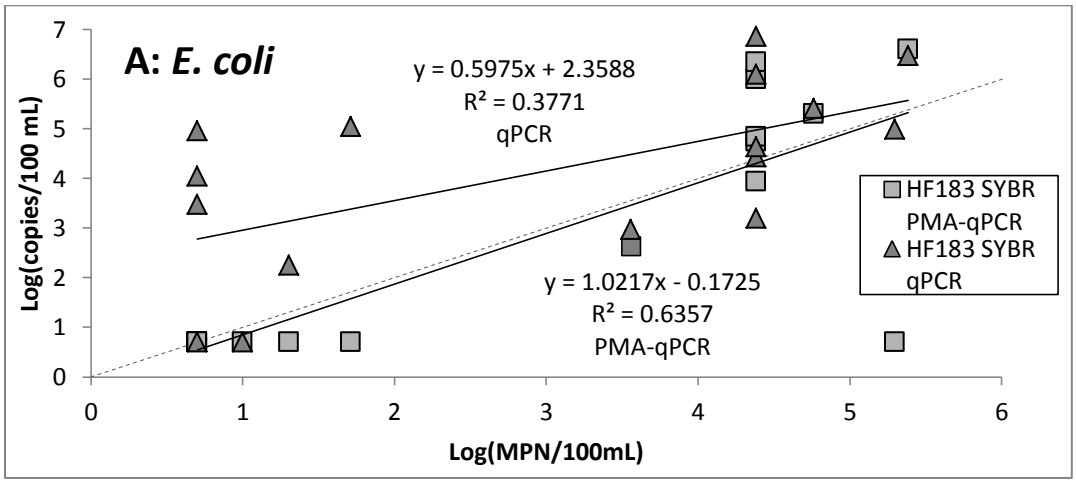


Figure 4 - 4. Results for qPCR and PMA-qPCR. Samples were analyzed for the human-associated HF183 SYBR marker with and without PMA treatment. PMA-qPCR and qPCR samples are plotted against A) *E.coli* and B) enterococci, enumerated with culture-based methods. Grey dashed line indicates a 1:1 relationship for reference.



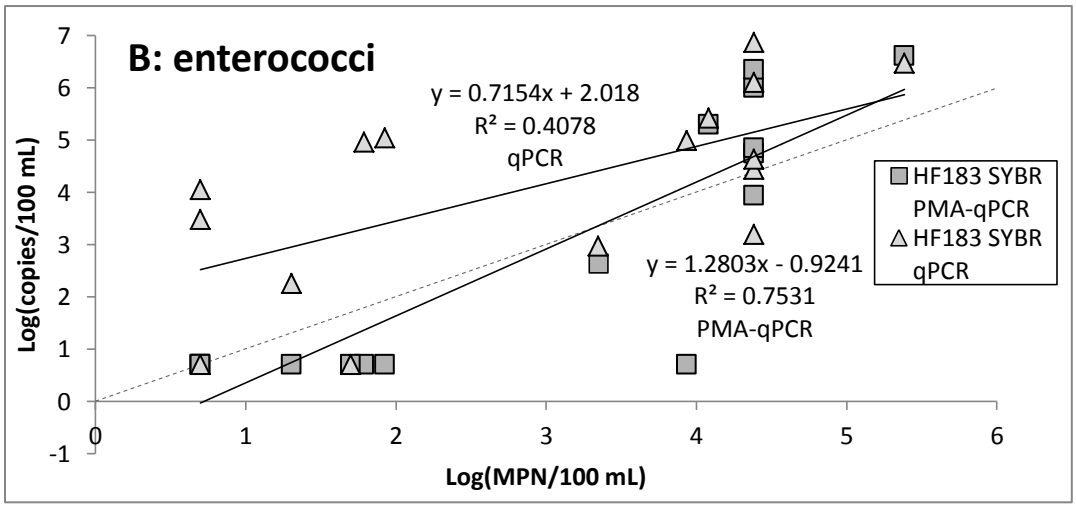


Figure 4 - 5. Concentrations of FIB, qPCR and PMA-qPCR are shown for A) SAB Creek and B) SAB Beach, for three different sample dates. Red markers indicate sample values are estimates, as the concentration was below sample range of quantification.

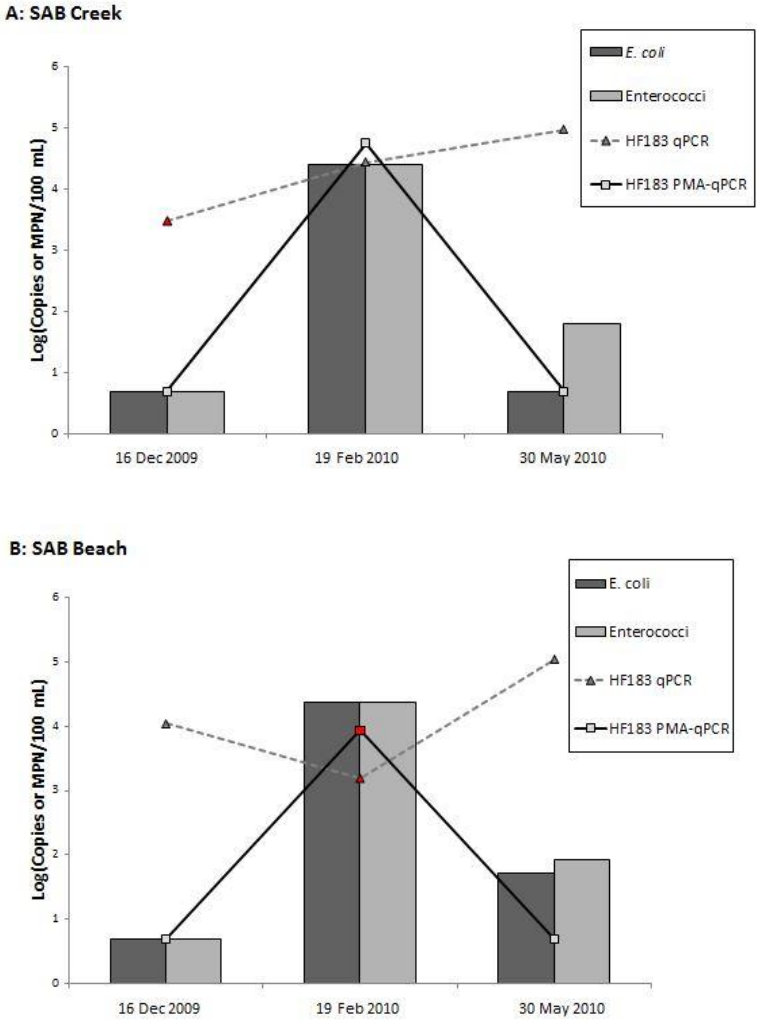
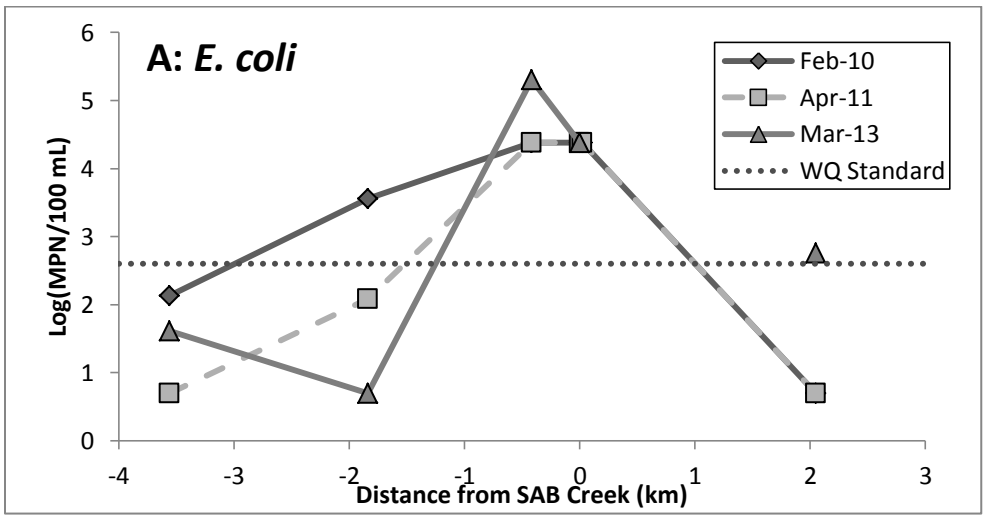
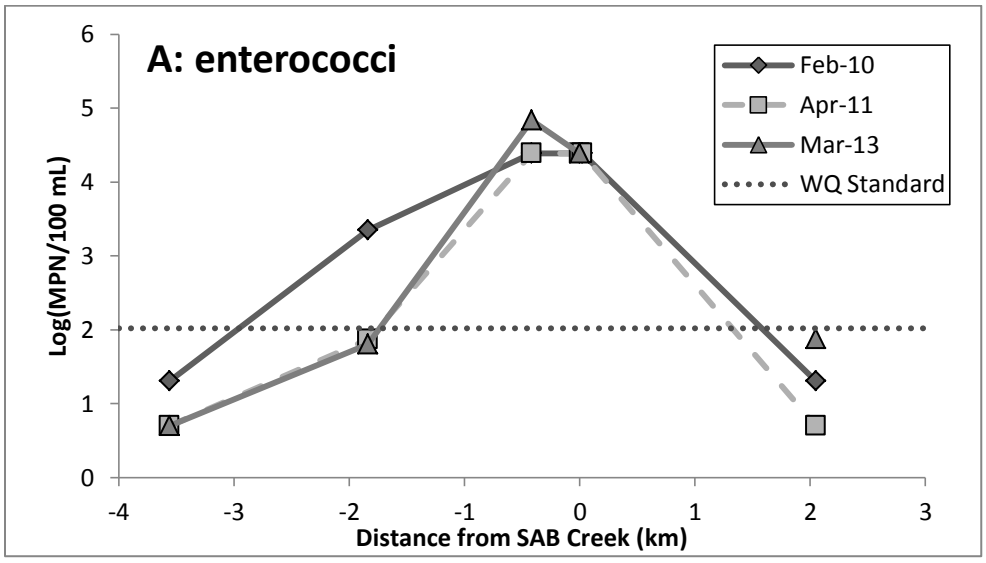


Figure 4 - 6. Concentrations of *E. coli* (panel A) and enterococci (panel B) are plotted against distance from the SAB Creek discharge site. Negative values represent distance downstream (south) of the SAB Creek.





.

Name	Source	Type	Target	Forward Primer / Reverse Primer	Probe/Dye	Reference
HF183 Taqman	Human	qPCR	*Bacteroides* 16S	ATCATGAGTTCACATGTCCG / CGTAGGAGTTTGGACCGTGT	FAM-CTGAGAGGAAGGTCCCCCACATTGGA-TAMRA	Haugland et al., 2010
HF183 SYBR	Human	qPCR	*Bacteroides* 16S	ATCATGAGTTCACATGTCCG / TACCCCGCCTACTATCTAATG	SYBR Green	Seurinck et al., 2005
BacHum	Human	qPCR	*Bacteroides 16S*	TGAGTTCACATGTCCGCATGA / CGTTACCCCGCCTACTATCTAATG	FAM-CTGAGAGGAAGGTCCCCCACATTGGA-TAMRA	Kildare et al., 2009
Gull2 Taqman	Gull	qPCR	*Catellicoccus marimammalium*	TGCATCGACCTAAAGTTTTGAG / GTCAAAGAGCGAGCAGTTACTA	FAM-CTGAGAGGGTGATCGGCCACATTGGGACT-BHQ1	Shibata et al., 2010
DogBact	Dog	qPCR	*Bacteroidales spp.*	CGC TTG TAT GTA CCG GTA CG / CAA TCG GAG TTC TTC GTG	FAM-ATTCGTGGTGTAGCGGTGAAATGCTTAG-BHQ1	Sinigalliano et al., 2012
HoF597	Horse	Endpoint	*Bacteroidales spp.*	CCA GCC GTA AAA TAG TCG G / CAA TCG GAG TTC TTC GTG	N/A	Dick et al., 2009
Enterococcus	General	qPCR	*Enterococcus spp.*	AGA AAT TCC AAA CGA ACT TG / CAG TGC TCT ACC TCC ATC ATT	6FAM-TGG TTC TCT CCG AAA TAG CTT TAG GGC TA-TAMRA	Haughland et al., 2005, USEPA Method A

Table 4 -1. Endpoint and qPCR assays used in study. Samples collected within the first phase of the study period (2009 – 2011) were analyzed for HF183 SYBR only. Samples collected from the second phase (2012 – 2013) were analyzed for all markers, excluding HF183 SYBR.

A Site Description	n	HF183 SYBR %ND	%Frequency detected	%ROQ	Median (ROQ) copies/100 mL	Range (ROQ) copies/100 mL lower	 upper
Baja Malibu	6	67	33	17	9.02E+02	-	-
SADM Beach	6	50	50	17	4.99E+03	-	-
SAB Creek	6	0	100	83	2.72E+04	2.87E+03	9.15E+04
SAB Beach	6	17	83	83	1.11E+04	2.28E+02	1.93E+05
RDM Creek	7	0	100	86	7.24E+04	1.19E+04	1.26E+06
Punta Bandera	6	67	33	0	-	-	-
All sites	41	29	71	54	4.46E+04	2.28E+02	7.30E+06

B Site Description	n (#)	HF183 Taqman % ND	%Frequency of detection	% ROQ	Median (ROQ) copies/ 100 mL	Range (ROQ) copies/ 100 mL lower	 upper
Baja Malibu	6	50	50	50	3.63E+03	2.99E+03	2.19E+04
SADM Beach	6	33	67	67	2.27E+04	1.48E+04	1.27E+05
SAB Creek	6	0	100	100	2.11E+06	2.14E+05	7.39E+06
SAB Beach	5	0	100	100	1.00E+06	6.86E+05	1.02E+07
RDM Creek	6	17	83	67	1.62E+06	2.48E+04	2.59E+06
Punta Bandera	6	50	50	33	4.98E+03	2.48E+03	2.75E+05
SADM Drain	6	0	100	100	4.00E+07	1.25E+06	2.69E+08
Isla Drain	6	17	83	83	9.69E+05	2.56E+04	5.62E+07

C Site Description	n (#)	BacHum % ND	%Frequency of detection	% ROQ	Median (ROQ) copies/ 100 mL	Range (ROQ) copies/ 100 mL lower	upper
Baja Malibu	6	33	67	67	1.46E+04	2.44E+03	4.85E+04
SADM Beach	6	33	67	67	5.72E+04	4.06E+04	2.52E+05
SAB Creek	6	0	100	100	3.48E+06	3.74E+05	9.00E+06
SAB Beach	5	0	100	100	2.70E+06	1.92E+06	2.36E+07
RDM Creek	6	17	83	83	2.98E+06	2.04E+05	4.48E+06
Punta Bandera	6	50	50	50	1.41E+04	5.50E+03	2.05E+05
SADM Drain	6	0	100	100	6.36E+07	2.71E+06	2.74E+08
Isla Drain	6	17	83	83	1.82E+06	6.38E+04	8.11E+07

D Site Description	n (#)	ENT % ND	%Frequency of detection	% ROQ	Median (ROQ) copies/ 100 mL	Range (ROQ) copies/ 100 mL lower	upper
Baja Malibu	6	2	67	67	1.21E+04	1.22E+03	3.65E+04
SADM Beach	6	1	83	83	3.29E+03	3.90E+02	8.49E+04
SAB Creek	6	0	100	100	1.53E+06	1.34E+05	7.18E+06
SAB Beach	5	0	100	100	2.03E+06	7.41E+05	1.07E+07
RDM Creek	6	1	83	83	1.03E+05	7.46E+04	5.29E+05
Punta Bandera	6	0	100	100	3.82E+03	6.58E+02	1.17E+05
SADM Drain	6	0	100	100	1.20E+07	8.69E+05	2.37E+07
Isla Drain	6	1	83	83	5.04E+05	8.91E+04	3.06E+06

E Site Description	n (#)	DogBact % ND	%Frequency of detection	% ROQ	Median (ROQ) copies/ 100 mL	Range (ROQ) copies/ 100 mL lower	upper
Baja Malibu	6	83	17	17	3.12E+04	-	-
SADM Beach	6	50	50	50	1.41E+04	1.14E+04	1.45E+05
SAB Creek	6	17	100	83	1.11E+06	5.32E+04	2.53E+06
SAB Beach	5	0	100	100	5.40E+05	4.28E+05	3.85E+06
RDM Creek	6	33	67	67	4.05E+05	2.99E+04	3.82E+06
Punta Bandera	6	50	50	50	9.02E+03	8.44E+03	1.04E+05
SADM Drain	6	17	83	83	1.36E+06	2.14E+05	4.89E+08
Isla Drain	6	33	67	67	1.60E+06	8.77E+04	7.39E+06

F Site Description	n (#)	Gull2Taq % ND	%Frequency of detection	% ROQ	Median (ROQ) copies/ 100 mL	Range (ROQ) copies/ 100 mL lower	upper
Baja Malibu	6	83	17	0	-	-	-
SADM Beach	6	67	33	17	2.79E+03	-	-
SAB Creek	6	100	0	0	-	-	-
SAB Beach	5	100	0	0	-	-	-
RDM Creek	6	100	0	0	-	-	-
Punta Bandera	6	83	17	17	2.57E+03	-	-
SADM Drain	6	100	0	0	-	-	-
Isla Drain	6	100	0	0	-	-	-

Table 4 - 2. Results of host-associated molecular markers. Summary of molecular markers tested in study for A) HF183 SYBR B) HF183Taqman C) BacHum D) ENT E) DogBact and F) Gull2Taqman. Number of samples included in analysis, percentages of samples detected for host markers and percentages for samples falling in the Range of Quantification (ROQ) and Non-Detect (ND) are provided. Median values and ranges of values for samples falling within ROQ are also shown. Samples with only one number falling in ROQ are reported as median value, but no range is given.

Sample Site	n	% Exceedance of USEPA Standard			% Exceedance of MX standard
		TC	EC	ENT	ENT
A	12	0	8	0	0
B	12	25	33	33	17
C	12	25	25	25	25
D	11	18	27	27	27
E	12	100	100	100	100
F	12	8	8	8	0
Bd	9	100	100	100	89
I	6	100	100	100	100
All sites	86	42	45	44	42

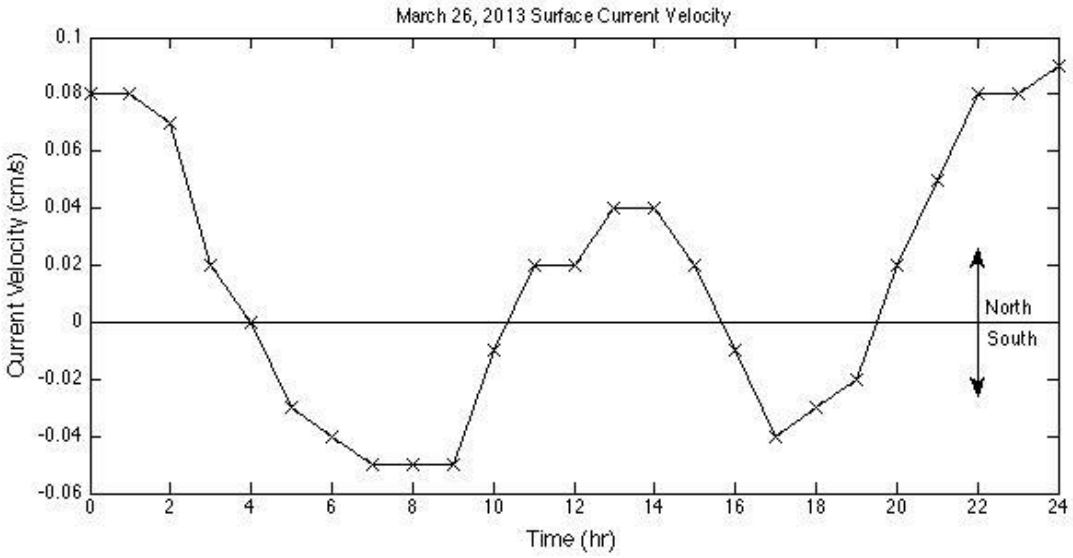
Table 4 - 3. Exceedance of FIB. Percent exceedance of FIB is listed for samples measured for total coliforms (TC), E. coli (EC) and enterococci (ENT) above the US EPA water quality standards and the Mexico enterococci.

6. Appendix C

Pooled Standard Curves Equations	Average HF183TaqMan	Average HF183SYBR	Average BacHum	Average DogBact	Average Gull2Taq	Average ENT
slope	-3.26	-3.48	-3.52	-3.52	-3.39	-3.66
R^2	1.00	0.99	0.99	0.98	0.98	1.00
y-intercept	37.41	36.57	38.14	40.35	39.01	37.44
LLOQ	32.36	32.36	33.61	33.24	34.05	33.31
CI (95%)	0.59	0.98	1.10	0.79	0.67	0.49
LOD (copies/rxn)	23.41	8.45	9.45	62.67	18.57	9.89
LLOQ (copies/rxn)	35.58	16.17	19.39	104.85	29.27	13.44

Table A. Master standard curve equations for molecular markers. Four standard curves were pooled to generate master standard curves for qPCR analysis (as recommended by Ebentier et al., 2013). Equations used, lower limit of quantification (LLOQ) and limit of detection (LOD) are reported for each assay. LLOQ was determined as average value of the lowest standard concentration used. The LOD was calculated using the 95% probability of detection of the lowest standard (Bustin et al., 2009).

Figure A. Current direction modeled using the Southern California Coastal Ocean Observing System (SCCOOS) for March 26, 2013 shows a northward current during time of sampling (10AM – 12PM). Plume from the SAB treatment plant may have been traveling in a northward direction on this date.



7. References

Bae, S and Wuertz, S., 2009a. Discrimination of Viable and Dead Fecal Bacteroidales Bacteria by Quantitative PCR with Propidium Monoazide Applied and Environmental Microbiology 75, 2940-2944.

Bae, S and Wuertz, S., 2009b. Rapid decay of host-specific Bacteroidales cells in seawater as measured by quantitative PCR with propidium monoazide Water Research 43, 4850-4859.

Border Environment Cooperation Commission (BECC), 2009a. Expansion of the Water and Wastewater Systems in Tijuana and Playas de Rosarito, Baja California. Board Document DB 2009-15. July 2009. BECC Certification Document.

Border Environment Cooperation Commission (BECC), 2009b. Environmental Assessment (EA) for the Expansion of the Water Distribution and Wastewater Collection System for Unserved Areas in Tijuana, B.C. Environmental Information Document. US Environmental Protection Agency.

Bernhard, A.E. and Field, K.G., 2000a. Identification of Nonpoint Sources of Fecal Pollution in Coastal Waters by Using Host-Specific 16S Ribosomal DNA Genetic Markers from Fecal Anaerobes. Applied and Environmental Microbiology 66(4), 1587-1594.

Bernhard, A.E. and Field, K.G., 2000b. A PCR Assay To Discriminate Human and Ruminant Feces on the Basis of Host Differences in Bacteroides-Prevotella Genes Encoding 16S rRNA. Applied and Environmental Microbiology 66(10), 4571-4574.

Boehm, A.B., Van De Werfhorst, L.C., Griffith, J.F., Holden, P.A., Jay, J.A., Shanks, O.C., Wang, D., Weisberg, S.B., 2013. Performance of Forty-Three Microbial Source Tracking Methods: A Twenty-Seven Lab Evaluation Study. Water Research In Press.

Border 2012. US-Mexico Environmental Program. US Environmental Protection Agency. EPA-160-R-03-001

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 55 (4), 611e622.

Cofepris 2012. Federal Commission for the Protection against Sanitary Risk. http://pfea.org/playaslimpiastijuana/wp-content/uploads/2012/08/COFEPRIS-bc RESULTADOS.pdf

Colford, J.M., Wade, T.J., Schiff, K.C., Wright, C. C., Griffith, J.F., Sandhu, S.K., Burns,

S., Sobsey, M., Lovelace, G., Weisberg, S B., 2007. Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination Epidemiology 18, 27-35.

Doyle, T. J., and R. T. Bryan., 2000. Infectious disease morbidity in the US region bordering Mexico, 1990-1998. J. Infect. Dis. 182:1503-1510.

Ebdon, J., Muniesa, M., Taylor, H., 2007. The application of a recently isolated strain of Bacteroides (GB-124) to identify human sources of faecal pollution in a temperate river catchment. Water Research, 41, 3683-3690.

Ebentier, D.L., Hanley, K.T., Cao, Y., Badgley, B.D., Boehm, A.B., Ervin, J.S., Goodwin, K.D., Gourmelon, M., Griffith, J.F., Holden, P.A., Kelty, C.A., Lozach, S., McGee, C., Peed, L.A., Raith, M., Ryu, H., Sadowsky, M.J., Scott, E.A., Santo Domingo, J., Schriewer, A., Sinigalliano, C.D., Shanks, O.C., Van De Werfhorst, L.C., Wang, D., Wuertz, S., Jay, J.A., 2013. Evaluation of the repeatability and reproducibility of a suite of qPCR-based microbial source tracking methods. Water Research In Press.

Field, K. and Samadpour, M.,2007. Fecal source tracking, the indicator paradigm, and managing water quality. Water Research, 41, 3517-3538.

Flood, C., Ufnar, J., Wang, S., Johnson, J., Carr, M. and Ellender, R., 2011. Lack of correlation between enterococcal counts and the presence of human specifc fecal markers in Mississippi creek and coastal waters. Water Research 45, 872-878.

Gawler, A.H., Beecher, J.E., Branda˜o, J., Carroll, N.M., Falcao, L., Gourmelon, M., Masterson, B., Nunes, B., 2007. Validation of host-specific Bacteriodales 16S rRNA genes as markers to determine the origin of fecal pollution in Atlantic Rim countries of the European Union. Water Research, 41, 3780–3784.

Gerba, C., Indicator microorganisms, in Environmental Microbiology, R. Maier, I. Pepper, and C. Gerba, Editors. 2000, Academic Press: San Diego, CA. p. 491-503.

Griffith, J., Weisberg, S. and McGee, C., 2003. Evaluation of microbial source tracking methods using mixed fecal sources in aqueous test samples. Journal of Water Health, 1, 141-151.

Haugland, R.A., Varma, M., Sivaganesan, M., Kelty, C., Peed, L., Shanks, O.C., 2010. Evaluation of genetic markers from the 16S rRNA gene V2 region for use in quantitative detection of selected Bacteroidales species and human fecal waste by qPCR. Syst. Appl. Microbiol. 33, 348–357.

Khan, I.U.H., Gannon, V., Kent, R., Koning, W., Lapen, D.R., Miller, J., Neumann, N., Phillips, R., Robertson, W., Topp, E., van Bochove, E., Edge, T.A.,2007. Development of a rapid quantitative PCR assay for direct detection and quantification of culturable and non-culturable Escherichia coli from agriculture watersheds. Journal of Microbiological Methods, 69, 480-488

Kildare, B.J., Leutenegger, C.M., McSwain, B.S., Bambic, D.G., Rajal, V.B. and Wuertz, S., 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: A Bayesian approach. Water Research 41(16), 3701-3715.

Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R. and Sayler, G., 2006. Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Applied and Environmental Microbiology 72(6), 4214-4224.

Layton, B., Cao, Y., Ebentier, D.L., Hanley, K.T., Van De Werfhorst, L.C., Wang, D., Madi, T., Whitman, R.L., Byappanahalli, M.N., Balleste, E., Meijer, W., Schriewer, A., Wuertz, S., Converse, R., Noble, R.T., Srinivasan, S., Rose, J., Soo Lee, C., Lee, J., Sheilds, J., Stewart, J., Reischer, G., Farnleitner, A., Sinigalliano, C.D., Rodrigues, R., Lozach, S., Gourmelon, M., Peed, L., Shanks, O.C., Jay, J.A., Holden, P.A., Boehm, A.B., Griffith, J.F., 2013. Performance of human fecal-associated PCR based assays: An international source identification method evaluation. Water Research In Press.

Lee, C.M., Lin, T.Y., Lin, C.C., Kohbodi, G.A., Bhattl, A., Lee, R. and Jay, J.A., 2006. Persistence of fecal indicator bacteria in Santa Monica Bay beach sediments. Water Research 40(14), 2593-2602.

Lee, C.M., J.G., G., W., K. and J.A., J., 2010. Covalently linked immunomagnetic separation/adenosine triphosphate technique (Cov-IMS/ATP) enables rapid, in-field detection and quantification of *Escherichia coli* and *Enterococcus* spp. in freshwater and marine environments. Journal of Applied Microbiology 109, 324-333.

Lee, J. Y. and Deimnger, R. A., 2004. Detection of E coli in beach water within 1 hour using immunomagnetic separation and ATP bioluminescence Luminescence 19, 31-36.

Litton, R., J. Ahn, B. Sercu, P. Holden, D. Sedlak and S. Grant, 2010. Evaluation of Chemical, Molecular, and Traditional Markers of Fecal Contamination in an Effluent Dominated Urban Stream. Environmental Science & Technology 44(19) 7369-7375

Nocker, A , Cheung, C and Camper, A., 2006. Comparison of propidium monoazide and ethidium monoazide for differentiation of live vs dead bacteria by selective removal of DNA from dead cells Journal of Microbiological Methods 67, 310-320

Nocker, A , Sossa-Fernandez, P , Bun, M and Camper, A., 2007. Use of propidium monoazide for live/dead distinctions in microbial ecology Applied and Environmental

Microbiology 73, 5111 -5117

McDaniels, A.E., Wymer, L., Rankin, C., Haugland, R., 2005. Evaluation of quantitative real time PCR for the measurement of Helicobacter pylori at low concentrations in drinking water. Water Research, 39, 4808-4816.

Mieszkin, S., Furet, J.P., Corthier, G., Gourmelon, M., 2009. Estimation of Pig Fecal Contamination in a River Catchment by Real-Time PCR Using Two Pig-Specific Bacteroidales 16S rRNA Genetic Markers. Applied and Environmental Microbiology, 75, 3045-3054.

Mika, K.B., Imamura, G., C. Chang, V. Conway, G. Fernandez, J.F. Griffith, R.A. Kampalath, C.M. Lee, C.-C. Lin, R. Moreno, S. Thompson, R.L. Whitman and Jay, J.A., 2009. Pilot- and bench-scale testing of faecal indicator bacteria survival in marine beach sand near point sources. Journal of Applied Microbiology 107(1), 72-84.

Okabe, S., Okayama, N., Savichtcheva, O., Ito, T., 2007. Identification and quantification of host-specific Bacteroides-Prevotella 16S rRNA genetic markers for assessment of fecal pollution in freshwaters. Appl. Microbiol. Biotechnol. 74 (4), 890–901.

Orozco-Borbon, V., Rico-Mora, R., Weisberg, S., Noble, R., Dorsey, J., Leecaster, M., McGee, C., 2006. Bacteriological water quality along the Tijuana-Ensenada, Baja California, Mexico shoreline. Marine Pollution Bulletin, 52, 1190-1196.

Santoro, A E and A B Boehm., 2007. Frequent occunence of the human-specific Bacteroides fecal marker at an open coast marine beach relationship to waves, tides and traditional indicators. Environmental Microbiology 9(8) 2038-2049.

Sassourbre L.M., Love, D.C., Silverman, A.I., Nelson, K.L. Boehm A.B., 2012a. Comparison of enterovirus and adenovirus concentration and enumeration methods in seawater from Southern California, USA and Baja Malibu, Mexico. Journal of Water and Health. 3, 419-30.

Sassourbre L.M., Love, Nelson, K.L. Boehm A.B., 2012b. Mechanisms for photoinactivation of seawater. Applied and Environmental Microbiology, 78(21) 7776-7785.

Segovia-Zavala, J.A, F. Delgadillo-Hinojosa, M.V. Orozco-Borbón, A. Muñoz-Barbosa, S.R. Canino-Herrera, 1995. Distribution of BOD and bacteria along the coast of the US-México border. Ciencias Marinas, 21 (4) (1995), pp. 415–426.

Seurinck, S., Defoirdt, T. Verstraete, W. and Sicilliano, S.D., 2005. Detection and quantification of the human-specific HF183 Bacteroides 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater. Environmental Microbiology 7(2), 249-259.

Shanks, O.C., Atikovic, E., Blackwood, A.D., Lu, J., Noble, R.T., Domingo, J.S., Seifring, S., Sivaganesan, M. and Haugland, R. A., 2008. Quantitative PCR for detection and enumeration of

genetic markers of bovine fecal pollution. Applied and Environmental Microbiology, 74, 745-752.

Shibata, T., Solo-Gabriele, H.M., Sinigalliano, C.D., Gidley, M.L., Plano, L.R.W., Fleisher, J.M., Wang, J.D., Elmir, S.M., He, G., Wright, M.E., Abdelzaher, A.M., Ortega, C., Wanless, D., Garza, A.C., Kish, J., Scott, T., Hollenbeck, J., Backer, L.C., Fleming, L.E., 2010. Evaluation of Conventional and Alternative Monitoring Methods for a Recreational Marine Beach with Nonpoint Source of Fecal Contamination. Environ. Sci. Technol. 44, 8175–8181.
Shuval, H., 2013. Estimating the global burden of thalassogenic diseases: human infectious diseases caused by wastewater pollution of the marine environment. Journal of Water and Health 1, 53–64.

Schriewer, A., Goodwin, K.D., Sinigalliano, C.D., Cox, A.M., Wanless, D., Bartkowiak, J. Ebentier, D.L., Hanley, K.T., Ervin, J., Deering, L.A., Shanks, O.C., Peed, L.A., Meijer, W.G., Griffith, J.F., SantoDomingo, J., Jay, J.A., Holden, P.A. Wuertz,S., 2013. Performance evaluation of canine-associated Bacteroidales assays in a multi-laboratory comparison study. Water Research In Press.

Siefring, S., Varma, M., Atikovic, E., Wymer, L., Haugland, R.A., 2008. Improved real-time PCR assays for the detection of fecal indicator bacteria in surface waters with different instrument and reagent systems. *Journal of Water and Health, **6**,* 225-237.

Sinigalliano, C.D., Ervin, J.S., Van De Werfhorst, L.C., Wang, D., Wanless, D., Bartkowiak, J., Layton, B., Raith, M., Schriewer, A., Badgley, B.D., Lee, C., Goodwin, K.D., Lee, J., Boehm, A.B., Noble, R.T., Holden, P.A., Jay, J.A., Wuertz, S., Byappanahalli, M.N., Whitman, R.L., Sadowsky, M.J., Meijer, W., Balleste, E., Gourmelon, M., Griffith, J.F., Ryu, H., Domingo, J.W.S., 2013. Multi-laboratory assessment on the performance of PCR assays targeting Catellicoccus marimammalium for microbial source tracking of coastal birds. Water Research In Press.

Sung Yung, K., Terrill, E., Cornuelle, B., 2009. Assessing Coastal Plumes in a Region of Multiple Discharges; The U.S.-Mexico Border. Environmental Science Technology, 43, 7450–7457.

Terrill, E., Pyle, R., Winslow, K., Kim, S. Y., Hazard, L., Otero, M., Jones, B., Furhman, J., 2009. Coastal Observations and Monitoring in South Bay San Diego IBWC/Surfrider Consent Decree. San Diego, CA, CH2M Hill.

USEPA (2010) Method A. Enterococci in Water by Taqman® Quantitative Polymerase Chain Reaction (qPCR) Assay.

Varma, M , Field, R , Stinson, M , Rukovets, B , Wymer, L and Haugland, R., 2009. Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater Water Research 43, 4790-4801

Wade, T J., Calderon R. L., Sams, E. Beach, M., Brenner, K. P., Williams, A. H. and Dufour A. P., 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-assocaited gastrointestinal illness. Environmental Health Prespectives 114, 24-28.

Chapter 5. Molecular marker data suggests that FIB inputs to coastal waters at Topanga State Beach are from multiple animal sources each with different seasonal patterns.

Abstract: High levels of fecal indicator bacteria (FIB) have been observed at Topanga State Beach resulting in exceedances of water quality standards and postings of beach advisory warnings. Although the origin of the bacteria impairing water quality at the beach is unknown, analysis of historical enterococci concentrations suggests the lagoon to be a main source of bacteria to the surf zone when the lagoon is breached and discharging to the ocean. This study utilizes molecular markers in spatial and temporal sampling to identify sources of fecal pollution within the Topanga Creek watershed. The relationships between upper and lower watershed sources, as well as possible contribution of FIB from both the creek and lagoon systems are explored. Spatial sampling showed that different sources of FIB impair Topanga creek and lagoon. A consistent decrease in indicator bacteria and source markers occurred between the upmost creek site and downstream lagoon sampling locations. Increased bacteria levels and presence of human, gull and dog-associated markers at lagoon sites suggest an independent source near the lagoon and eliminated the creek as the source of FIB exceedances at Topanga State Beach. Dog, gull and human-associated markers were found to be important sources in the lagoon and ocean. Dog and gull-associated markers were also detected at high frequencies when exceedances in FIB were measured in ocean samples. Seasonal variability was seen for both markers, with highest levels occurring in winter. Additionally, transient activity and contaminated groundwater inputs may be responsible for contributing human fecal pollution to marine waters. Microbial source tracking presented different trends in FIB and source markers and shows the importance of the application of a suite of markers over long term spatial and temporal sampling to identify a complex combination of chronic sources of contamination.

1. Introduction

Topanga State Beach, California is frequently listed as one of the most impacted beaches in the state of California (Heal the Bay 2013) based on FIB levels, despite numerous projects within the lower watershed intended to improve water quality (Dagit et al., 2013). Ranked 9th most polluted California beach in 2006, and 4th in 2011, Topanga State Beach has FIB exceedances well into the summer season, but the cause and source of FIB to the ocean has been unknown. Potential sources of fecal contamination to the watershed include malfunctioning septic systems, transients, horses, dogs, gulls and other wildlife specific to the region. Topanga Creek and lagoon may also be potential sources of FIB to the surfzone; microbial contamination may be transported from the upper watershed via the creek to the lagoon and beach. Studies have also shown beach sand and sediments can harbor bacteria and serve as a source of FIB to the water column (Ishii et al., 2007; Yamahara et al., 2009).

A 21month microbial source tracking (MST) study was initiated on the Topanga watershed that measured FIB levels and also utilized culture-independent molecular markers for detection of host-associated fecal contamination. Unlike FIB, which can originate from multiple hosts, MST methods can help identify unique sources of fecal pollution through use of host-associated primers that allow for identification of the original host of fecal pollution to environmental waters (Harwood et al., 2013; Boehm et al., 2013). MST methods are often deployed using a tiered approach. The first tier typically involves identifying locations impaired for FIB or other general water quality parameters (Noble et al., 2006; Boehm et al., 2003). Locations of high FIB are analyzed using spatial and temporal sampling. After which, sites impaired by FIB are also analyzed for host markers to identify sources contributing to elevated concentrations of FIB. However, results from studies using a tiered approach with FIB as a first tier to locate human-associated pollution have been mixed (Sercu et al. 2009, Reischer et al.

2008, Boehm et al. 2003, Bower et al. 2005, Flood et al. 2011). Use of FIB to infer locations for follow up analysis with source markers is confounded by the fact that FIB has been known to vary on short timescales (Boehm et al., 2002). Further, seasonal trends of host-associated markers have not been well documented and warrant further research.

This study investigates sources of FIB to the watershed and reports on the applicability of using MST technology over longer time scales. A combination of snapshot surveys, long-term monitoring during wet and dry weather seasons, and the use of a suite of markers at all sites was utilized to identify sources of FIB. Spatial sampling was used to determine if contamination is conveyed downstream via the creek to the ocean. Impacted sites and sources are identified to provide suggestions for targeted and effective remediation efforts to reduce number of exceedances and improve water quality at Topanga State Beach.

2. Materials and Methods

2.1 Field Site

2.1.1 Topanga Creek Watershed

Topanga State Beach receives over 750,000 annual visitors and suffers from poor water quality. Due to the Mediterranean climate, this region experiences a dry season (April – October) and wet season (November – March), with typical rainfall averaging 20 inches a year. However, rainfall during the course of this study was below average levels (9.9 inches of rainfall in 2012 – 2013). Topanga Creek watershed (approximately 47 km2) is 70% undeveloped (GeoPentech, 2006) and includes a creek and lagoon system (Figure 5-1). Topanga Creek drains the upper watershed and cumulates in Topanga Lagoon, a dynamic lagoon system that breaches and berms throughout the year, contributing variable flow to Topanga State Beach.

2.1.2 Sampling sites

Initial snapshot monitoring within the Topanga Creek watershed began 5 October 2011. In addition, certain sampling locations throughout the watershed were chosen for long-term monitoring over a nine month period between 17 November 2012 and 1 July 2013. In order to document any input coming from the upper watershed, samples were collected at five sites within the creek. Creek sites include (from upstream to downstream): Owl Falls (6500m), Scratchy Trail (4800m), Topanga Boulevard Bridge (3600m), Brookside Drive (1700m) and Snake Pit (300m). The most upstream site Owl Falls (OF), is located nearest to the town of Topanga and lies just below the confluence of the two major tributaries draining the upper portions of the watershed. Downstream lies Scratchy Trail (ST), a remote sample location approximately 15 minute hike from the main road. With large boulders and a steep slope, the creek is least impacted by any development in the area. Next is Topanga Bridge (TB), located approximately halfway between the town and lagoon, this location experiences some level of human activity. Rock climbing and graffiti are regular occurrences at this site. Brookside Drive (BR) lies just downstream; this sample location is easily accessible from the road. Trailers have been known to empty waste tanks along the dirt bank of the road, adjacent to this site. The last of the creek sites is Snake Pit (SP). This location has also seen evidence of transient encampments nearby.

A lagoon transect was also sampled at three sites (Highway Bridge, Topanga Lagoon and Lagoon Outlet). Highway Bridge (HB) was sampled on the east end of the Pacific Coast Highway Bridge, using a bridge sampling device that was lowered with a rope into the middle of the lagoon. The Topanga Lagoon (TL) sample was collected near the east wall of the lagoon, and the Lagoon Outlet (LO) was sampled at the mouth of the discharge point, before the mixing zone of lagoon and ocean water.

Two marine samples were collected from Topanga State Beach at Beach Outlet (BO) and Beach Upper (BU). BO was sampled as the outlet of the lagoon, whether the lagoon was bermed or breached. BO is also a Los Angeles County sampling site. Beach Upper (BU) was collected approximately a quarter mile north of the lagoon, to represent marine water quality conditions upstream of the lagoon discharge point.

Additional samples were also occasionally collected from Falls Drive (FD), which is located at the top of Owl Falls and collects all flow from Dix Creek, and Behind Abuelita's Restaurant (BA), which is a site used in earlier studies, and drains everything from the upper watershed below the confluence of the main creek and Old Topanga Creek sub-drainages (Dagit et al., 2004).

2.2 Sample Collection

All marine and lagoon samples were collected before sunrise to eliminate the effects of photoinactivation on FIB (Boehm 2009). Samples collected within Topanga Creek were taken before exposure to direct sunlight. All samples were stored in containers on ice within approximately 15 minutes of collection and transported back to the lab for analysis. Sample bottles (polypropylene plastic) were washed with 10% HCl and rinsed three times with source water before use. Approximately two liter surface samples were collected from the creek in actively flowing sections, from the lagoon utilizing either a 12' pole sampler or a bridge sampler, and from the ocean in knee deep surf.

2.3 Rain Events and First Flush Surveys

Four sampling events occurred during active rainfall over the course of the study (10/5/11 (FF), 11/17/12 (FF), 1/24/13, 3/8/13), of which two were categorized as first flush and two as rain

events during small storms that lasted 2 - 5 days. First flush (FF) was defined as the first storm of the season to reach at least ¾ inches in rainfall. Between eight to nine samples were collected during FF within the upper and lower watershed. Total rainfall recorded varied for the two rain events in 2013, however both amounts were relatively low (1.65 in and 1.06 in) due to a dry year; annual rainfall for 2013 was only half as much as the typical average for that region. A total of 35 samples were collected during active rainfall.

2.4 In Town Survey

Inputs from the town of Topanga, California were investigated over a two week sampling period from 23 May – 7 June 2012. Samples were collected on three visits over the two week period from two tributaries draining the upper reaches of Topanga, and below the confluence in the main stem of Topanga Creek (Figure 5-2). A bracketed sampling approach was used to hone in on locations with high levels of FIB. Samples were analyzed for host markers and FIB using a culture based method (IDEXX).

2.5 Long-term Seasonal Watershed Survey

Ten sampling locations were chosen for a long-term survey to identify sources of fecal contamination and to investigate seasonal trends in FIB and host marker concentrations within the Topanga Creek watershed. Samples were collected from 17 November 2012 to 1 July 2013. Sites were sampled monthly in the dry weather season (April – July) and bi-monthly during the wet weather season (November – March). Five samples were taken along the main stem of Topanga Creek, three lagoon samples were collected along an inland to ocean transect, and two marine samples were collected from Topanga State Beach (Figure 5-1). Additional samples were

collected occasionally within the creek and ocean. Full descriptions of sampling locations are described above (*Section 2.1.2.*).

2.6 Septic System Testing

2.6.1. Testing Methods

Existing tanks within Topanga State Park were pumped out, then tested by backfilling to a minimum depth of 2" above the riser seam to prevent damage from hydrostatic uplift. They were then monitored for two hours. No tank was accepted if there was any leakage over the two hour period. Topanga Underground conducted testing in Spring and Summer 2013.

2.6.2 Testing of Beach Restrooms and Lifegaurd system

Water samples were pulled from the Los Angeles County Department of Beaches and Harbors Restrooms and Lifeguard station septic system at a sampling port where fluids pass between the UV disinfection system and the distribution box, which connects to a subsurface leach field. Samples were collected monthly from January 2013 through July 2013 by technicians from BioSolutions. Each 2 liter sample was collected and processed according to the sampling protocol, within the normal holding time by the Jay Lab at UCLA. A 500 ml sample was collected and tested for nutrients as well. BioSolutions also collected samples tested by Pat-Chem Inc, Moorpark, California for: Biological Oxygen Demand (BOD), Dissolved Oxygen, Total Suspended Solids (TSS), pH, Turbidity, Total Alkalinity, Carbonate Alkalinity, Bicarbonate Alkalinity, and Hydroxide Alkalinity.

2.6 FIB Enumeration

Samples were processed for FIB within six hours of collection. Three types of FIB, Total Coliform (TC), *Escherichia coli* (EC), and enterococci (ENT) were measured with Colilert-18™ and Enterolert™ (IDEXX, Westbrook ME) reagents and protocols to determine the most probable number (MPN) of cells per 100 mL sample. Briefly, 10 mL of sample water was diluted in 90 mL Milli-Q water containing IDEXX Colilert-18™ or Enterolert™ reagents, sealed in a Quanti-Tray/2000 and incubated at 35°C for 18 hours (for TC and EC) or 41°C for 24 hours (for ENT). The lower limit of detection (LOD) for these assays is 10 MPN/100 mL and any sample below the limit of detection was assigned a value of 5 MPN/100 mL for analysis. Samples greater than the upper LOD were set to double the maximum possible value (if the max was 24916 MPN/100 mL then set to 49832).

2.7 DNA Extraction

Two hundred mL of sample water was filtered through 0.4 µm polycarbonate filters (EMD Millipore, Billerica MA) in triplicate. These filters were folded inwards and transferred into individual 2 ml screw cap tubes (Sarstedt Inc., Newton NC) preloaded with acid-washed glass beads (Sigma-Aldrich, St. Louis MO) and archived at -80°C until extracted. DNA extraction from filters were conducted with the DNA-EZ ST1 Extraction Kit (GeneRite, North Brunswick NJ) following the manufacturer's protocol. Eluted DNA samples were stored at -20°C until analysis of molecular host-associated markers with qPCR.

2.8 Quantitative PCR

Four host-associated markers were deployed to identify sources of fecal pollution present within the watershed. The human-associated *Bacteroidales* HF183 TaqMan (HF183) assay was

used to detect presence of human fecal contamination, and the BacHum TaqMan (BacHum) assay was used as a second human-associated marker to confirm results. The Gull2 TaqMan (Gull), a gull-associated *Catellicoccus*, and the dog-associated *Bacteroidales* DogBact TaqMan (Dog) markers were used to detect presence of non-human sources. In addition, a subset of samples were analyzed for the horse-associated HF0597 PCR assay. The qPCR reactions were performed as previously developed by the USEPA and NOAA (Haugland et al. 2010, Shanks et al. 2009, Dick et al. 2005, Lu et al. 2008). A list of primers and probes used are detailed in (Appendix D-Table C). Quantitative PCR reactions were run in triplicate with 2 µl of extracted sample DNA as a template and averaged concentrations were reported in copies / 100 mL. For qPCR, samples were scored as detected when an amplification signal greater than a fluorescence threshold of 0.03 (ΔRn) was detected within 40 thermal cycles. A PCR maximum cycle number of 35 and qPCR of 40 were determined as optimal (Boehm et al., 2013). Non-detects were assigned to samples if one or less replicates were positive (ND), or if samples did not amplify within the 40 cycles.

2.9 Water chemistry/Geochemical Parameters

For marine waters, measurements of water temperature and conductivity were collected in-situ (Orion conductivity meter). Ambient water chemistry measurements including pH, dissolved oxygen (mg/L and % sat), specific conductivity (µS/cm), water temperature (ºC), air temperature (ºC), and salinity (ppt) (YSI 55 DO meter, Oakton pH meter, Oakton conductivity meter, and refractometer) were taken in the field for creek and lagoon samples. Analysis of turbidity (NTU) was tested with the LaMotte Turbidimeter 2020 and nutrients (nitrate, nitrite, ammonia and orthophosphates (ppm)) were tested using the LaMotte Smart3 Colorimeter for a subset of samples. Flow (ft/s) was measured in creek sites using a Marsh-McBirney Flowmate

3000. In-situ water quality testing equipment was calibrated before each sampling event. The membranes and solution in the DO meters were checked and replaced as needed.

3. Results

3.1 Historical analysis of FIB

In a review of historical enterococci data taken by the Los Angeles County Department of Public Health, a pattern of bacterial exceedances occurring at Topanga Beach well into the dry season (as late as mid-July) was noted. When this data was compared to creek flow data collected by the County at the same time as the bacterial data, it was apparent that bacterial exceedances correlated strongly with breaches in the Topanga Creek Lagoon (Appendix D). Geomean of enterococci concentrations were significantly higher when the lagoon was breached and discharging to the ocean (110 – 120 MPN/100ml) for wet and dry season samples. Geomean concentrations were a magnitude less (10-20 MPN/100mL) when the lagoon was bermed and not connected to the ocean. The Topanga Lagoon discharges episodically into the ocean as late as July. This correlation between Lagoon discharges and high FIB values in ocean water samples strongly suggested that Topanga Lagoon was the primary source of bacterial pollution to the ocean.

Based on this historical analysis and the identification of lower Topanga Creek and lagoon as the likely most significant source of FIB to the ocean, a microbial source tracking study of those areas was initiated. A full watershed snapshot in October 2011 was conducted. Locations with elevated FIB were identified with this snapshot and further analyzed with additional sampling to investigate the upper watershed for fecal pollution and to determine if sources were being conveyed from the creek to the ocean.

3.2 Snapshot sampling results

3.2.1 Results of initial snapshot during first flush

The first flush storm event occurred on 5 October 2011. Samples were collected from the upper watershed downstream to the ocean. Results of the first flush sampling reflected a pattern of hot spots of fecal contamination in the upper watershed. The lagoon was not connected to the ocean at the time of sampling (although it did break through later that day) and samples along the shoreline did not detect bacteria.

Results from the 2011 first flush sampling event indicated high bacterial levels throughout the watershed and, in most cases, human-associated markers were found within these bacterial populations. Subsequent snapshot sampling events once the creek had been flowing identified at least four hot spots of bacterial levels with human signatures. These were Entrado/Highvale Road (not re-verified in 2012 due to lack of flow), a sample site in the town of Topanga (Behind Abuelitas), Topanga Bridge, and the Lagoon. For each of the hot spots bacterial levels experienced exceedances and all showed the presence of human marker on more than one occasion.

3.2.2. Results from follow-up snapshot monitoring

The watershed snap-shot sampling efforts in 2012 indicated that bacterial sinks exist in two reaches of the creek. The first sink in the upper Narrows reach, located between Owl Falls (OF) and the Scratchy Trail (ST), and the second sink, between the Topanga Bridge and the Rodeo Grounds area. Both sinks occurred in areas with little human development, although Topanga Canyon Blvd. road is adjacent to the creek. The upper Narrows sink, between OF and

ST, processes a larger bacterial load in a shorter creek reach (from 6500-4800m = 1700m) than the lower canyon sink (3600 – 300m = 3300m).

The sampling effort of 2012 identified a hot spot of high ENT levels and related human-associated marker in the town region of the Topanga watershed. To better understand the nature and extent of this hot spot, samples were taken three times over a two week period in an attempt to bracket in the source (Figure 5-2).

The intensive sampling with the FIB enumeration methods indicated that the FIB source was coming from both the main stem of the creek and the Old Topanga Creek tributary. The main stem source did not appear to extend above site 6 (School Road crossing) while the Old Topanga source was not definitively bracketed as exceedance levels were found at the northern most site sampled (site 1, Backbone Trail Crossing). These sample events were further analyzed for human, dog, and horse-associated markers (Table 5-3). No horse-associated marker was detected at any site.

Sites along the main stem (site 6, 7 and 8) did not show markers except for one hit for BacHum at a level too low to quantify at the School Road site (Table 5-3). High levels of human-marker were detected along the Old Topanga Creek stem near site 1, in addition to high levels of dog marker detected at site 3. These sources may explain the concurrent hits of human and dog markers at site 4, located below the confluence.

3.2 *Upper watershed sampling*

Except for a few occasions associated with either rain events or observed transient activity, data indicated bacteria levels decreased as the flow moved downstream to the lowest creek sampling site at the Snake Pit, located 300 meters upstream from the Topanga Lagoon.

Samples collected from the PCH Bridge, within the lagoon and along the beach in the ocean had clearly different patterns than those observed upstream within Topanga Creek. FIB levels are frequently elevated in the upper watershed, particularly at Owl Falls. However, elevated FIB levels seen in the upper watershed decreased throughout the watershed and are consistently lower at downstream sites including Scratchy Trail and Topanga Bridge. Nutrient levels in Topanga creek and lagoon were overall low, and despite the very low flow conditions in 2012-2013, the pattern of decreasing levels of nutrients as the creek flows downstream were consistent with those observed in previous studies (Dagit et al. 2004). Exceptions to this pattern were observed during rain events and associated with transient activities.

3.3 Rain sampling events

Four sampling events occurred during active rainfall (10/5/11, 11/17/12, 1/24/13, 3/8/13). When geometric means of the watershed FIB and marker values of samples were taken during rain and compared to the geometric means of non-rain samples, the rain samples were 10 to 15 times higher than non-rain samples with the exception of Gull marker, which had lower values during rain, and Dog, which was 35 times higher when raining (Table 5-1). Clearly active rainfall increased the bacterial levels in the watershed, which is typical of other studies throughout Southern California (Noble et al. 2003, Boehm et al. 2002, Surbeck et al. 2006).

3.4 Seasonal marker and FIB analysis

3.4.1 Topanga Creek marker and FIB analysis

This study aimed to understand sources of FIB and host-associated markers in dry weather, as compared to wet weather. Therefore, rain samples were not included in the analyses unless otherwise stated.

The seasonal differences in FIB and marker geometric means over the watershed were compared in Table 5-2. A variety of responses to the season were observed. TC winter season values were half of the dry season's while EC, ENT and the human-associated markers showed little to no seasonal variation. Gull marker was s six times higher in the winter while Dog marker was28 times higher in the winter. The differences observed in seasonal patterns for the various FIB and source markers indicate that FIB may come from a variety of sources.

To better understand how the season and site location affect water quality, we compared FIB and markers on a site- by-site basis. For TC, the highest values were observed at Owl Falls and then these levels dropped at Scratchy Trail and remained below Owl Falls levels for the remainder of the creek sites. TC values were consistently higher in the drier season than the wet season. EC and ENT levels were lower throughout the creek throughout the year. Dry season values were also consistently higher than wet season values (Figure 5-3 A and B). For example, Human marker values (HF183 and BacHum) were the highest in the creek at OF and were again detected at about $1/10^{th}$ the Owl Falls levels at Topanga Bridge. There were no obvious seasonal trends to the human marker data (Figure 5-4 A and B). Gull marker levels were detected at consistently lower levels throughout the creek with no detection of Gull at the most inland and upper watershed site Owl Falls. Dog marker during the wet season at Owl Falls was the highest value for any marker measured. Site Scratchy Trail has not shown any Dog marker during the study while sites further downstream did show low levels of this marker (Figure 5-5 A and B). It is clear that the upper site closed to town, Owl Falls, was the most impacted in terms of FIB, human, and dog markers.

3.4.2 Topanga Lagoon marker and FIB analysis

Very consistent TC values were observed across all sites and seasons. EC and ENT values were also consistent throughout the lagoon, with a possible slight elevation towards Lagoon Outlet and slightly higher values in the winter. Human marker values (HF183 and BacHum) were consistently detected throughout the lagoon at low levels with a possible slight elevation during the dry season (Figure 5-4 C and D). Gull marker is detected at consistently high levels throughout the lagoon with no consistent seasonal trend and a possible elevation in values towards site, Lagoon Outlet.

Seasonal affects were most pronounced for the Dog marker. Dog marker was the most dynamic marker in the lagoon with winter values, two orders of magnitude higher than dry season values (Figure 5-5 C and D). Additionally there was an order of magnitude increase in Dog marker level going from site PCH Highway Bridge to site Lagoon Outlet. The lagoon also had consistent high levels of FIB and Gull markers and a low level of human-associated marker.

3.4.3 Topanga State Beach marker and FIB analysis

TC levels at Beach Outlet were about five times higher than Beach Upcoast values without any seasonal variation indicated. EC values between the sites were more consistent while ENT values were slightly elevated at Beach Outlet. Neither EC nor ENT showed a consistent seasonal pattern across the two sites (Figure 5-3 E and F). Human marker values (HF183 and BacHum) were not detected during the dry season, but were present in the winter season at higher levels at Beach Outlet (Figure 5-4 E and F). Gull marker was detected at both sites with higher levels detected during the winter season. Dog marker clearly was the most dynamic marker in the ocean with winter values two orders of magnitude higher than dry season values at both sites. Additionally there were higher levels of Dog marker at site Beach Upcoast

than site Beach Outlet (Figure 5-5 E and F). Markers levels were all higher in the winter season. Additionally, human and gull were elevated at Beach Outlet over Beach Upcoast.

3.5. Time series analysis

In order to understand how the creek influenced the lagoon, which subsequently influenced the ocean, three representative sites, (Snake Pit, Topanga Lagoon and Beach Outlet) were examined for FIB and source markers in a time series plot. FIB levels appeared to be highest in the winter months at SP with a possible spike occurring in October of 2011 and 2012 (Figure 5-9 A and 5-10 A). FIB levels were seen to peak in October and then gradually decrease until February. Increased concentrations of FIB occurred between February to October in 2012 and from February to July in 2013. FIB values were observed to increase in spring and summer months in both 2012 and 2013, suggesting a possible seasonal trend with levels peaking in November at SP.

Dog marker increased in the winter months at SP as well, and appeared to decrease beginning in June through September (Figure 5-6 C). The highest concentration of the Dog marker was seen in October 2012, the same month that FIB levels were observed to spike at this site. Gull was rarely detected at this site, however one sample that was positive for the Gull marker in April 2012 corresponded with exceedances of EC and ENT (Figure 5-6 B). HF183 was not detected at this site; however three samples did amplify for the BacHum assay. Two samples were positive for BacHum in January 2013, one during a rain event and another in a follow up sampling three days later. The follow up sample had even greater levels than the rain event sample.

Within the lagoon, consistently high levels of FIB (Figure 5-9 B and 5-10 B) and gull marker were observed throughout the sampling season. There did not appear to be any seasonal trend for the Gull marker (Figure 5-8 B). Similar to Snake Pit, a peak in FIB levels was seen in October 2012. A possible seasonal trend was observed with FIB levels decreasing in Winter (Oct – Feb) and increasing in Spring and Summer months (Feb – Oct). There appeared to be two orders of magnitude drop in TC and EC levels from the Winter to Summer season. High levels of Dog marker were present in the Winter months. There were also periodic detects for human-associated marker for both the HF183 Taqman and BacHum qPCR assays (Figure 5-7 A). Samples scored as positive for one replicate above the LOD conveyed frequent presence of human marker in samples tested. Ten samples out of 23 (43%) were positive for the HF183 marker and 14 samples out of 23 (61%) were positive for BacHum. BacHum concentration was consistently higher than measured HF183 concentrations, and both human-associated assays correlated well.

Beach Outlet exhibited similar trends as Topanga Lagoon, but with lower FIB concentrations (Figure 5-9 B and C, Figure 5-10 B and C). FIB levels and Gull marker levels were fairly consistent throughout the year. Dog marker was present at higher levels during the Winter months. Sporadic human hits seemed more frequent during the Winter months (Figure 5-8A).

3.6. Septic System Testing Results

3.6.1. Septic system locations

The septic systems located along Pacific Coast Highway (PCH) were identified as potential sources of FIB to Topanga Lagoon and Beach. It was not feasible to test the privately

owned systems west of Topanga Lagoon, but it was possible to test the four systems managed by the California Department of Parks and Recreation in Topanga State Park on the north-side of PCH, as well as the Lifeguard Station Restrooms managed by the Los Angeles County Department of Beaches and Harbors. These systems were the closest to the lagoon and examining their condition and function were critical to understanding the potential of these systems to its contribution FIB to downstream waters. Aerial and ground surveys to map the locations of the septic systems and their potential connectivity were completed in Summer 2013. The topographic survey was conducted by Chris Nelson and Associates.

The septic systems along Pacific Coast Highway within Topanga State Park were evaluated as described above. These septic systems were being pumped at least weekly. They were all older systems and even though they were no longer connected to leach fields or seepage pits, the potential for leakage was present.

The following on-site wastewaster treatment systems tested along Pacific Coast Highway include the Malibu Feed Bin, Reel Inn Restaurant, Ranger Station, and Cholada's Restaurant. Three additional businesses were also evaluated for waste collection facilities (Rosenthal Winery, Wiley's Bait Shop, and Something Fishy). Wiley's Bait shop had no restroom, and the Something Fishy store were no longer in business. Although their septic system existed, it was not in use and was ruled out as a possible source. Rosenthal Winery bathroom had no fixtures, and instead utilized several portable toilets outside the building, which appeared to be maintained properly. Reel Inn and Cholada's OWTS were tested and were functioning properly. Malibu Feed Bin and the Ranger Station were both found to have leaks and may be potential sources of FIB and host markers to the creek and lagoon.

5.2.1. Malfunctioning septic systems

Topanga Canyon Blvd- Malibu Feed Bin

The Topanga Canyon-Malibu Feed Bin system was not functional at time of inspection. The seepage pit was 100% full and backed up into the tank. The tank and the pit needed to be pumped. Two 8" pumping risers were installed on the tank and the pit has an existing 4" pumping riser.

Pacific Coast Highway-Ranger Station

The Pacific Coast Highway Ranger Station system had several components. There was a 4" clean out on the sewer line behind the partially occupied Ranger cabin. There was also an abandoned 14 foot cesspool located between a palm tree and the nearby small tree adjacent to the ranger house. This was apparently not connected to anything and was dry. By water testing and some pipe cleaning, it was determined that the Ranger cabin was draining to a tank (tank #1) that was cracked and, as a result, effluent was seeping out. This tank was pumped empty and 6" of concrete placed over the existing floor. This was done to prevent any future leakage. It was determined that if tank#1 was full, it would overflow to a larger tank (tank#2). Although tank#2 had some water at time of inspection, no additional flow into the tank was observed. Nonetheless, tank#2 is situated close to the creek and could be a contributor to bacteria levels.

5.3.1 Harbors Restrooms and Lifeguard Station Results

FIB and Marker results were summarized in Appendix D. Even after advanced septic processing with UV disinfection that kept FIB levels quite low, molecular assays detected both human markers in almost every sample. Only human markers were detected in septage from this

system, except for one positive sample for the Dog marker. Based on the FIB results, it does not appear that the lifeguard septic system contributed FIB to either the lagoon or ocean.

Nutrient levels in the lifeguard septage samples tested were consistently high and most required a 1/100 dilution in order to even test. Nitrate-N, ammonia – N, orthophosphates were in exceedance in all samples. On 5 June 2013, the samples were not diluted and thus were completely over-range (Appendix D).

4. Discussion

These results presented a broad look at the weather, seasonal, and site data to show any major trends within the watershed. Active rainfall in the watershed increased all FIB and marker levels except the Gull marker. Watershed Total coliform (TC) winter season values were also lower than dry season values, while watershed *E. coli* (EC) and enterococci (ENT) values showed little seasonal variation. Watershed human markers indicated little seasonal variation, while Gull and Dog markers increased in the Winter season.

An adaptive sampling approach was successfully used to track locations within the town of Topanga that were elevated for enterococci concentrations. Additional analysis with source markers allowed for identification of several possible sources contributing to observed ENT exceedances, Dog and human-associated markers. Detection of the human-associated marker in four of eight spatially intensive sites tested suggested human fecal contamination is one important source near the town of Topanga, corresponding with high FIB levels in the creek.

When just Topanga Creek was considered, site Owl Falls (6500m OF), located just downstream of all upper watershed inputs, was found to be the most impacted, with high levels of TC, Winter EC, ENT, human markers and Winter Dog marker. These high levels were not propagated down creek as evidenced by the site, Scratchy Trail (4800m ST) having the lowest

FIB and markers levels within the sampled creek sites . Sources contributing fecal contamination within the upper watershed were independent from the lower watershed sources. Therefore, effective mitigation efforts aimed to improve water quality at Topanga State Beach should focus on lower watershed sources.

If just the lagoon was considered, the PCH Bridge (HB), Topanga Lagoon (TL) and Lagoon Outlet (LO) sites were found to be consistent both by site and season, with the exception of Dog marker which was almost 100 times higher in the Winter season. Lagoon FIB and Gull marker values were higher in magnitude than creek and ocean sites. If just the ocean was considered, then FIB values were consistent by season, but may be slightly higher at site Beach Outlet (BO) than Beach Upcoast (BU). All marker values were higher in the winter season than the dry season, with Dog marker increasing by as much as 100 times. Additionally Dog marker could have been higher at site BU than BO (Figure 5-5F).

Long term microbial source tracking, over a 21 month period, allowed for analysis of seasonal fluctuations in molecular markers. Both Gull and Dog-associated markers were present at high frequency and were identified as important sources to Topanga Lagoon and State Beach. Gulls and other waterfowl have been found to impair water quality at other beaches and may be responsible for exceedances of FIB in surfzone and lagoon samples (Lu et al., 2008; Sinigalliano 2013). Although the gull marker was detected consistently in the lagoon, it is possible that increased number of beach visitors at BU and BO prevented gulls from roosting on the sand; therefore a reduction in gull is observed at ocean sites in the dry season. Lafferty and colleagues (2013) found reduced shorebird populations present in beaches with increased human activity. Some gulls exhibit migrating behaviors, and it has been documented that larger shorebird populations are present in Southern California beaches in winter months (Lafferty 2001;

Hubbard and Dugan 2003). This study found presence of gull waste most frequently corresponding to typical peaks in shorebird abundance (Oct – Dec months). Greater frequency of detection and magnitude of the Dog-associated marker in winter months may also be related to the lifeguard presence at Topanga State Beach. Decreased lifeguard hours in winter months correspond to a peak of this marker in the winter season. Higher Dog marker levels in BU over BO could be due to the fact that the lifeguard patrol ended just east (downcoast) of BU, and many of the residences along the beach upcoast have dogs. Although it is difficult to control for fecal contributions from wildlife such as gulls, fecal waste from pets can be mitigated by watershed managers as current regulations prohibit dogs on this beach. Increased enforcement by lifeguards, community education and awareness, along with better signage may help reduce contamination associated with domestic dogs.

On the other hand, human-associated markers were detected periodically in lagoon samples. Presence of markers corresponded with recorded visual observations of human feces and transient activity (Table 5-4). A mass balance of one direct deposit (~200g of human feces) was calculated to result in an exceedance of ENT in the lagoon. Homeless encampments were found and dismantled throughout the watershed, this a continuing issue for the city. Transient activity near the lagoon was recoded for several months (January to March 2013) adjacent to the lagoon (HB and TL sites) and found to directly impact water quality near these sites.

Testing of the septic systems along Pacific Coast Highway found that the system at the Ranger residence located in the State Park, was possibly leaking so repairs were completed in Summer 2013. The system at the Feed Bin was also found to be a potential source of leakage and required repair and further testing to evaluate the input of potential contamination into Topanga Creek. The other systems within Topanga State Park did not appear to be leaking, nor did the

County Lifeguard facility. Although testing in Summer 2013 indicated that the majority of septic systems from businesses adjacent to Topanga Lagoon were not likely to be actively contributing any leakage during this study period, there have been several studies that indicate long lag time between input into the ground water table and emergence in either the ocean or a lagoon (Stone Environmental 2004). Therefore, human fecal contamination detected at the lagoon could possibly be partially due to two leaking septic systems (Ranger residence and Malibu Feed Bin), tested in this study. Since most of these systems have only been capped since 2008, additional dye testing in the future may be required in order to conclusively document any potential inputs. Additionally, the Los Angeles County Lifeguard Station restroom facility at Topanga Beach was upgraded in 2008 with a state of the art Advantex treatment system (Dagit et al., 2013). The renovated system incorporated chlorination, de-chlorination, and UV treatment to eliminate bacterial contamination; consistently low to non-detectable levels of FIB sampled from the lifeguard station eliminated this OWTS as a source of human pollution to the beach. Detection of the human marker from treated septage (77 – 2,874,282 copies/100mL) was expected as human-associated markers have been measured from treated wastewater effluent at similar levels in other studies (Bae and Wuertz 2009).

5. Conclusions

Snap shot sampling, coupled with long-term microbial source tracking at several locations in the Topanga Creek watershed allowed for identification of several problem areas requiring remediation efforts. Spatial sampling revealed that human sources are prevalent near the town of Topanga. Additionally, a reduction in FIB and source markers downstream implied a decoupling of sources in creek sites and downstream lagoon and ocean sites. Dog and Gull markers presented seasonal trends, with higher levels in Winter months. Human marker was detected in lagoon and ocean samples, coinciding with presence of transient activity and two

leaking septic systems. Mitigation efforts to reduce exceedances of FIB at Topanga State Beach should prioritize potential sources from the lower watershed. Testing and repairs of local sewage infrastructure, along with better enforcement regarding presence of dogs on the beach may help to improve water quality. This study showed the need for long-term water quality monitoring efforts with multiple host markers when trying to identify sources of fecal contamination.

Acknowledgements

Numerous Resource Conservation District of the Santa Monica Mountains (RCDSMM) and University of California at Los Angeles (UCLA) staff, students and volunteers helped with sample collection and processing. Further assistance and support was provided by the rangers and staff at Topanga State Park, as well as the lifeguards at Topanga Beach, and the staff of Los Angeles County Department of Beaches and Harbors. BioSolutions and Topanga Underground provided technical assistance with the septic system monitoring and examination. Funding was provided in part by Los Angeles County and by the California State Water Resources Control Board through the Source Identification Protocol Project grant.

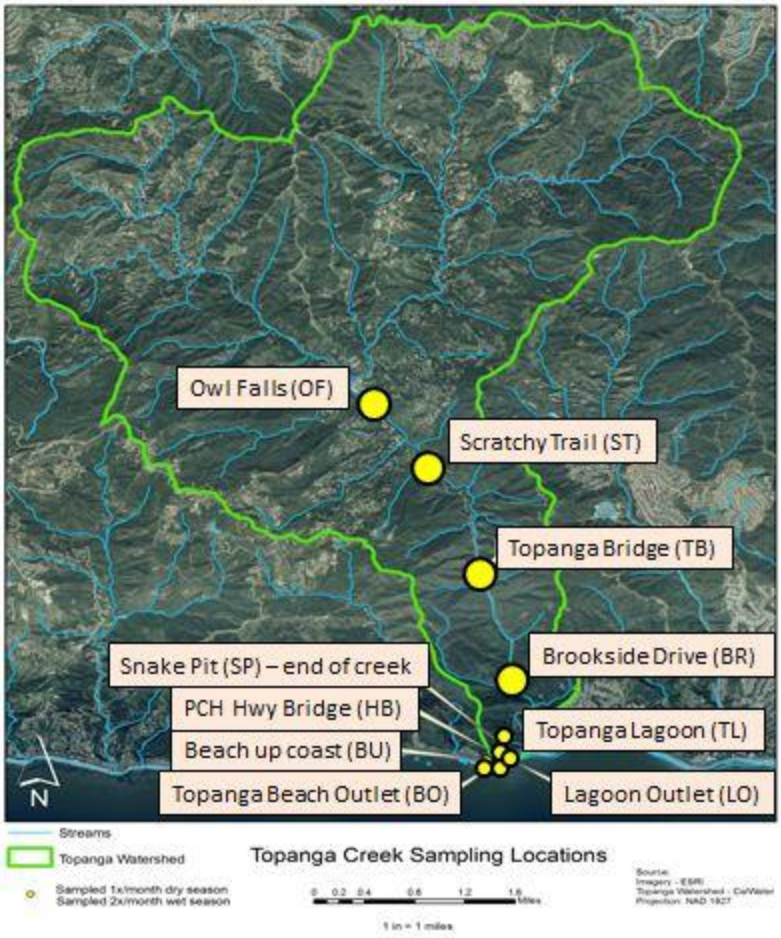


Figure 5 - 1. Map of the Topanga Creek Watershed and sampling locations. Five samples were collected from the lower half of the watershed along the main stem of Topanga Creek. Creek sites include Owl Falls (OF), Scratchy Trail (ST), Topanga Bridge (TB), Brookside Drive (BR) and Snake Pit (SP). Three samples were collected within Topanga Lagoon at the Pacific Coast Highway Bridge (HB), Topanga Lagoon (TL) at the east end, and at the Lagoon outlet (LO), just before the lagoon discharge point. Two marine samples were taken, one directly out from the lagoon (BO), this sample represents the mixing point if the lagoon is breached, and an upcoast beach site (BU) north of the lagoon.

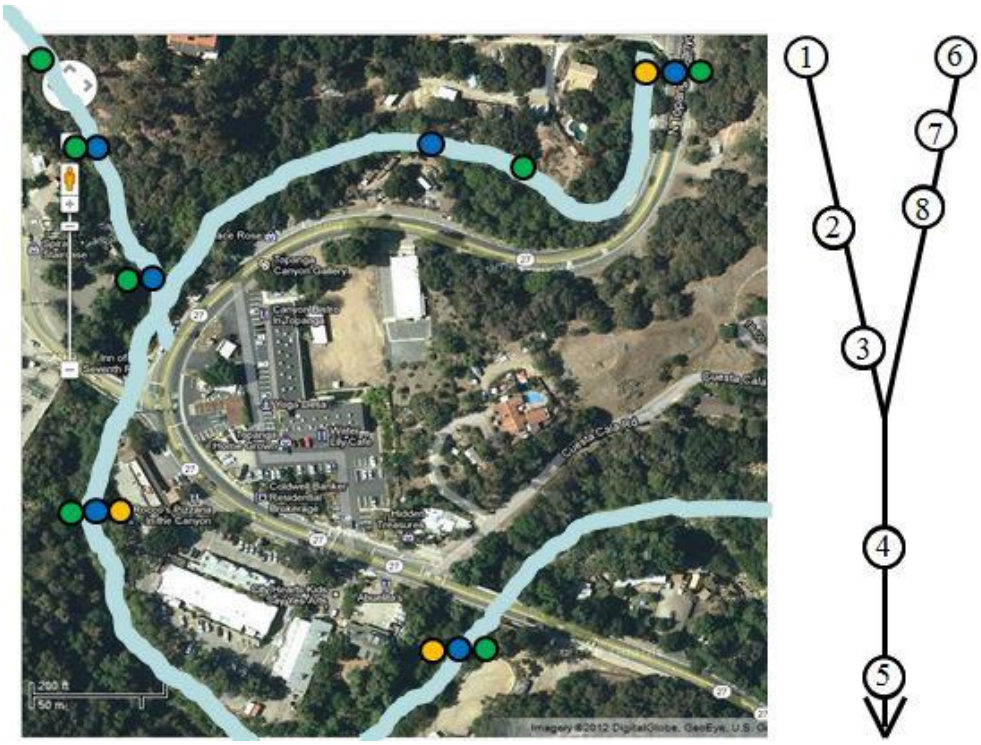


Figure 5 - 2. Map of sampling locations in Topanga town. Samples were collected from 8 sites within the creek on three separate days, within a 2 week period (May 23 – June 7 2012). Brackets sampling approach was used, therefore total samples collect from each site varies. Satellite image provided by Google Maps (© Google 2013).

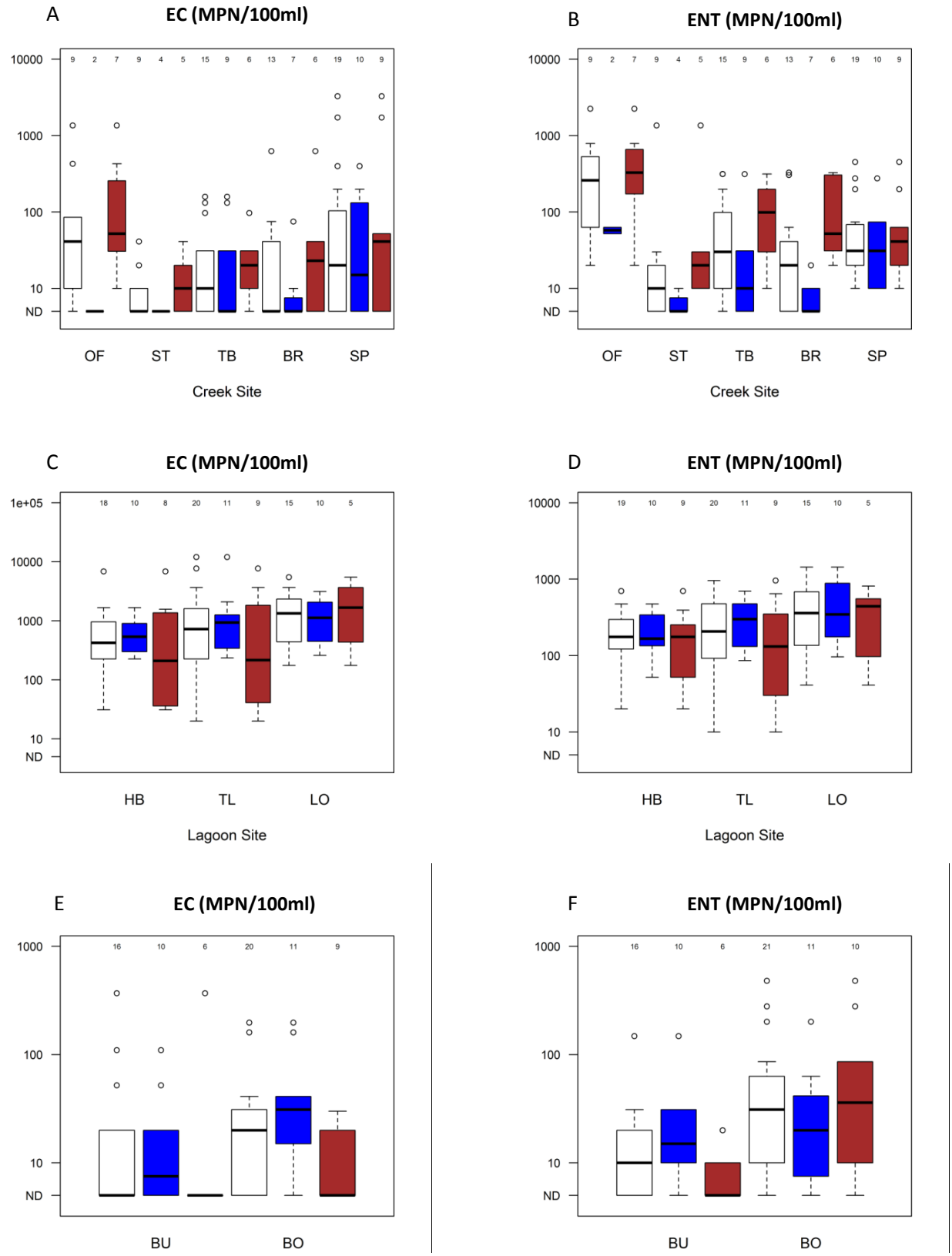


Figure 5 - 3. Box and whisker plots of *E. coli* and enterococci concentrations from Topanga Creek (A & B), Lagoon (C & D) and State Beach (E & F). The box signifies the 25th, 50th and 75th , while whiskers are 10th and 90thpercentiles.

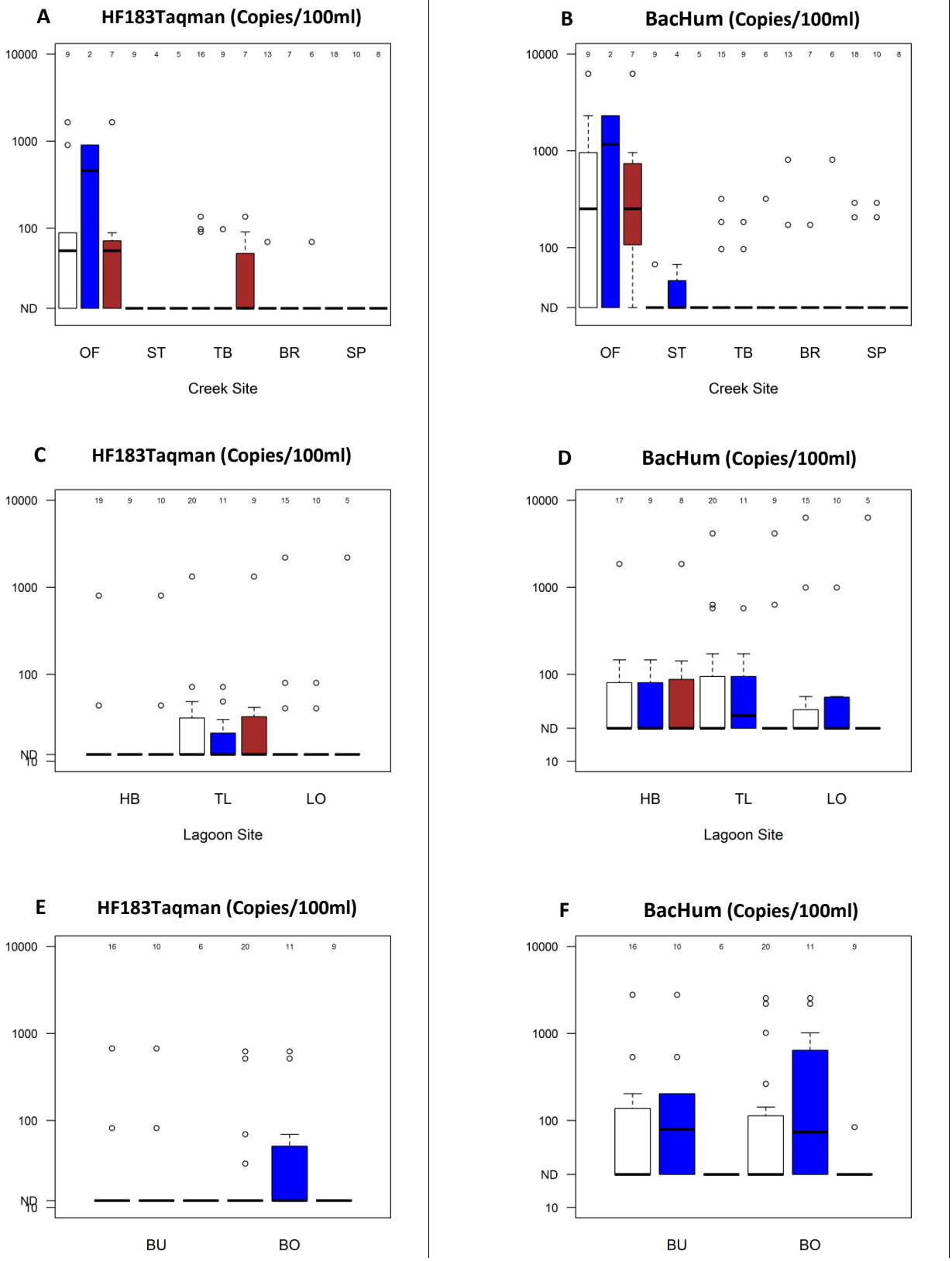


Figure 5 - 4. Box and whisker plots of human markers (HF183 Taq and BacHum) from Topanga Creek (A & B), Lagoon (C & D) and State Beach (E & F). The box signifies the 25th, 50th and 75th, while whiskers are 10th and 90th percentiles.

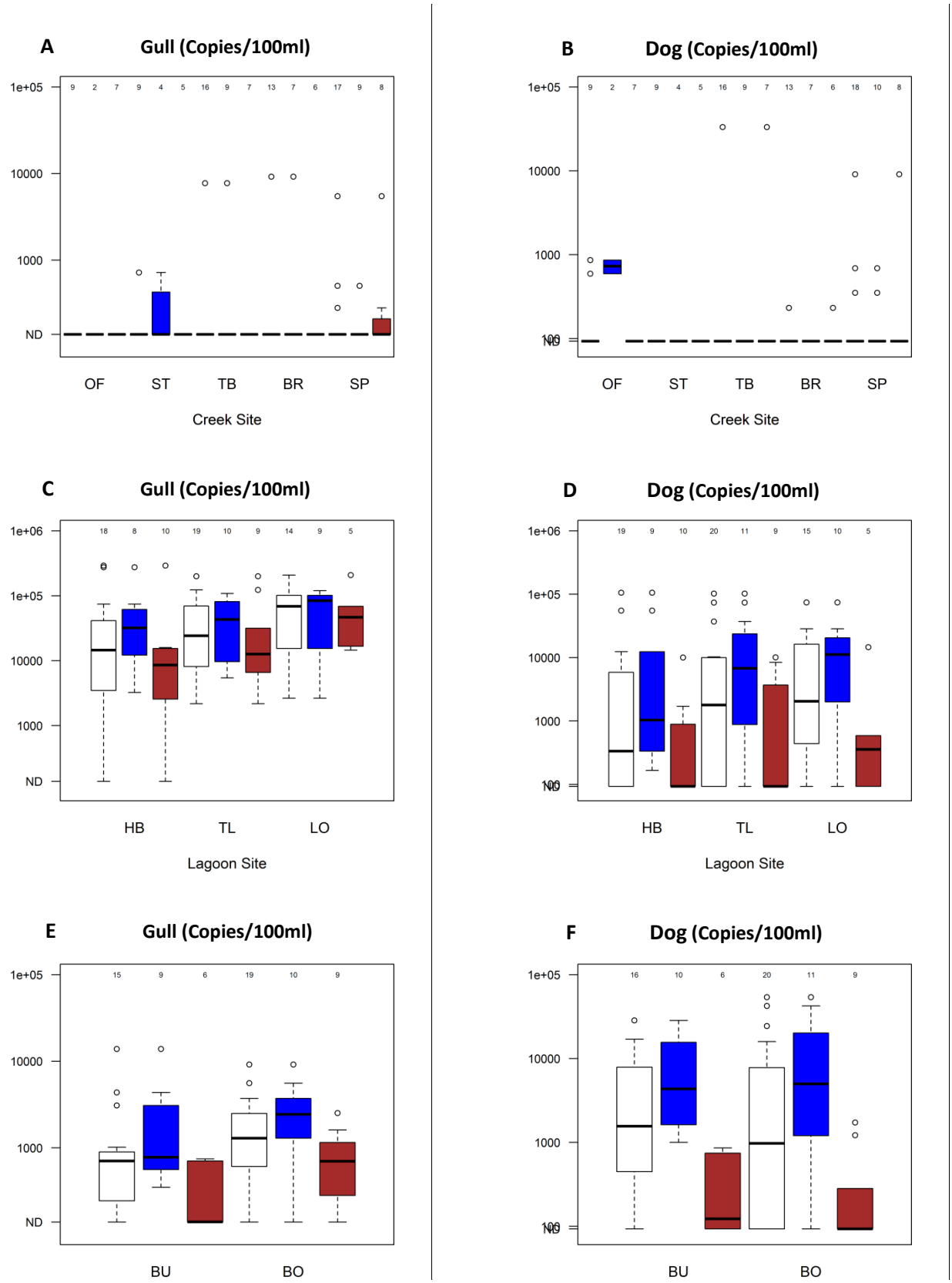


Figure 5 - 5. Box and whisker plots of host-associated markers (Gull and Dog) from Topanga Creek (A & B), Lagoon (C & D) and State Beach (E & F). The box signifies the 25th, 50th and 75th, while whiskers are the 10th and 90th percentiles.

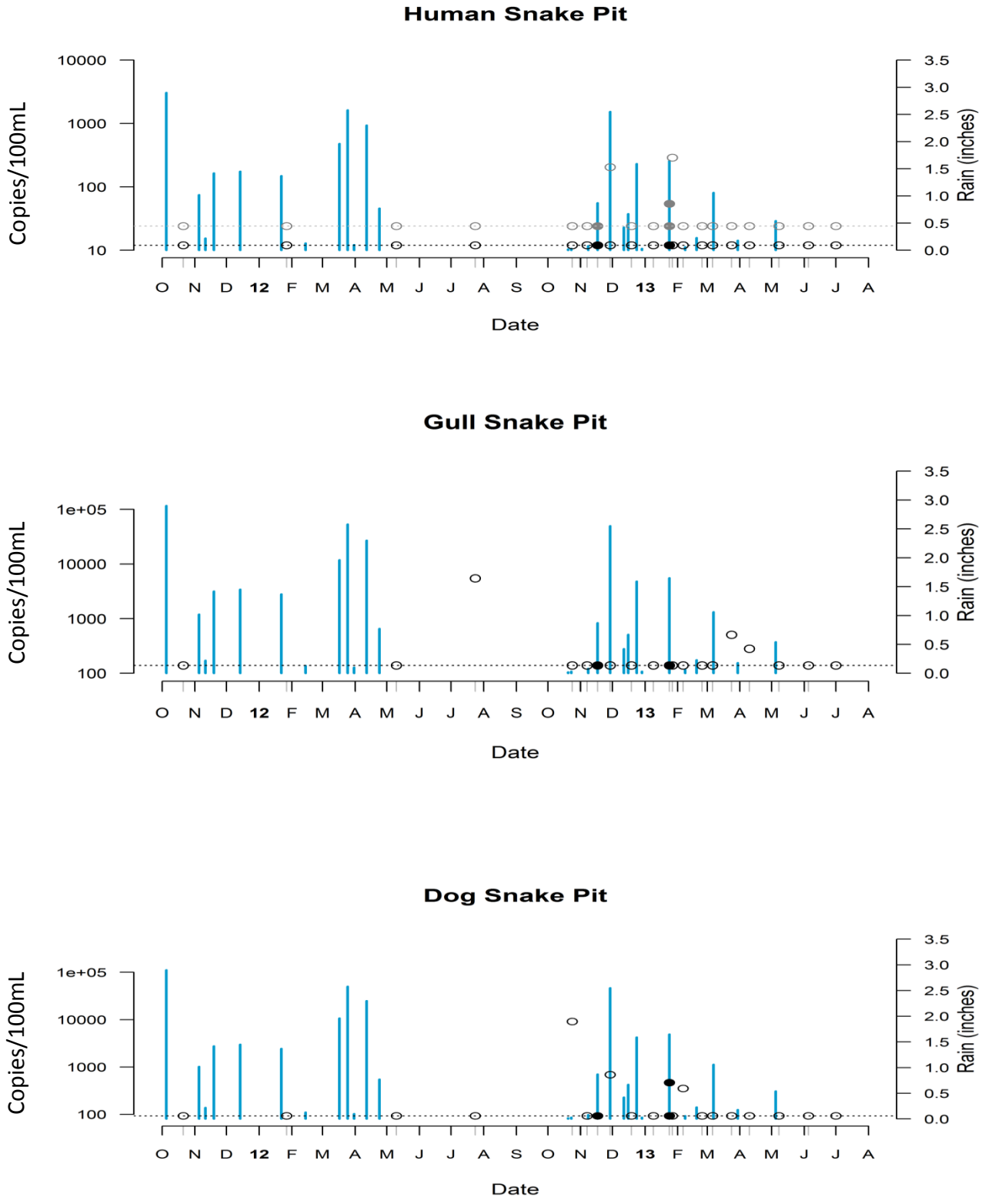


Figure 5 - 6. Time-series plots for Snake Pit creek site showing trends in A) human (HF183-black circles, and BacHum-grey circles), B) gull and C) dog-associated markers. Dotted line represents the limit of detection.

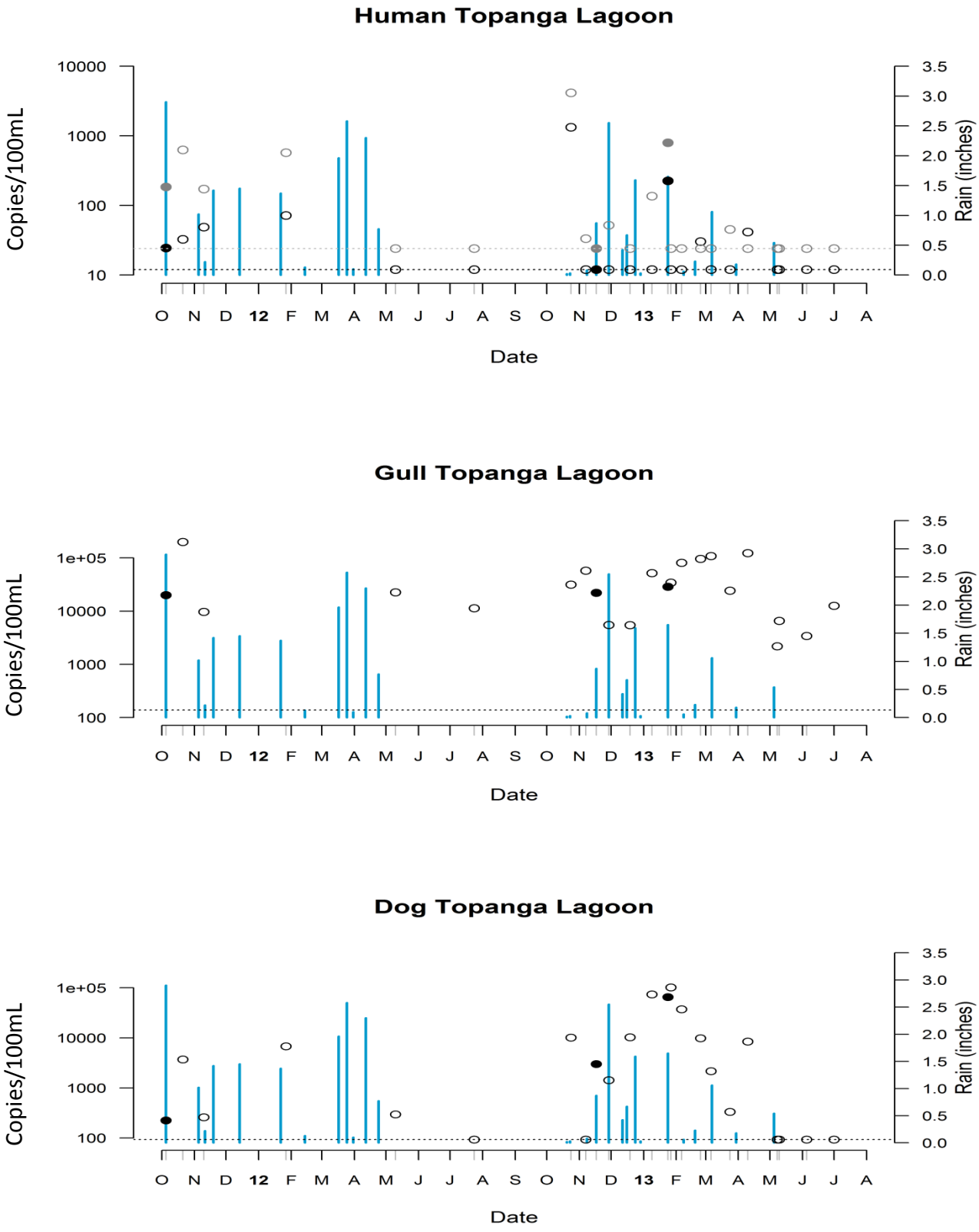


Figure 5 - 7. Time-series plots for Topanga Lagoon (TL) showing trends in A) human (HF183-black circles, and BacHum-grey circles), B) gull and C) dog-associated markers. Dotted line represents the limit of detection.

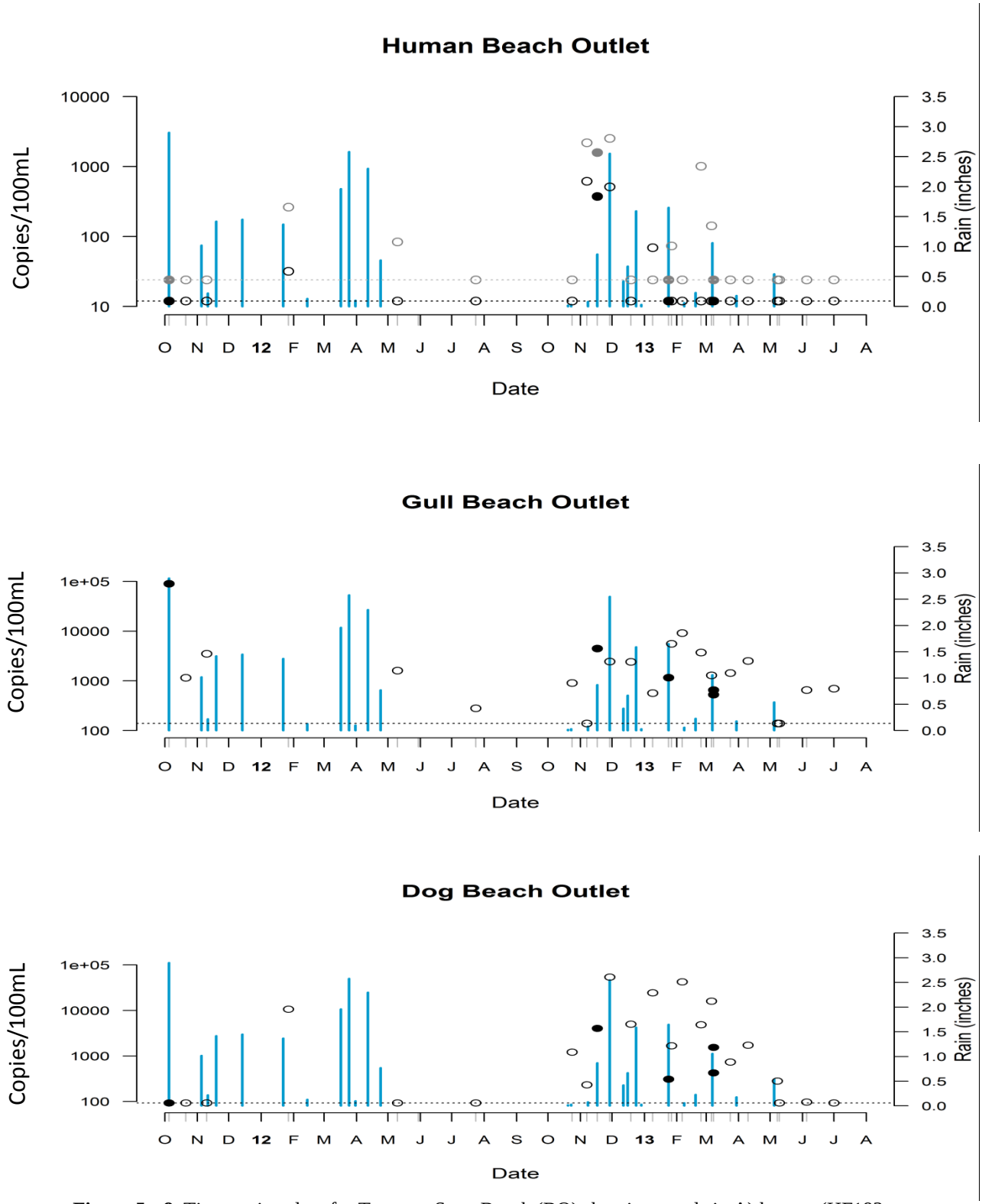


Figure 5 - 8. Time-series plots for Topanga State Beach (BO) showing trends in A) human (HF183-black circles, and BacHum-grey circles), B) gull and C) dog-associated markers. Dotted line represents the limit of detection.

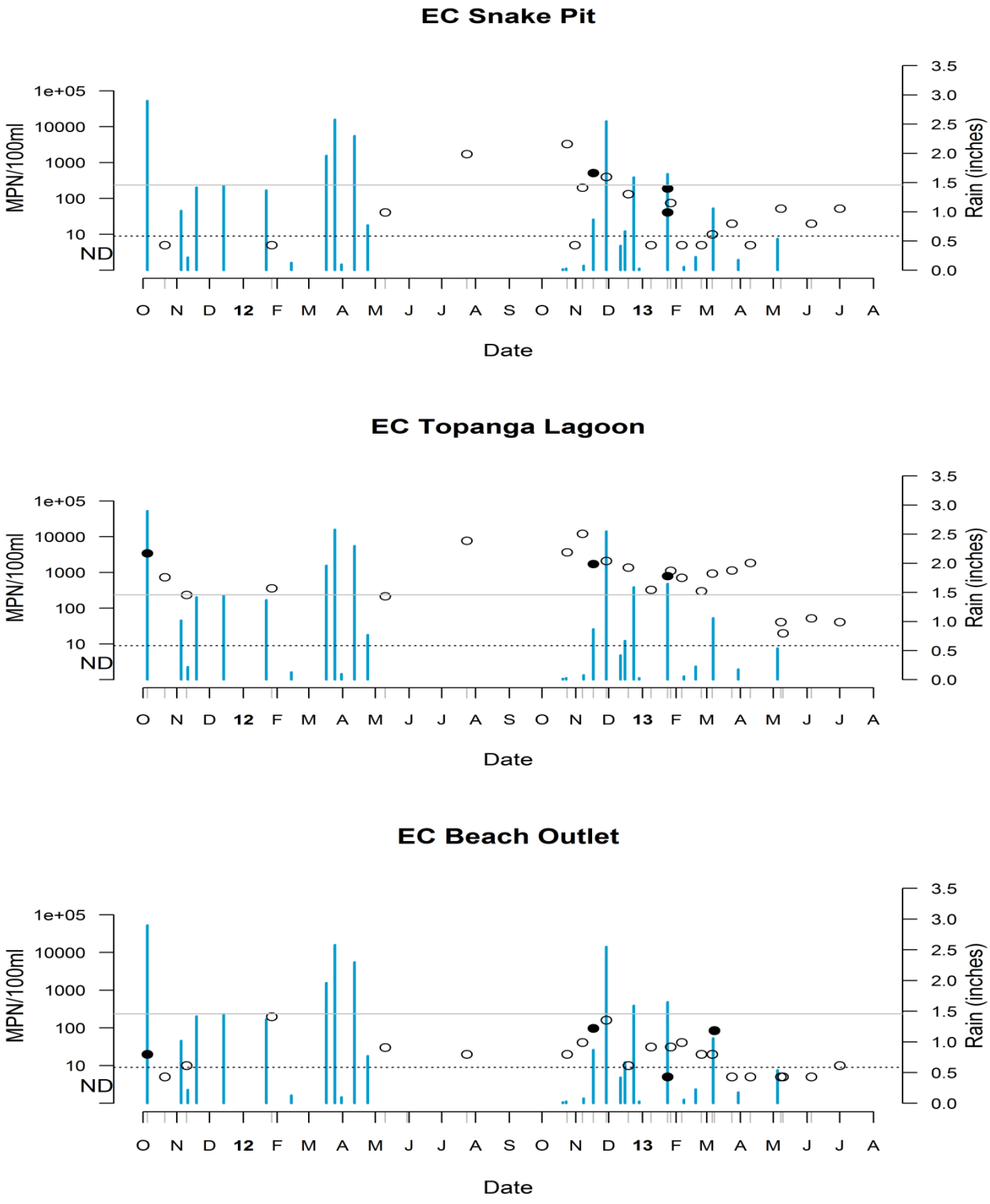


Figure 5 - 9. Concentrations of *E. coli* (EC) are plotted for the study period (black circles). Rain is represented by blue bars. Trends in EC concentrations are shown for A) Snake pit B) Topanga Lagoon and C) Beach Outlet sampling sites. Dotted line represents the limit of detection. Solid grey line shows EC water quality limit.

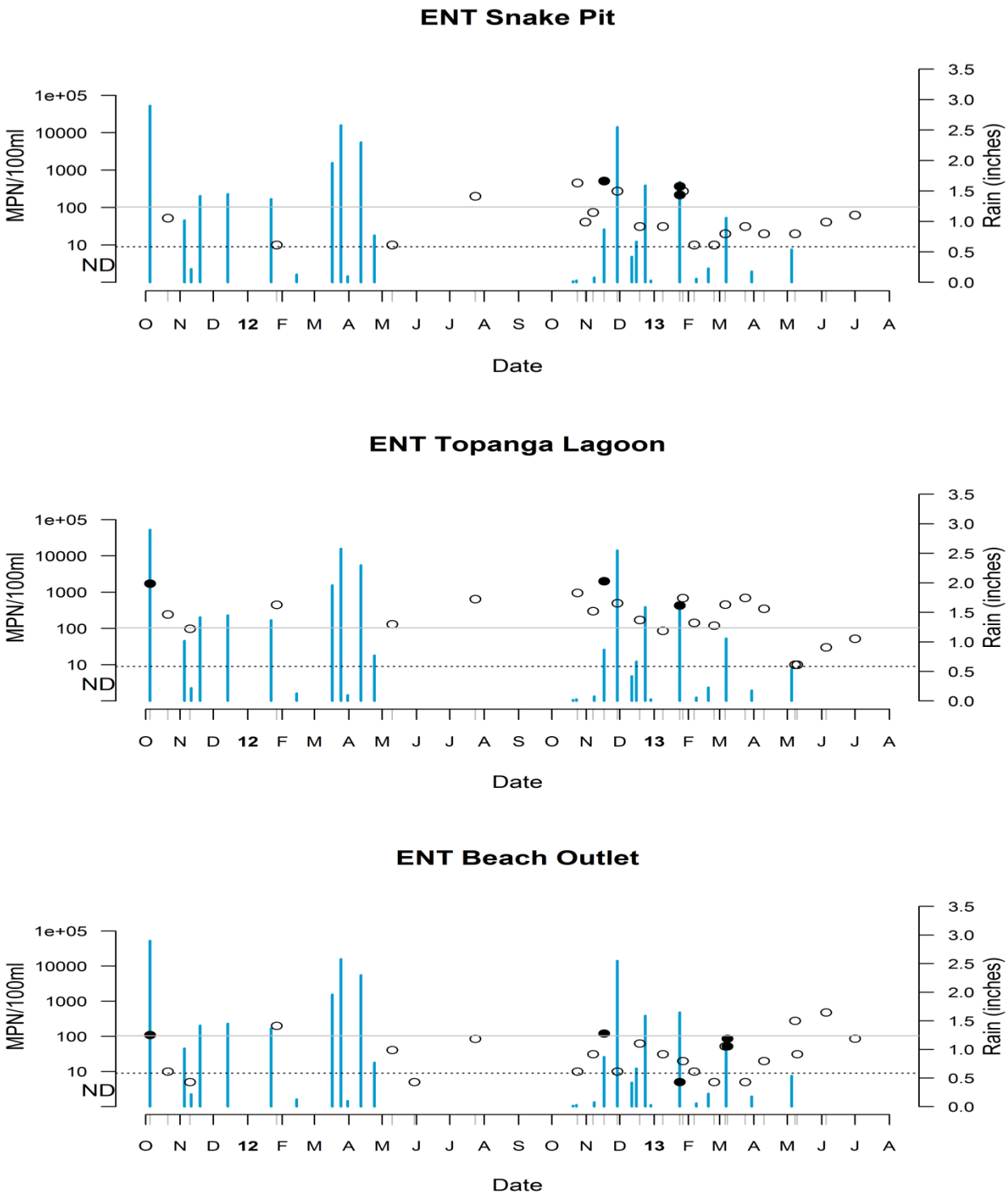


Figure 5 - 10. Concentrations of enterococci (ENT) are plotted for the study period (black circles). Rain is represented by blue bars. Trends in ENT concentrations are shown for A) Snake pit B) Topanga Lagoon and C) Beach Outlet sampling sites. Dotted line represents the limit of detection. Solid grey line shows ENT water quality limit.

Table 5 - 1. Geometric means and marker values of FIB for all samples collected during active rainfall versus all samples collected when not actively raining from 5 Oct 2011 to 1 July 2013. Values in parentheses indicate number of data points (N).

	TC MPN/100ml	**EC** MPN/100ml	**ENT** MPN/100ml	**HF** gene copies/100ml	**BH** gene copies/100ml	**Gull** gene copies/100ml	**Dog** gene copies/100ml
Raining	10568 (27)	756 (27)	863 (27)	26 (23)	56 (27)	66 (23)	986 (17)
Not Raining	940 (194)	51 (194)	54 (217)	2 (197)	5 (197)	126 (165)	28 (203)

Exceedances: TC>10,000 MPN, EC >235 MPN, ENT >104 MPN Marine, >61 MPN freshwater

Table 5- 2. Winter (Nov. 1 to May 31) and Recreation Season (April 1 to Oct. 31) geometric means of FIB and marker values raining from 5 Oct 2011 to 1 July 2013. Values in parentheses indicate number of data points (N). Rain data not used in this analysis.

	TC MPN/100ml	**EC** MPN/100ml	**ENT** MPN/100ml	**HF** gene copies/100ml	**BH** gene copies/100ml	**Gull** gene copies/100ml	**Dog** gene copies/100ml
Winter	615 (96)	51 (96)	41 (96)	2 (95)	6 (95)	290 (85)	169 (95)
Recreation	1427 (98)	50 (98)	66 (121)	2 (102)	5 (102)	52 (80)	6 (108)

Table 5- 3. Topanga town sampling results. A bracketed sampling approach with IMS/ATP and source markers was used. Sample values for enterococci, human- and dog-associated markers are shown. "*" represents sample collected was not processed for that specific marker.

Site	Map #	ENT			HF183			BacHum			Dog		
		5/23	5/30	6/7	5/23	5/30	6/7	5/23	5/30	6/7	5/23	5/30	6/7
Backbone Trail	1			135			*			*			ND
	2		275	414		2400	*		4700	*			ND
	3		393	185		ND	*		D	*			*
School Road	6	52	65	30	ND	ND	*	ND	D	*	ND	ND	ND
	7			30			*			*			ND
	8		780			ND			ND			ND	
Post Office	4	256	223	146	D	D	*	50	ND	*	590	135	ND
Behind Abuelita's	5	233	132	63	ND	ND	*	ND	ND	*	ND	220	*

Table 5 - 4. Summary of Feces observations by Sampling Date and Location, where H= Human, D=Dog, and B=Bird. (grey boxes indicate transient activity observed).

Site	19 Dec 12	9 Jan 13	24 Jan 13	27 Jan 13	6 Feb 13	24 Feb 13	6 Mar 13	24 Mar 13	8 May 13	1 Jul 13	31 Jul 13
Lagoon Outlet (LO)	D										
Topanga Lagoon (TL)	H,D,B	B	H,B	H,B	H,B	H,B	H,B	B	H,B	B	B
PCH Bridge				H	H	H	H				
Snake Pit											
Brookside Dr.	H										
Topanga Bridge											
Scratchy Trail											
Owl Falls											
Behind Abuelita's											

6. Appendix D

Table A. Sampling Locations (Coordinate System: UTM , Zone 11N). Samples were collected once per month in the dry season (Apr – Oct) and twice a month in the wet season (Nov – Mar). In addition, samples were collected once in a first flush event in the wet weather season.

Site Name	Easting (m)	Northing (m)	Elevation (ft)	Number Samples collected in Wet Season	Number Samples Dry Season
Beach Upcoast (BU)	353726	3767515	0	2/mo + first flush	1/mo
Beach Outlet (BO)	353896	3767506	0	2/mo + first flush	1/mo
Lagoon Outlet (LO)	353872	3767529	0	2/mo + first flush	1/mo
Lifeguard Station Beach (LG)	353968	3767553	0	2/mo + first flush	1/mo
Topanga Lagoon (TL)	353887	3767573	0	2/mo + first flush	1/mo
PCH Bridge - 0m (HB)	353868	3767649	0	2/mo + first flush	1/mo
Lifeguard Station Septic (LS)	353994	3767655	0	1/mo	1/mo
Snake Pit – 300m (SP)	354015	3767841	0	2/mo + first flush	1/mo
Brookside Drive – 1700m (BR)	354075	3768713	0	2/mo + first flush	1/mo
Topanga Bridge – 3600m (TB)	353522	3770391	200	2/mo + first flush	1/mo
Scratchy Trail – 4800m (ST)	353518	3771500	500	1/mo + first flush	1/mo
Owl Falls – 6500m (OF)	352673	3772373	700	1/mo + first flush	1/mo
Falls Drive (FD)	352535	3772259	750	occasional	
Behind Abuelita's (BA)	351570	3772891	700	occasional	

Table B -1. Total coliforms levels in Lifeguard treatment system, Topanga Lagoon, Beach Outlet, Beach Upcoast. (Exceedance >10,000 MPN/100ml)

Date	**Lifeguard** TC MPN/100ml	**Ocean in front of Lifeguard** **Copy/100ml**	**Topanga lagoon** TC MPN/100ml	**Beach Outlet** TC MPN/100 ml	**Beach Upcoast** TC MPN/100ml
11/17/12 First Flush	ND*	52	29090	341	341
12/19/12	ND	Not collected	13760	98	75
1/9/13	<10	Not collected	1664	41	20
2/6/13	ND	Not collected	4611	327	605
3/6/13	2400	Not collected	4352	41	10
4/10/13	794	Not collected	4786	75	<10
5/8/13	185	97	2254	63	52
6/5/13	2224	199	2282	2489	10
7/1/13	414	86	2098	141	20
7/31/13	2987	Not available	7270	201	960

*ND= Not detectable

Table B - 2 *E. coli* levels at Lifeguard treatment system, Topanga Lagoon, Beach Outlet, Beach Upcoast. (Exceedance <235 MPN/100ml)

Date	**Lifeguard** MPN/100ml	**Ocean in front of Lifeguard** **Copy/100ml**	**Topanga lagoon** MPN/100ml	**Beach Outlet** MPN/100ml	**Beach Upcoast** MPN/100ml
11/17/12 First Flush	ND*	<10	2098	160	110
12/19/12	ND	Not collected	1376	10	20
1/9/13	<10	Not collected	327	31	<10
2/6/13	ND	Not collected	712	41	52
3/6/13	<10	Not collected	933	20	<10
4/10/13	<10	Not collected	1835	<10	<10
5/8/13	<10	<10	41	<10	<10
6/5/13	<10	<10	52	<10	<10
7/1/13	41	<10	41	10	<10
7/31/13	<10	Not available	171	<10	187

*ND= Not detectable

Table B-3. Enterococcus levels at Lifeguard treatment system, Topanga Lagoon, Beach Outlet, Beach Upcoast. (Exceedance >104 MPN/100ml saltwater and >61 MPN/100ml for freshwater)

Date	**Lifeguard** MPN/100ml	Ocean in front of Lifeguard Copy/100ml	**Topanga lagoon** MPN/100ml	**Beach Outlet** MPN/100ml	**Beach Upcoast** MPN/100ml
11/17/12 First Flush	ND*	10	495	10	31
12/19/12	ND	Not collected	171	63	20
1/9/13	<10	Not collected	86	31	20
2/6/13	ND	Not collected	142	10	31
3/6/13	50	Not collected	455	52	148
4/10/13	30	Not collected	350	20	20
5/8/13	10	3873	10	279	<10
6/5/13	327	4106	30	480	10
7/1/13	399	52	52	86	<10
7/31/13	657	Not available	5794	75	231

*ND= Not detectable

Table B-4. Human Marker (HF183 copy/100ml) levels at Lifeguard treatment system, Topanga Lagoon, Beach Outlet, Beach Upcoast.

Date	**Lifeguard** **Copy/100ml**	**Ocean in front of Lifeguard** **Copy/100ml**	**Topanga lagoon** **Copy/100ml**	**Beach Outlet** **Copy/100ml**	**Beach Upcoast** **Copy/100ml**
11/17/12 First Flush	Not collected	Not collected	ND*	286	673
12/19/12	Not collected	Not collected	ND	D	ND
1/9/13	2616	Not collected	D	70	D
2/6/13	Not collected	Not collected	ND	D	ND
3/6/13	Not yet run	Not collected	ND	ND	ND
4/10/13	9224	Not collected	13	ND	ND
5/8/13	ND	ND	ND	ND	D
6/5/13	1335	ND	ND	ND	ND
7/1/13	357985	ND	ND	ND	ND

*ND= Not detectable

Table B-5. BacHum Marker levels at Lifeguard treatment system, Topanga Lagoon, Beach Outlet, Beach Upcoast.

Date	Lifeguard Copy/100ml	Ocean in front of Lifeguard Copy/100ml	Topanga lagoon Copy/100ml	Beach Outlet Copy/100ml	Beach Upcoast Copy/100ml
11/17/12 First Flush	Not collected	Not collected	D	2538	2777
12/19/12	Not collected	Not collected	ND*	ND	ND
1/9/13	23238	Not collected	46	18	69
2/6/13	Not collected	Not collected	ND	ND	48
3/6/13	29701	Not collected	ND	ND	ND
4/10/13	246174	Not collected	ND	ND	ND
5/8/13	77	ND	D	ND	D
6/5/13	8512	ND	ND	ND	D
7/1/13	2874282	ND	ND	ND	ND

*ND= Not detectable

Table C – List of qPCR and end-point assays used in the study.

Name	Source	Type	Target	Forward Primer / Reverse Primer	Probe/Dye	Reference
HF183 Taqman	Human	qPCR	*Bacteroides* 16S	ATCATGAGTTCACATGTCCG / CGTAGGAGTTTGGACCGTGT	FAM-CTGAGAGGAAGGTCCCCCACATTGGA-TAMRA	Haugland et al., 2010
BacHum	Human	qPCR	*Bacteroides 16S*	TGAGTTCACATGTCCGCATGA/ CGTTACCCCGCCTACTATCTAATG	FAM-CTGAGAGGAAGGTCCCCCACATTGGA-TAMRA	Kildare et al., 2009
Gull2 Taqman	Gull	qPCR	*Catellicoccus marimammalium*	TGCATCGACCTAAAGTTTGAG/ GTCAAAGAGCGAGCAGTTACTA	FAM-CTGAGAGGGTGATCGGCCACATTGGGACT-BHQ1	Shibata et al., 2010
DogBact	Dog	qPCR	*Bacteroidales spp.*	CGC TTG TAT GTA CCG GTA CG CAA TCG GAG TTC TTC GTG	FAM-ATTCGTGGTGTAGCGGTGAAATGCTTAG-BHQ1	Sinigalliano et al., 2012
HoF597	Horse	Endpoint	*Bacteroidales spp.*	CCA GCC GTA AAA TAG TCG G CAA TCG GAG TTC TTC GTG	N/A	Dick et al., 2009

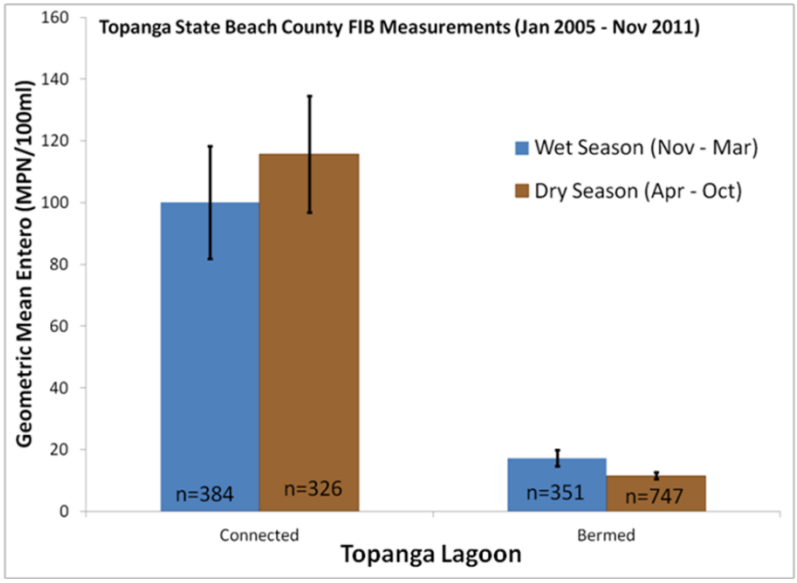


Figure A. Geometric mean of Enterococcus (MPN/100ml) connected and bermed conditions for wet and dry seasons between January 2005 – November 2011.

7. References

Boehm, A.B., Grant, S.B., Kim, J.H., Mowbray, S.L., McGee, C.D., Clark, C.D., Foley, D.M. and Wellman, D.E., 2002. Decadal and shorter period variability of surf zone water quality at Huntington Beach, California. Environmental Science & Technology 36(18), 3885-3892.

Boehm, J. Fuhrman, R. Mrse, and S. Grant., 2003. Tiered approach for identication of a human fecal pollution source at a recreational beach: case study at Avalon Bay, Catalina Island, California. Environmental Science &Technology, (37):673 – 680.

Boehm AB, et al., 2009. Covariation and photoinactivation of traditional and novel indicator organisms and human viruses at a sewage-impacted marine beach. Environ. Sci. Technol. 43:8046–8052.

Boehm, A. B., L. Van De Werfhorst, J. Griffith, P. Holden, J.A. Jay, O. Shanks, D. Wang and S. Weisberg., 2013 Performance of forthey-three microbial source tracking methods: A twenty-seven lab evaluation study. Water Research. In Press.

Bower, P.A., Scopel, C.O., Jensen, E.T., Depas, M.M. and McLellan, S.L. (2005) Detection of genetic markers of fecal indicator bacteria in Lake Michigan and determination of their relationship to Escherichia coli densities using standard microbiological methods. Applied and Environmental Microbiology 71(12), 8305-8313.

Dagit, R., S. Williams and J. Fuhrman. 2004. Topanga Creek Watershed Water Quality Study Final Report. Resource Conservation District of the Santa Monica Mountains, Topanga, CA.

Dagit, R., Krug, J., Adamek, K., Riedel, T., Zimmer-Faust, A.G., Thulsiraj, V., Jay, J.A., Braband, S., Tufto, D., Sherman, R. 2013. Topanga Source ID Study Annual Report 2012 - 2013 Report. Resource Conservation District of the Santa Monica Mountains, Topanga, CA.

Dick L.K., Bernhard A.E., Brodeur T.J., Santo Domingo J.W., Simpson J.M., Walters S.P., Field K.G., 2005. Host distributions of uncultivated fecal *Bacteroidales* bacteria reveal genetic markers for fecal source identification. Appl Environ Microbiol. 71(6):3184-91.

Dugan J.E., Hubbard, D.M., McCrary, M.D., Pierson, M.O., 2003. The response of macrofauna communities and shorebirds to macrophyte wrack subsidies on exposed sandy beaches of southern California. Estuarine Coastal Shelf Sci 58:25-40.

Flood C., Ufnar, J., Wang, S., Johnson, J., Carr, M. and Ellender, R. (2011) Lack of correlation between enterococcal counts and the presence of human specifc fecal markers in Mississippi creek and coastal waters. Water Research 45, 872-878.

GeoPentech, 2006. Hydrogeologic Study, Lower Topanga Creek Watershed, Los Angeles County. California. Resource Conservation District of the Santa Monica Mountains.

Harwood, V.J., Staley, C., Badgley, B.D., Borges, K., Korajkic, A., 2013. Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. FEMS Microbiology Reviews, 1-40. doi:10.1111/1574-6976.12031

Haugland, R.A., M. Varma, M. Sivaganesan, C. Kelty, L. Peed, O.C. Shanks. 2010. Evaluation of Genetic Markers from the 16S rRNA gene V2 Region for Use in Quantitative Detection of Selected *Bacteroidales* species and Human Fecal Waster by Real Time PCR. Syst Appl Microbiol. 33(6), 348-57.

Heal the Bay. 2013. 2012-2013 Annual Beach Report Card. Heal the Bay, Santa Monica, CA pg 79.

Ishii, S., Hansen, D.L., Hicks, R.E. and Sadowsky, M.J. (2007) Beach sand and sediments are temporal sinks and sources of Escherichia coli in lake superior. Environmental Science & Technology 41(7), 2203-2209.

Lafferty K.D., 2001. Birds at a Southern California beach: seasonality, habitat use and disturbance by human activity. Biodivers Conserv 10(11):1949-1962.

Lu J, Santo Domingo JW, Lamendella R, Edge T, Hill S. (2008) Phylogenetic diversity and molecular detection of bacteria in gull feces. Appl Environ Microbiol. Jul;74(13):3969-76.

Noble, R.T., Moore, D.F., Leecaster, M.K., McGee, C.D. and Welsberg, S.B. 2003. Comparison of total coliform, fecal coliform, and enterococcus bacterial indicator response for ocean recreational water quality testing. Water Research 37(7), 1637-1643.

Noble, R.T., Griffith, J.F., Blackwood, A.D., Fuhrman, J.A., Gregory, J.B., Hernandez, X., Liang, X.L., Bera, A.A. and Schiff, K., 2006. Multitiered approach using quantitative PCR to track sources of fecal pollution affecting Santa Monica Bay, California. Applied and Environmental Microbiology 72(2), 1604-1612.

Reischer, G.H., Haider, J.M., Sommer, R., Stadler, H., Keiblinger, K.M., Hornek, R., Zerobin, W., Mach, R.L. and Farnleitner, A. (2008) Quantitative microbial faecal source tracking with sampling guided by hydrological catchment dynamics. Environmental Microbiology 10(10), 2598-2608.

Sercu, B., VanDeWerfhorst, L., Murray, J. and Holden, P. (2009) Storm Drains are Sources of Human Fecal pollution During Dry Weather in Three Urban Southern California watersheds. Environmental Science and Technology 43 (2), 293-298.

Shanks, O.C., C.A. Kelty, M. Sivaganesan, M. Varma, and R.A. Haugland. 2009. Quantitative PCR for Genetic Markers of Human Fecal Pollution. Applied and Environmental Microbiology 75:5507-5513.

Sinigalliano, C.D., Ervin, J.S., Van De Werfhorst, L.C., Wang, D., Wanless, D., Bartkowiak, J., Layton, B., Raith, M., Schriewer, A., Badgley, B.D., Lee, C., Goodwin, K.D., Lee, J., Boehm, A.B., Noble, R.T., Holden, P.A., Jay, J.A., Wuertz, S., Byappanahalli, M.N., Whitman, R.L., Sadowsky, M.J., Meijer, W., Balleste, E., Gourmelon, M., Griffith, J.F., Ryu, H., Domingo, J.W.S., 2013. Multi-laboratory assessment on the performance of PCR assays targeting Catellicoccus marimammalium for microbial source tracking of coastal birds. Water Research In Press.

Stone Environmental. 2004. Risk Assessment of Decentralized Wastewater Treatment Systems in High Priority Areas in the City of Malibu, California. Final Report August 30, 2004. Prepared for the Santa Monica Bay Restoration Commission by Stone Environmental Inc. Montpelier, VT.

Surbeck, C.Q., Jiang, S.C., Ahn, J.H. and Grant, S.B. (2006) Flow fingerprinting fecal pollution and suspended solids in stormwater runoff from an urban coastal watershed. Environmental Science & Technology 40(14), 4435-4441.

Yamahara, K.M., Walters, S.P. and Boehm, A.B. (2009) Growth of Enterococci in Unaltered, Unseeded Beach Sands Subjected to Tidal Wetting. Applied and Environmental Microbiology 75(6), 1517-1524.

Chapter 6. Summary: Evaluation and performance of rapid methods for identifying and tracking sources of fecal pollution in coastal watersheds

This work aims to bridge gaps in current research on effective methods for assessing water quality and tracking sources of fecal contamination. Several methods show promise to improve monitoring practices, including those presented in this study. At many beaches, sources contributing to exceedances in FIB water quality standards are unknown. qPCR assays can help differentiate between animal and human sources, allowing for more targeted remediation efforts. The addition of a propidium monoazide (PMA) treatment step to qPCR enables viable quantification, further advancing the science to quantify recent contamination events in environmental waters. In addition, the covalently-linked immunomagnetic separation/adenosine triphosphate (Cov-IMS/ATP) technique is another rapid viability-based method that can be used for near real time detection of fecal pollution, and shows promise for assessing water quality in complex watersheds along the Southern and Baja California coastline.

Application of microbial source tracking (MST) techniques has gained popularity in the field, with the emergence of several new end-point and quantitative PCR assays for identification of host-associated markers. The Source Identification Protocol Project (SIPP) was a study used to test 41 different MST methods across 27 different labs, including the Jay Lab. A critical step in evaluating the application of host markers is to test performance across different water matrices. In Chapter 2, we extend upon previous work conducted in the SIPP study (which tested assays in artificial water only). We compared performance of two human and two gull assays for transferability across different water matrices (artificial, fresh and marine waters), modeled limits of detection and provided suggestions on benefits of use for watershed managers based on cost-analysis of each assay.

In Chapters 3 and 4, we show the Cov-IMS/ATP method was used successfully used in quantifying levels of enterococci for assessing water quality. We optimized the Cov-IMS/ATP assay and present a predictable empirical relationship between the Cov-IMS/ATP method and traditional culture-based methods, which allows for more widespread application of this rapid and field portable technique. To our knowledge, this is the first study to test the specificity of Cov-IMS/ATP against *Enterococcus* species and cross-reacting organisms.

In Chapters 4 and 5, we validate the use of a suite of host-markers to identify important sources impairing water quality in two different watersheds. Although limited studies have applied source tracking in Mexico, this study shows host-associated markers worked well in a complex sub-drainage basin with multiple source inputs at a field site in Tijuana, MX. In chapter 4 we also show that rapid viability-based methods are important for accurately assessing fecal contamination adjacent to a wastewater treatment plant. Reduced correlation between FIB and human-associated markers was likely the result of qPCR measuring dead DNA from treated wastewater effluent at our field site. The viability based PMA-qPCR assay for HF183 SYBR tested in this study was able to capture dynamic changes in FIB and shows improved performance in assessing recent fecal contamination events in environmental waters associated with sewage treatment.

In the future we hope to test the Cov-IMS/ATP for quantifying more human-associated organisms such as *Bacteroides thetaiotaomicron*. A field-portable rapid method for detecting human contamination can greatly improve monitoring practices to rapidly detect sewage spills and inadequate sewage treatment. In addition, we hope to test the PMA-qPCR method for a range of human and animal associated qPCR assays. This will help validate the use of the viability-based PMA-qPCR technique to detect recent contamination from a variety of fecal

hosts, and can provide critical information about the relationship of qPCR markers, FIB and aging fecal material.