Monitoring and Mitigation to Address Fecal Pathogen Pollution along California Coast

Proposition 50 Coastal Management Program
California State Water Board Agreement No. 06-076-553

May 31, 2011

By

Applied Marine Sciences, Inc.

University of California Davis

California Department of Fish and Game
Marine Wildlife Veterinary Care and Research Center

Under the auspices of

Central Coast Long-term Environmental Assessment Program

CLEAN

Final Report

Monitoring and Mitigation to Address Fecal Pathogen Pollution along California Coast

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Submitted to:

California Regional Water Quality Control Board

Region 3

895 Aerovista Place, Suite 101

San Luis Obispo, CA 93401

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1. Executive Summary

1.1. Report Organization

Coastal waters worldwide have been significantly influenced by human activities, as they are adjacent to densely populated areas and support a wide range of transport, commercial and recreational uses. Urbanization-associated impairments of nearshore water quality can result from enrichment of nearshore marine waters by nutrients and chemical and biological pollutants that are transported from terrestrial watersheds to the ocean in ever-increasing quantities. . Even after reaching the ocean this pollutant load poses health risks to humans and animals, and the degree of risk from marine dispersal of anthropogenic chemicals and pathogens may be greatly under-estimated. Fecal indicator bacteria (FIB) that normally reside in the gastrointestinal tract of humans and animals are used throughout the world to assess the microbiological quality of drinking and recreational waters. In the United States, FIB are used to define bacterial water quality standards aimed at reducing health risks in waters used for recreation and aquaculture. Groups of standard FIB monitored in water include total and fecal coliforms, *Escherichia coli*, and enterococci, and are considered as indicators of health risk in epidemiologic and quantitative microbial risk assessment (QMRA) studies.

To date, many monitoring programs have focused only on FIB measurements and do not test for the presence or absence of known pathogens, partly due to associated costs and expertise required for pathogen testing. However, substantial evidence has been collected challenging the usefulness of FIB data as a predictor of actual disease risk from contact with polluted water. A few limitations of using standard FIB to represent pathogens in water include the fact that FIB have the ability to multiply in the environment, they are not host-specific, and absence of FIB does not necessarily mean that pathogens are also absent. Consequently, alternative indicators of fecal pollution are needed that address the weaknesses of standard FIB for protection of human health. Ideally, these indicators would decay at similar rates as pathogens, be present at high concentrations in fecal sources, and be present at low concentrations in unpolluted environments. An added benefit of using alternative indicators is that in some cases host sources of fecal contamination can be identified.

The goals of this research program were to use both laboratory and field approaches to investigate issues related to water quality monitoring and mitigation of fecal pathogen pollution along the central California coast. Our specific objectives were to 1) evaluate water quality monitoring approaches by characterizing the relationships between FIB and enteric pathogen detection in a broad range of freshwater and marine surfacewaters along the central coast, 2) consider the relative importance of fecal pathogen loading from different sources, 3) evaluate whether filter-feeding estuarine or marine invertebrates (mussels) may be better indicators of water quality than direct water testing, 4) evaluate microbial source tracking techniques to distinguish between human and animal sources of fecal pollution, 5) characterize patterns of fecal pathogen shedding among terrestrial and marine animals, and 6) evaluate wetlands as a possible Best Management Practice (BMP) to mitigate impairments and improve surface water quality with respect to fecal pathogen pollution. The results are organized according to a series of priority questions that relate to the study goals and objectives, and are summarized in the following section, with additional details provided throughout the report.

1.2. Results for each Question

- *What are the spatial and temporal patterns in fecal indicator bacteria and pathogens along the central California coast, and what is the relationship between fecal indicator bacterial concentrations and fecal pathogen detection in: wastewater influent and effluent; rivers and streams; ocean and mussels; stormwater; and wetlands?*

Fecal indicator bacteria (total coliform, fecal coliform, *Enterococcus,* and *Bacteroidales* counts) were compared with direct detection of target bacteria (*Campylobacter* spp., *Salmonella* spp., *Escherichia coli* O157, and *Vibrio* spp.) and protozoa (*Giardia* and *Cryptosporidium*) in wastewater and surface water samples during a two-year period. Wastewater influent and effluent samples were tested quarterly from four wastewater treatment facilities. These facilities varied in the volume of wastewater handled per day, as well as the technologies utilized for wastewater processing. A significant reduction of pathogens between pre- and post-treatment was noted for all 4 facilities, but with differences among facilities in removal efficiency of FIB and enteric pathogens.

Water samples from ten coastal river sites were tested monthly over two years. FIB and enteric pathogen detection was both common and widely distributed between the ten coastal rivers, although detection was not highly correlated with sampling during the wet or dry seasons. Stormwater was sampled from three sites, and ocean water and mussels were sampled quarterly from six sites. FIB and specific pathogen detection was less common in these nearshore marine samples, when compared to river or stormwater. Pathogen trends in stormwater were similar to other sample types, with the protozoa *Cryptosporidium* and *Giardia* detected most often, followed by *Salmonella* and *Vibrio* spp., and little or no *Campylobacter* or *E. coli*-O157:H7 detection.

Quarterly testing of water collected from multiple sites in the Tembladero Slough constructed wetland showed that pathogens were detected most often in the slough sourcewater and at the inflow site, and less frequently as water moved down through the wetland. The ability to predict pathogen occurrence in relation to FIB threshold levels was evaluated using a weighted PQ measure that showed the universal *Bacteroidales* genetic marker had a comparable or greater mean predictive potential than standard FIB. We found that measures of traditional indicator bacteria, including coliforms and enterococci, correlated detection of some, but not all bacterial and protozoal pathogens in this study. Collectively our study findings suggest that monitoring for indicator bacteria alone may not provide sufficient information to minimize public contact with fecal pathogens in surface waters. We recommend utilizing a combination of FIB and specific pathogen assays to provide the most useful and accurate perspective regarding the presence, relative abundance, and contributing sources of fecal contamination in environmental water samples.

- *How can fecal pathogen loading of nearshore ecosystems be compared across the full range of surface water inputs to the ocean?*

This question emerged during the course of the study as a key synthesis question that could help resource managers more accurately assess local risks from water contact, and prioritize management strategies to minimize coastal pollution. We used data collected from different sources (i.e., streams and rivers, storm runoff and wastewater), combined with data on flow rates

and the number of potential loading sources by area, to make preliminary estimates of relative loading and to identify gaps where future study and data compilation is needed to improve the risk estimates. In some cases, fecal pathogen inputs were identified from all three sources (streams and rivers, storm runoff and wastewater) and were not dominated by any single source. Given the inherent uncertainty in our load estimates and considering only the days on which sampling occurred, the average daily ocean input of *Vibrio cholerae* was significantly greater for streams and rivers, while wastewater was the major contributor for *Giardia.* In contrast, *Cryptosporidium* and *V. parahaemolyticus* inputs were not significantly different among sources. Collectively our data suggest that discharge of pathogens in water originating from streams, rivers and storm runoff poses a greater risk to human health than offshore, deep-water discharges of wastewater effluent along the central California coast due to the absence of water treatment, limited pathogen dilution, and direct shoreline discharge patterns associated with the inland surface water sources.

- *What is the relationship between exceedences of water quality objectives for fecal indicator bacteria (FIB) and fecal pathogen detection in surface water samples?*

California has set cutoffs for FIB counts to ensure public safety during water contact recreation and consumption of shellfish harvested from surface water bodies. In the current study, stormwater samples most commonly exceeded water quality (FIB) criteria, followed by river/stream/slough samples, and finally ocean water. Associations between FIB exceedances and specific pathogen detection varied by water sample type and pathogen group. Of all target pathogens, only *Cryptosporidium* detection was significantly associated with total coliform levels that exceeded current water quality criteria cutoffs. High fecal coliform counts were more closely associated with the presence of specific pathogens in surface water: *Cryptosporidium, Giardia, Salmonella*, and *V. parahaemolyticus* detection were all significantly associated with fecal coliform exceedences, while high enterococcal counts were predictive of *Giardia* and *V. parahaemolyticus* detection in surface water. These findings generally support the continued use of water quality criteria using FIB cutoffs for predicting health risks during recreational water contact and shellfish harvest. However, the lack of association between presence of some pathogens and FIB exceedences supports the concept that "absence of evidence is not necessarily evidence of absence", meaning that enteric pathogens may still be present in surface waters with acceptable FIB levels, as was observed in the current study. This finding underscores the need to consider using multiple, or alternative water quality monitoring practices to improve our ability to predict pathogen presence and minimize health risks associated with water contact. Quantitative Microbial Risk Assessment is one framework that can be used to more comprehensively consider, characterize, and predict health risks associated with different beneficial uses.

- *Are mussels better indicators of ocean microbial water quality than seawater?*

In the current study we compared time- and location-matched mussel sampling with collection and processing of 20 L volumes of seawater. Shellfish, including mussels, clams and oysters have all been suggested as more sensitive bioindicators of water quality in aquatic ecosystems, when compared to water "grab" samples. However, using whole mussel homogenates we found no significant difference in pathogen detection between time- and location-matched mussels and seawater. For example, the prevalence of *Giardia* and *Vibrio* species detection differed by less than 2%. However, some notable differences were observed:

Campylobacter and *Salmonella* were detected in seawater when mussels tested negative, with 10% and 5% pathogen prevalence, respectively, in seawater. Similarly, *Cryptosporidium* oocysts were detected in 26% of seawater samples, but only 6% of mussel batches. Based on the weather and water quality characteristics present during our sampling efforts, we suggest that bivalves may be most useful as bioindicators when sampled during "high-risk" periods for fecal contamination of aquatic ecosystems, such as during or after storm events.

- *Which of three microbial source tracking methods is most promising and what can be learned about trends in human versus animal sources of fecal pollution?*

Evaluating microbial source tracking (MST) techniques for distinguishing human from animal sources of fecal pollution along the central California coast was deemed important because the approaches for remediating human as compared to animal sources of fecal contamination are different, and because new molecular approaches are providing insights on source tracking that were previously unavailable with traditional phenotypic characterization methods. The three MST methods evaluated were 1) *Bacteroidales* assays, 2) an *Enterococcus* surface protein (esp) gene assay, and 3) total to fecal coliform ratios in water samples. The comparative study showed that the *Enterococcus esp* assay and total: fecal coliform ratios did not perform as well and do not show as much promise as *Bacteroidales* for future MST work. Based on the comparative MST results, *Bacteroidales* host-specific qPCR was then used to quantify fecal bacteria in water and provide insights into contributing host fecal sources. More than140 surface water samples from 10 major rivers and estuaries within the Monterey Bay region were tested during 14 months with four *Bacteroidales*-specific assays (universal, human, dog, and cow). Bayesian conditional probability analysis was used to characterize the performance of *Bacteroidales* assays, and the ratios of concentrations determined using host-specific to universal assays indicated that fecal contamination from human sources was more common than livestock or dog sources in the coastal study sites.

- *What are the patterns and risk factors for fecal pathogen shedding from central coast animals, and are the same types of fecal pathogens detected in sea otters as are detected in other marine and terrestrial animals?*

Feces from domestic and wild animals were tested to determine the prevalence and genotypes of selected pathogens in the Monterey Bay region. Of 808 fecal samples tested between 2007 and 2010, 28% were positive for one or more target pathogens, and many of the same species detected in terrestrial animals were also isolated from sea otters. *Giardia* spp. were isolated most frequently, with an overall animal prevalence of 15%, followed by *Campylobacter* spp. (11%), *Vibrio cholerae* (9%), *Cryptosporidium* spp. (6%), *Salmonella* spp. (6%), and *Vibrio parahaemolyticus* (5%). Molecular characterization of *Giardia* and *Cryptosporidium* revealed both zoonotic and host-specific genotypes. Fifteen different *Salmonella* serotypes were detected, 11 of which were isolated from opossums, a non-native species introduced to coastal California. Risk factors associated with pathogen detection in animal feces included animal group, age class, gender, live-dead status, and season. These study findings provide insights that may be used to help prioritize animal management and water quality monitoring strategies.

- *Are wetlands effective in reducing fecal pathogen loads in surface water, and, if so, what wetland characteristics are most important to achieve pathogen reduction?*

Wetlands evaluation involved both controlled laboratory trials and field experiments. First, laboratory mesocosm tank models that simulated coastal wetlands were used to study specific variables believed to reduce the load of fecal pathogens present in contaminated runoff as it flows through a wetland. By introducing known quantities of specific pathogens at the inflow, and collecting samples under varying climatic and wetland restoration conditions (e.g., wetland length, vegetation configuration, salinity, flow rate), we determined the effects of these variables on reduction of pathogen concentrations in water traveling through the model wetlands. These studies revealed that the presence of vegetation enhanced removal of oocysts from fecally-polluted water at both fast and slow flow rates. The important role of vegetation in removal of waterborne protozoa should be considered in wetland reconstruction and management efforts for coastal ecosystems. Similar water measurements were conducted during quarterly testing at a reconstructed wetland at Tembladero Slough, providing a larger scale, "real world" model of the ability of coastal wetlands to reduce fecal pathogen loads in surface waters. These larger-scale findings indicate that both the distance from various point source(s) of contamination and periodic rainfall events influence the efficiency of pathogen reduction in natural systems.

Considered collectively, our study findings provide important new insights for water quality managers working at all levels and in multiple disciplines. These include specific suggestions for improving water quality monitoring and mitigation efforts in order to optimize the balance between coastal development and safety of coastal marine waters for recreation, shellfish harvest and other beneficial uses such as threatened and endangered species protection. Publications and outreach materials related to this project will be posted on the website www.pathogenpollution.org as they become available.

2. Background

2.1. CCLEAN Program Description

2.1.1. Objectives

The Management Plan for the Monterey Bay National Marine Sanctuary includes a Memorandum of Agreement among eight federal, state, and regional agencies (including the Central Coast Regional Water Quality Control Board) to develop an ecosystem-based Water Quality Protection Program for the Sanctuary. The CCLEAN (Central Coast Long-term Environmental Assessment Network) monitoring program was designed to assist these agencies and fulfill several regulatory objectives. The Regional Board has developed a framework for partial fulfillment of this Water Quality Protection Program called the Central Coast Ambient Monitoring Program (CCAMP). This multidisciplinary program includes sampling in watersheds that flow into coastal regions, in estuarine coastal confluences, and at coastal sites. The goal of CCAMP is to "collect, assess, and disseminate scientifically-based water quality information to aid decision-makers and the public in maintaining, restoring, and enhancing water quality and associated beneficial uses." CCLEAN provides the initial nearshore component of CCAMP. It is being funded by the City of Santa Cruz, City of Watsonville, Moss Landing Power Plant, Monterey Regional Water Pollution Control Agency, and Carmel Area Wastewater District, under the direction of the Regional Board. CCLEAN satisfies the NPDES receiving water monitoring and reporting requirements of program participants.

Within the framework of CCAMP, the goal of the CCLEAN program is to assist stakeholders in maintaining, restoring, and enhancing nearshore water and sediment quality and associated beneficial uses in the Central Coast Region. The specific objectives of the program are as follows:

- Obtain high-quality data describing the status and long-term trends in the quality of nearshore waters, sediments, and associated beneficial uses.
- Determine whether nearshore waters and sediments are in compliance with the Ocean Plan.
- Determine sources of contaminants to nearshore waters.
- Provide legally defensible data on the effects of wastewater discharges in nearshore waters.
- Develop a long-term database on trends in the quality of nearshore waters, sediments and associated beneficial uses.
- Ensure that the nearshore component database is compatible with other regional monitoring efforts and regulatory requirements.
- Ensure that nearshore component data are presented in ways that are understandable and relevant to the needs of stakeholders.

CCLEAN has historically supported and collaborated on research related to water quality issues in the Monterey Bay area. These have included a study of the relationship between

persistent organic pollutants and morbidity and mortality in southern sea otters, funded through a grant from the California State Water Resources Control Board (Miller et al, 2007), as well as the current study.

2.2. Proposition 50 Grant Program

2.2.1. History

In the November 5, 2002 election, California voters considered a ballot measure named Proposition 50, which asked the following question: "Should the state borrow three billion four hundred forty million dollars ($3,440,000,000) through the sale of general obligation bonds for a variety of water projects including coastal protection, the CALFED Bay-Delta Program, integrated regional water management, safe drinking water, and water quality?" Voters approved Proposition 50 by a vote of 55.3% to 44.7%.

2.2.2. Focus

Of the total funds authorized by Proposition 50, $100,000,000 was allocated for the purpose of financing projects that restore and protect the water quality and environment of coastal waters, estuaries, bays, near-shore waters and groundwater. Of this sum, not less than $20,000,000 was required to be expended to “implement priority actions specified in the Santa Monica Bay Restoration Plan.” The remaining $80,000,000 allocated for coastal water quality was required by the proposition to be divided 60/40 between southern and northern coastal California, respectively, for distribution to successful grant applicants through a competitive application process. Funds were allocated for annual grant application cycles only as money from bond sales was available and in the 2005–2006 consolidated grants program of the Division of Financial Assistance of the State of California Water Resources Control Board, $26,900,000 was available for projects in coastal counties north of Ventura County.

2.2.3. Application Process

Shortly before the 2005–2006 grant application cycle, CCLEAN became aware of recent studies that reported a correlation between proximity to coastal freshwater discharges and an increased risk of *Toxoplasma gondii* infection in southern sea otters (*Enhydra lutris nereis*) (Miller et al., 2002). Because of the wide public interest in this pathogen at the time, CCLEAN participants decided to seek grant funding under Proposition 50 to study sources of *T. gondii* and other fecal pathogens along the central California coast. Following exploratory discussions with researchers in the field of marine wildlife pathogens, a team was formed from scientists at University of California at Davis, California Department of Fish and Game Marine Wildlife Veterinary Care and Research Center and Applied Marine Sciences, Inc. to apply for a Proposition 50 grant in the 2005–2006 application cycle. Through the efforts of State Water Board staff, the conceptual proposal for this study was considered under a special segment of the Proposition 50 funds designated for ocean protection, for which applications received expedited review. On February 9, 2006, a concept proposal was submitted and on March 14, 2006 the conceptual proposal was approved for submittal of a full proposal. This proposal was submitted on May 9, 2006 and on June 21, 2006, following a recommendation from the California Ocean Protection Council, the California State Water Resources Control Board approved a resolution to fund the project.

The original grant agreement required that all work be completed by March 1, 2010. However, when the California economic crisis occurred in 2008, sales of State bonds declined and work on all grants was suspended on December 22, 2008. With the eventual improvement in

the State's fiscal condition, the fecal pathogen project was given approval to re-start on January 6, 2010. Subsequently, a request was made and approved for a no-cost extension of the project through May 31, 2011. Individual responsibilities and contact information for members of the fecal pathogen project are listed in tables 1 and 2.

3. Project Description

3.1. Team

Table 1. Project team.

Responsibility	Name (Affiliation)	Telephone Number
Project Manager	Dane Hardin (CCLEAN)	831-426-6326
QA Officer	Bill Ray (SWRCB)	916-341-5583
State Board Contract Manager	Mark Magtoto	916-341-5481
City of Watsonville, Project Contact	Bob Geyer (City of Watsonville)	831-768-3179
Co-PI, Bacteriology Faculty	Barbara Byrne (UCD)	530-754-6286
Co-PI, Protozoology Faculty	Patricia Conrad (UCD)	530-752-7210
Co-PI, Project Coordinator	Woutrina Miller (UCD)	530-219-1369
Co-PI, Coastal Fieldwork, Mitigation Testing	David A. Jessup (CDFG retired)	831-469-1726
Co-PI, Coastal Fieldwork, Mitigation Testing	Melissa Miller (CDFG)	831-469-1746

Table 2. Monitoring and Mitigation to Address Fecal Pathogen Pollution along California Coast personnel responsibilities.

Name	Affiliation	Title	Contact Information
Dane Hardin	CCLEAN	Program Manager	(831) 426-6326 hardin@amarine.com
Woutrina Miller	UC Davis	Co-PI, Program Coordinator	(530) 219-1369 wamiller@ucdavis.edu
Melissa Miller	CDFG	Co-PI, Coastal Fieldwork	(831) 469-1746 mmiller@ospr.dfg.ca.gov
Barbara Byrne	UC Davis	Co-PI Bacteriology	(530) 754-6286 bbyrne@ucdavis.edu
Patricia Conrad	UC Davis	Co-PI, Protozoology	(530) 752-7210 paconrad@ucdavis.edu
David A. Jessup	CDFG (retired)	Co-PI, Coastal Fieldwork	(831) 469-1726
Stefan Wuertz	UC Davis	Co-investigator, Microbial Source Tracking	(530) 754-6407 swuertz@ucdavis.edu
Stori Oates	CDFG	Staff Research Associate, Coastal Fieldwork	(831) 234-7478 storioates@yahoo.com

Name	Affiliation	Title	Contact Information
Ann Melli	UC Davis	Staff Research Associate, Protozoology	(530) 754-6144 acmelli@ucdavis.edu
Nadira Chouicha	UC Davis	Staff Research Associate, Bacteriology	(530) 752-8211 nchouicha@ucdavis.edu
Clare Dominik	Applied Marine Sciences Inc.	Consulting Research Associate, Coastal Fieldwork	(831) 426-6326 dominik@amarine.com
Juanita Lupercio Ortega	UC Davis	Outreach Coordinator	(530) 220-2864 jlupercio7@msn.com

3.2. Goals and Objectives

The central California coast has an especially rich tourism industry, attracted by the diverse estuarine and marine resources of the region, including extensive sandy beaches and scenic, rocky coastlines. In addition, this area offers ample opportunity for water-contact sports such as swimming, surfing, kayaking, and wildlife viewing. Fisheries also exist locally for harvest of marine-origin foods for human consumption including shellfish, crustaceans, squid, fish, and kelp.

Fecal pollution by terrestrial-origin bacteria and parasites significantly impairs coastal beneficial uses throughout California by causing beach closures and disease of humans and protected marine species. Between 2000 and 2002, the number of days of beach closure for Santa Cruz and Monterey County almost tripled, from 3.9 to 11.8 beach-mile days. This finding is substantiated by recent CCLEAN data indicating that most coastal streams between the San Lorenzo and Salinas Rivers, inclusive, have exceeded the proposed Basin Plan Amendment for concentrations of *Escherichia coli* (CCLEAN, 2005). Fecal-origin biological pollutants also appear to be negatively impacting the health of southern sea otters. As a federally protected threatened species, the survival and maintenance of southern sea otters must be supported by the quality of California coastal waters. Just as importantly, through their biology and diet, these animals serve as key biological indicators of nearshore coastal pollution and could be ideal sentinels for water-associated human health risks.

Despite decades of protection, the southern sea otter population has demonstrated an alarmingly slow rate of recovery and multiple periods of population decline. Elevated mortality due to infectious disease, including disease associated with terrestrial-origin protozoa and bacteria appears to be one factor limiting southern sea otter recovery (Kreuder et al., 2003; Thomas and Cole, 1996). Fecal pathogens isolated from dead and dying sea otters from nearshore marine ecosystems appear to have terrestrial origins and could be associated with coastal development, wetlands ablation, and coastal wastewater discharge (Conrad et al., 2005; Miller et al., 2002, 2005c, 2006). Baseline data collected on selected enteric bacteria and protozoa from live and dead sea otters throughout the central California coast since 2000 has confirmed the presence of a wide range of enteric bacterial pathogens in sea otters, including *Campylobacter* spp., *Salmonella* spp., and *Vibrio* spp. (including *V. parahaemolyticus* and *V. cholerae*) (Miller et al., 2009), and the protozoal pathogens *Cryptosporidium* and *Giardia*

(Miller, unpublished). Fatal systemic infections due to some of these pathogens have also been documented in sea otters (M. Miller, B. Byrne unpublished). Many sea otter pathogens are similar or identical to fecal pathogens that cause illness in humans and terrestrial animals. Collectively these data indicate significant impairment to the water-contact recreation beneficial use and sea otters along the central California coast, though contributing sources and sustainable solutions to mitigate fecal pollution are not well understood.

The goals of this research were to utilize both laboratory and field approaches to help clarify the extent of these problems, to evaluate both traditional and non-traditional measures of coastal water quality and to optimize efforts for mitigation of fecal pathogen pollution along the central California coast. These study findings are also relevant to other coastal watersheds in California and beyond, and will produce practical approaches and preliminary data that may indirectly benefit stakeholders and researchers around the world. Our specific objectives were to 1) evaluate water quality monitoring approaches by characterizing relationships between fecal indicator bacteria and specific pathogen detection in different types of surface water along the central coast, 2) consider the relative importance of fecal pathogen loading from different sources, 3) evaluate whether mussels may be better indicators of water quality than water testing, 4) characterize pathogen removal efficiency in wastewater treatment plants, 5) evaluate microbial source tracking techniques to distinguish between human and animal sources of fecal pollution, 6) characterize patterns of fecal pathogen shedding among terrestrial and marine animals, and 7) evaluate wetlands as a possible Beneficial Management Practice (BMP) to mitigate and improve surface water quality with respect to fecal pathogen pollution along the central coast.

3.3. Pathogen Indicators and Fecal Pathogens Studied

The project first compared traditional indicators of fecal contamination (total coliform, fecal coliform, and *Enterococcus* counts) with methods for direct detection of bacterial (*Campylobacter* spp., *Salmonella* spp., *Escherichia coli* O157, and *Vibrio* spp.) and protozoal (*Cryptosporidium* and *Giardia* spp.) enteric pathogens in wastewater, surface water, ocean water and mussels, and stormwater. Wastewater influent and effluent samples were tested quarterly from four treatment facilities along the central California coast. Water from 10 coastal rivers was tested monthly, while paired ocean water and shellfish samples from 6 sites were tested quarterly. Additionally, a stormwater study focused on characterizing the fecal pathogen content of street runoff from three urban communities in the Monterey Bay region during consecutive wet seasons. For each coastal sample site, stormwater was collected 3 times sequentially during a single storm event, with 3 storms sampled in this manner for each site. Where possible the sampling at all three sites was completed during a single storm event to facilitate comparison. Three additional storms were sampled, however due to inconsistent weather timing and intensity among sample locations, sampling effort varied by storm event and sample site (1 or 2 samples per site or less could be sampled per storm). Data from all sources were analyzed for trends in indicator and pathogen detection based on environmental variables such as local precipitation, as well as clustering in space and time. Bacterial detection was conducted using standard methodologies that utilize membrane filtration and dilutional plating onto selective media (APHA, 2005). Detection of *Cryptosporidium* and *Giardia* spp. utilized Envirocheck filtration, immunomagnetic separation and direct immunofluorescent quantification according to EPA Method 1623 (Miller 2005 a, b; USEPA, 2005). Isolates from the environmental monitoring were compared molecularly to isolates from terrestrial animals and sea otters to determine

whether the same types of pathogens were present, supporting the hypothesis that fecal pathogen pollution was flowing from land to sea, and that sea otters act as sentinels of nearshore ecosystem health.

A complementary aim was to evaluate several promising microbial source tracking (MST) techniques for distinguishing human from animal sources of fecal pollution along the central California coast. This was important because the approaches for remediating human, as compared to animal, sources of fecal contamination are different, and because new molecular approaches are providing tools for source tracking that may be more precise than with traditional phenotypic characterization methods. The first MST method was based on a recent report that fecal *Bacteroidales* bacteria from humans have unique signatures that are distinct from animal fecal sources (Shanks et al., 2006). The second technique evaluated the utility of amplifying the *Enterococcus* surface protein (*esp*) gene to differentiate human from animal fecal sources (Scott et al., 2005). The third technique was based on a correlation between the ratio of total to fecal coliforms that has been associated with human health risks and sources of fecal pollution (Haile et al., 1999). The MST techniques initially were evaluated and compared using published literature, elicited expert opinions, and known isolates collected during the terrestrial animal/otter/human fecal studies described above. Additionally, water samples for MST analysis were collected from all 10 of the freshwater monitoring sites and analyzed for patterns in space and time. This work extends previous findings by application to the marine environment in a new geographic region, and discusses the strengths and limitations of each method. In addition to the bacterial MST techniques, the genotyping of *Cryptosporidium* and *Giardia* protozoa provided additional insights into human versus animal sources of fecal contamination in environmental samples.

Wetlands evaluation involved both controlled laboratory trials and field experiments. First, laboratory mesocosm tank models that simulated coastal wetlands were used to study specific variables believed to reduce the load of fecal pathogens present in contaminated runoff as it flows through a wetland. By introducing known quantities of specific pathogens at the inflow, and taking samples under varying climactic and wetland restoration conditions (e.g. wetland length, vegetation configuration, vegetation type, salinity, flow rate), we studied the effects of these variables on microbe concentrations in water traveling through the model wetlands. Similar measurements were then performed during two years of quarterly testing at field sites at the Tembladero Slough wetlands project near Moss Landing, CA, to provide a more natural evaluation of the ability of coastal wetlands to reduce fecal pathogen loads in surface water. Studies in other parts of the United States have suggested that wetlands may be effective tools for reducing non-point source pollution flowing from land to sea (Karim et al., 2004; Kay et al., 2005; Quinonez- Diaz et al., 2001; Thurston et al., 2001). We also examined how differences in the ecology of bacterial and protozoal pathogens affected their removal from water flowing through wetlands.

3.4. Questions to be answered

- What are the spatial and temporal patterns in FIB and enteric pathogen detection along the central California coast, and what is the relationship FIB concentrations and enteric pathogen detection in: wastewater influent and effluent; rivers and streams; ocean and mussels; stormwater; and wetlands?
- How can fecal pathogen loading of nearshore ecosystems be compared across the full range of surface water inputs to the ocean?

- What is the relationship between exceedences of water quality objectives for FIB and enteric pathogen detection in surface water samples?
- Are mussels better indicators of ocean microbial water quality than seawater?
- Which of three microbial source tracking methods is most promising and what can be learned about trends in human versus animal sources of fecal pollution?
- What are the patterns and risk factors for fecal pathogen shedding from central coast animals, and are the same types of fecal pathogens detected in sea otters as are detected in other marine and terrestrial animals?
- Are wetlands effective in reducing fecal pathogen loads in surface water, and, if so, what wetland characteristics are most important to achieve pathogen reduction?

3.5. Project Scope

3.5.1. *Field Methods*

Multiple sampling and sample processing methods were used to complete the proposed research, depending on the sample type and project objectives. Table 3 summarizes the sample types and collection techniques used to complete the proposed work. The full range of field sampling efforts that were undertaken during this study are summarized in Table 4.

Table 3. Overview of sample types and collection techniques.

Sample Type	Sampling Method
Wastewater Influent and Effluent	Grab sample, 24 Hour Composite
Stormwater	Grab sample
Mussels	Hand collected
Surface water	Grab sample
Terrestrial animal & sea otter feces	Hand collected
Wetlands water	Grab sample

Table 4. Sample types, test parameters, sampling frequency and applicable water-quality stressors.

Sampling Activity	Parameters Sampled at Each Site	Frequency of Sampling	Applicable Water-quality Stressors
Surface Water and Effluent			
Collect monthly water samples from approximately 10 field sites in the Monterey Bay region for comparison of fecal indicators and direct pathogen testing and nutrient testing.	FIB: total coliform, fecal coliform, and *Enterococcus* counts. Pathogens: *Cryptosporidium* spp., *Giardia* spp., *Campylobacter* spp., *Escherichia coli*-O157, *Salmonella* spp., and *Vibrio cholerae*. Isolates archived for use in the molecular characterization studies.	Monthly	Pathogens
Collect stormwater samples from 3 storm drains in each of 3 urban communities along the Monterey Bay during 3 storms in Year 2.	FIB: total coliform, fecal coliform, and *Enterococcus* counts. Pathogens: *Cryptosporidium* spp., *Giardia* spp., *Campylobacter* spp., *Escherichia coli*-O157, *Salmonella* spp., and *Vibrio cholerae*. Isolates archived for use in the molecular characterization studies.	Focused sampling during 3 storms	Pathogens
Obtain quarterly wastewater influent and effluent samples from 4 treatment plants along the central California coast for analysis of treatment plant removal efficiency and comparison of FIB and pathogen testing.	FIB: total coliform, fecal coliform, and *Enterococcus* counts. Pathogens: *Cryptosporidium* spp., *Giardia* spp., *Campylobacter* spp., *Escherichia coli*-O157, *Salmonella* spp., and *Vibrio cholerae*. Isolates archived for use in the molecular characterization studies.	Quarterly	Pathogens
Paired Mussel and Water			
Collect quarterly paired mussel and water samples from three sites in the Monterey Bay region and three sites in the Estero Bay region for comparison of detection of fecal indicators and actual pathogens in water and mussels.	FIB: total coliform, fecal coliform, and *Enterococcus* counts. Pathogens: *Cryptosporidium* spp., *Giardia* spp., *Campylobacter* spp., *Escherichia coli*-O157, *Salmonella* spp., and *Vibrio cholerae*. Isolates archived for use in the molecular characterization studies.	Quarterly	Pathogens
Feces			
Obtain fecal samples from sea otters and selected terrestrial animals.	*Cryptosporidium* spp., *Giardia* spp., *Campylobacter* spp., *Escherichia coli*-O157, *Salmonella* spp., and *Vibrio cholerae*. Isolates archived for use in the molecular characterization studies.	Opportunistic	Pathogens
Wetlands			
Collect water samples for a month quarterly at the Tembladero Slough Constructed Wetland over the course of a year. Additionally, collect water samples during the controlled wetland microcosm pathogen exposure experiments.	FIB: total coliform, fecal coliform, and *Enterococcus* counts. Pathogens: *Cryptosporidium* spp., *Giardia* spp., *Campylobacter* spp., *Escherichia coli*-O157, *Salmonella* spp., and *Vibrio cholerae*. Isolates archived for use in the molecular characterization studies.	Quarterly	Pathogens

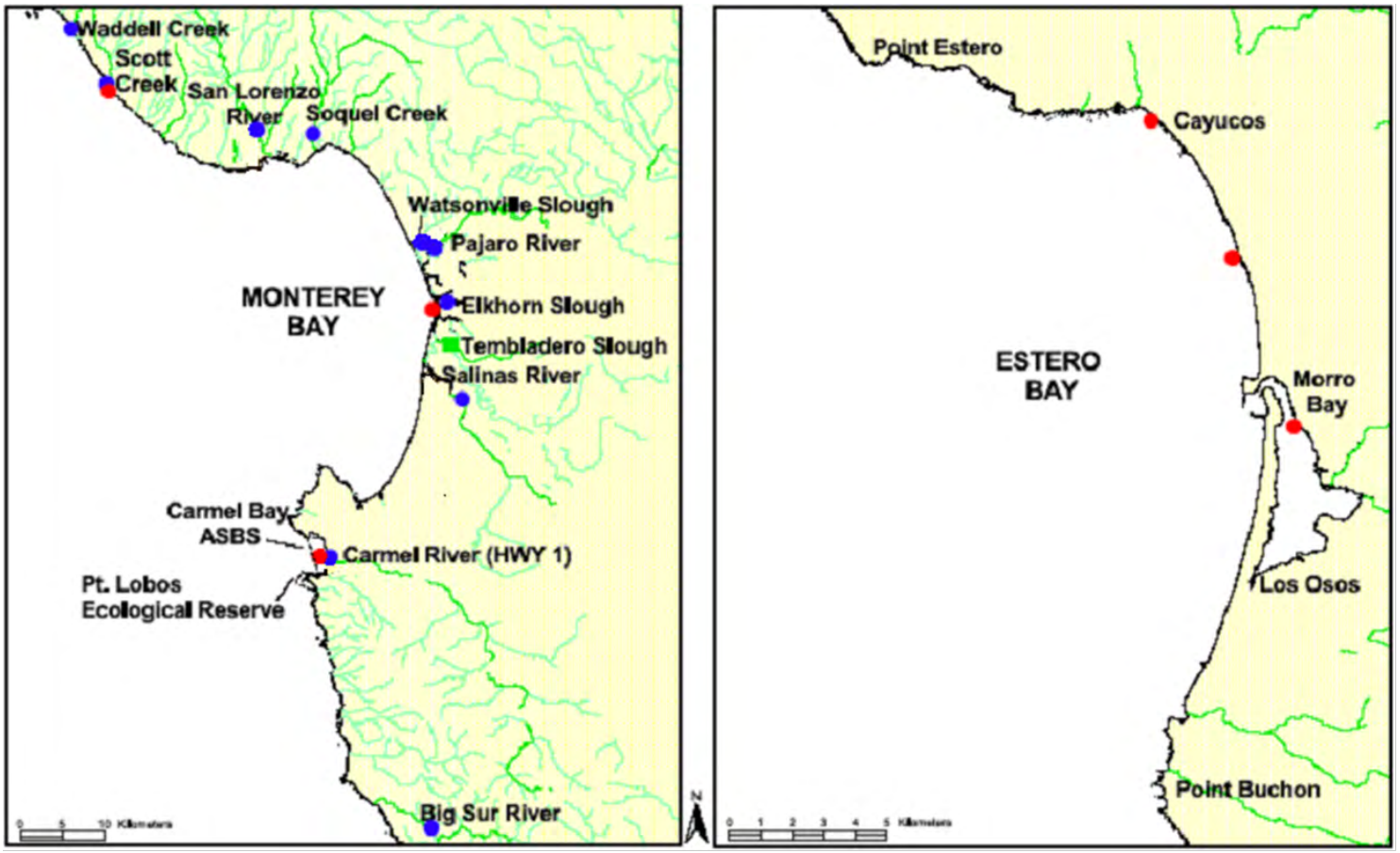


Figure 1. Location of sampling sites for wastewater, surface water, ocean water, mussels, and constructed wetland

3.5.1.1. Influent and Effluent Sampling of Wastewater

Quarterly samples of wastewater influent and effluent from each of the four treatment plants were collected for analysis of bacterial indicators and bacterial and protozoal pathogens. The four wastewater treatment plants will only be identified by number (1-4). Grab samples were collected by treatment plant personnel at spigots built into each treatment plant for sample collection. Prior to sampling all sterile 2 L sample bottles were labeled and had 1mL per 1 L of sample of sodium thiosulfate (for dechlorinization) added prior to delivery of the bottles to each plant. After collection in the field, samples were placed directly into a cooler with wet or blue ice and delivered to the California Department of Fish and Game (CDFG) Marine Wildlife Veterinary Care and Research Center (MWVCRC) for bacterial and protozoal filtration. Protozoal testing was performed based on EPA Method 1623 that involves using a peristaltic pump to filter the 2 L wastewater samples through Envirochek filters to concentrate the oocysts and cysts before transport to the UC Davis laboratories for further processing. Bacterial testing used standard membrane filtration methods to filter a series of dilution volumes through 45 μm filters that were then placed on transport media and kept chilled and delivered for further processing at the UC Davis laboratories the next day. Protozoal and bacterial filtration was performed in a hood using sterile technique. All wastewater samples were collected and filtered on the same day. The bacterial plates and protozoal filters were packed on blue ice and delivered to the laboratory at UC Davis for analysis within 24 hours.

3.5.1.2. Rivers and Streams

Stream sampling included collection of monthly surface water grab samples from 10 sites for analysis of fecal indicators and specific bacterial and protozoal pathogens. Locations of each sample site are shown in Table 5 and Figure 1. Samples were collected during daylight hours when outgoing tides reduced the marine influence at the stream mouths, where applicable. Three two-person teams (North, Middle, and South) were used to ensure that all samples were collected at about the same time and on the same day. Each two-person team had dedicated coolers for each of their assigned sites with 10 pre-labeled 2 L sample bottles (5 for bacteria and 5 for protozoa) and double-bagged wet ice. After collection in the field, samples were immediately processed at the CDFG-MWVCRC the same day. Water samples from field collection bottles were combined and mixed in 10L sterile carboys before bacterial and protozoal testing. Protozoal testing for *Cryptosporidium* and *Giardia* was performed based on EPA Method 1623, filtering the 10L surface water samples through Envirochek filters to concentrate the oocysts and cysts before transport to UC Davis for further processing. Bacterial testing for standard indicator bacteria (fecal coliforms, total coliforms and *Enterococcus*), as well as for bacteria with pathogenic potential (*Campylobacter jejuni, Salmonella enterica, Escherichia coli* O157:H7, *Vibrio cholerae*, and *Vibrio parahaemolyticus)* utilized standard membrane filtration methods to filter a series of dilution volumes through 45 μm filters that were then placed on transport media, refrigerated and delivered for further processing at the UC Davis laboratories the following day.

Additionally, water samples for *Bacteroidales* microbial source tracking analysis were collected in 2L sterile bottles, refrigerated, and transported overnight to UC Davis for processing and testing. *Bacteroidales* present in the water samples were concentrated using hollow-fiber ultrafiltration (HFF) as described previously (Rajal et el., 2007). The surrogate *Acinetobacter baylyi* ADP1 was added into all samples. Filtration recoveries were calculated by measuring concentrations of *A. baylyi* in subsamples of pre-filtration and post filtration samples. The water was pumped through an ultrafiltration unit with a 50,000 MW membrane cut-off (Microza AHP 2010, Pall Life Sciences, East Hills, NY, USA), until the volume was reduced to approximately

50 mL. Two elution steps of the filter module and the system with 0.05 M glycine/NaOH and 0.1% Tween 80 were performed to increase recovery of organisms. The combined retentate and eluates yielded a volume of 100-200 mL and were subjected to DNA extraction and subsequent quantification using qPCR techniques.

Table 5. Locations of stream water monitoring sites for fecal pathogen project.

Site	Location	Latitude	Longitude
Waddell Creek	at Hwy 1	37.09635	-122.27780
Scott Creek	at Hwy 1	37.04070	-122.22910
San Lorenzo River	at Laurel Street Bridge	36.96960	-122.02268
Soquel Creek	at Nob Hill Market	36.97313	-121.95392
Watsonville Slough	at Beach Road	36.86917	-121.81678
Pajaro River	at Thurwacher Bridge	36.87993	-121.79307
Elkhorn Slough	at Hwy 1 bridge	36.80988	-121.78480
Salinas River	at Davis Road	36.64678	-121.70233
Carmel River	at Hwy 1	36.53630	-121.91270
Big Sur River	at Andrew Molera State Park	36.28247	-121.85782

3.5.1.3. Ocean Water and Mussels

Paired ocean water and mussel samplings were conducted quarterly. The objectives of this program element was to determine the extent to which humans and sea otters might be exposed to pathogens from consumed components of the food web and to compare the utility of ocean water grab samples and bivalves for assessment of coastal water quality. Ocean water samples were collected from six sites (Table 6) for assessment of fecal indicator bacteria and detection of specific bacterial and protozoal pathogens. Mussels from the area directly adjacent to the location of ocean water sampling were collected at the same time the water was sampled. A duplicate water sample was collected at one of the six sites and was processed as a field duplicate for QA/QC purposes. Protozoal testing for *Cryptosporidium* and *Giardia* was performed based on EPA Method 1623, filtering the 10L ocean water samples through Envirochek filters to concentrate the oocysts and cysts before transport to UC Davis for further processing. Testing for FIB (fecal coliforms, and total coliforms, and *Enterococcus*), and enteric pathogens (*Campylobacter jejuni, Salmonella enterica, Escherichia coli* O157:H7, *Vibrio cholerae*, and *Vibrio parahaemolyticus)* utilized standard membrane filtration methods to pass a series of dilution volumes through 45 μm filters. The inoculated filters were then placed on transport media, chilled and delivered for further processing at UC Davis the following day.

At least thirty mussels of 40-60 mm shell length were collected at each site. Mussel collection and processing was consistent with the California Department of Fish and Game Standard Operating Procedures (CDFG, 2001). Mussels were collected from the rocks using gloved hands, with gloves changed between sample locations. Mussels collected from each site were stored in a plastic bag that had been prelabeled with a waterproof marking pen. After collection, each mussel sample was double-bagged, placed in an ice chest with double-bagged

gloved hands, with gloves changed between sample locations. Mussels collected from each site were stored in a plastic bag that had been prelabeled with a waterproof marking pen. After collection, each mussel sample was double-bagged, placed in an ice chest with double-bagged blue ice packets and maintained at <10°C for transfer to the laboratories and all subsampling was completed the following day. Mussel subsampling was performed at UC Davis. Mussels were individually removed from the bag and cleaned of epiphytic organisms under running deionized water. A fresh stainless steel scalpel was used to sever the adductor mussel, remove the byssal threads and scoop out the remaining soft tissues. These tissues were homogenized at the lowest speed possible to avoid heating the sample or spattering. The homogenate had a paste-like consistency with no chunks of clearly defined tissue. Samples were tested in accordance with the American Public Health Association (1970) procedures. Sterile, protective gloves were worn during mussel processing. Sampling procedures for collection and handling of nearshore water samples, with the exception of the timing relative to tidal processes, followed those of the river and stream sampling.

Table 6. Site names and coordinates for paired mussel and nearshore water sampling locations for P3 Project.

Site Name	Latitude	Longitude
Scott Creek	37.04190	-122.23370
Elkhorn Slough	36.80988	-121.78480
Carmel River Beach	36.53897	-121.93193
Cayucos	35.44353	-120.90100
Estero Bay	35.40682	-120.87223
Morro Bay	35.35778	-120.85080

3.5.1.4. Stormwater Sampling

Objectives for stormwater sampling included collection of stormwater samples during three separate storm events from a single storm drain located in each of three urban communities along the Monterey Bay, during Year 2 of the project, for analysis of indicator bacteria and pathogens. The goal was to sample hourly for 3 consecutive hours at each site during the same storm event. Sampling was initiated when it was raining enough at all three sites for the water to be sheeting on the road. At some sites a catchpole was required to safely collect stormwater samples, while in other areas the bottle could be held manually for sample collection, using the same process as for seawater and surfacewater collection. The first sample for each site was designated as A and hourly sampling continued over the next 2 hours (samples B, and C) as long as rain continued at all three sites. Each sample was placed in a cooler with double-bagged ice for delivery to MWVCRC. Protozoal testing for *Cryptosporidium* and *Giardia* was performed based on EPA Method 1623, which requires processing 10L stormwater samples with Envirochek filters to concentrate the oocysts and cysts prior to transport to UC Davis for further processing. Bacterial testing for fecal indicator bacteria (fecal coliforms, and total coliforms, and *Enterococcus*), as well as for specific enteric pathogens (*Campylobacter jejuni, Salmonella enterica, Escherichia*

coli O157:H7, *Vibrio cholerae*, and *Vibrio parahaemolyticus)* employed standard membrane filtration methods to filter a series of dilution volumes through 45 μm filters that were then placed on transport media, kept chilled and delivered to UC Davis for further processing the next day. The locations of each stormwater sampling site are shown in Table 7.

Table 7. Locations of stormwater monitoring sites for P3 Project.

Sample site	Location	Latitude	Longitude
Santa Cruz	End of Woodrow Avenue	36.95255	-122.03675
Pacific Grove	Greenwood Park	36.62230	-121.91383
Carmel	End of Ocean Avenue	36.55500	-121.93000

3.5.1.5. Scat Collections

Collection of ≥ 50 fecal samples from terrestrial and marine animals was conducted over three years. Sampled terrestrial animals included domestic animals such as dogs (*Canis lupus familiaris*), cats (*Felis domesticus*) and beef cattle (*Bos taurus*). Sampled terrestrial wildlife included coyotes (*Canis latrans*), grey foxes (*Urocyon cinereargenteus*), mountain lions (*Puma concolor*), bobcats (*Lynx rufus*), and Virginia opossums (*Didelphis virginiana*). Marine animals sampled included gulls (*Larus* spp.) and sea otters (*Enhydra lutris neries*).

Fecal samples from live domestic dogs, cats, beef cattle, gulls, and sea otters were collected from the ground, or off baited plastic sheets laid down to facilitate sampling within minutes to hours post-defecation. Samples from live-caught opossums were collected from the interior surfaces of Tomahawk traps (Tomahawk, WI, USA). Freshness of samples was ascertained by observation of defecation and/or the presence of a high moisture sheen (Cox et al., 2005). Feces also were collected during necropsy of domestic cats, wild canids, wild felids, opossums, and sea otters (≤ 72 hour postmortem interval under refrigeration) at the MWVCRC. All domestic cats included in the study were humanely euthanized, non-adoptable animals, obtained through local animal shelters. Fecal samples were collected aseptically in sterile 50ml conical vials and refrigerated. Samples for bacterial isolation and identification were refrigerated and shipped overnight to the University of California, Davis School of Veterinary Medicine (Davis, California, USA) (Miller et al., 2009). Samples for protozoal identification and enumeration were processed at MWVCRC.

3.5.1.6. Lab and Field Wetland Experiments

Pathogen Tank Exposure Experiments

A mesocosm tank laboratory model was designed to mimic the flow of a wetland ecosystem where key factors could be manipulated in a relatively controlled setting that also incorporated typical central California coastal wetland water and vegetation types. Three tanks were built that measured three meters long by half a meter wide and re-circulated a volume of 450 L of water at a depth of 30 cm. Water used in these mesocosm tank experiments was collected from Tembladero Slough, which also supplies the constructed wetland utilized in the field sampling experiments detailed below. The mesocosm tanks were constructed from polypropylene sheets and an outer wood frame, with PVC pipe serving as the tank input and outflow. A 3-5 cm deep layer of commercial sand (Number 30 Silica Sand, Cemex) was placed on the bottom of each tank as substrate prior to each experimental run.

Wetland characteristics that were evaluated included both hydrological and ecological factors (Table 8). The hydrological parameters examined include factors that may be affected by climate change, such as salinity and flow rate. The baseline salinity of the Tembladero Slough water (0.1%) was artificially increased to 3% salinity using Coralife® Scientific Grade Marine Salt to emulate an estuary with higher seawater influence. Flow rates of input water were also manipulated, and flow rates of 0.1 cm/second and 1.0 cm/sec were selected as reference values for all subsequent studies to represent the broad range of flow rates observed in wetlands and estuaries along the central California coast.

Table 8: Experimental conditions used in the pathogen tank exposure experiments. All conditions involved three replicates.

Condition	Vegetation	Salinity	Flow Rate
1	Absent	0.1%	0.1 cm/sec
2	Absent	0.1%	1 cm/sec
3	Absent	3%	0.1 cm/sec
4	Absent	3%	1 cm/sec
5	California Bulrush-Buffer configuration	0.1%	0.1 cm/sec
6	California Bulrush-Buffer configuration	0.1%	1 cm/sec
7	California Bulrush-Channel configuration	0.1%	0.1 cm/sec
8	California Bulrush-Channel configuration	0.1%	1 cm/sec
9	Slough Sedge-Buffer configuration	0.1%	0.1 cm/sec
10	Slough Sedge-Buffer configuration	0.1%	1 cm/sec

The ecologic parameters examined include factors that are used to restore coastal wetlands such as vegetation presence (or absence), vegetation type, vegetation configuration, and wetland length (Figure 2). Two native wetland plants were evaluated in this study: California bulrush (*Schoenoplectus californicus*) (Stevens and Hoag, 2003) and slough sedge (*Carex obnupta*) (Stevens and Hoag, 2006), and both were transplanted from the Tembladero Slough Constructed Wetland. Both plants are members of the Sedge Family (*Cyperaceae*), that are native to California and are commonly used in wetland and marsh sites for pollution remediation and embankment stabilization. California bulrush typically has long, thick stems, whereas slough sedge has a thinner, more grass-like appearance. California bulrush was planted in two different conditions (Figure 2), buffer or channel, and slough sedge was planted with a buffer configuration. The buffer strip had vegetation transplanted in a 15 cm wide strip across the entire center of the tank so water flows between all stems. The channel configuration had the California bulrush transplanted in two 15 cm^2 bushels in the center of the tank, but with a 20 cm wide strip between so water could to flow between the bushels. These configurations were chosen to emulate wetland rehabilitation structures.

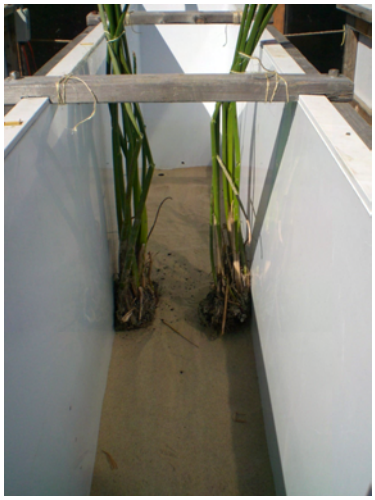
a b 

Figure 2: Comparison of California bulrush configuration. a: Buffer configuration, b: Channel configuration.

For each experimental condition, $4.5x10^6$ each of heat-killed *Cryptosporidium parvum* oocysts (Wisconsin State Laboratory of Hygiene, Madison, WI) and *Giardia lamblia* cysts (Waterborne, Inc, New Orleans, LA) were released as a bolus 8 cm downstream from inflow at a depth of 15 cm below the water surface. The number of parasites inoculated was determined such that if parasites were homogenously mixed throughout the tank, the concentration would be 10 oocysts or cysts per mL. Using a serological pipette, 50 mL of water was collected at 76 cm and 226 cm downstream from inflow at a depth of 20 cm below the water surface over a 48 hour period. Time points for sample collection were: pre-release, 1 minute, 5 min, 10 min, 15 min, 30 min, 60 min, 90 min, 2 hour, 4 hr, 6 hr, 24 hr, 48 hr, and 72 hours post-release. Samples were concentrated via immunomagnetic separation (IMS) and stained using a direct fluorescent antibody (DFA), followed by protozoal enumeration using fluorescent microscopy. Results are expressed as the concentration of oocysts or cysts per 50 mL.

Field Sampling at Tembladero Slough Constructed Wetland

Over two years of sampling in the Tembladero Slough Constructed Wetland, 92 samples were collected for protozoal analysis, 78 samples were collected for FIB analysis using colilert-18 testing, and 48 samples were collected for FIB analysis using membrane filtration from six sites within the wetland, and one from the source water (Tembladero Slough) (Figure 3). Samples were collected once per week over four weeks, twice each year, from April, 2008 through June, 2010. For each sample, 10 L of water was collected in a sterile plastic container for protozoal analysis and 100 mL was collected in a second container for bacterial analysis.

Samples for protozoal analysis were analyzed according to the EPA Method 1623 for detection of *Cryptosporidium parvum* and *Giardia lamblia* in water through filtration using Envirochek ® cartridges, immunomagnetic separation (IMS) for purification, and direct fluorescent antibody tests (DFA) for parasite identification and quantification using fluorescent microscopy. Results are expressed as the oocyst or cyst count per 10 L of water. Samples for bacterial analysis were tested using membrane filtration and Colilert-18 tests (the standard, EPA-approved, 18-hour testing method for detection of total coliforms and *Escherichia coli*). For

membrane filtration, water samples were filtered and incubated overnight. Total coliform, fecal coliform, and *Enterococci* colony forming units (cfu) were enumerated and expressed as cfu/100 mL. For the Colilert-18 tests, water samples were incubated at 35˚C in a quanti-tray overnight and total coliforms were estimated by number of luminescent wells, while *E. coli* was estimated by number of fluorescent wells. Results are expressed as most probable number (MPN) per 100 mL.

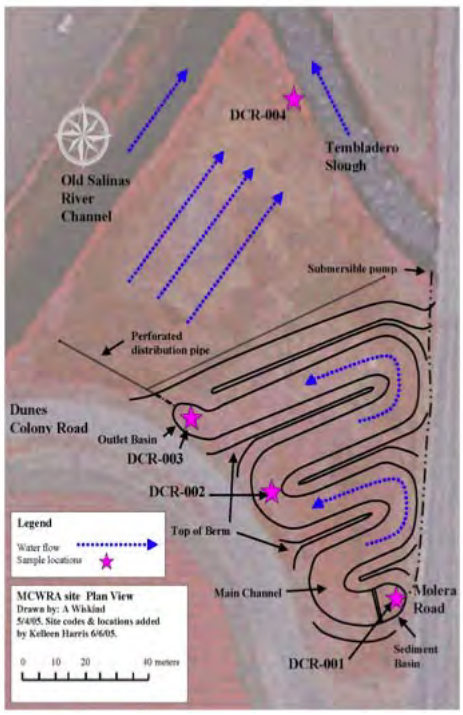


Figure 3: Map of the Tembladero Slough Constructed Wetland Sampling Sites. The pink stars represent the main sampling locations. The Tembladero Slough Constructed Wetland consists of a "zig zag" water channel where water flows at a determined rate (center right), and a natural flood plain at the end (top center). Blue arrows represent the direction of water flow.

A variety of factors were assessed to determine their effect on in the numbers of protozoa or indicator bacteria in the circulating water. These factors included sample site, season, rainfall and specific water quality parameters. Seasonality in the central California coast includes the rainy season from November-April and the dry season from May-October. Rainfall events were determined by rain occurring on the sampling date or within three days prior to sampling, as determined by the NOAA weather station in Watsonville, CA. Water quality parameters were also recorded at each sample site and date. These parameters include water temperature (˚C), turbidity (NTU), salinity (ppt), conductivity (µm), total dissolved solids, dissolved oxygen (mg/L and %), pH, and wetland water depth (cm).

3.5.2. Analytical Methods

3.5.2.1. Bacterial Identification and characterization

Indicator bacterial enumeration. Detection of indicator bacteria was performed using a delayed incubation modification of the standard membrane filtration method, as described in the

Standard Methods (SM) for the Examination of Water and Wastewater (APHA, 2005). Biochemical, serologic, and/or polymerase chain reaction (PCR) techniques were used for final bacterial identification, as described below. Table 9 shows the surface water volumes processed for each bacterial group.

Table 9. Volumes and dilutions for membrane filtration process and bacterial culture in river (R) and seawater (S) samples

	Volume Filtered (ml)				
	10^{-1}	1	10	100	500
Total coliform		R	R, S	R, S	
Fecal coliform		R	R, S	R, S	
Enterococcus			R, S	R, S	
Campylobacter spp.			R, S	R, S	R, S
E. coli O157 (ml)			R, S	R, S	R, S
Salmonella spp.	R		R, S	R, S	R, S
Vibrio spp.			R, S	R, S	R, S

Total coliforms (TC) were enumerated as red colonies with a metallic sheen on m- Endo agar LES (Hardy Diagnostics) after 24 h incubation at 35.5ºC. Fecal coliforms (FC) were identified as blue colonies on m-FC medium (Hardy Diagnostics) after 48 h of incubation at 44.5ºC. *Enterococcus* spp. (ENT) were identified as small light and dark red colonies grown on m-*Enterococcus* agar after 48 h incubation at 35.5ºC. Suspect *Enterococcus* colonies were further characterized with biochemical testing as esculin-positive colonies on bile esculin agar (BEA) plate with growth in 6.5% NaCl broth. All counts were standardized to a 100 mL sample volume for statistical analyses.

For *Bacteroidales* and *Acinetobacter* qPCR, genomic DNA was extracted from 10 mL of HFF concentrate Obtained from river and estuarine water samples. QIAamp DNA Stool kits (Qiagen, Valencia, CA, USA) were used according to the manufacturer's directions, with slight modifications by using a homemade Guanidine Isocyanate-based lysis buffer instead of Buffer AL, and centrifuging samples prior to application to spin filters (Carey et al., 2004; Kildare et al., 2007).

Each 25 µL PCR reaction contained 12.5 µL of commercially available TaqMan PCR mastermix (Eurogentec, San Diego, CA, USA) with 400 nM each of forward and reverse primers and 80 nM probe for the respective TaqMan system. For all TaqMan reactions, 10 µL of the diluted gDNA sample was assayed in a final reaction volume of 25 µL. In order to suppress inhibitors, bovine serum albumin (BSA) was added to each reaction in a final concentration of 50 ng/µL, and four serial dilutions were performed to assess inhibition factors. Cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C, using an ABI Prism 7000 (Applied Biosystems, Carlsbad, CA, USA). *Bacteroidales* assay primers (Table 10) and specificity were established previously (Kildare et al., 2007). Primers are listed in Table 10.

Table 10. Primers used for qPCR assays.

Primers	Oligonucleotide sequence (5'–3')	Reference

Universal *Bacteroidales*:		
BacUni-520f	CGTTATCCGGATTTATTGGGTTTA	(34)
BacUni-690r1	CAATCGGAGTTCTTCGTGATATCTA	
BacUni-690r2	AATCGGAGTTCCTCGTGATATCTA	
BacUni-656p	6-FAM-TGGTGTAGCGGTGAAA-TAMRA-MGB	
Human *Bacteroidales:*		
BacHum-160f	TGAGTTCACATGTCCGCATGA	(34)
BacHum-241r	CGTTACCCCGCCTACTATCTAATG	
BacHum-193p [C]	6-FAM-TCCGGTAGACGATGGGGATGCGTT-TAMRA	
Cow *Bacteroidales*:		
BacCow-CF128f	CCAACYTTCCCGWTACTC[1]	[1](6), [2](34)
BacCow-305r	GGACCGTGTCTCAGTTCCAGTG[2]	
BacCow-257p	6-FAM-TAGGGGTTCTGAGAGGAAGGTCCCCC-TAMRA[2]	
Dog *Bacteroidales*:		
BacCan-545f1	GGAGCGCAGACGGGTTTT	(34)
BacUni-690r1	CAATCGGAGTTCTTCGTGATATCTA	
BacUni-690r2	AATCGGAGTTCCTCGTGATATCTA	
BacUni-656p	6-FAM-TGGTGTAGCGGTGAAA-TAMRA-MGB	
Acinetobacter:		
Acinet-137F	GATGCAACGCGAAGAACCTTA	Schreiwer 2010
Acinet-210R	TTCCCGAAGGCACCAATC	
Acinet-159p	6-FAM-CTGGCCTTGACATAGTAGAAACTTTCC-TAMRA	

3.5.2.2. Pathogenic bacterial enumeration.

Isolation of pathogenic bacteria from riverine and estuarine samples targeted *Campylobacter* spp., *E. coli*-O157:H7, *Salmonella* spp. and *Vibrio* spp. The general methodology for pathogen detection and enumeration primarily utilized serial dilutions with membrane filtration of water and delayed incubation of filters on selective media. Enumeration of *Campylobacter* spp. was as outlined in the *SM for the Examination of Water and Wastewater* with slight modifications (el-Sherbeeny et al., 1985). Briefly, a 100-mL volume of water was filtered through a 0.45 μm pore-size, 47 mm diameter cellulose nitrate membrane filter. A second, similar filter was placed in Campy thioglycollate broth (Hardy Diagnostics) for 24 h under microaerophilic conditions at 37ºC. Each filter was placed facedown on Campy-cefoperazone, vancomycin, and amphotericin B (Campy-CVA) agar with 5% sheep blood (Hardy Diagnostics) and incubated for 24 h at 37ºC under microaerophillic conditions (Pack-Micro Aero, Mitsubishi Gas Chemical Company). The filter was then placed face up on another CVA agar plate and both plates were incubated again at 37ºC. Plates were read for *Campylobacter* spp. presence after 48 h. Suspect *Campylobacter* spp. colonies were small grayish-brown-smooth or flat-mucoid-gray, and irregularly edged

translucent colonies that Gram-stained as Gram-negative curved rods. Biochemical testing including oxidase, catalase, hippurate, nalidixic acid (NA) sensitivity, and cefoxitin (CF) sensitivity, as well as *Campylobacter* genus-specific PCR were used to confirm isolates (Bang et al., 2001).

Screening for *Escherichia coli*-O157:H7 with immunomagnetic separation (IMS) began in September, 2007. Briefly, a Dynabeads (Invitrogen Corporation, Carlsbad, California) anti-*E coli*-O157:H7 IMS technique was used to concentrate suspect bacteria, followed by plating of separated bacteria on CHROMagar O157:H7 (Hardy Diagnostics) according to the manufacturer's instructions. Suspect colonies were further characterized using biochemical testing and PCR with primers specific to the O157 and H7 genes (Gannon et al., 1997; Hu et al., 1999).

Salmonella spp. detection used filter incubation on xylose lysine desoxycholate (XLD) medium and 24 h incubation at 35.5ºC. A second filter was pre-enriched in selenite broth for 24 h at 37ºC prior to subculture on XLD. *Salmonella* were initially identified by the presence of red colonies with black centers on XLD. An additional modification of the EPA method (USEPA, 2006) involved filtering 100 mL of water, enriching the filters in tryptic soy broth, and subculturing onto Criterion-modified, semi-solid Rappaport Vassiliadis agar. Suspect *Salmonella* were confirmed with triple sugar iron (TSI) agar (alkaline:acid:H_2S production), and were o-nitrophenyl-beta-D-galactopyranoside (ONPG)-negative, oxidase-negative, urease-negative and indole-negative. *Salmonella* were further characterized with Poly O (A-E) antiserum and group-specific antiserum (BD Difco).

Vibrio spp. were isolated by incubating filters on thiosulfate-citrate-bile salt-sucrose agar (Hardy Diagnostics) for 24 h at 35.5ºC. Large and small yellow colonies were consistent with the isolation of *Vibrio cholerae* and *V. alginolyticus*; small green colonies were consistent with isolation of *V. parahaemolyticus*. Upon further biochemical testing, *V. cholerae* strains showed alkaline slant/acid butt or acid slant/acid butt on TSI agar after incubating at 37°C for 18-24 h and were oxidase-positive, ONPG-positive, and fermented sucrose. *Vibrio cholerae* confirmation on a subset of isolates utilized API 20E strips, O1-specific antisera, and PCR assays targeting *Vibrio* spp., *V. cholerae*, and ctx toxin genes (Koch et al., 1993; Vezzulli et al., 2009). *Vibrio parahaemolyticus* was confirmed for production of oxidase, reaction in TSI agar (alkaline slant/acid butt), urease production, hemolysis on sheep blood agar, and species-specific PCR (Vezzulli et al., 2009). All counts were standardized to per 100 ml for statistical analysis.

Virulence gene screening of *V. cholerae* and *V. parahaemolyticus* isolates was performed for selected isolates using PCR assays that target specific pathogenicity markers. To test for enterotoxogenic *V. cholerae*, a *ctx*-specific PCR method was used and the genomic DNA from *V. cholerae* was isolated by boiling method. Briefly, 2-3 colonies were suspended in 200 µl of molecular biology-grade water, vortexed, and placed in boiling water for 15 min. Samples were then centrifuged at 12,000 X g for 5 min and supernatant was collected and used as DNA template. An aliquot of 2 µl of template was subjected to PCR testing done with oligonucleotide primers for the encoding *ctx*- specific gene as described previously (Koch et al., 1993). PCR reaction products were resolved on a 1.2% agarose gel in lx Tris- EDTA, pH 8.3 and visualized with ethidium bromide using a Gel-Doc 2000.

Virulence gene screening for *tdh*-positive *V. parahaemolyticus* isolates was determined using a *tdh*-specific qPCR protocol, based on methods described by Nordstrom et al. (2007). Each 25-µl PCR mixture contained 12.5 µl of commercially available Taqman Environmental Master Mix 2.0 (Applied Biosystems, Carlsbad, CA) with 400 nM of the *tdh* forward and reverse primers

(Anaspec, Fremont, CA), 80 nM probe for *tdh* (Applied Biosystems, Carlsbad, CA), and 10 uL of gDNA sample. Real-time PCR thermal cycling was conducted using a StepOne Plus system from Applied Biosystems (Carlsbad, CA). Cycling parameters consisted of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 59°C. Positive controls, consisting of a *V. parahaemolyticus* strain possessing the *tdh* gene, and a negative control (nuclease-free H_2O added as a template) were prepared for each real-time PCR run.

Salmonella Typhimurium isolates from different terrestrial and marine animal sources were fingerprinted to permit finer discrimination among strains and elucidate potential land-sea connections. Chromosomal fingerprinting using pulsed-field gel electrophoresis (PFGE) was performed on selected *S.* Typhimurium and *S.* Typhimurium (Copenhagen) isolates as described previously (Smith, 2002). Briefly, bacteria were incorporated into agarose plugs and digested with *Xba*I. Digested plugs were run on a Bio-Rad Chef DRIII PFGE apparatus (Bio-Rad Laboratories, Hercules, CA) for 18 hours at 6 volts/cm with an initial switch time of 2.16 seconds and a final switch time of 63 seconds and 120^{o} included angle at 14^{o}C. The banding pattern was analyzed using BioNumerics, Version 4.6 (Applied Maths, Austin, TX) unweighted pairgroup analysis with arithmetic means and Dice's coefficient (1% optimization and 2% position tolerance). Isolates with 80% or greater similarity were considered closely related and constituted a PFGE type. Isolates with 90% or greater genotypic similarity were considered identical as this represented a one band or less difference between isolates and accounted for small amounts of variability between gels (Shaaly, 2005).

The Kirby Bauer method of disk diffusion was used to test for antimicrobial resistance and was followed as stated in *Performance Standards for Antimicrobial Disk Dilution Susceptibly Tests for Bacteria Isolated from Animals; Approved Standard—Third Edition* (CLSI, 2006). Briefly, paper disks impregnated with specific antibiotics were distributed evenly across large Muller-Hinton plates that had been previously streaked with bacterial broth. The plates were incubated for 24 to 48 hours and the zone of no bacterial growth around each disk was measured and compared to ranges in the literature that corresponded to "susceptible", "intermediate" or "resistant" to the effects of that antibiotic. Selection of antibiotics for testing was based on those most commonly used in domestic animal and wildlife medicine and results of prior studies: antibiotics that were evaluated included amoxicillin/clavulanic acid (clavamox) (30µg), ampicillin (10µg), cefazolin (30µg), enrofloxicin (5µg), erythromycin (15µg), gentamicin (10µg), penicillin (10µg), rifampin (5µg), tetracycline (30µg), ceftazidime (30µg), imipenem (10µg), trimethoprim/sulphamethoxazole (TMS) (1.25µg /23.75µg).

3.5.2.3. Protozoal Identification and characterization

Cryptosporidium oocysts and *Giardia* cysts were quantified according to EPA Method 1623 (USEPA, 2005). This involved pumping up to 10 L of water through the Envirochek HV filter (Pall Gelman Laboratory, Ann Arbor, Michigan), eluting the filter using a hand-wrist shaker, concentrating the parasites using Dynabeads (Invitrogen Corporation) immunomagnetic separation (IMS), and finally enumerating oocysts and cysts using a direct fluorescent antibody (DFA) technique with BTF kit reagents (BTF, Precise Microbiology, Sydney, Australia). *Cryptosporidium* oocysts were identified as ~5 µm spheres outlined in apple green and often with a mid-line seam, whereas *Giardia* cysts were also apple green but oval and 9-14 µm long. Slides were viewed with FITC and DAPI stains on an Axioscop epifluorescent microscope. Organisms were visualized at 20x and identification confirmed at 40x. All slides were read by the same microscopist.

To genotype protozoa isolated from fecal samples, an 18S rDNA PCR protocol (Morgan et al., 1997) in conjunction with DNA sequence analysis that targets all *Cryptosporidium* spp was used. *Giardia* spp were analyzed via amplification of the glutamate dehydrogenase (*gdh*) genes (Read et al., 2004). For DNA extraction, the 100-µL purified immunomagnetic separation product was centrifuged and the supernatant removed. The pellet was mixed with 180 µL of lysis buffer solution (ATL buffer, QIAGEN Inc, Valencia, California) in a cryotube, placed in liquid nitrogen for 4 minutes, and then placed in boiling water for 4 minutes to break open oocysts and cysts. A DNA extraction kit (QIAmp DNA mini kit, QIAGEN Inc, Valencia, California) was used to digest each sample and extract DNA according to the manufacturer's instructions. DNA was eluted with 50 µL of 95°C DNAase-free PCR water. The Morgan primers targeted a 298bp DNA region for *Cryptosporidium* spp; for *Giardia* spp, while GDH primers targeted a 432bp region. Gel electrophoresis was used to separate the PCR products on a 2% agarose gel that contained 0.005% ethidium bromide. The PCR products were purified using EXOzapIT (USB Corporation, Cleveland, Ohio) or from the gel by use of a kit (QIAquick gel extraction kit, QIAGEN Inc, Valencia, California) according to the manufacturer's instructions, and sequences were obtained and analyzed as previously described.

3.5.2.4. Quality assurance.

A field blank and sample blank were run with membrane filtration techniques for each time point and sample site, respectively. For hollow-fiber ultrafiltration, laboratory blanks were processed with each sampling set. For each second qPCR plate a negative control was measured. One duplicate field sample was run during each sampling period. Spiking experiments were performed using deionized water and environmental matrix samples to determine method recoveries. Minimum detection limits were determined based on spiking experiments and the volume of sample analyzed per test for each site and time point.

3.5.2.5. Statistical Methods

Each indicator bacterium was categorized in three groups with its specific cutoff value based on water agency regulations. For TC, according to the California Ocean Plan, the cutoffs were 1,000 and 10,000 cfu per 100ml (SWRCB, 1990a). For FC, categories were defined using 200 and 400 cfu/100ml as cutoffs based on the criteria defined by State Water Resources Control Board (SWRCB, 1990b). For EN, 35 and 104cfu/100ml were used as cutoffs set by the U.S. Environmental Protection Agency (Cabelli, 1983). These cutoffs were consistent with regularly water quality monitoring standards for public beaches: maximum 10,00cfu/100ml for TC, 400cfu/100ml for FC, and 104cfu/100ml for EN (http://www.co.monterey.ca.us/health/beaches/).

For most environmental variables, the geometric means were selected as cutoff values for categories, with a turbidity of 4.75ntu in river and seawater samples and ambient temperature of 14ºC. For covariates such as the seawater temperature category, 12ºC was the cutpoint defined by previous studies (Burkhardt et al., 1992; Miller et al., 2006). For descriptive analysis, four seasons were categorized as follows: spring (March-May), summer (June-August), fall (September-November), and winter (December-February). For the risk factor analysis, dry season was categorized from June to November and wet season was categorized from December to May.

Descriptive measures showed space and temporal trends for river samples throughout the course of the study. For further analyses, datasets of river and seawater samples were combined into one dataset used for further statistical analyses. Spearman's rank correlation coefficient (r_s)

was used to assess correlation between indicator and pathogenic bacteria recorded as continuous data that required non-parametric tests. For dichotomous bacterial outcomes, simple logistic regression evaluated the strength of association between individual risk factors and the presence or absence of bacterial pathogens. Forward-stepping multivariable models were evaluated to simultaneously assess the association between environmental predictors, detection of the indicator bacteria, and fecal pathogen presence. The criterion for attempting regression models was more than five ($n \geq 5$) occurrences of pathogenic bacteria detection in the study. Prevalences of bacteria in different sample types were compared using Chi-square test. Prevalence of bacteria in mussel batches and seawater samples were compared using Wilcoxon signed-rank test for continuous outcome data and McNemar's test for dichotomous outcomes. Statistical analyses were performed using STATA version 10.0 (StataCorp., College Station, TX, USA). Results were considered significant at $P < 0.05$.

Concentrations and sample limits of detection (SLOD) for *Bacteroidales* and *Acinetobacter* detected by real-time PCR were analyzed according to Rajal et al. (2007). These SLODs are individual limits of detection for each sample and account for concentration factors, recoveries and qPCR inhibition to help evaluating non-detects. The SLOD in gene copies/mL (gc/mL) values are calculated as follows:

$$SLOD = \left(\frac{1000 \cdot ALOD \cdot I}{C_{filtr} \cdot C_{extr} \cdot R} \right)$$

Where ALOD (gc/uL) is the assay limit of detection for the applied assay and specific conditions, C indicates concentration factors for filtration (C_{filtr}) or nucleic acid extraction (C_{extr}). The overall recovery proportion, R, is assessed by measurement of known spike doses of a bacterial surrogate, *Acinetobacter baylyi* strain ADP1 (Vezzulli et al., 2009), previously referenced as *Acinetobacter* sp. strain ADP1 (Johnson et al., 1997).

Data were compiled and analyzed using Excel 2007 (Microsoft Corp., USA). Box plots were created to show the medians, 25th and 75th percentiles, 10th and 90th percentiles, and data outliers using SIGMAPLOT® version 10 (Systat Software Inc., USA). Kendall and Spearman correlations, as well as logistic regression analyses using Nagelkerke's R-square (Harwood et al., 2005), were performed using SPSS version 14 (SPSS Inc., USA).

As a weighted measure of how well pathogen occurrence can be predicted by the presence of an indicator organism, a Predictive Qualifier was calculated for different threshold cutoff levels of the indicator, using indicator and pathogen presence/absence:

$$Predictive\ Qualifier\ (\%) = \frac{TOT_{true}(\%) + POS_{true}(\%)}{2}$$

In the equation, TOT_{true} is the total percentage of matches that represent either simultaneous indicator and pathogen presence or simultaneous indicator and pathogen absence, and POS_{true} is the percentage of matches when pathogens were positive and matched correctly by indicators. For example, at a low threshold cutoff concentration level of 1 gc/mL for universal *Bacteroidales,* the prediction would be that 100% of the samples are positive for pathogens, because universal *Bacteroidales* markers were detected in 100% of the samples above 1 gc/mL. At a certain threshold, most of the indicator samples will predict a pathogen non-detect and therefore the percentage of total matches will be rather high when most of the samples are also non-detects for pathogens. At some optimal threshold, both pathogen presence and absence will

be matched by indicator occurrence at or above this threshold. This simple equation helps to qualify the predictive value for thresholds of an indicator with emphasis on pathogen occurrence.

Conditional probability analysis was used to determine whether the host-specific assays correctly measure the organism for which they were designed, Bayes' Theorem was used as previously reported (Kildare et al., 2007) for the calculation of the probability of a given source of contamination existing in a water sample, given that a positive test result is obtained; likewise, the probability that a given source of contamination in a water sample exists when a negative test result is obtained was also calculated. To apply Bayes' Theorem, the assays were assumed to be independent discrete random variables, which is appropriate for presence/absence data.

Conditional probabilities for each of the assays, predictive values, and prevailing (background) rates for each set of assay results for this study were calculated using validation results obtained by Kildare et al. (2007). The conditional probability gives the probability that the signal of the tested assay actually results from the targeted host source. The diagnostic sensitivity is the proportion of samples that correctly tested positive over all the samples that actually experienced fecal contamination from the targeted host source. Diagnostic specificity is the proportion of samples that correctly tested negative over the total number of samples that actually did not experience fecal contamination from the targeted host source. The positive predictive value of the test is the proportion of samples that correctly tested positive over the total number of samples that tested positive. Lastly, the negative predictive value of the test is the proportion of samples that are correctly negative over the total number of samples that test negative.

Total to fecal coliform ratios were calculated for all river, ocean, stormwater, and wastewater samples in the study. The associations between these ratios and pathogen detection were evaluated in a similar manner to Haile et al. (1999). We expected that the detection of bacterial pathogens might be greater when the ratio of total to fecal coliforms was smaller, indicating a relatively greater proportion of fecal contamination. We selected *Salmonella*, *Cryptosporidium*, and *Giardia* for this analysis, as they are notable pathogens of public health interest. We used a ratio defined by a cutpoint of 5 (where 5 corresponds to there being 5 times as much total as fecal coliform in the water) (Haile et al., 1999). Because Haile et al. (1999) reported increased risks with decreasing cutpoints; we also used a cutpoint of 2 for all analyses. Associations between total to fecal coliform ratios and pathogen detection were evaluated using simple logistic regression. Analyses were performed using Stata/LC 11.1 (Stata- Corp.). We then restricted our analysis to samples with total to fecal coliform ratios in excess of 1000 or 5000 cfu as Haile et al. (1999) reported that any effect of a lower total to fecal coliform ratio should be stronger when there was a higher degree of contamination, indicated by total coliform counts in excess of 1,000 or 5,000 cfu. Associations were evaluated using Fisher's Exact Test and analyses were performed using Stata/LC 11.1 (Stata- Corp.).

Statistics used to summarize and evaluate pathogen detection in terrestrial and marine mammal fecal samples were similar to surface water data analyses described above in that the prevalence and risk factors associated with pathogen detection were determined over the course of multiple years. Fecal shedding prevalence of *Campylobacter* spp., *Salmonella* spp., *E. coli* O157:H7, *Vibrio* spp. (including *V. cholerae*, *V. parahaemolyticus*, and *V. alginolyticus*) and *Cryptosporidium* spp. and *Giardia* spp. were calculated based on risk factors including animal group (domestic dogs, domestic cats, beef cattle, wild canids, wild felids, opossums, gulls, sea otters), gender (male, female, unknown), age (≤ 6 months, subadult, adult, unknown), season (wet, dry), location (Elkhorn Slough watershed, Carmel River watershed, Monterey Bay area),

collection year (2007, 2008, 2009-10), and status (live, dead) by dividing the number of pathogen-positive fecal samples by the total number of samples collected for each risk factor (Callaway et al., 2005). The 95% exact binomial confidence intervals (CI) around each point estimate were calculated using Stata/LC 11.1 (Stata- Corp., College Station, TX, USA). Seasons were based on approximate climactic seasonality of the Monterey Bay area in terms of rainfall, air and water temperatures, and salinity (Broenkow, 1977; Yoklavich at al., 1991; Caffrey, 2002). Wet season samples were collected during November through April and dry season samples were collected during May through October. Because fewer samples were collected during 2009 (n=1) and 2010 (n=15) and atmospheric conditions were similar, samples from 2009 and 2010 were pooled.

Univariate and multivariate logistic regression approaches were then used in a forward selecting manner to investigate associations between bacterial detection and species-specific, demographic, or environmental risk factors. Univariate analyses evaluated the associations between each risk factor individually with regard to presence or absence of the bacterial outcome of interest. Multivariate models produced adjusted odds ratios that simultaneously measured the strength of associations between multiple risk factors and the bacterial outcome of interest. The regression analyses were adjusted for data dependence within each watershed by including a cluster variable for sampling area. Analyses were performed using Stata/LC 11.1 (Stata-Corp.).

The wastewater, rivers and streams, ocean and stormwater samples had many non-detects throughout the data. In order to incorporate the potential for a non-detect to account for pathogen concentrations that were below the minimum detection limit of each analytical method, regression order statistics (ROS) were used to analyze the data. Many times water quality data have multiple detection limits for different methods used, and this allows incorporation of those limits that may be biased when using a typical log-normal transformation (Lee Helsel, 2005). Caltrans has a data analysis tool that it developed for using ROS statistics in Excel as an add-in. The mean and 95% confidence intervals are presented in tables at the end of each section.

For the pathogen mesocosm tank exposure experiments, multivariate longitudinal negative binomial regression was used for analysis of wetland factor effects on *Cryptosporidium* and *Giardia* counts. Covariance structures were modeled as compound symmetry and autoregressive, respectively. Wald tests were used to evaluate differences between factors for each model. For the field sampling experiments, univariate analysis for protozoa was determined based on geometric mean values, while univariate analysis for FIB was performed using Regression on Ordered Statistics (ROS) to account for samples falling below the minimum detection limits of 100 MPN and 0.5 CFU. Given the small sample sizes and non-normal distribution of parameters, a non-parametric Spearman's rho was calculated to evaluate relationships between *Cryptosporidium* and *Giardia* concentrations, bacterial counts and hydrologic parameters. A Wilcoxon-Mann-Whitney test was used to evaluate significance of rainfall events and season with *Cryptosporidium* and *Giardia* detection and relative concentrations. P-values of <0.05 were considered statistically significant. Statistical analysis was completed using SAS 9.2 software.

4. Results of each Question

4.1. What are the spatial and temporal patterns in pathogen indicator concentrations and what is the relationship between pathogen indicators and actual fecal pathogens in: Influent and Effluent, Rivers and Streams, Ocean Water and Mussel Tissue , Storm Runoff, and Wetlands?

4.1.1. Wastewater Influent and Effluent

Ten paired influent and effluent samples were collected from four wastewater treatment facilities (Table 11, Table 14). The analysis of these samples provided the opportunity to examine 1) differences between wastewater treatment facilities and sampling dates in the concentrations of indicator of bacteria and fecal pathogens, 2) how efficiently indicator bacteria and fecal pathogens are removed from wastewater through the treatment process, which can vary by facility, and 3) variations in the relationships between indicator bacteria and fecal pathogens. Table 12 lists the percent prevalence for the organisms detected. These data, as well as those from rivers, streams and storm runoff, also permit rough estimates of loads to marine waters (see Section 4.2). The first seven sets of wastewater samples were analyzed for the full list of indicator bacteria and fecal pathogens, whereas the last three samples were used only to measure the recovery of protozoal oocysts spiked into field samples to enable estimates of protozoal detection limits.

Recoveries of spiked oocysts of *Cryptosporidium* and *Giardia* varied substantially between influent and effluent, as well as between sites. Mean recoveries of *Cryptosporidium* and *Giardia* in influent ranged between 0.6% and 3.3%, and between 0.6% and 1.8%, respectively (Figure 4a). Mean recoveries of *Cryptosporidium* and *Giardia* in effluent ranged between 15.0% and 62.5%, and between 2.5% and 14.0%, respectively (Figure 4b). These percentages are comparable with a prior report of ranges for recovery of *Cryptosporidium* oocysts (from 0–36% in raw influent and from 3–42% in secondary effluent) (McCuin and Clancy, 2005). The lower percentage of oocyst recovery in influent is probably due to the greater proportion of particulate matter in these samples, when compared to effluent; variability in oocyst recovery was associated with the relative concentration of suspended particles and other, less well characterized factors by previous investigators (DiGiorgio et al., 2002; Krometis et al., 2009). Site 1 had the greatest mean recoveries for both protozoa in both influent and effluent.

Analysis of variance on arcsine-transformed data revealed significant differences among sites in the percentage recovery of both *Cryptosporidium* and *Giardia* in effluent (Table 13). The mean recoveries noted at Site 1 (Figure 4b) were also significantly greater than at other sites. These differences in spike recoveries among sites led to the application of site-specific recovery values for estimates of protozoal concentrations and loads. Even with the application of site-specific recovery values, the low recoveries and relatively large standard errors for influent and effluent at some sites introduced substantial uncertainty into recovery-corrected estimates of protozoal concentrations and loads. For example, corrected concentrations of *Cryptosporidium* and *Giardia* in effluent from sites 3 and 4 have estimated errors of plus or minus 30–40%.

Table 11. Sample dates, type and flow on given sample date as well as weather information close to sample date.

Date	Site (Treatment Facility)	Sample Type	Flow, MGD	Precipitation up to 3 days prior to sampling y= yes, n=no m=missing
5/7/07	1	24-Hour Comp	8.8	y
6/5/07	1	24-Hour Comp	10.2	n
9/24/07	1	24-Hour Comp	11.9	y
12/17/07	1	24-Hour Comp	11.4	y
3/31/08	1	24-Hour Comp	8.1	y
6/2/08	1	24-Hour Comp	7.8	n
9/28/08	1	24-Hour Comp	9.2	n
3/7/11	1	Grab	12.9	y
3/8/11	1	Grab	11.8	y
3/9/11	1	Grab	11.7	y
5/7/07	2	Grab	6.67	m
6/5/07	2	Grab	5.89	n
9/24/07	2	Grab	7.08	y
12/17/07	2	Grab	7.11	y
3/31/08	2	Grab	6.41	y
6/2/08	2	Grab	6.66	n
9/28/08	2	Grab	5.4	n
3/1/11	2	24-Hour Comp	6.64	y
3/2/11	2	24-Hour Comp	6.69	n
3/3/11	2	24-Hour Comp	6.5	n
5/7/07	3	Grab	2.7	n
6/5/07	3	Grab	0.2	n
9/24/07	3	Grab	9.0	m
12/17/07	3	Grab	18.5	y
3/31/08	3	Grab	0.7	y
6/2/08	3	Grab	0.0	n
9/28/08	3	Grab	6.81	n
3/1/11	3	Grab	19.8	y
3/2/11	3	Grab	20.0	y
3/3/11	3	Grab	18.9	n
5/7/07	4	24-Hour Comp	0.271	m
6/5/07	4	24-Hour Comp	0.263	n
9/24/07	4	24-Hour Comp	1.448	m
12/17/07	4	24-Hour Comp	0.369	m
3/31/08	4	24-Hour Comp	0.57	y
6/2/08	4	24-Hour Comp	0.446	n
9/28/08	4	24-Hour Comp	0.316	n
3/1/11	4	24-Hour Comp	1.589	y
3/2/11	4	24-Hour Comp	1.512	n
3/3/11	4	24-Hour Comp	1.427	n

Table 12. Percent prevalence of each organism in all effluent and influent samples for each of the 4 treatment plants. ND= non-detect.

Site	Inf[1] Eff[2]	TotColi[3]	FecColi[4]	Enteroco[5]	Crypto[6]	Giardia	Salm[7]	Vpara[8]	Vchol[9]
1	1	100	100	86	57	100	71	29	29
2	1	100	100	57	57	100	71	29	14
3	1	100	100	100	71	100	71	29	29
4	1	100	100	100	57	100	71	43	29
1	2	100	100	100	71	86	43	14	14
2	2	100	100	100	29	100	86	29	ND
3	2	100	100	100	43	100	57	29	ND
4	2	100	100	100	57	86	57	ND	ND

[1] = Influent; [2] = Effluent; [3] = total coliform; [4] = fecal coliform; [5] = *Enterococcus*; [6] = *Cryptosporidium*; [7] = *Salmonella;* [8] = *Vibrio parahaemolyticus*; [9] = *Vibrio cholerae*

Table 13. Results from analysis of variance for differences among sites in the recovery of spiked protozoal oocysts. Arcsine-transformed data used.

Variable	R^2	*P*	*A posteriori*
Cryptosporidium in influent	0.4635	0.1536	
Cryptosporidium in effluent	0.5591	0.0747	1=3, 1>4=2, 3=4=2
Giardia in influent	0.1974	0.6016	
Giardia in effluent	0.6976	0.0179	1=2, 1>3=4, 2=3=4

Seven out of 11 fecal pathogens of interest for the current study were detected at all wastewater treatment facilities during the 2007–2008 sampling (Table 14). *Vibrio alginolyticus*, *Campylobacter* and Ecoli-O157:H7 were not detected in any wastewater sample. Modifications of analytical methods following the first one or two sampling periods affected results for two microbes: First, greater dilutions were required to accurately quantify *Vibrio parahaemolyticus* and, secondly, use of the US EPA method (USEPA 2006; Method 1682) was required to detect *Salmonella* in wastewater samples. These two issues resulted in enumerations for *V. parahaemolyticus* of "too numerous to count" (TNTC), as well as and non-detect results for *Salmonella* that could be attributed to analytical problems in earlier samples. ROS data for wastewater are shown in Table 15.

Concentrations of both indicator bacteria and fecal pathogens were much higher in wastewater influent than in effluent. Mean densities of indicator bacteria were greater than those of all fecal pathogens in both influent and effluent. *Giardia* had greater mean concentrations than *Cryptosporidium* in both influent and effluent, and *Vibrio parahaemolyticus* had the greatest mean concentrations of the *Vibrio* bacteria.

Table 14. Influent and effluent loads of selected fecal pathogens (microbe concentration/ 100 ml or 10 L) for 4 wastewater treatment facilities in the Monterey Bay region of central California. Concentrations of *Cryptosporidium* and *Giardia* were corrected, using percent recovery of spikes.

Date	Site	Source[1]	TotColi[2] 100ml	FecColi[3] 100ml	Enteroco[4] 100ml	Crypto[5] 10L	Giardia 10L	Vibpar[6] 100ml	Vibchol[7] 100ml	Vibalg[8] 100ml	Campy [9]100ml	Salm[10] 100ml	Ecoli-O157 100ml
5/7/07	1	1	14400000	5000000	9300000	300	162000	TNTC[11]	<1000	<1000	<100	NR[12]	<1000
6/5/07	1	1	19800000	9000000	630000	1800	972000	10000	<1000	<1000	<100	NR	<1000
9/24/07	1	1	5000000	32000000	450000	<15	16364	<1000	55000	<1000	<100	35	<1000
12/17/07	1	1	42000000	15000000	1600000	<15	1623273	<1000	900	<1000	<100	2	<1000
3/31/08	1	1	3000000	7200000	500000	5400	693818	<1000	<1000	<1000	<100	43	<1000
6/2/08	1	1	8700000	8400000	2000000	<15	494182	<1000	<1000	<1000	<100	35	<1000
9/29/08	1	1	2000000	1100000	<100	27300	568909	<1000	<1000	<1000	<100	92	<1000
3/7/11	1	1	NA[12]	NA	NA	<15	515455	NA	NA	NA	NA	NA	NA
3/8/11	1	1	NA	NA	NA	<15	216273	NA	NA	NA	NA	NA	NA
3/9/11	1	1	NA	NA	NA	<15	2487273	NA	NA	NA	NA	NA	NA
5/7/07	2	1	99000000	36000000	900000	667	47538	TNTC	<1000	<1000	<100	NR	<1000
6/5/07	2	1	187000000	88000000	3500000	3333	889846	71000	<1000	<1000	<100	NR	<1000
9/24/07	2	1	52000000	38000000	900000	<33	1170462	<1000	<1000	<1000	<100	161	<1000
12/17/07	2	1	53000000	69000000	4500000	667	1357846	<1000	1000	<1000	<100	5	<1000
3/31/08	2	1	3400000	8400000	470000	8000	548308	<1000	<1000	<1000	<100	1	<1000
6/2/08	2	1	40000000	10000000	590000	<33	605538	<1000	<1000	<1000	<100	11	<1000
9/29/08	2	1	64000000	31000000	45000	<33	3744462	<1000	<1000	<1000	<100	8	<1000
3/1/11	2	1	NA	NA	NA	<33	25763077	NA	NA	NA	NA	NA	NA
3/2/11	2	1	NA	NA	NA	103333	8967692	NA	NA	NA	NA	NA	NA
3/3/11	2	1	NA	NA	NA	74667	12221538	NA	NA	NA	NA	NA	NA
5/7/07	3	1	9000000	1800000	600000	182	547714	TNTC	<1000	<1000	<100	NR	<1000
6/5/07	3	1	86000000	26000000	180000	627273	1218857	53000	<1000	<1000	<100	NR	<1000
9/24/07	3	1	4500000	3600000	300000	<18	10440000	100	<1000	<1000	<100	161	<1000
12/17/07	3	1	12000000	39000000	6300000	727	5629714	<1000	600	<1000	<100	16	<1000
3/31/08	3	1	4900000	4400000	110000	<18	51429	<1000	<1000	<1000	<100	5	<1000
6/2/08	3	1	34000000	4400000	200000	5818	13302857	<1000	<1000	<1000	<100	161	<1000
9/29/08	3	1	22000000	10000000	200000	1091	293143	<1000	<1000	<1000	<100	16	<1000

Date	Site	Source[1]	TotColi[2] 100ml	FecColi[3] 100ml	Enteroco[4] 100ml	Crypto[5] 10L	Giardia 10L	Vibpar[6] 100ml	Vibchol[7] 100ml	Vibalg[8] 100ml	Campy [9]100ml	Salm[10] 100ml	Ecoli-O157 100ml
3/1/11	3	1	NA	NA	NA	<18	45162857	NA	NA	NA	NA	NA	NA
3/2/11	3	1	NA	NA	NA	<18	44777143	NA	NA	NA	NA	NA	NA
3/3/11	3	1	NA	NA	NA	24727	26084571	NA	NA	NA	NA	NA	NA
5/7/07	4	1	70000000	18000000	1500000	<86	154286	TNTC	<1000	<1000	<100	NR	<1000
6/5/07	4	1	29000000	20000000	2400000	<86	475714	180000	<1000	<1000	<100	NR	<1000
9/24/07	4	1	47000000	17000000	90000	22286	156000	<1000	<1000	<1000	<100	16	<1000
12/17/07	4	1	47000000	4500000	18000	13714	541714	15000	190	<1000	<100	10	<1000
3/31/08	4	1	4200000	6900000	230000	12000	6558000	<1000	<1000	<1000	<100	16	<1000
6/2/08	4	1	26000000	3400000	380000	<86	2514857	<1000	<1000	<1000	<100	161	<1000
9/29/08	4	1	750000000	6200000	<100	211543	64312886	<1000	1200	<1000	<100	6	<1000
3/1/11	4	1	NA	NA	NA	23143	1288286	NA	NA	NA	NA	NA	NA
3/2/11	4	1	NA	NA	NA	<86	2082000	NA	NA	NA	NA	NA	NA
3/3/11	4	1	NA	NA	NA	8571	895714	NA	NA	NA	NA	NA	NA
5/7/07	1	2	<100	<100	900	80	643	<1	<1	<1	<100	NR	<10
6/5/07	1	2	<100	<100	1800	8	643	196	<1	<1	<100	NR	<10
9/24/07	1	2	<100	<100	<100	<1	143	<1	<1	<1	<100	<1	<10
12/17/07	1	2	100	100	100	1	643	<1	<1	<1	<100	<1	<10
3/31/08	1	2	800	1200	100	8	<4	<1	<1	<1	<100	1	<10
6/2/08	1	2	1100	320	310	48	71	<1	<1	<1	<100	1	<10
9/29/08	1	2	2500	1200	350	1576	750	<1	2	<1	<100	1	<10
3/7/11	1	2	NA	NA	NA	176	2786	NA	NA	NA	NA	NA	NA
3/8/11	1	2	NA	NA	NA	144	1214	NA	NA	NA	NA	NA	NA
3/9/11	1	2	NA	NA	NA	112	1536	NA	NA	NA	NA	NA	NA
5/7/07	2	2	4200000	1700000	20000	33	130	126	<1	<1	<100	NR	<10
6/5/07	2	2	1400000	900000	47000	33	130	3000	<1	<1	<100	NR	<10
9/24/07	2	2	5700000	3400000	72000	<3	478	<1	<1	<1	<100	8	<10
12/17/07	2	2	9000000	2700000	6300	<3	1739	<1	<1	<1	<100	1	<10
3/31/08	2	2	27000	35000	7000	<3	87	<1	<1	<1	<100	1	<10
6/2/08	2	2	550000	110000	2300	<3	43	<1	<1	<1	<100	2	<10
9/29/08	2	2	2800000	460000	6200	<3	348	<1	<1	<1	<100	54	<10

Date	Site	Source[1]	TotColi[2] 100ml	FecColi[3] 100ml	Enteroco[4] 100ml	Crypto[5] 10L	Giardia 10L	Vibpar[6] 100ml	Vibchol[7] 100ml	Vibalg[8] 100ml	Campy [9]100ml	Salm[10] 100ml	Ecoli-O157 100ml
3/1/11	2	2	NA	NA	NA	<3	1609	NA	NA	NA	NA	NA	NA
3/2/11	2	2	NA	NA	NA	233	1043	NA	NA	NA	NA	NA	NA
3/3/11	2	2	NA	NA	NA	<3	1391	NA	NA	NA	NA	NA	NA
5/7/07	3	2	4200000	1300000	1400000	<2	2842	TNTC	<1	<1	<100	NR	<10
6/5/07	3	2	180000	90000	8000	156	4263	24000	<1	<1	<100	NR	<10
9/24/07	3	2	540000	5000000	36000	39	2053	<1	<1	<1	<100	54	<10
12/17/07	3	2	47000000	84000000	1000	<2	7421	<1	<1	<1	<100	<1	<10
3/31/08	3	2	300	55000	2300	<2	3316	<1	<1	<1	<100	1	<10
6/2/08	3	2	270000	6500	1300	<2	1895	<1	<1	<1	<100	2	<10
9/29/08	3	2	680000	320000	<100	292	9474	<1	<1	<1	<100	92	<10
3/1/11	3	2	NA	NA	NA	58	15158	NA	NA	NA	NA	NA	NA
3/2/11	3	2	NA	NA	NA	117	8053	NA	NA	NA	NA	NA	NA
3/3/11	3	2	NA	NA	NA	2	12316	NA	NA	NA	NA	NA	NA
5/7/07	4	2	200000	90000	900	23	3600	<1	<1	<1	<100	NR	<10
6/5/07	4	2	1700000	180000	8100	23	<20	<1	<1	<1	<100	NR	<10
9/24/07	4	2	<100	<100	<100	<2	200	<1	<1	<1	<100	<1	<10
12/17/07	4	2	4700000	<100	<100	<2	4000	<1	<1	<1	<100	<1	<10
3/31/08	4	2	2000	2000	100	<2	17800	<1	<1	<1	<100	1	<10
6/2/08	4	2	<100	300000	100000	113	7000	<1	<1	<1	<100	1	<10
9/29/08	4	2	600	250	<100	23	1600	<1	<1	<1	<100	1	<10
3/1/11	4	2	NA	NA	NA	113	4400	NA	NA	NA	NA	NA	NA
3/2/11	4	2	NA	NA	NA	361	54400	NA	NA	NA	NA	NA	NA
3/3/11	4	2	NA	NA	NA	541	25200	NA	NA	NA	NA	NA	NA
	Influent Mean		59962069	18689286	1353329	29430	7238840	13713	2103	<1000	<100	48	<1000
	Influent Standard Error		21893768	3271347	341279	16462	2273706	6261	1640	-	-	10	-
	Effluent Mean		2969818	3594717	61520	109	5011	2047	<1	<1	<100	11	<10
	Effluent Standard Error		1409885	2498869	41649	42	1533	1125	-	-	-	4	-

[1] = 1 = influent, 2 = effluent; [2] = total coliform; [3] = fecal coliform; [4] = *Enterococcus*; [5] = *Cryptosporidium*; [6] = *Vibrio parahaemolyticus*; [7] = *Vibrio cholerae*; [8] = *Vibrio alginolyticus*; [9] = *Campylobacter*; [10] = *Salmonella*; [11] = too numerous to count, dilutions changed for subsequent sampling events; [12] = not reported; [13] = not analyzed

Table 15. Influent and effluent loads of selected fecal pathogens (microbe concentration/ 100 ml or 10 L) for 4 wastewater treatment facilities in the Monterey Bay region of central California: Measures of central tendency using ROS methods.

Site	Influent or Effluent	Parameter	*Cryptosporidium* Per10L	*Giardia* Per10L	*Salmonella* Per 100mL	*Vibrio parahaemolyticus* Per 100mL	Total Coliform Per 100mL	Fecal Coliform Per 100mL	*Enterococcus* Per 100mL
1	Effluent	Mean	431	1482	DNS	DNS	686	446	523
1	Effluent	95% CI	0-1255	504-2460	DNS	DNS	0-1430	48-843	0-1046
2	Effluent	Mean	DNS	1004	13.20	DNS	3382429	1329286	22971
2	Effluent	95% CI	DNS	391-1616	0-39.79	DNS	898931-586526	327688-2330883	2027-43916
3	Effluent	Mean	DNS	2644	29.54	DNS	7552900	12967357	206957
3	Effluent	95% CI	DNS	1491-3796	0-75.12	DNS	0-25269266	0-45347289	0-761564
4	Effluent	Mean	DNS	3716	DNS	ND	943257	81779	15629
4	Effluent	95% CI	DNS	38-7394	DNS	ND	0-2482461	0-174904	0-53622
1	Influent	Mean	5815	1420750	41.24	DNS	13557143	11100000	2079155
1	Influent	95% CI	0-17210	505389-2336111	9.75-72.73	DNS	1881289-25232997	2536159-19663841	0-5148242
2	Influent	Mean	14310	5992600	37.13	DNS	71200000	40057143	1557857
2	Influent	95% CI	0-32917	0-12087734	0-120.64	DNS	23187224-119212776	17568282-62546004	230125-2885590
3	Influent	Mean	90727	8604650	41.64	DNS	24628571	12742857	1127143
3	Influent	95% CI	0-330499	1990822-15218478	0-121.68	DNS	0-49764326	1520176-23965538	0-3467929
4	Influent	Mean	8662	9214270	41.64	54642	139028571	10857143	660135
4	Influent	95% CI	0-24509	0-29011123	0-121.68	0-177942	0-414521153	5531081-16183204	0-1397385

DNS= Data not sufficient, ND=Non-detect

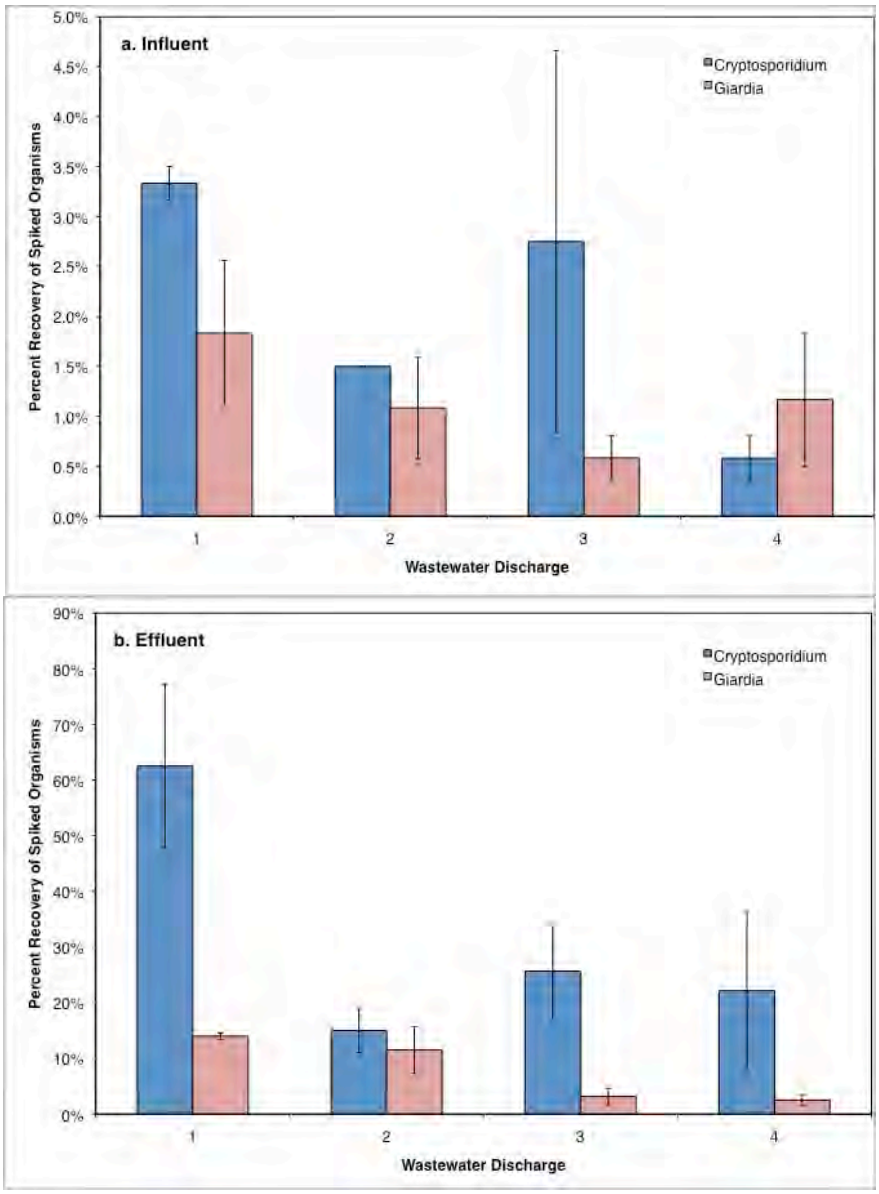


Figure 4 (a, b). Mean recoveries of spiked protozoal oocysts into influent and effluent samples (n = 3) from four wastewater treatment facilities. Bars indicate standard errors.

Influent concentrations of some indicator bacteria for wastewater treatment facilities in central California were similar to those reported elsewhere. For example, Scott et al (2003) reported total coliform counts of approximately 60,000,000 cfu/100ml in influent from three wastewater treatment facilities in Florida, which compares to the mean of nearly 60,000,000 in the current study (Table 14). *Enterococcus* counts reported by Scott et al (2003) ranged approximately 800,000 cfu/100ml and 4,000,000 cfu/100ml, which compares to the mean concentration in the current study of 1,353,329 cfu/100ml.

Influent concentrations of fecal protozoa for wastewater treatment facilities in central California were also similar to those reported elsewhere. Scott et al. (2003) reported *Cryptosporidium* concentrations between 800 and 3,000 organisms/L, whereas Payment et al. (2001) reported *Cryptosporidium* at average concentrations of 26 organisms/L. These compare to

a mean influent *Cryptosporidium* concentration of 2,943 organisms/L in the current study. Influent *Giardia* concentrations reported by Scott et al (2003) ranged between approximately 10,000 organisms/L and 100,000 organisms/L, whereas those reported by Payment et al (2001) averaged 1,552 organisms/L. These influent *Giardia* concentrations are comparable to the mean of 72,388 organisms/L reported in the current study. However, both of previous studies reported raw protozoal concentrations that were not corrected using data from spiked sample recoveries. Consequently, the raw concentrations in the current study were similar to or below those from prior studies (Scott et al (2003 Payment et al 2001). If the data in Table 14 were replaced with raw data without correction from the spiking studies, the mean concentration of *Cryptosporidium* would be reduced to <90 organisms/L and *Giardia* to <22,000 organisms/L.

The mean concentrations of most FIB and fecal pathogens did not show variation with sampling date in either influent or effluent, except for an apparent decline over time in *Enterococcus* in influent, and high concentrations of *V. parahaemolyticus* during the June, 2007 sampling period in both influent and effluent (Figure 5). There were no apparent differences among sites with respect to concentrations of microbes in influent, although sites 1 and 2 had much lower concentrations of indicator bacteria in effluent (Figure 6).

Two one-way ANOVAs (JMP, SAS Campus Drive Building S Cary, NC 27513) using transformed [log (x+1)] data were performed to determine whether the differences in mean densities of indicator bacteria and fecal pathogens between wastewater treatment facilities (Figures 5 and 6, Table 16) were statistically significant. All values below the detection limit were included at one half the detection limit to account for pathogen presence at <MDL (Arnone and Walling, 2006). One influent indicator bacterium and one fecal pathogen exhibited significant temporal variation, but no spatial variation: Influent *Enterococcus* concentrations in December 2008 were lower than in any other month, while influent *Vibrio parahaemolyticus* concentrations were greater in June 2007 than in any other month. Effluent *V. parahaemolyticus* concentrations were also greater in June 2007 than in any other month. Site 1 had significantly lower effluent concentrations of total coliform, fecal coliform and *Enterococcus*, while sites 1 and 2 had significantly lower concentrations of *Giardia* than did either Site 3 or Site 4. Concentrations of *V. parahaemolyticus* were significantly greater in June 2007 in both influent and effluent, while influent concentrations of *Enterococcus* were lowest in September 2008.

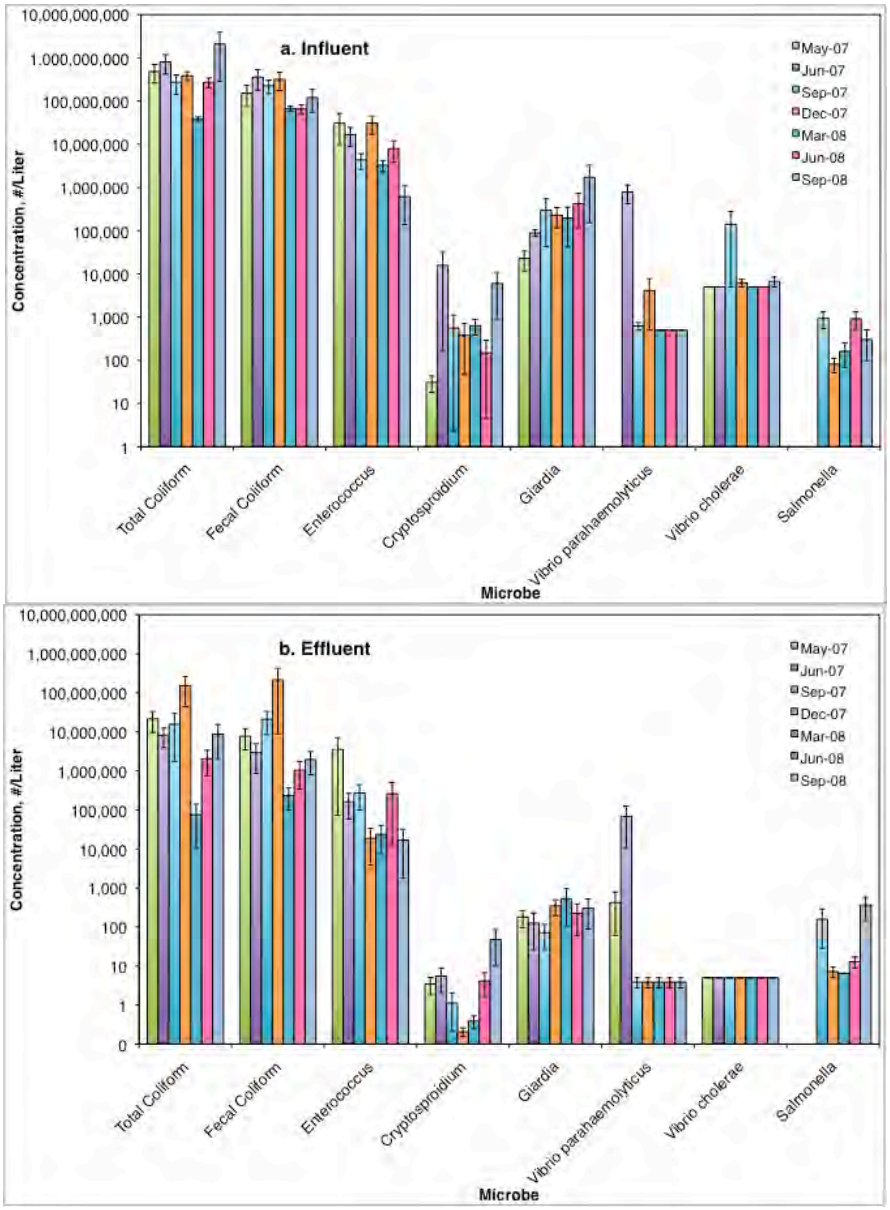


Figure 5 (a, b). Mean densities of indicator bacteria and fecal pathogens in samples collected from four wastewater treatment facilities in the Monterey Bay area. Bars indicate standard errors.

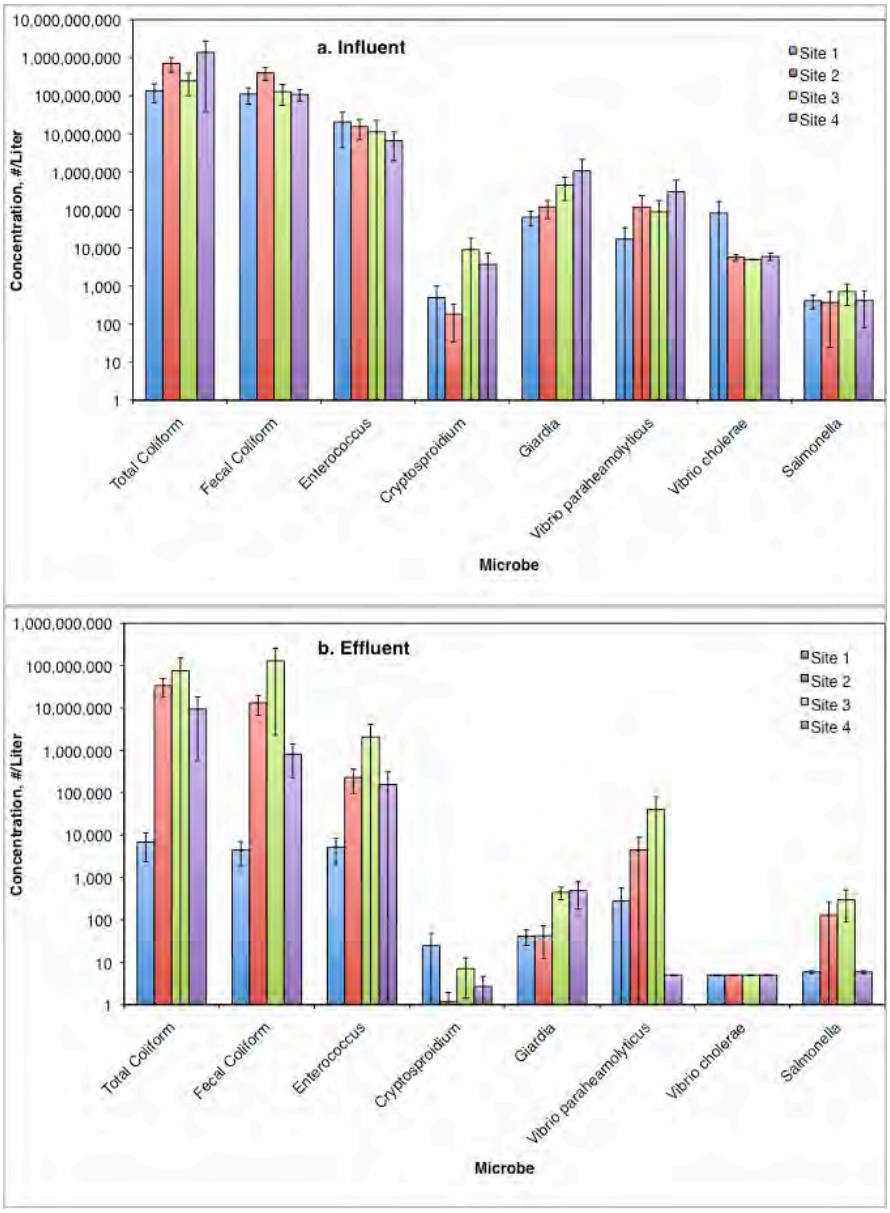


Figure 6 (a, b). Mean densities of indicator bacteria and fecal pathogens collected approximately quarterly in 2007 and 2008 from four wastewater treatment facilities in the Monterey Bay area. Bars indicate standard errors.

Table 16. Temporal and spatial differences among sites for concentrations of indicator bacteria and fecal pathogens at four wastewater treatment facilities in central California. (Analysis of variance using transformed data [log (x+1)])

	Main Effect	R^2	P	*A posteriori*[1]
Influent				
Enterococcus	Time[1]	0.5339	0.0113	2=4=6=3=5>7
Vibrio parahaemolyticus	Time	0.8279	<0.0001	2>4=7=5=6=3
Effluent				
Total Coliform	Site	0.5332	0.0058	2=3, 3=4, 2>4=1
Fecal Coliform	Site	0.6454	0.0007	3=2>4=1
Enterococcus	Site	0.3383	0.0177	2=3=4, 3=4=1, 2>1
Giardia	Site	0.4097	0.0049	3=4> 2=1
Vibrio parahaemolyticus	Time	0.6110	0.0022	2=1, 1=3=4=5=6=7, 2>3=4=5=6=7

[1] = 1= 5/7/07, 2 = 6/5/07, 3 = 9/24/07, 4 = 12/17/07, 5 = 3/31/08, 6 = 6/2/08, 7 = 9/29/08; [2] = Site 1, Site 2, Site 3, Site 4; sites or times with the highest concentrations are on the left and those with the lowest concentrations are on the right.

To explore potential causes of the temporal variation observed in influent pathogen concentrations, the relationship between local rainfall and influent concentrations of FIB and fecal pathogens was assessed for each site. Cumulative rainfall amounts for two, five and seven days prior to wastewater sampling were obtained for nearby rain gauges from the California Data Exchange Center, operated by the California Department of Water Resources (http://cdec.water.ca.gov/). The rainfall data were used as independent variables in model building exercises using stepwise multiple regressions. All rainfall and microbe data were transformed [log (x+1)]. To expand the conditions tested for protozoa, the March 2011 sampling dates were included in the regressions for *Cryptosporidium* and *Giardia*. The stepwise regressions revealed no significant associations between cumulative precipitation during two, five or seven days prior to sampling and influent concentrations of indicator bacteria, *Vibrio* spp. or *Salmonella*. Both protozoa, however, exhibited significant associations with rainfall, on various time scales and at some sites (Table 17). *Cryptosporidium* counts were significantly associated with cumulative rainfall over the previous five days at sites 3 and 4, and *Giardia* counts were significantly associated with cumulative rainfall over the previous seven days at Site 3.

Table 17. Results of stepwise multiple regressions to determine relationships between indicator bacteria or fecal pathogens in influent and local rainfall. Transformed [log (x+1)] data used.

Variable	Site	R^2	*P*	Model
Cryptosporidium	3	0.5275	0.0174	*Cryptosporidium* = 4.08 + 18.01(5-day rainfall)
Cryptosporidium	4	0.3502	0.0715	*Cryptosporidium* = 5.34 + 8.95(5-day rainfall)
Giardia	3	0.8479	0.0002	*Giardia* = 13.31 + 10.81(7-day rainfall)

Estimates of protozoal removal efficiencies by the wastewater treatment facilities were affected by the large differences protozoal detection between influent and effluent in oocyst spike recovery studies. Because of the effects of total solid concentrations and other factors on efficiency of oocyst recovery and detection, there were sometimes greater estimated concentrations of *Cryptosporidium* and *Giardia* in effluent compared to influent from the same time and site (Table 14). Consequently, estimates of percent removal through the wastewater treatment process were based on concentration values summed over all sampling periods at each site.

Percent removal of indicator bacteria and fecal pathogens often exceeded 99% (Table 18). Moreover, all wastewater treatment facilities appeared to remove more than 99% of the actual fecal pathogens measured. Across all sites, *V. cholerae*, *Giardia* and *Cryptosporidium* were most easily removed, with average removals exceeding 99.6%. *Salmonella* had the lowest average removal at 76.94%, with no site achieving 99% removal, and fecal coliform had the second lowest average removal, in part due to very high concentrations measured in the December 2007 effluent sample at Site 3 (see Table 14). Site 1 had the best overall removal, averaging 99.99% over all microbes measured and 99.86% for fecal pathogens. Site 3 had the lowest overall removal, averaging 65.24%, although it removed 99.94% of fecal pathogens.

Removal efficiencies reported here are comparable to those found in other investigations of wastewater treatment processes. Scott et al (2003) reported 100.0% removal of total and fecal coliforms and *Enterococcus* from three Florida wastewater treatment facilities, while Que et al (2009) reported 99.5% removal of total coliforms and 99.0% of *Enterococcus* at a municipal wastewater treatment facility in Australia. Scott et al (2003) also reported removal efficiencies of 96.8% - 99.75% for *Cryptosporidium* and 99.94% - 99.98% for *Giardia*. Wen et al (2009) reported removal efficiencies of 80% for *Cryptosporidium* and 96.3% for *Giardia*. However, accurate comparisons of removal efficiencies for FIB and fecal pathogen concentrations are potentially problematic due to inconsistent inclusion of non-corrected (raw) data from previous studies and data that have been corrected for spike recoveries (as was done in the current study).

Table 18. Percent removal of indicator bacteria and fecal pathogens from wastewater during treatment in four wastewater treatment facilities in the Monterey Bay area. Red text indicates >99% removal efficiency.

	Percent Removal									
Site	TotColi[1] 100ml	FecColi[2] 100ml	Enteroco[3] 100ml	Crypto[4] 10L	Giardia 10L	Vibpar[5] 100ml	Vibchol[6] 100ml	Salm[7] 100ml	Fecal Pathogens	All
1	>99.99%	>99.99%	99.98	93.83	99.89	98.41	99.99	98.57	99.86	99.99
2	95.25	96.68	98.53	99.83	99.99	95.74	99.91	64.14	99.98	96.08
3	69.33	-1.76	81.64	99.90	99.95	56.44	99.90	58.57	99.94	65.24
4	99.32	99.25	97.63	99.59	99.85	>99.99%	99.91	98.58	99.85	99.35
All	95.22	80.77	95.45	99.63	99.93	91.92	99.98	76.94	99.92	92.83

[1] = total coliform; [2] = fecal coliform; [3] = *Enterococcus*; [4] = *Cryptosporidium*; [5] = *Vibrio parahaemolyticus*; [6] = *Vibrio cholerae*; [7] = *Salmonella*

4.1.2. Rivers and Streams

Fecal indicator bacteria. Fecal indicator bacteria (FIB) measured in riverine and estuarine samples included total coliforms, fecal coliforms, *Enterococcus*, and *Bacteroidales*. As expected for more developed watersheds, total coliforms and *Bacteroidales* were detected in all samples. Fecal coliforms were detected in 99% of samples, and *Enterococcus* in 75% of samples. The greatest FIB concentrations were observed in surface waters from the high-use central bay tributaries including the San Lorenzo River, Watsonville Slough, Pajaro River, and Salinas River. The mean concentrations of indicator bacteria for the 10 river sites ranging from north to south along the central coast are shown in Figure 7 and Figure 8. Mean concentrations of all four indicator bacteria were greatest at Watsonville Slough, while the lowest total coliform, fecal coliform, and *Enterococcus* concentrations were observed at Elkhorn Slough, a site with high tidal flushing. All four FIBs were relatively low in the two rivers on the extreme north (Waddell Creek and Scott Creek) and south (Carmel River, Big Sur River) ends of the sampling region.

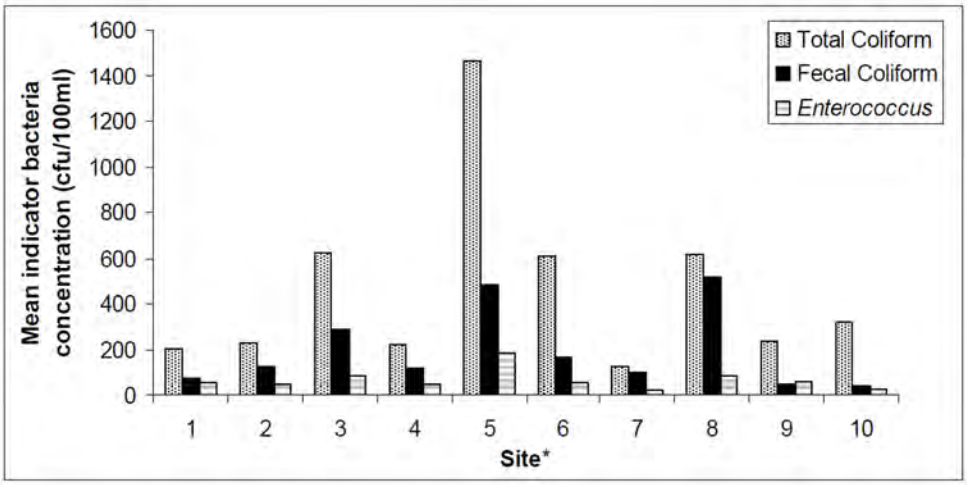


Figure 7. Mean indicator bacteria concentrations at 10 rivers along the central California coast from April, 2007 to September, 2008. ***Site ID: 1=Waddell Creek, 2=Scott Creek, 3=San Lorenzo River, 4=Soquel Creek, 5=Watsonville Slough, 6=Pajaro River, 7=Elkhorn Slough, 8=Salinas River, 9=Carmel River, 10=Big Sur River (from North to South)**

Seasonal dynamics of FIB concentrations are summarized by month in Figure 8. Total coliform bacterial concentrations were greatest during the summer months (June through September), and were lowest during the winter months of January through March. However, fecal coliform, *Enterococcus*, and *Bacteroidales* concentrations did not demonstrate clear trends by season (Figure 8 and Figure 9). The median values of fecal coliforms ranged from 30 cfu/100 mL in spring, to 84 cfu/100 mL in fall, whereas the median values of *Enterococcus* ranged from 10 cfu/100 ml in summer, to 20 cfu/100 mL in winter.

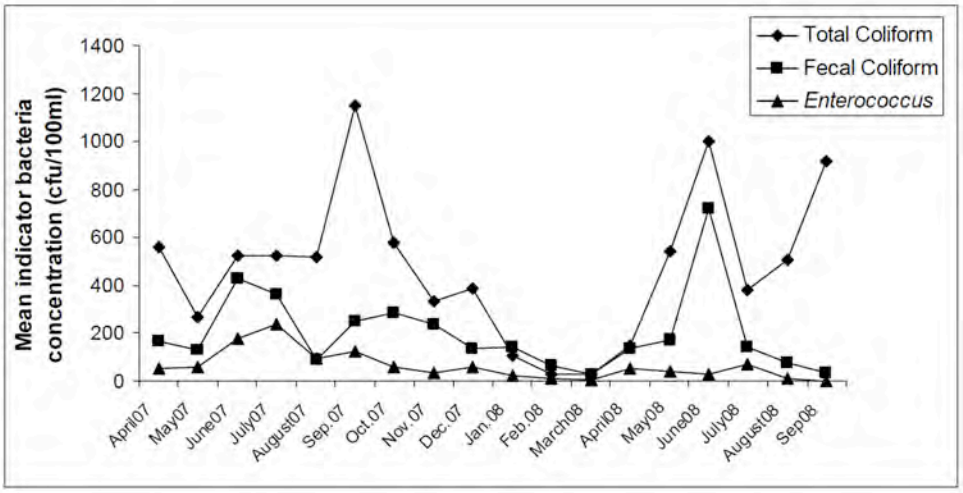


Figure 8. Mean monthly indicator bacterial mean concentrations at 10 rivers along the central California coast from April, 2007 to September, 2008.

Pathogens in surface water. Pathogens were detected at freshwater sites as well as estuarine sites with a tidal marine influence (Table 19). *Vibrio cholerae* was detected most frequently from surface water samples (44%), followed by *Giardia* spp. (21%), *Cryptosporidium* spp. (18%), *V. alginolyticus* (8%), *Salmonella* spp. (7%), *V. parahaemolyticus* (6%), and *Campylobacter* spp. (5%). *Escherichia coli*-O157:H7 was not detected in any surface water samples.

Vibrio cholerae were detected at all study sites except the Big Sur River, with a median concentration of 23 cfu/100 mL in positive samples. No *ctx*-positive or O1-positive *V. cholerae* were detected. *Vibrio parahaemolyticus* was detected at half of the study sites, with a median concentration of 55.5 cfu/100 mL. Overall, the concentrations ranged from 0.2 to 1,980 cfu/100 mL for *V. cholerae*, from 0.2 to 720 cfu/100 mL for *V. parahaemolyticus,* and from 0.4 to 720 cfu/100 mL for *V. alginolyticus*. None of the target bacteria were detected at the Big Sur River, the southernmost and least developed site. Target bacteria did not exhibit a seasonal distribution, however *Vibrio* spp. were most prevalent during summer. The occurrence of lower FIB counts in winter coincided with a lower detection of *Vibrio* spp. The greatest concentrations of all FIB, as well as *Vibrio* spp., were observed at Watsonville Slough. Duplicate samples were not significantly different among sites and timepoints ($P > 0.05$).

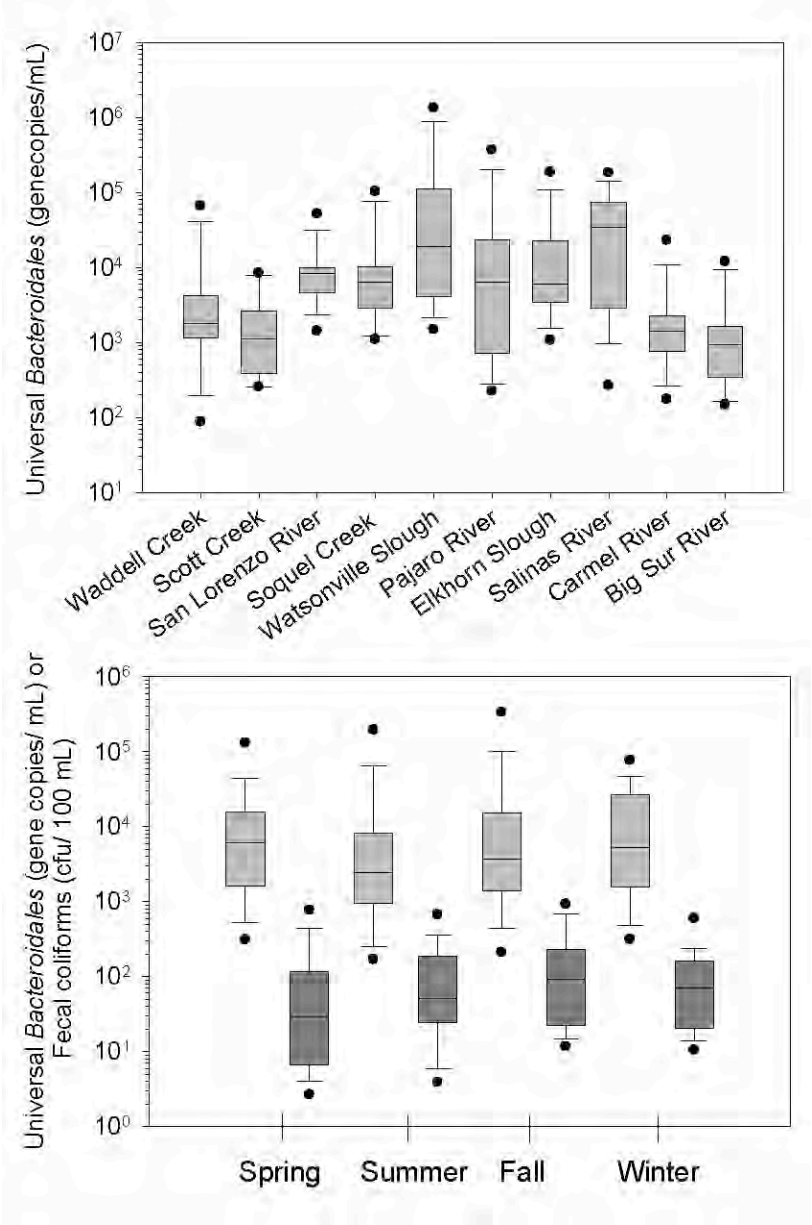


Figure 9. Box plots of (top) site-specific variation of universal *Bacteroidales* concentration from North to South, and (bottom) seasonal variation for universal *Bacteroidales* (light grey) and fecal coliforms (dark). Upper and lower bounds of boxes denote the 75th and 25th percentiles. Upper and lower bounds bars are the 90th and 10th percentiles, with outliers represented by filled circles. Note logarithmic vertical axis.

Figure 10 shows the mean *Vibrio* counts by site; the remaining target pathogens were not detected frequently or at high concentrations. *Vibrio cholerae* concentrations were greatest in Waddell Creek, the San Lorenzo River, Watsonville Slough and Pajaro River. Low *V. parahaemolyticus* and *V. alginolyticus* concentrations were detected in most rivers with the exception of the San Lorenzo. Seasonal dynamics of *Vibrio* concentrations are summarized by month in Figure 11. Concentrations of *V. cholerae*, *V. parahaemolyticus*, and *V. alginolyticus* peaked in August when the average water temperature was 16.4ºC.

Cryptosporidium oocysts were detected at nine of the ten study sites and *Giardia* cysts were found at all ten sites. *Cryptosporidium* parasites were detected most often (29% of samples) at Soquel Creek and Watsonville Slough, two sites in the central Monterey Bay region that have urban and rural uses upstream of sampling sites. *Giardia* cysts were detected most often (43% of positive samples) in water samples from the San Lorenzo River, another highly developed tributary to Monterey Bay. For *Cryptosporidium* spp. the range of oocyst concentrations detected in positive samples was low, from 1 to 9 /10 L, with a median value within positive samples of 2 /10 L. The concentrations of *Giardia* cysts in positive samples ranged from 1 to 58 /10 L, with a median value of 1 /10 L. *Cryptosporidium* spp. contamination was observed year-round, with the greatest number of positive samples detected in November and February (40%). In contrast, no seasonal trends were observed for *Giardia* spp., with cysts detected in 10-40% of samples throughout the year.

ROS analysis was performed on river and stream surface water data and the calculated means and 95% confidence intervals are listed in Table 23.

Table 19. Prevalence of bacterial and protozoal pathogens in monthly surface water samples by site in the Monterey Bay region of California (2007-2008).

Site ID	Marine Influence[a]	Proportion of water samples positive (%)							
		V.p.[b]	*V.ch.*[c]	*V. alg.*[d]	*Salm.*[e]	*Campy.*[f]	*E.c.*-O157[g]	*Crypto.*[h]	*Giardia*[i]
Waddell Creek	Yes	-[j]	50	-	21	-	-	-	21
Scott Creek	No	-	43	-	13	7	-	21	14
San Lorenzo River	Yes	14	64	-	21	-	-	14	43
Soquel Creek	No	-	29	21	7	7	-	29	29
Watsonville Slough	No	21	79	14	-	21	-	29	7
Pajaro River	Yes	7	79	-	-	-	-	21	29
Elkhorn Slough	Yes	14	21	43	7	7	-	23	15
Salinas River	No	-	64	-	-	7	-	21	21
Carmel River	No	7	14	-	-	-	-	14	21
Big Sur River	No	-	-	-	-	-	-	8	8
Overall % positive		6	44	8	7	5	-	18	21

aMarine-influenced if site mean salinity above 0.5 ppt

b*Vibrio parahaemolyticus*; c*V. cholerae*; d*V. alginolyticus*; e*Salmonella* spp; f*Campylobacter* spp.;

g*Escherichia coli*-O157:H7; h*Cryptosporidium* spp.; i*Giardia* spp., not detected.

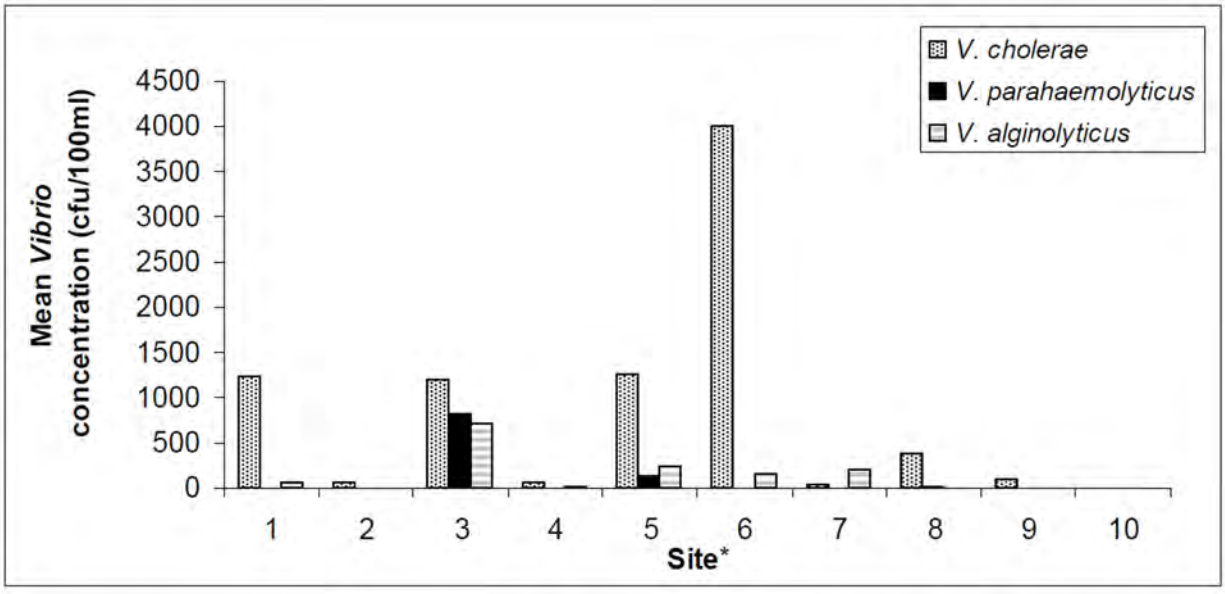


Figure 10. Mean *Vibrio* concentrations at 10 rivers along the central California coast from April, 2007 to September, 2008. ***Site ID: 1=Waddell Creek, 2=Scott Creek, 3=San Lorenzo River, 4=Soquel Creek, 5=Watsonville Slough, 6=Pajaro River, 7=Elkhorn Slough, 8=Salinas River, 9=Carmel River, 10=Big Sur River (from North to South)**

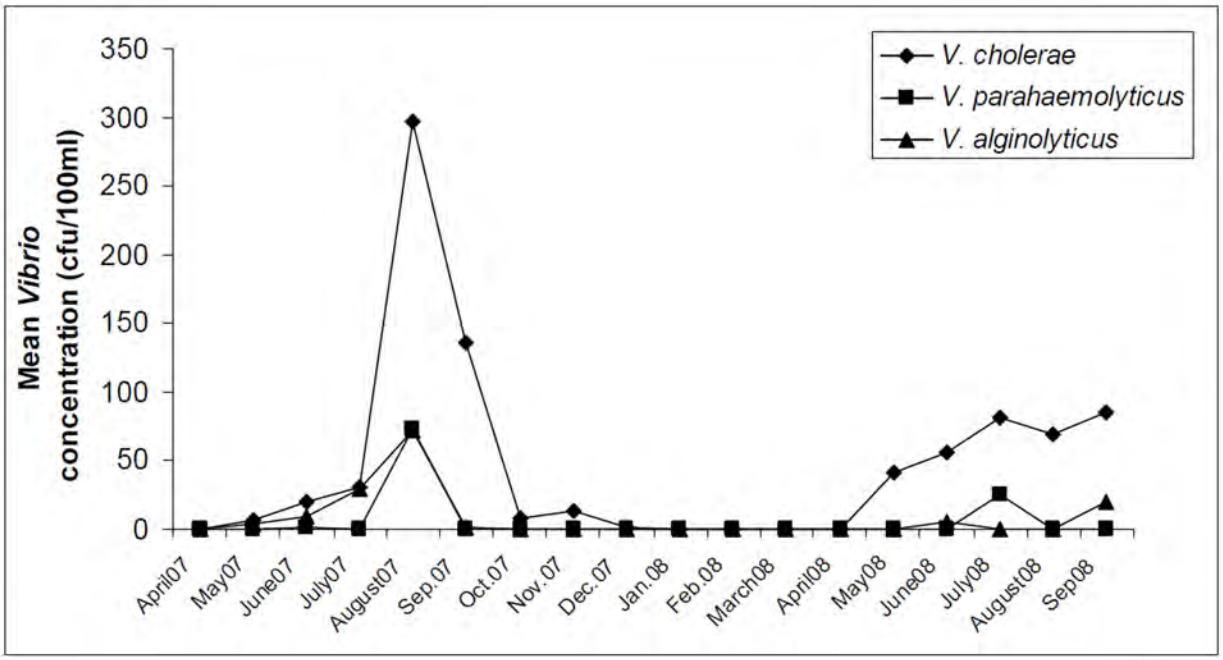


Figure 11. Mean monthly *Vibrio* concentrations at 10 rivers along the central California coast from April, 2007 to September, 2008.

The strength of association between environmental variables (like temperature and salinity) and the presence of the target pathogens in surface waters (e.g., river, estuaries, ocean) was initially addressed using simple logistic regression analyses. Only *Vibrio* spp. were used for these analyses because they are endemic in surface waters and have been associated with disease. Table 20 shows the univariate odds ratios and 95% confidence intervals for variables significantly associated with the detection of *V. cholerae*, *V. parahaemolyticus*, and/or *V. alginolyticus*. Based on univariate analysis, *V. cholerae* was 6 times more likely to be detected in surface water if the water temperature was >12ºC and if fecal coliforms were > 400cfu/10ml. *Vibrio cholerae* also was 3 times more likely to be detected if total coliforms were between 1,000 to 10,000cfu/100ml, and if *Enterococcus* concentrations were >104cfu/100ml. Overall, surface water samples were significantly more likely to test positive for *V. cholerae* during the dry season and if the ambient temperature was >14ºC. In contrast, *V. parahaemolyticus* was approximately 6 times more likely to be cultured from water samples collected on rainy days, if fecal coliform concentrations in the same water sample were between 200-400cfu/100ml, and if *Enterococcus* concentrations were between 35-104cfu/100ml. *Vibrio alginolyticus* detection also was associated with rain, demonstrating a 2.5 times greater likelihood than during sunny conditions.

Following univariate analysis, environmental variables that were significantly associated with target bacteria detection in surface waters were analyzed using a forward-stepping multivariable logistic regression approach to simultaneously consider the risk contributions of multiple variables. Table 21 shows the final logistic regression models that contained the risk factor variables significantly associated with detection of *V. cholerae*, *V. parahaemolyticus*, or *V. alginolyticus* in surface water samples. In the final model, *V. cholerae* detection was significantly associated with greater fecal coliform concentration (>400cfu/100ml), greater water temperature (>12ºC), and was less likely to be detected in ocean compared to river/estuary water samples. There was no association between *V. cholerae* detection and recent rainfall, in contrast to *V. parahaemolyticus*, where the odds of detection were almost 7 times greater if rain had occurred on the day that the sample was collected. Similarly, *V. alginolyticus* was more likely to be detected when rain had occurred within 24 hours prior to sampling.

Correlation of indicators and pathogens. Datasets were non-normally distributed, partly due to the fact that the majority of samples tested negative for the selected pathogens. Thus, non-parametric Kendall and Spearman correlation analyses were performed and showed the same trends when calculated a) using the complete data sets with non-detects (negative test results) replaced by zeros, and b) using only positive test result data. For case a), detection of the universal *Bacteroidales* marker correlated significantly with confirmation of *Cryptosporidium* spp., while total coliforms and fecal coliform detection correlated significantly with that of *Cryptosporidium* spp. and *V. cholerae* (Table 22). In contrast, enterococcal counts were not correlated with detection of any specific pathogens.

Table 20. Simple logistic regression analysis of variables with significant association of pathogenic bacteria detection in surface water samples from April, 2007 to September, 2008.

	V. cholerae			*V. parahaemolyticus*			*V. alginolyticus*		
Variables	Odds Ratio	95%CI	*P* value	Odds Ratio	95%CI	*P* value	Odds Ratio	95%CI	*P* value
Indicator Bacteria									
Total Coliform (cfu/100 ml)									
≤ 1,000	1	-	-	1	-	-	1	-	-
> 1,000-10,000	3.60	(1.86, 6.98)	<0.001*	0.83	(0.18, 3.87)	0.82	0.73	(0.23, 2.36)	0.59
Fecal Coliform (cfu /100 ml)									
≤ 200	1	-	-	1	-	-	1	-	-
> 200-400	2.86	(1.24, 6.61)	0.014*	5.8	(0.96, 34.7)	0.05*	0.34	(0.10, 1.14)	0.08
> 400	4.9	(1.9, 12.6)	0.001*	NA	NA	NA	0.88	(0.35, 2.23)	0.80
Enterococcus (cfu/100 ml)									
≤ 35	1	-	-	1	-	-	1	-	-
> 35-104	2.93	(1.48, 5.76)	0.002*	5.18	(0.97, 27.6)	0.05*	1.09	(0.40, 2.94)	0.87
> 104	NA	NA	NA	1.44	(0.37, 5.59)	0.60	1.09	(0.24, 4.98)	0.91
Water Condition									
Water Temperature									
≤12℃	1	-	-	1	-	-	1	-	-
>12℃	6.48	(3.15, 13.3)	<0.001*	NA	NA	NA	1.84	(0.76, 4.46)	0.18
Turbidity									
≤ 4.75ntu	1	-	-	1	-	-	1	-	-
> 4.75ntu	1.45	(0.66, 3.21)	0.36	0.83	(0.20, 3.37)	0.79	1.18	(0.40, 3.44)	0.76
Weather Condition at the sampling day									
Ambient Temperature									
≤14℃	1	-	-	1	-	-	1	-	-
>14℃	2.05	(1.02, 4.09)	0.04*	0.86	(0.26, 2.88)	0.81	0.55	(0.31, 0.98)	0.04*
Weather Condition									
Clear/Sunny	1	-	-	1	-	-	1	-	-
Overcast/cloudy	NA	NA	NA	NA	NA	NA	NA	NA	NA
Shower	1.43	(0.86, 2.41)	0.17	6.08	(1.37, 26.9)	0.02*	2.47	(1.46, 4.16)	0.001*
Weather Condition in past 24 hours									
Clear/Sunny	1	-	-	1	-	-	1	-	-
Overcast/cloudy	1.43	(0.79, 2.58)	0.24	NA	NA	NA	2.99	(1.41, 6.32)	0.004*
Shower	0.74	(0.13, 4.09)	0.73	3.38	(1.20, 9.55)	0.02*	2.99	(0.56, 16.0)	0.20
Site Characteristic									
Faeces									
No	1	-	-	1	-	-	1	-	-
Yes	1.68	(1.00, 2.84)	0.05*	1.50	(0.44, 5.10)	0.51	0.83	(0.35, 1.99)	0.68
Animal tracks									
No	1	-	-	1	-	-	1	-	-
Yes	0.93	(0.34, 2.55)	0.89	0.49	(0.14, 9.09)	0.12	0.60	(0.30, 1.04)	0.06
Animal presence									
No	1	-	-	1			1	-	-
Yes	1.55	(0.79, 3.03)	0.20	1.64	(0.36, 7.55)	0.53	3.22	(1.69, 6.17)	<0.001*
Garbage									
No	1	-	-	1	-	-	1	-	-
Yes	2.24	(1.16, 4.35)	0.001*	2.31	(0.62, 8.57)	0.21	0.39	(0.20, 0.77)	0.007*
Other									
SampleSite									
River	1	-	-	1	-	-	1	-	-
Ocean	0.13	(0.02, 0.83)	0.03*	0.52	(0.05, 5.49)	0.59	2.5	(0.83, 7.53)	0.10
Season									
Wet	1	-	-	1	-	-	1	-	-
Dry	2.35	(1.40, 3.94)	0.001*	1.64	(0.69, 3.89)	0.28	2.29	(1.18, 4.44)	0.01*

*$P < 0.05$, consider significant

NA = Not Applicable

Table 21. Multivariate logistic regression analysis of risk factors significantly associated with *Vibrio* spp. detection in surface water samples form April, 2007 to September, 2008.

Bacterial species	Significant risk factor	Odds Ratio	95%CI	*P* value
V. cholerae	Fecal Coliform (cfu/100 ml)			
	≤ 200	1		-
	> 400	3.56	(1.39, 9.11)	0.008*
	Water Temperature			
	≤12ºC	1	-	-
	>12ºC	3.63	(1.52, 8.65)	0.004*
	SampleSite			
	River	1	-	-
	Ocean	0.20	(0.04, 1.02)	0.05*
V. parahaemolyticus	Weather Condition			
	Clear/Sunny	1	-	-
	Shower	6.85	(1.77, 26.2)	0.005*
V. alginolyticus	Weather Condition in past 24 hours			
	Clear/Sunny	1	-	-
	Overcast/cloudy	2.15	(0.99, 4.64)	0.05*

*$P < 0.05$, consider significant

When organisms are detected in a minority of samples, some of the many non-detects will inevitably match during correlation analysis between organisms. Given the fact that not detecting a certain parameter at the minimum detection limits of a given test does not mean that the parameter is absent, all non-detects were removed for case b) to see if relationships existed between positive test results, when considered separately. We considered this a valid approach under the assumption that "not detected, but present" parameters might also correlate. For the three standard FIB, the only correlation that remained was between fecal coliforms and *Cryptosporidium* spp. (Spearman R=0.453, *P*=0.023), while levels of enterococci and host-specific *Bacteroidales* markers were not significantly correlated with enteric pathogen detection. The only significant correlation between *Bacteroidales* and specific pathogen detection was for the ratio of human to universal *Bacteroidales* (using any ratio >0) and *Vibrio cholerae* detection, with R = 0.529 (*P*=0.017). Sparse data precluded correlation analysis for other *Vibrio* species (n ≤ 5). Using binary logistic regression with presence/absence data, no association was found between detection of the human *Bacteroidales* marker and isolation of any *Vibrio* spp. (maximum Nagelkerke's R-square: 0.057).

Table 22. Significant Spearman correlations between *Bacteroidales* markers, indicator bacteria, and specific pathogen detection for all sites combined, either with non-detects (test-negative samples) included as zero values (top) or omitted (bottom).

		n	Spearman	
			R	*P*-value[a]
		all data		
Universal *Bact.*[b]	Dog *Bact.*[b]	143	0.227	0.006
	Cryptosporidium spp.	137	0.213	0.013
Human *Bact.*[b]	Total coliforms	137	0.264	0.002
	Fecal coliforms	137	0.310	< 0.001
	Enterococci	137	0.224	0.008
Cow *Bact*[b]	Dog *Bact.*[b]	143	0.240	0.004
Total coliforms	Fecal coliforms	138	0.719	< 0.001
	Enterococci	138	0.331	< 0.001
	V. cholerae	138	0.245	0.004
	Vibrio spp.	138	0.254	0.003
Fecal coliforms	Enterococci	138	0.434	< 0.001
	Cryptosporidium spp.	138	0.180	0.035
	V. cholerae	138	0.240	0.005
	Vibrio spp.	138	0.225	0.008
		detects only		
Universal *Bact.*[b]	Human *Bact.*	53	0.412	0.002
	Fecal coliforms	142	0.200	0.017
Total coliforms	Fecal coliforms	142	0.725	< 0.001
	Enterococci	112	0.626	< 0.001
Fecal coliforms	Enterococci	112	0.602	< 0.001
	Cryptosporidium spp.	25	0.453	0.023

[a] *P*-value is the probability of obtaining a result that is at least as extreme as the one that was actually observed, assuming that the null hypothesis is true.
[b] universal, human, or dog *Bacteroidales* marker.

Predictive Qualifier. Pathogens were categorized as positive or negative in surface water samples in order to calculate Predictive Qualifier (PQ) values at various thresholds of indicator bacterial concentrations. This PQ approach provided insights into how threshold cutoffs for categorizing indicator (FIB and *Bacteroidales*) data are related to specific pathogen detection (presence/absence). Categorizing indicator data can be useful, for example, when total coliforms or universal *Bacteroidales* signals were detected in almost every sample, and so presence/absence data alone would not provide informative data. The PQ gives a pathogen

presence-weighted percentage of how often FIB presence co-occurs with specific pathogen detection, when certain indicator threshold concentrations are applied.

For environmental samples the question regarding what percentage of association exceeds randomness is not trivial, as different factors can co-occur. For example, co-occurrence of indicators and pathogens at a certain sampling point in a river might simply be the result of a confluence of two streams with separate origins of indicators and pathogens further upstream. In this study we considered Predictive Qualifier values ≥66% (PQ66) as noteworthy, because they are above what might be considered random. If a specific pathogen was only present in a few samples from a given location, and the FIB was never detected at that site, then total matches would be close to 100% (TOT_{true}). However, pathogen occurrence in relation to indicators would be low (POS_{true} = 0%), leading to a PQ value <50%. To achieve a PQ ≥66% with averaging, both terms TOT_{true} and POS_{true} must exceed 66%, indicating that two in of three cases are predicted correctly.

The PQ66 target level (or greater) was reached for specific threshold concentration ranges of universal *Bacteroidales* and FIBs, but not human-specific *Bacteroidales,* with several pathogens (Figures 12 and 13). PQ66 ranges were observed for universal *Bacteroidales* with *V. cholerae* (100 - 800 gc/mL), *V. alginolyticus* (1,400 – 1,499 gc/mL), and *Vibrio* spp. (100 – 1,599 gc/mL) for all study sites combined. When separating sites by average salinity, the most apparent change was that prediction of *Cryptosporidium* spp. presence increased from a PQ of 66% up to over 77% (between 1,100 and 6,299 gc/mL) for marine-influenced sites. Given the small number of dog- and cow-positive *Bacteroidales* samples, their PQ performance was not evaluated.

Total coliforms showed the highest PQ values, with overlapping ranges for all three *Vibrio* species analyzed individually as well as for their combined signals in fresh water. The PQ66 range was from 10 to 399 cfu/100 mL in fresh water sites only, and from 10 to 199 cfu/ 100 mL, when fresh and salt water sites were pooled. Both ranges span beyond the median value of 117 cfu/100 mL of total coliform-positive samples. A similar range (20 - 239 cfu/100 mL) was observed for *Salmonella* spp. in marine-influenced sites.

Fecal coliforms were the only FIBs that reached PQ66 thresholds for specific pathogen detection in both salinity-separated and pooled sites, specifically for *Cryptosporidium* spp., *Salmonella* spp., *V. parahaemolyticus* and *V. cholerae*. For *Salmonella* detection, the highest PQ value (88.4 %) and the largest PQ66 range (10 -239 cfu fecal coliforms/ 100 mL) were reached at marine-influenced sites. Similar trends were observed at fresh water sites for detection of *V. cholerae* (10 – 179 cfu/100 mL, PQ_{max}: 76.4%), *V. parahaemolyticus* (20 – 149 cfu/100 mL, PQ_{max}: 83.5%), and combined *Vibrio* spp. (10 – 169 cfu/100 mL, PQ_{max}: 79.1%). When all sites were pooled, enterococci reached PQ66 values for detection of *Salmonella spp.* (10 – 39 cfu/ 100 mL, max. PQ 74.2%) and *V. parahaemolyticus* (40 – 79 cfu/ 100 mL, max PQ 73.6 %).

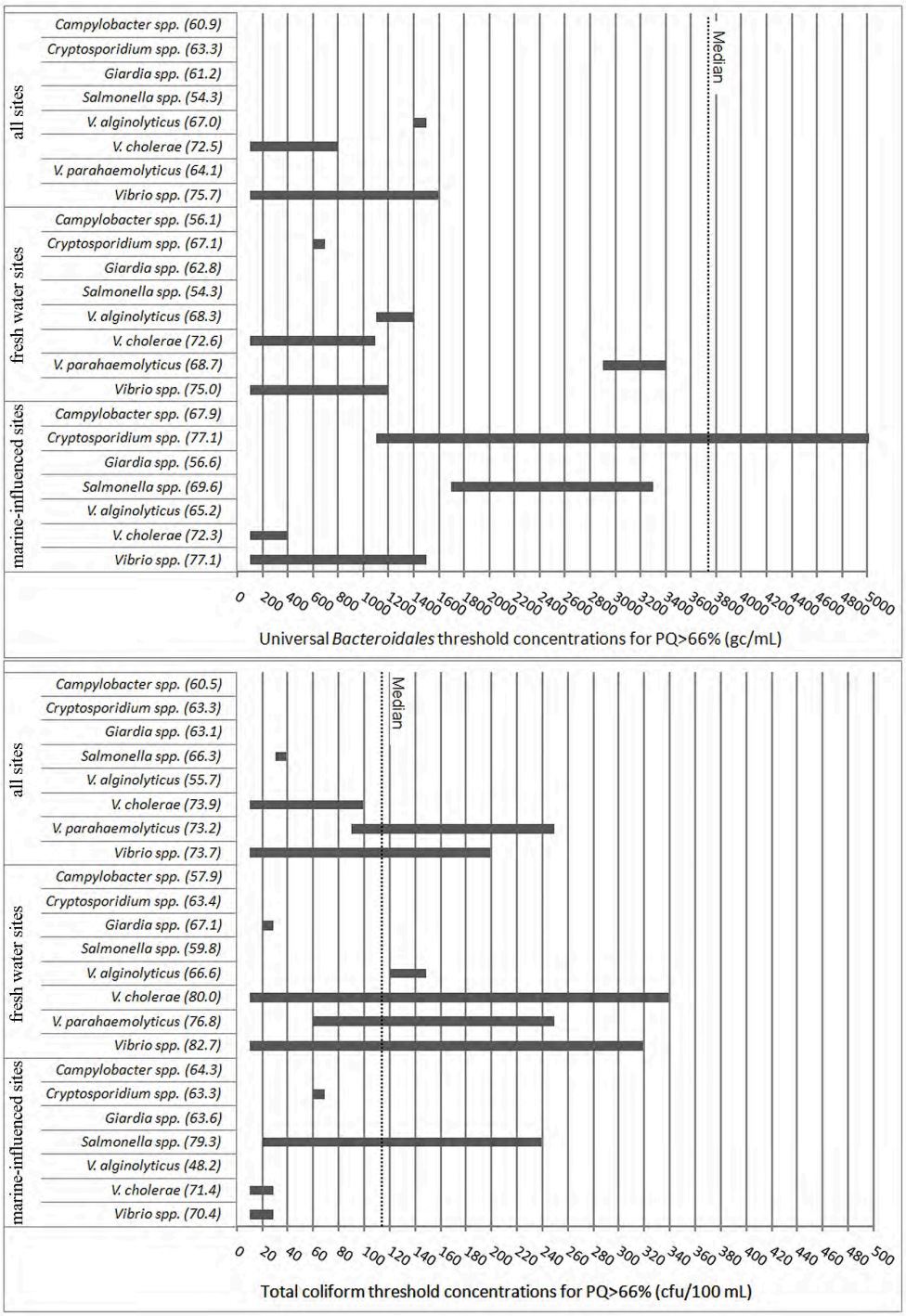


Figure 12. Threshold concentration ranges for universal *Bacteroidales* (top) and total coliforms (bottom), with resulting PQ values greater than 66% for data from all sites combined and separated by average site salinity (freshwater salinity: <0.5 ppt). Maximum PQ values are in parentheses. *Vibrio parahaemolyticus* are not displayed for marine-influenced sites because only one positive sample was detected. The vertical dotted line indicates the median value of all measured samples.

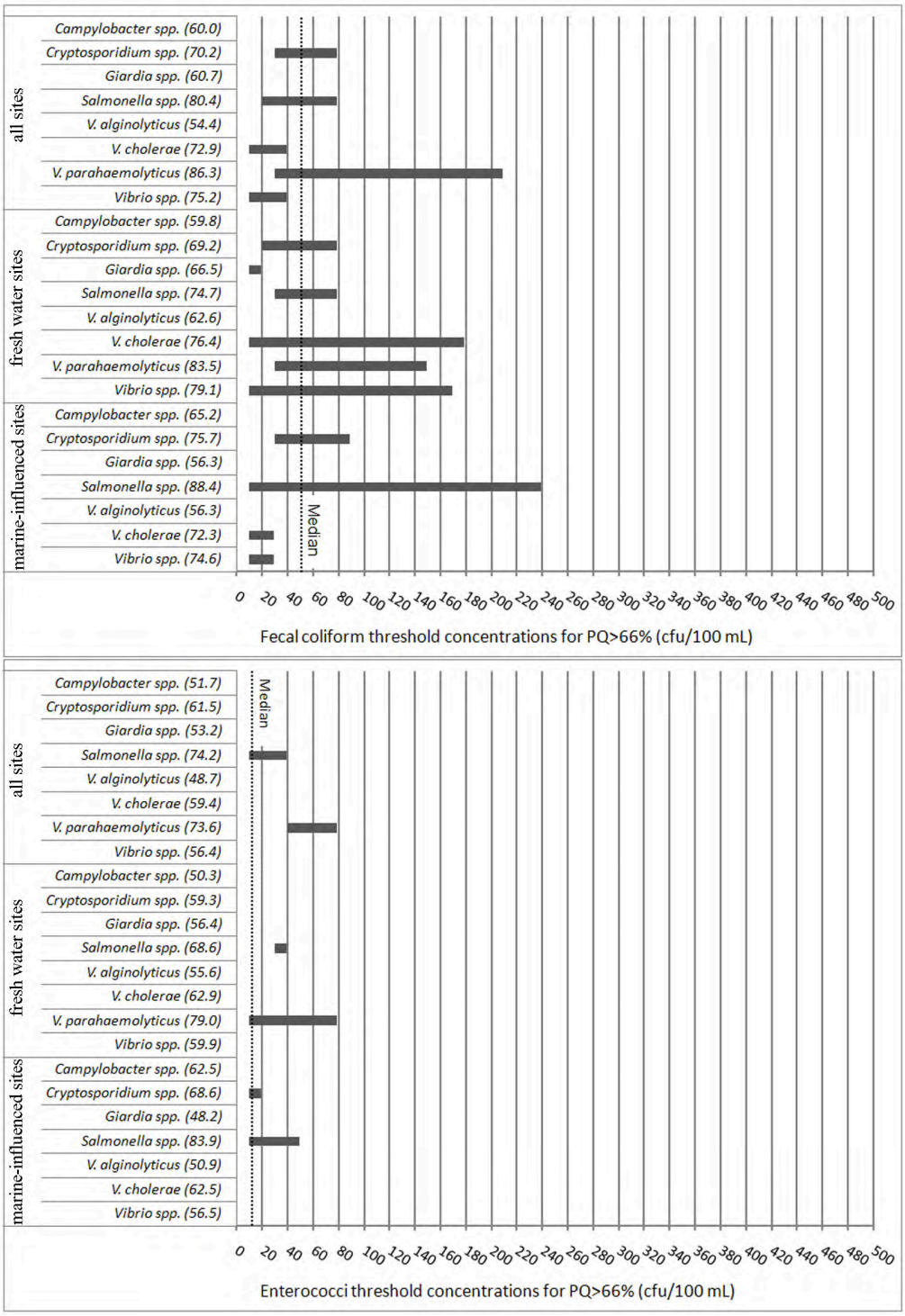


Figure 13. Threshold concentration ranges for fecal coliforms (top) and enterococci (bottom), with resulting PQ values greater than 66% for all sites combined, and separated by average salinity (freshwater salinity: <0.5 ppt). Maximum PQ values are shown in parentheses. The vertical dotted line indicates the median value of all measured samples.

To compare the predictive ability of indicators, mean PQ values for *V. cholerae*, *Cryptosporidium* spp. and *Giardia* spp. were chosen for evaluation because these pathogens were detected most commonly. For the elimination of outliers, PQ values of each indicator were considered within a threshold concentration range from zero (non-detect) to the 75th percentile of the measured concentrations. Fecal coliforms exhibited the highest overall mean PQ value for this comparison (55.6%), followed closely by enterococci (54.5%), total coliforms (54.0%), and universal *Bacteroidales* (51.1%). When the same criteria were applied to the subset of marine-influenced sites, universal *Bacteroidales* showed the highest mean PQ (52.3 %), followed by enterococci (50.8%), fecal coliforms (48.9%) and total coliforms (47.0%). For single pathogens, the mean PQ values can also vary significantly. For example, the highest mean PQ for *Cryptosporidium* spp. detection was 66.7 % when predicted by universal *Bacteroidales* for marine-influenced sites, compared to 55.2% for fresh water samples.

In this study we found that there was widely distributed pathogen occurrence across the study sites. The protozoa *Cryptosporidium* and *Giardia* were detected at low levels at most study sites, similar to findings from other environmental studies by Miller et al. (2005c) and those reviewed by Fayer et al. (2004). Contributing to the seemingly ubiquitous nature of these fecal protozoa in surface waters are the fact that protozoal oocysts and cysts are known to persist in aquatic environments (Juni and Janik 1969; Samadpour et al., 2002), and are shed in the feces of a wide range of human and animal hosts (Fayer et al., 2004). With respect to bacterial pathogens, *Salmonella* and *Campylobacter* spp. were detected infrequently, while *Vibrio* species were commonly detected, with variations in abundance linked to seasonal fluxes in salinity, temperature and other local environmental conditions similar to previous reports(Boom et al., 1990; Karim et al., 2004; Robertson et al., 1992). The fact that protozoa were detected more frequently than bacterial pathogens could be due to differences in host loading dynamics, variations in environmental persistence, differences in detection methods and sample volumes and competitive growth of other microorganisms.

One key objective of the current study was to investigate relationships between conventional FIB metrics and specific pathogen detection in order to improve predictive tools and minimize health risks associated with recreational water contact. We had hypothesized that FIB measurements would be good predictors of the presence of pathogenic bacteria in water and mussels. Time and location-matched sampling revealed that total coliforms and fecal coliform counts had a significant positive association with the occurrence of *V. cholerae*, though not with other *Vibrio* spp. No such association was identified for enterococci. Unfortunately, target bacterial pathogens other than *Vibrio* spp. were not detected often enough to facilitate similar analyses.

These findings were similar to a report by Blackwell et al. (2008) who determined that total coliform concentrations were positively correlated with the occurrence of *V. cholerae*, *V. parahaemolyticus* and other *Vibrio* spp. In contrast, Ristori et al. (2007) reported no correlation between standard FIB detection methods and *Vibrio* presence, while Koh et al. (1994) demonstrated either no relationship or a negative relationship between standard FIB detection methods and *Vibrio* counts. Overall, conventional FIB levels did not consistently predict specific bacterial pathogen presence or absence, and the precision of risk detection varied between different FIB assays and enteric bacterial pathogens. As a result, correlations between FIB and specific pathogen detection were weak, and no single FIB assay could perfectly predict pathogen presence or absence, similar to findings from previous studies (Harwood et al., 2005; Yates, 2007; Ortega et al., 2009; Wilkes et al., 2009).

Vibrio cholerae and *V. parahaemolyticus* were the two potential pathogens detected most commonly in surface waters (Table 22). *Vibrio cholerae* is the causative agent of cholera in some regions of the world, but many non-pathogenic *V. cholerae* strains are autochthonous to rivers, estuarine, and coastal water in California and elsewhere (Louis et al., 2003; Constantin de Magny, 2008). Temperature, salinity, rainfall and plankton are all important variables in the ecology of *V. cholerae*, influencing the transmission of human epidemic cholera (Kaysner et al., 1987; Louis et al., 2003; Constantin de Magny, 2008). In the current study, *V. cholerae* was 7 times more likely to be detected in rivers than in ocean water, similar to findings a study in Japan (Uchiyama, 1998). In addition to environmental variables, another reason *V. cholerae* detection was higher in river water was due to fecal inputs from terrestrial animals and humans that can contaminate overland runoff. Once introduced into a waterway, *Vibrio* bacteria may multiply and find niches facilitate long-term survival, and may persist in both culturable and non-culturable states. In this study, no pathogenic strains of *V. cholerae* were found using ctx-specific PCR for toxin genes or O1-antiserum. However, non-O1 *V. cholerae* have been occasionally associated with human diarrheal illness along the central California coast (Chandrasekhar et al., 2008; Ottaviani et al., 2009), so we cannot exclude the possibility of health risks for humans and animals that have contact with recreational waters.

Vibrio parahaemolyticus also survives well in estuarine environments, and disease in humans has been sometimes associated with consumption of raw bivalve shellfish (Kaneko and Colwell, 1975; CDC, 1999). A positive correlation between water temperature and *V. parahaemolyticus* counts in water and mussels has been shown (DePaola et al., 2003; Parveen et al., 2008). Interestingly, *V. parahaemolyticus* was 6 times more likely to be detected when rain had occurred on the sampling day, but *V. parahaemolyticus* detection was not significantly associated with temperature in the current study. No prior studies have reported associations between rain events and *V. parahaemolyticus* detection, though Miller et al. (2006) found a negative association between rain and *V. alginolyticus* detection in mussels. *Vibrio alginolyticus* was frequently detected in our study and is commonly reported in seawater and seafood (Cavallo and Stabili, 2002; Lhafi and Kuhne, 2007). In contrast to *V. cholerae* and *V. parahaemolyticus*, *V. alginolyticus* has only rarely been associated with clinical disease and is considered an opportunist. Our findings concur with reports from Italy and Indonesia in which *V. alginolyticus* was detected in both mussels and nearby ocean areas (Molitoris et al., 1985; Cavallo and Stabili, 2002). These contrasting findings for different *Vibrio* spp. highlight the diversity in ecological adaptations and niches occupied by different *Vibrio* spp.

The greatest FIB concentrations occurred during summer and the lowest occurred during winter, consistent with previous reports (Plummer and Long, 2007). Increased FIB densities in summer months could reflect higher environmental temperatures, providing favorable bacterial growth conditions and/or enhanced fecal inputs from livestock, pets, wildlife, or humans (Harwood et al., 2005; Plummer and Long, 2007). This is also the season when nearshore fecal inputs from tourists and their pets, leaking septic systems, irrigated lawns and vessel traffic could peak in many coastal areas. The highest densities of all *Vibrio* species was found during summer, when the mean water temperature was 16.3ºC. Optimal water temperatures for *V. alginolyticus*, *V. cholerae*, and *V. parahaemolyticus* proliferation are >8ºC, >10°C, and 12-20°C, respectively (Kaper et al., 1979; Blake et al., 1980; Martinez-Urtaza et al., 2008). The significant association between peak concentrations of these three *Vibrio* spp. and water temperature >12ºC was matched temporally with mean FIB enumerations and monthly temperatures, consistent with previous findings (Kaper et al., 1979; Blake et al., 1980; Mezrioui et al., 1995; Barbieri et al., 1999;

DePoala et al., 2003; Blackwell and Oliver 2008; Martinez-Urtaza et al., 2008). During colder months, concentrations of *V. alginolyticus*, *V. parahaemolyticus*, and *Vibrio* spp. declined. This finding can be explained by the ability of many bacteria to enter into a viable but nonculturable (VBNC) state (Bates and Oliver, 2004), a dormant state in which bacterial cells are metabolically active, but cannot be isolated using standard laboratory methods (Ravel et al., 1995).

The nadir for *Campylobacter* detection also occurred during winter when average temperatures dropped to <12°C. Low temperatures in winter and high solar radiation in summer affects survival and recovery of *Campylobacter* spp. (Carter et al., 1987; Horman et al., 2004), and inactivation of *Campylobacter* spp. by solar radiation may contribute to lower recovery in summer (Carter et al., 1987; Horman et al., 2004; Boyle et al., 2008). Our findings are consistent with reports from Norway and Finland, where environmental *Campylobacter* spp. detection was seasonal, with the majority of isolates obtained during cool, but not cold months (Horman et al., 2004).

Salmonella can be released into the environment from infected humans, farm animals, pets, and wildlife, and these opportunistic pathogens can persist in wetlands, wastewater discharges and storm runoff (Baudart et al., 2007). In the current study, *Salmonellae* were detected in approximately 5% of river and seawater samples year-round, consistent with reports by other authors (Morinìigo et al., 1993; Polo et al., 1998). *Salmonella* was more likely to be detected in colder months, and detection peaked in December. No seasonal variation of *Salmonella* spp. detection in marine environments was observed by Alonso et al. (1992).

Eschericha coli O157:H7 was not detected in this study. Previous studies have also reported no *E. coli* O157:H7 from invertebrates and sea otters along the central California coast (DePaola et al., 2003; Ristori et al., 2007; Miller et al., 2005; 2006; 2009). The widely accepted method of immunomagnetic separation with CHROMagar O157 detection used in the current study had a mdl of 10cfu/100ml in spiked river samples, and 1cfu/100ml in spiked ocean samples (De Boer and Heuvelink, 2000; Church et al., 2007). Potential explanations for the lack of *E. coli* O157:H7 detection in this and prior studies from coastal California include that no *E. coli* O157:H7 was present, or that the bacterium was present in a VBNC state due to reduced nutrient status, high salinity, pH extremes or other factors that interfered with pathogen detection (Ramaiah et al., 2002; Na et al., 2006; Soto et al., 2009).

Significant Spearman and Kendall correlations were observed for some, but not all FIB/ pathogen combinations. Analyses were performed as an all-inclusive data set with non-detect samples set to 0, and after excluding non-detects from the data set. Results from the two approaches differed slightly, but overall correlations ranged from 0.14 to 0.73 (perfect correlation would = 1). Significant FIB/ pathogen correlations are summarized in Table 22. The general pattern of values < 0.5 may reflect the range of environments tested, seasonal variations, differences in survivorship and complex niches for both FIBs and pathogens. In a prior study (Field and Samadpour 2007), enterococci enumeration was not highly correlated with *Campylobacter* spp., *Cryptosporidium* spp. *Giardia* spp., or *Salmonella* spp. detection in surface waters. For most environmental studies, pathogens were only detected in a minority of samples, but these detects are the most important cases for characterization of relationships between FIBs and pathogens. One limitation of using the full dataset to perform correlation analysis is that the effect of pathogen-positive samples can be diluted. Conversely, if only pathogen-positive samples are assessed, critical spatio-temporal and seasonal information regarding pathogen prevalence is lost that could help clarify relationships between FIB values and risk of specific pathogen

presence or absence. Additionally, correlation analysis does not permit different indicator threshold concentrations to be used for categorizing results, as can be done in PQ analysis.

Calculation of Predictive Qualifier levels employed a weighted average to determine how often pathogen occurrence was correctly predicted or missed when selecting different threshold cutoff concentrations for FIB detection. We extended the approach of Harwood et al. (2005), who expressed the relationship between FIB and pathogen detection as the percentage of true/false, positive/negative combinations, while also weighting different FIB concentrations by the actual pathogen occurrence in paired samples. Binary logistic regression is also useful for stepwise analysis of signal thresholds, but it does not permit weighted evaluation of pathogen occurrence (Seurinck et al., 2005; Wiedenmann et al., 2006). We found no associations between detection of any specific pathogen and identification of the human-specific *Bacteroidales* marker in paired water samples; this finding is consistent with the low PQ percentages observed for threshold concentrations of the human marker for all pathogens.

The fact that 100% of samples were positive for universal *Bacteroidales* markers demonstrates why establishment of threshold cutoffs is necessary for estimation of predictive values: presence/absence is often uninformative. Threshold cutoffs could be varied depending on the pathogen of interest; with respect to *V. cholerae* detection, the highest Predictive Qualifier percentage was associated with a signal threshold of 500 gc/mL. For wastewater and river samples in Sapporo Japan, good predictive values for total and human *Bacteroidales* markers were found in association with *E. coli*-O157:H7 and *Salmonella* detection. Unfortunately, both pathogens were either never detected or rare in the current study (Seurinck et al., 2005). In Canada, ruminant *Bacteroidales* presence was predictive for *E. coli* O157:H7 detection (Wiedenmann et al., 2006). Salinity plays an important role for the predictive ability of FIB. While *Cryptosporidium* spp. were distributed equally at freshwater- and marine-influenced sites in association with universal *Bacteroidales* detection, a range of >10% between water types could be observed for mean PQ values. Additional studies are needed to fill-in data gaps and provide guidance as to the best approaches for predicting health risks.

In conclusion, conventional indicator enumeration methods have imprecise relationships for predicting the risk of specific pathogen exposure in water samples. Total and fecal coliform concentrations were predictive only for the presence of *V. cholerae*, and no pathogenic strains of *V. cholerae* were found in the current study. High *Vibrio* counts were significantly associated with higher water temperatures, as reported previously. Low occurrences of *Campylobacter*, *Salmonella*, and *E. coli* O157:H7 precluded robust statistical assessment of potential FIB/ pathogen associations. This study assessed the utility of traditional FIB measures to predict the risk of specific pathogen presence or absence in freshwater, seawater, and mussels at the land-sea interface of central California, and to begin the process of tracing pathogen disbursement from the land to surface water and marine ecosystems. Understanding the dynamics of indicator bacteria and pathogens as they flow from the human or animal host into watersheds and oceans will facilitate future studies aimed at minimizing health risks related to surface water consumption and occupational or recreational water contact.

Table 23. Summary statistics for pathogen concentrations in rivers using regression on order statistics (ROS).

	Cryptosporidium Per10L	*Giardia* Per10L	Total Coliform Per 100mL	Fecal Coliform Per 100mL	*Enterococci* Per 100mL	*Vibrio cholerae* Per 100 mL	*Vibrio alginolyticus* Per 100mL
Waddell Creek							
Mean	445	DNS	202	71	51	70	4
95% CI	0-1683	DNS	51-354	26-116	15-86	0-166	0-8
Scott Creek							
Mean	8	DNS	227	125	45	4	DNS
95% CI	0-18	DNS	0-492	13-238	26-64	0-10	DNS
San Lorenzo River							
Mean	47	52	622	291	86	68	DNS
95% CI	0-110	0-117	323-9211	161-420	40-131	0-167	DNS
Soquel Creek							
Mean	13	8	226	120	52	3	DNS
95% CI	3-23	3-13	148-303	81-159	37-67	0-8	DNS
Watsonville Slough							
Mean	12	5	1466	487	182	71	13
95% CI	4	2-8	744-2187	288-685	43-322	0-164	0-41
Pajaro River							
Mean	4	6	609	164	52	222	DNS
95% CI	1	3-10	0-1344	16-313	15-90	0-481	DNS
Elkhorn Slough							
Mean	7	4	127	101	19	2	11
95% CI	3	0-8	17-237	15-187	0-38	0-5	0-23
Salinas River							
Mean	DNS	34	615	519	85	23	DNS
95% CI	DNS	0-71	288-941	0-1278	0-203	5-40	DNS
Carmel River							

	Cryptosporidium Per10L	*Giardia* Per10L	Total Coliform Per 100mL	Fecal Coliform Per 100mL	*Enterococci* Per 100mL	*Vibrio cholerae* Per 100 mL	*Vibrio alginolyticus* Per 100mL
Mean	8	DNS	235	47	57	DNS	DNS
95% CI	3-14	DNS	34-436	20-74	0-121	DNS	DNS
Big Sur River							
Mean	DNS	DNS	361	43	27	ND	ND
95% CI	DNS	DNS	0-825	21-64	8-45	ND	ND

DNS = Data not sufficient; ND=Non-detect

4.1.3. Ocean and Mussels

Ocean and mussel samples (n=42) were collected quarterly from April, 2007 to September, 2008. Similar to river water, *Vibrio* spp., *Cryptosporidium*, and *Giardia* were the most common target pathogens detected in ocean water and mussels (Table 24). *Campylobacter* and *Salmonella* spp. were detected in ocean water, but not mussels. *Vibrio cholerae, V. alginolyticus*, *Cryptosporidium*, and *Giardia* were isolated from both ocean water and mussels, while *Escherichia coli* O157:H7 and *V. parahaemolyticus* were not detected in either sample type. Interestingly, *V. cholerae* was detected significantly less often in ocean water mussels, when compared to river samples (P<0.001).

Table 24. Pathogen prevalence in ocean water and mussels in central California.

Pathogen	Ocean water (%)*	Mussels (%)*
Campylobacter	10	0
Salmonella	5	0
Escherichia coli O157:H7	0	0
Vibrio cholerae	10	7
Vibrio parahaemolyticus	0	0
Vibrio alginolyticus	24	24
Cryptosporidium	26	6
Giardia	19	17

*n=42 time and location-matched samples of ocean water and mussels. Mussels were collected in batches of 30 mussels/ sample.

In addition to determining pathogen prevalence in paired ocean water and mussels, we evaluated FIB versus pathogen detection in the same sample set. The relationship between FIB concentrations and specific pathogen detection was variable, but good correlation was found between higher FIB concentrations and *Vibrio* spp. detection. Spearman's rank correlation revealed a significant correlation between FIB counts and *V. cholerae* detection in ocean water, with r_s = 0.44 for total coliforms (P =0.005), r_s = 0.42 for fecal coliforms (P =0.007), and r_s = 0.44 for enterococci (P =0.004). Similar results were obtained for mussels; FIB counts were significantly correlated with *V. alginolyticus* detection, with r_s =0.41 for total coliforms (P =0.008), r_s =0.61 for fecal coliforms (P <0.001), and r_s =0.68 for enterococci (P <0.001). ROS analysis was also performed for ocean water and the calculated means and 95% confidence intervals are listed in table 25.

Table 25. Summary statistics for pathogen concentrations in ocean samples using regression on order statistics (ROS).

	Cryptosporidium Per10L	*Giardia* Per10L	Total Coliform Per 100mL	Fecal Coliform Per 100mL	*Enterococci* Per 100mL	*Vibrio cholerae* Per 100 mL	*Vibrio alginolyticus* Per 100mL
Scott Creek							
Mean	DNS	DNS	30	9	DNS	ND	ND
95% CI	DNS	DNS	0-74	0-32	DNS	ND	ND
Elkhorn Slough							
Mean	DNS	DNS	794	386	120	13	
95% CI	DNS	DNS	272-1316	52-721	5-235	0-28	
Carmel River Beach							ND
Mean	DNS	DNS	8	5	DNS	ND	ND
95% CI	DNS	DNS	0-18	0-10	DNS	ND	
Cayucos Pier							
Mean	23	DNS	6	8	5	ND	11
95% CI	4-41	DNS	2-10	0-19	0-12	ND	0-23
Motel Pt							
Mean	DNS	DNS	5	DNS	DNS	ND	DNS
95% CI	DNS	DNS	2-8	DNS	DNS	ND	DNS
Morro Bay							
Mean	11	DNS	45	40	11	ND	4572
95% CI	0-23	DNS	8-82	13-68	2-19	ND	0-17518

DNS = data not sufficient; ND=Non-detect

4.1.4. Stormwater

Six storm events were sampled during the rainy seasons of 2008 and 2010. The three stormwater sample sets obtained on January 8, 2008, January 19, 2010 and April 12, 2010 included samples from all sample sites and at all three one-hour time intervals. Sampling was attempted during three additional storms on November 1, 2008, December 15, 2008, and April 5, 2010; however the rain either ceased before all three time points could be sampled or geographic separations resulted in rain that was not consistent between all three sites, resulting in incomplete sampling in 2010. Flow estimates made on April 5 and 12, 2010 at a culvert upstream from the Pacific Grove site provided raw data to determine approximate values for pathogen outflow to the ocean. Summary statistics of raw data are listed in Table 26 for all stormwater samples, and ROS analysis was performed to estimate corrected measures of central tendency along with 95% confidence intervals in Table 28.

Table 26. Summary table of stormwater pathogen concentration means and ranges for each site.

MEAN	***Cryptosporidium* 10L**	***Giardia* 10L**	**Total Coliform 100ml**	**Fecal Coliform 100ml**	***Enterococcus* 100ml**	***Vibrio parahaemolyticus* 100ml**	***Salmonella* 100ml**
Woodrow, SC	143	51	18820	11973	3507	5	1
Greenwood, PG	291	45	15554	11277	6900	10	0
Ocean, Carmel	51	34	21342	4983	5228	0	0
MAX							
Woodrow, SC	1195	264	97000	56000	14000	55	10
Greenwood, PG	2958	229	72000	70000	16200	60	2
Ocean, Carmel	233	143	31000	12000	15900	1	0
MIN							
Woodrow, SC	0	0	3400	1700	0	0	0
Greenwood, PG	0	0	1000	1000	0	0	0
Ocean, Carmel	0	0	12000	600	0	0	0
Prevalence (%)	56%	98%	100%	100%	68%	20%	17%

Differences in FIB and pathogen loading by sample sequence (e.g. hour 1, 2 or 3) at each site were assessed for significance using ANOVA (JMP). Sample sequence was not significantly different for any organism in any storm event, so samples were pooled and tested for variation among the storms and sample sites using ANOVA (Table 27). Concentrations of total and fecal coliforms, and *Cryptosporidium* were significantly greater during the November 1, 2008 storm, when compared to any other storms. *Salmonella* and *Enterococcus* counts were significantly higher during the January 19, 2010 storm event than any other storm. Significantly higher *Salmonella* concentrations were detected at the Santa Cruz site, when compared to the Carmel site across all storm events. However, the Carmel site, exhibited significantly higher total coliform concentrations than all other sites, and across all storm events. Significantly higher *Enterococcus* counts were detected at the Pacific Grove site, when compared to the Santa Cruz

site. A regional program measures FIBs in storm runoff, providing data for comparison with the current study: The Monterey Bay National Marine Sanctuary (MBNMS) sponsors a volunteer monitoring program called "First Flush". A major objective of this program is to measure constituents in the first substantial rainfall of the storm season to estimate contaminants from urban landscapes that enter the ocean during the first storm event. Storm drains at Woodrow Avenue (Santa Cruz) and Greenwood Park (Pacific Grove) were sampled through this program in 2008 (Monterey Bay Sanctuary Citizen Watershed Monitoring Network, 2008).

FIB concentrations for Santa Cruz and Monterey were comparable between the current study and First Flush values. On November 1, 2008, slightly lower concentration of *Enterococcus* and fecal coliforms were measured at Woodrow Avenue than were reported by First Flush on October 4, 2008. Enterococcus concentrations from the current study and First Flush were 7.6×10^4 and 1.5×10^5, respectively. Fecal coliform concentrations were 5.6×10^4 and 2.0×10^5, respectively. On November 1, 2008, both the current study and First Flush sampled the Greenwood Park site in Monterey, although sampling times differed (samples for this study were collected 0800–1000 hrs, while First Flush began sampling at 2000 hrs; maximum fecal coliform concentrations were 7.0×10^4 and 3.5×10^4, respectively and maximum *Enterococcus* detection was 2.2×10^4 and 7.5×10^4, respectively. These values are comparable to maximum concentrations of fecal coliform and *Enterococcus* in storm runoff in North Carolina (1.2×10^5 and 1.0×10^5, respectively) (Parker et al., 2010).

The stormwater data demonstrate that although storms can have varying levels of flow, the concentrations sampled over several hours were not significantly different. This could indicate that concentrations of FIBs and fecal pathogens in stormwater may be sufficiently characterized by one sample in this area. Nevertheless, estimates of loadings require an estimate of total flow during the discharge period. This study also demonstrates that estimates of protozoa concentrations in stormwater are subject to large uncertainties due to low percent recovery in stormwater matrix.

Table 27. ANOVA results for stormwater data with significant differences by pathogen. *A Posteriori* test used was Tukey HSD (Honestly Significant Difference) test. Red values indicate a p<0.05.

ANOVA Results	Cryptosporidium 10L	Giardia 10L	Salmonella 100ml	Total Coliform 100ml	Fecal Coliform 100ml	Enterococcus 100ml	Vibrio parahemolyticus 100ml
PROB F	<0.0001	0.0366	0.0013	<0.0001	<0.0001	<0.0001	0.0612
DATE	<0.0001	0.0161	0.0014	<0.0001	<0.0001	<0.0001	0.0615
SITE ID	0.1605	0.4849	0.0292	0.0106	0.1391	0.0486	0.1535
LS Means TUKEY HSD by DATE	11/1/08 > REST		1/19/10 > REST	11/1/08 > REST	11/1/08 > REST	1/19/10 > REST	
LS Means TUKEY HSD by SITE ID			SITE 1 > SITE 3	SITE 3 > All others		SITE 2 > SITE 1	

Table 28. Summary of central tendency using regression on order statistics (ROS) for stormwater samples.

	***Cryptosporidium* Per10L**	***Giardia* Per10L**	**Total Coliform Per 100mL**	**Fecal Coliform Per 100mL**	***Enterococci* Per 100mL**	***Vibrio parahaemolyticus* Per 100 mL**
Santa Cruz: Woodrow Ave						
Mean	144	51	18820	11973	3565	DNS
95% CI	0-331	9-94	2805-34835	2938-21009	747-6383	DNS
Pacific Grove: Greenwood Park						
Mean	292	48	15554	11277	7630	11
95% CI	0-887	7-89	3796-27312	0-23988	4700-10560	0-23
Carmel State Beach						
Mean	52	40	21342	4983	5248	ND
95% CI	6-98	10-70	17547-25137	2946-7020	1794-8703	ND

DNS = Data not sufficient; ND = Non-detect

4.1.5. Wetlands

Field Sampling at Tembladero Slough Constructed Wetland

A total of 87 water samples from the Tembladero Slough Constructed Wetland were analyzed for the detection of *Cryptosporidium* and *Giardia* (Table 29). *Cryptosporidium* and *Giardia* were both detected in surface water samples, with each sample site testing positive for at least one of these protozoa during the study. *Cryptosporidium* oocysts were more prevalent (44%) than *Giardia* cysts (12%), and protozoal prevalence was highest in the source water, with 50% of source water samples testing positive for either *Cryptosporidium* or *Giardia*. For both *C. parvum* and *G. lamblia,* the first sample site within the wetland channel (Figure 3) at the upper inflow had the highest prevalence of protozoa (47% and 17%, respectively) for all samples collected within the constructed wetland, and the prevalence was lower at the next two sites down the channel. Interestingly, for *Cryptosporidium*, the prevalence increased within the lower flood plain section of the wetland, possibly due to Tembladero Slough water overflowing its banks into this lower wetland during heavy storm events. Across all sample sites, concentrations of *Cryptosporidium* and *Giardia* in water samples ranged from 0-148 oocysts/10 L and 0-33 cysts/10 L*,* respectively.

Table 29. Prevalence of *Cryptosporidium parvum* and *Giardia lamblia* in the Tembladero Slough Constructed Wetland

Site	n*	*Cryptosporidium*			*Giardia*		
		Prevalence (%)	Mean Concentration (10 L^{-1})	Range (10 L^{-1})	Prevalence (%)	Mean Concentration (10 L^{-1})	Range (10 L^{-1})
Tembladero Slough	4	50	2.7	0-6	50	1.2	0-33
Channel Inlet	23	48	2.4	0-12	17	1.2	0-14
Mid-Channel 1	5	20	0.8	0-4	0	0	0
Mid-Channel 2	18	22	2.6	0-28	11	0.1	0-1
Channel Outflow into Floodplain	22	50	3.6	0-35	5	0.2	0-4
Floodplain Outflow	15	60	22.8	0-148	13	0.6	0-7
Mean Prevalence		44			13		

*n=total number of samples tested for each pathogen.

The presence of target bacteria was also examined. *Vibrio* spp. were cultured from 61% (27/44) of water samples tested by membrane filtration, with one isolate confirmed as *V. cholerae* O1, 25 confirmed as non-O1 *V. cholerae,* and one *V. alginolyticus* isolate. All *V. cholerae* isolates were negative for the ctx toxin gene. *Salmonella* was detected in 5% (2/44) of samples, including serotypes Heidelberg (Group B) and Braenderup (Group C1). *Campylobacter* was isolated from 5% (2/44) of samples, with one *C. jejuni* isolate. No *E. coli* O157:H7 was detected from water samples collected from the source water or the reconstructed wetland.

In addition to specific bacterial pathogens, FIB were also enumerated in wetland samples. A total of 76 samples were analyzed for total coliform counts and *E. coli* using Colilert-18 tests (Table 30). A subset of 51 samples was also analyzed for total coliforms, fecal coliforms and

Enterococcus using membrane filtration (Table 31). Among all sample sites, Tembladero Slough source water had the highest total coliform and *E. coli* counts. Within the wetland, the first sample site had the highest FIB counts, and these counts decreased progressively at more distant sites. Similar to the trend seen with protozoa, the last sample site within the lower floodplain exhibited higher MPN or CFU counts for all FIB except fecal coliforms, possibly due to periodic flooding by the source water.

Two methods for total coliform detection were compared: membrane filtration, which produces colony forming unit (CFU) data, and the Colilert-18 (IDEXX) chromogenic substrate method, which produces most probable number (MPN) data. Of 76 samples tested using Colilert-18 and 51 samples using membrane filtration, 28 were processed using both methods. All 28 were positive for total coliforms, and CFU values were generally lower than paired MPN value for the same water sample. Spearman rank correlations revealed that MPN and CFU values were not correlated ($r=0.043$, $p>0.05$).

Table 30. Total coliform and *Escherichia coli* detection in water samples collected from source water and the Tembladero Slough Constructed Wetland using the Colilert-18 test

Site	n*	Total coliforms		*E. coli*	
		Mean MPN (100 mL^{-1})	Range (100 mL^{-1})	Mean MPN (100 mL^{-1})	Range (100 mL^{-1})
Tembladero Slough	4	716508	92080- 2420000	11883	200-46400
Channel Inlet	20	176179	4870- 2420000	3038	100-22600
Mid-Channel 1	4	53365	25300-123300	NT	NT
Mid-Channel 2	16	83677	5460- 517200	1298	100- 6300
Channel Outflow into Floodplain	19	50288	2790-224700	2059	4.3-16070
Floodplain Outflow	13	242574	1320- 2420000	4970	100-29500
Total Samples	76				

*n=total number of samples tested; NT = Not tested.

Table 31. Total coliform, fecal coliform, and enterococci detection in water samples collected from source water and the Tembladero Slough Constructed Wetland using membrane filtration

Site	n*	Total coliforms		Fecal coliforms		Enterococci	
		Mean CFU (100 mL^{-1})	Range (100 mL^{-1})	Mean CFU (100 mL^{-1})	Range (100 mL^{-1})	Mean CFU (100 mL^{-1})	Range (100 mL^{-1})
Tembladero Slough	4	NT	NT	NT	NT	NT	NT
Channel Inlet	12	1913	70-7200	1796	30-8300	576	75-3200
Mid-Channel 1	4	520	30-1600	283	200-500	ND	ND
Mid-Channel 2	11	818	50-2400	580	15-1700	239	9-1100
Channel Outflow into Floodplain	12	901	100-2800	996	190-2600	450	35-2200
Floodplain Outflow	8	2214	40-16000	338	4-1540	1077	165-6000
Total Samples	51						

NT = Not tested.

To evaluate the importance of season for pathogen detection in the coastal wetland, Wilcoxon scores were calculated to test the association between protozoal counts and seasonality or rainfall events. Positive *Cryptosporidium* samples were weakly associated with both season (U=1520.5, p =0.0002) and rainfall within three days of sampling (U=1320.0, p-value=0.0070, Table 32). *Giardia* counts were not significantly associated with season or rainfall events.

Numerous water quality variables were assessed at each sampling date at each site, as shown in Table 33. Using Spearman rank correlations, *Cryptosporidium* counts were inversely, although weakly correlated, with lower salinity ($r = -0.35985$, $p < 0.01$), total dissolved solid concentrations ($r = -0.35274$, $p < 0.01$) and wetland depth ($r = 0.56196$, $p < 0.01$), and were positively correlated with % dissolved oxygen ($r = 0.3412$, $p < 0.01$). *Giardia* counts were inversely correlated with wetland depth ($r = -0.46003$, $p < 0.05$). This inverse association for wetland water characteristics (e.g. salinity, sediment concentrations and depth) and pathogen transport is in contrast to our findings from the pathogen tank exposure experiments. A California Sea Grant that builds on preliminary data from this State Water Resources Control Board study is now in progress to further clarify the effect of salinity and sediment on pathogen transport in tank, constructed, and tidal wetlands in central California. In the current project, *Cryptosporidium* and *Giardia* were also examined for their correlation with total coliforms and *E. coli*, but no association was observed between indicator and pathogen levels.

Table 32. Wilcoxon Score (U) of seasonality and rainfall events with protozoa in Tembladero Slough Constructed Wetland.

	Cryptosporidium	***Giardia***
Season	1521 **	1858
Rain event on sampling date	1274 *	1044
Rain event on sampling date or within 3 days prior	1320 **	1665

*p <0.05, **p <0.01.

Table 33. Spearman's rho correlation of water quality parameters with protozoa in Tembladero Slough Constructed Wetland.

Parameter	***Cryptosporidium***	***Giardia***
Temperature (*C)	-0.0313	0.1213
Turbidity (NTU)	0.0830	0.2022
Salinity (ppt)	-0.3599 **	-0.0936
Conductivity (um)	-0.2874 *	-0.1059
Total Dissolved Solids	-0.3527 **	-0.0884
Dissolved Oxygen (mg/L)	0.3122 **	0.0487

Parameter	*Cryptosporidium*	*Giardia*
Dissolved Oxygen (%)	0.3412 **	0.0732
pH	-0.0605	0.0077
Wetland depth (cm)	-0.5620**	-0.4600 *

*p-value <0.05 **p-value<0.01

4.2. Loading Estimates

Load estimates were calculated using flow data provided by the participating wastewater treatment plants, USGS stream gages on rivers where available, and direct measurements of stormwater. Estimates were made at all other sites using the floating object method. Sample concentrations were scaled up to organisms/oocysts per liter and flow was converted from MGD (millions of gallons per day) or cfs (cubic feet per second) to liters per day to provide an estimate of organisms per day. Stormwater measurements were obtained during two storms (April 5 and 12, 2010) within the culvert that empties directly into the stormwater sample site at Greenwood Park, Pacific Grove, just before it flows into the ocean (Figure 14, 15). A flow meter was deployed inside the culvert to measure flow during the entire 2-hour period that was sampled at 3, 1 hour intervals.

Direct comparison of stormwater flow assessment methods revealed that the floating object method consistently overestimated the stormwater discharges obtained using a flow meter by 2 orders of magnitude. This overestimate was utilized as a correction factor for calculating load estimates at the Woodrow Ave site at Santa Cruz, while loads at the Pacific Grove site at Greenwood Park were determined via direct measurement using the flow meter data.

The Carmel stormwater sampling site was initially located beneath an overhead rectangular spillway, but the samplers were at risk of exposure to the splashing, contaminated water while sampling. To ensure staff safety, further samples were collected at a culvert located at beach level, approximately 92 m down the beach. Both culverts were considered catchments of the same stormwater plume and were pooled for subsequent analyses. The beach-level culvert was cracked, however, causing stormwater to flow over rip-rap, then directly onto the beach and into the ocean, making it impossible to determine flow in any precise or accurate way at this site. By comparison, the Woodrow Ave and Greenwood Park sites were both trapezoidal earthen channels with a discernable cross-sectional area that facilitated estimates of water volume, and thus, water discharges for each stormwater plume.

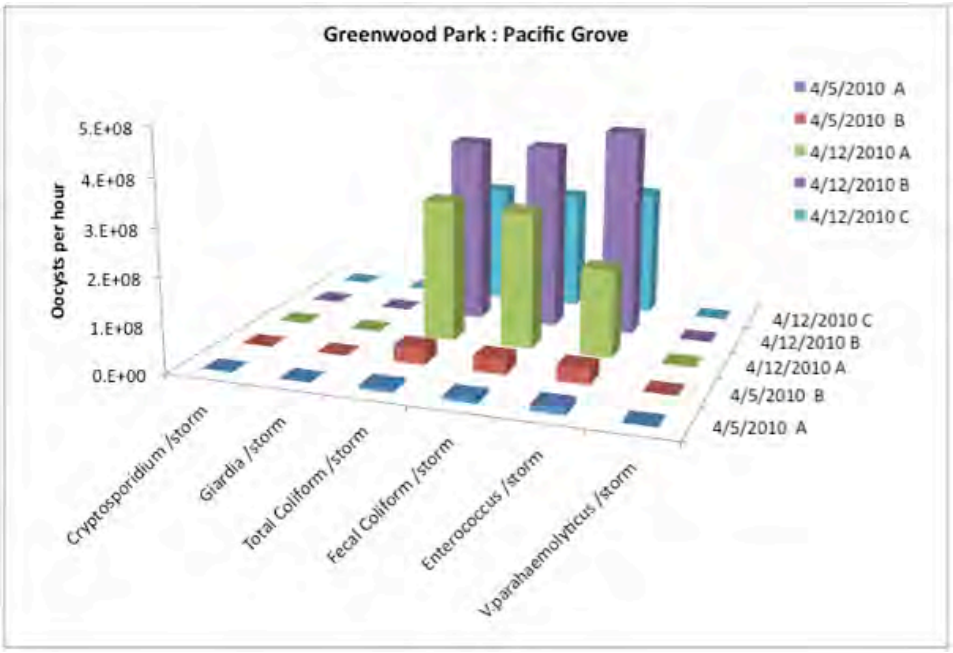


Figure 14. Stormwater load estimates for the Pacific Grove site at Greenwood Park.

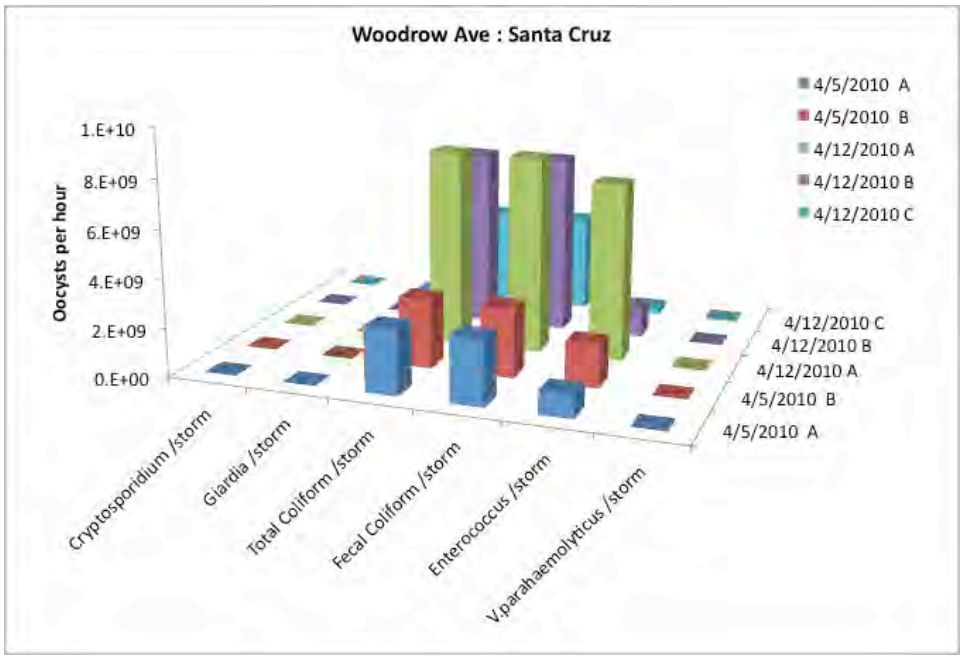


Figure 15. Stormwater load estimates for the Santa Cruz site at Woodrow Ave.

While not an explicit objective of the current study, estimates of pathogen loads contributed by various freshwater sources entering nearshore ocean waters (e.g. river flows, storm runoff and wastewater effluent) can help pinpoint the information required to produce more accurate load estimates in the future, as well as assist water quality managers with prioritizing activities to reduce coastal discharges of fecal pathogens. However, estimating loads of fecal pathogens is fraught with challenges and requires measurement of concentrations, and ideally synoptic flow volumes, from a wide range of water sources. The various sources that contribute to fecal pathogen loads to ocean waters each present unique obstacles for safely and accurately measuring pathogen concentrations and water flows, including limitations with currently available methods for pathogen detection in environmental samples.

As the current and previous studies have demonstrated, the precision of protozoal enumeration in environmental samples is subject to substantial variation that may be attributable to source-specific properties (e.g. salinity, water depth and total dissolved solids); this characteristic was clearly demonstrated during spike-recovery studies. For example, recoveries of spiked protozoal oocysts from surface water samples ranged from 2% to 26% for *Cryptosporidium*, and from 0% to 30% for *Giardia* (Table 34). Within adjacent coastal creeks (i.e., Waddell Creek and Scott Creek), protozoal recoveries varied between 2% and 26% for *Cryptosporidium* and between 1% and 15% for *Giardia*. These counts varied significantly even for water samples obtained from the same source. For example, spiked water samples collected from Molera Wetland, in February and April 2008 ranged from 3% to 24% recovery for *Cryptosporidium,* and 1% to 21% for *Giardia*. As demonstrated previously, much of this variation can be attributed to variations in water properties, such as salinity and total dissolved solids that directly impact oocyst recovery. As a result of this high level of imprecision in protozoal recovery, when overall mean recoveries are used to estimate protozoal concentrations in surface water, such estimates have an inherent error of 27–32%. This error is due to the large degree of spatial and temporal variation in water composition. Consequently, over- or under-estimates of protozoal concentrations contribute substantial uncertainty to load estimates for protozoa. This within- and between-site variation in spike recoveries may be a greater problem for load estimates for protozoa in natural surface waters and storm runoff than for wastewater discharges, because the quality of wastewater discharge is far more consistent through time.

Table 34. Percent recovery of spiked protozoal oocysts in subsamples of coastal surface water from the Monterey Bay area.

Site	Sample date	*Cryptosporidium*	*Giardia*
Waddell Creek	5/22/07	2%	1%
Scott Creek	6/11/09	26%	15%
San Lorenzo River	7/9/07	8%	0%
Watsonville Slough	9/4/07	18%	30%
Salinas River	12/4/07	5%	30%
Storm Drain	1/8/08	4%	3%
Dairy wetland	2/20/08	17%	24%
Molera Wetland	2/26/08	24%	21%
Molera Wetland	4/21/08	3%	1%
Mean		12%	14%
Standard Error		3.2%	4.3%

Obtaining precise flow data to determine pathogen loading can also be challenging, particularly for coastal streams and rivers. Several of the coastal streams and rivers sampled in this project were not equipped with recording flow gauges, so our load estimates are based on extrapolated data from sites with flow gauges. An additional challenge is accounting for the full range in outflow conditions that occurs by stream or river, and by storm event. Without specifically targeting high-flow conditions for sampling, the load estimates presented here for streams and rivers must be considered conservative. In addition, none of the sampled storm drains were equipped with flow gauges. Through a loan from the Monterey Bay National Marine Sanctuary, flows from one storm drain were measured during two storm events, and discharge volume from a second storm drain was estimated by measuring the travel velocity of a floating object, along with water depth. In addition, we are unaware of a region-wide inventory of the full compliment of storm drains and other non-point sources that discharge runoff into the ocean.

Given the wide range in intrinsic variation outlined above, we used available data to develop a list of assumptions and standardize efforts for estimating fecal pathogen loads: 1) Recoveries of *Cryptosporidium* and *Giardia* from surface waters averaged 12% and 14%, respectively; 2) Recoveries of *Cryptosporidium* and *Giardia* from storm runoff were 4% and 3%, respectively; 3) The discharge flows from the San Lorenzo River, Soquel Creek, Pajaro River, Salinas River, Carmel River and Big Sur River (http://cdec.water.ca.gov/) comprise the majority of surface water discharges to ocean waters in the Monterey Bay region, and are comparable to other surface water discharges in fecal pathogen concentrations; 4) The storm drains at Woodrow Street in Santa Cruz and Greenwood Park in Pacific Grove provide a reasonable representation of storm runoff quantity and quality from urbanized areas around Monterey Bay.

The resulting estimates of pathogen loads due to storm runoff are rough, due to extrapolation from two storm runoff sites located on the northern (Santa Cruz), and southern (Pacific Grove) edges of Monterey Bay. While storm runoff discharges differ in their catchment areas and proportion of impervious surfaces, our extrapolation assumes that the discharges in Pacific Grove and Santa Cruz are typical for the region. This extrapolation is supported by data in an inventory of storm runoff discharges on the Monterey Peninsula published by the California State Water Board in 2011 (Appendix 5 of the Draft Program Environmental Impact Report associated with proposed discharge regulations for storm runoff into Areas of Special Biological Significance) (State Water Resources Control Board, 2011). For the Monterey Peninsula, there are 49 discrete conveyances that discharge storm runoff from urban watersheds into the ocean, with an aggregate width of 121.5 meters that is scattered across a coastline of approximately 23 kilometers. The entire coastline of Monterey Bay with adjacent urban land uses is approximately 55 kilometers. Applying the ratio of 121.5 meters of storm runoff conveyance/ 23 km of coastline provides an estimate of 290 meters of storm runoff conveyance for the entire Monterey Bay region. The estimated flows for the Woodrow Avenue, Santa Cruz, and Greenwood Park, Pacific Grove, storm drains have a combined width of 2.3 meters, which represents 0.8% of all estimated conveyances.

Another challenge encountered when estimating loads from storm runoff is that runoff is episodic and is location- and storm-specific. The total annual runoff from Woodrow Avenue and Greenwood Park was estimated by comparing hourly rainfall data from nearby rain gauges (http://cdec.water.ca.gov/) for the days when storm sampling was performed. For both locations, a minimum of 0.1 inch of rain/ 2 hrs was the lowest rate of rainfall that occurred during sampling events. The numbers of hours that satisfied the ≥0.1-inch criterion were totaled for both years

when stormwater sampling occurred (2008 and 2010), resulting in estimates for the average number of hours of precipitation with probable surface runoff/ year for each site. These average runoff hours were divided by 365 to estimate the average daily pathogen loads contributed by each site. The estimated loads from the two sites were then combined and divided by 0.8% (i.e., the ratio of the sizes of these two discharges to the estimated total width of discharges around the entire bay), to provide an estimate of total storm runoff from urbanized areas into the ocean waters of greater Monterey Bay.

The mean concentrations of indicator bacteria and fecal pathogens from wastewater, surface water, and storm runoff exhibited strikingly different profiles (Figure 16). Compared to the other sources, wastewater contributed higher concentrations of *Giardia* and *Vibrio parahaemolyticus* to total pathogen loads, whereas storm runoff contributed a higher percentage of *Cryptosporidium* and surface waters contributed a higher percentage of *Vibrio cholerae*. Similar trends were observed for analysis of variance on transformed data [log (x+1)] for fecal pathogens (Table 35).

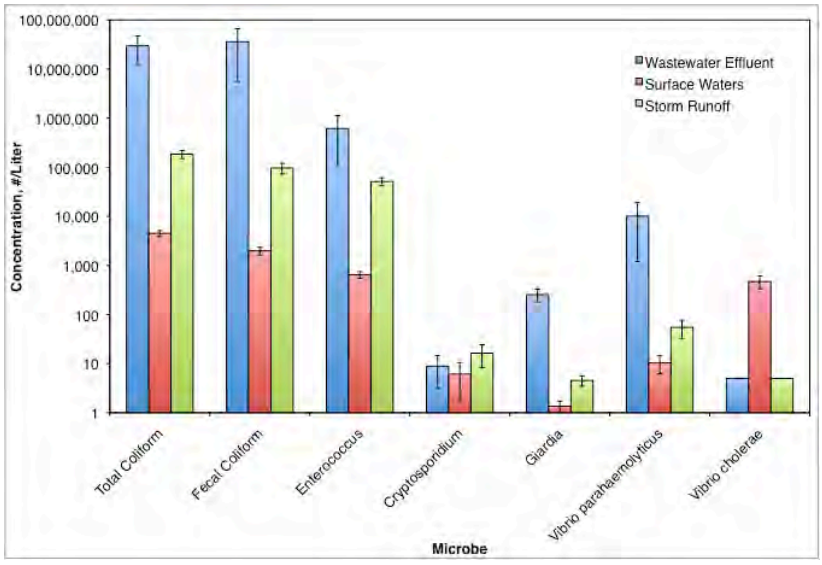


Figure 16. Mean concentrations of indicator bacteria and fecal pathogens from wastewater effluent, surface waters (streams and rivers) and storm runoff. Wastewater and streams and rivers were sampled in 2007 and 2008, whereas storm runoff was sampled in 2008 and 2010. Concentrations are not flow-weighted. Error bars indicate standard errors.

The general patterns noted in concentrations of indicator bacteria and fecal pathogens were also reflected in loads. Not surprisingly, loads of indicator bacteria/L from wastewater effluent were higher than those from surface waters or storm runoff. However, when corrected for estimated flow rates, proportionally greater flows from streams and rivers and higher FIB concentrations in storm runoff produced very similar load estimates for these two sources that, when combined, exceeded total pathogen loading from wastewater for all fecal pathogens except *Giardia* (Figure 17). Similar findings were revealed for analysis of variance using transformed data, with significantly higher loads of *Giardia* contributed by wastewater effluent, significantly higher environmental loading of *V. cholerae* by streams and rivers, and no

significant differences among sources for environmental loading by *Cryptosporidium* and *V. parahaemolyticus* (Table 36).

Table 35. ANOVA results to determine significant differences in concentrations of indicator bacteria and fecal pathogen concentrations among discharge sources using transformed data [log (x+1)].

Microbe	R^2	P	*A posteriori*[1]
Indicator Bacteria			
Total Coliform	0.4994	<0.0001*	1>3>2
Fecal Coliform	0.5529	<0.0001*	1>3>2
Enterococcus	0.2953	<0.0001*	1>3>2
Fecal Pathogens			
Cryptosporidium	0.0790	<0.0001*	3=1, 3>2, 1=2
Giardia	0.6469	<0.0001*	1>3>2
Vibrio parahaemolyticus	0.0747	<0.0001*	1=3>2
Vibrio cholerae	0.0934	<0.0001*	2>1=3

* = If P <0.05, result is statistically significant.
[1] = 1 = wastewater; 2 = surface water; 3 = storm water

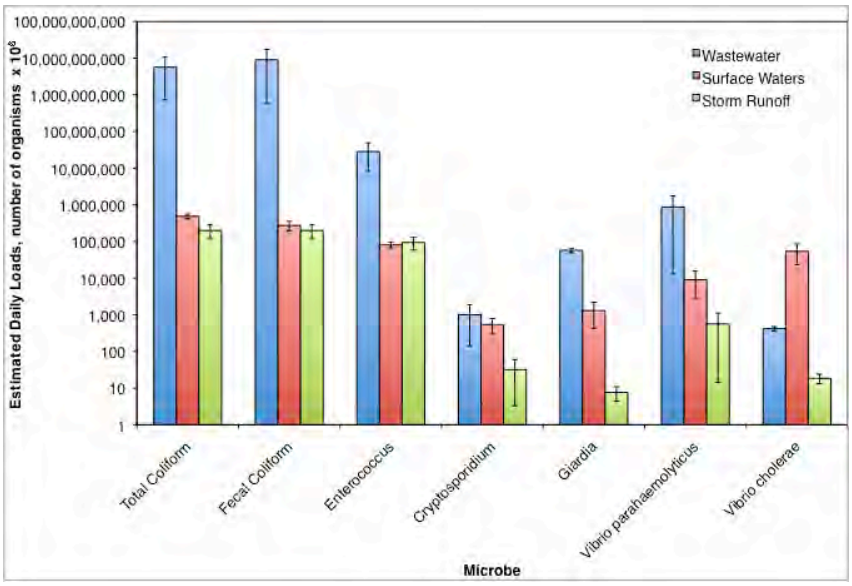


Figure 17. Mean daily loads (concentration x flow) of indicator bacteria and fecal pathogens from wastewater effluent, surface waters (streams and rivers) and storm runoff. Wastewater and streams and rivers were sampled in 2007 and 2008, whereas storm runoff was sampled in 2008 and 2010. Error bars indicate standard errors.

Table 36. ANOVA results to determine significant differences in daily loads of indicator bacteria and fecal pathogens among discharge sources using transformed data [log (x+1)].

Microbe	R^2	P	*A posteriori*[1]
Indicator bacteria			
Total Coliform	0.8545	<0.0001*	1>2=3
Fecal Coliform	0.8214	<0.0001*	1>3=2
Enterococcus	0.7951	<0.0001*	1>3=2
Fecal pathogens			
Cryptosporidium	0.1317	0.2115	1=2=3
Giardia	0.6135	<0.0001*	1>2>3
Vibrio parahaemolyticus	0.1032	0.2019	1=2=3
Vibrio cholerae	0.6801	<0.0001*	2>1>3

* = If P <0.05, result is statistically significant.
[1] = 1 = wastewater; 2 = surface water; 3 = storm water

Estimated loads of indicator bacteria and specific pathogens should not be the sole criterion for selecting management options to reduce fecal contamination in receiving waters. The location and mode of discharge from each source also affect their risk of causing human and wildlife health concerns. For example, wastewater discharges are required to be located in deep water, such that high dilution of the discharge is likely to occur before it reaches areas frequented by humans. Conversely, streams, rivers and storm drains, discharge directly to the nearshore environment with little dilution, especially for discharges that occur within embayments and sloughs. The similarity in environmental loading for all fecal pathogens except *Giardia* and *V. parahaemolyticus* suggest that efforts to lower human health risk for recreational water contact in nearshore marine waters should focus first on coastal water sources with direct shoreline discharges: surface water and storm runoff. However, more accurate pathogen loading estimates provided by future studies could modify this conclusion.

Our initial estimate of environmental loading by fecal indicator bacteria and specific pathogens by streams, rivers and storm runoff has both highlighted the limitations of current water surveillance systems, and provided preliminary data to pinpoint water sources where more detailed testing and focused mitigation efforts could be most beneficial for protecting public health. Sample-specific recovery estimates would provide more accurate measurements of pathogen concentrations by reducing the uncertainty associated with use of mean recoveries composed of widely varying values, though this would add significant costs to the study. Broader efforts to measure outflows from storm drains would help clarify whether environmental loads of fecal pathogens from stormwater are similar to those from streams and rivers, as is suggested by this study.

4.3. What is the relationship between exceedences of water quality objectives for bacteria and actual concentrations of known fecal pathogens?

California has set cutoffs for fecal indicator bacteria (FIB) counts; levels falling below these cutoffs are considered acceptable for water contact recreation in surface water bodies. For example, the Basin Plan states that fecal coliform concentrations shall not exceed a log mean

count of 200/100 ml water, nor shall more than ten percent of total samples during any 30 day period exceed 400/100 ml. Similarly, while streams and river water quality criteria fall under the Basin Plan, ocean water quality criteria for fecal indicator bacteria are listed in the Ocean Plan, for example total coliforms are not to exceed 10,000/100 ml, fecal coliforms are not to exceed 400/100 ml, and *Enterococci* are not to exceed 104/100 ml. Table 37 shows the proportion of surface water samples from the current study that exceeded water quality criteria cutoffs. The general trend was that stormwater samples exceeded water quality criteria most often (up to 100% of the time for some FIB measures), followed by river/stream/slough samples, and finally ocean water samples.

Table 37. Proportion of fecal indicator bacterial counts exceeding standard water quality criteria in surface water samples collected along the central coast of California (2008-2010).

	n	Total coliforms >10,000/100ml	Total coliforms >230/100ml*	Fecal coliforms >400/100ml	Enterococci >104/100ml
Stormwater	40	60%	53%	100%	100%
River	178	4%	42%	11%	17%
Ocean	42	0%	0%	5%	7%
Overall	260	12%	37%	24%	28%

*Ocean Plan: Total coliforms not to exceed 230/100ml in >10% of water samples in coastal areas where shellfish are harvested.

The relationship between FIB exceedences and enteric pathogen detection was evaluated for the standard FIB measures of total coliforms, fecal coliforms, and *Enterococci* using univariate and multivariate logistic regression. Table 38 demonstrates the univariate relationships between FIB counts and enteric pathogen detection for pooled data from river and ocean samples. In univariate analyses, *Cryptosporidium* and *Giardia* were detected two to five times more often when FIB levels of any type exceeded water quality criteria cutoffs. *Salmonella* was detected approximately three times more often when fecal coliforms or *Enterococci* exceeded water quality cutoffs, but not total coliforms. *Campylobacter* detection was not significantly associated with FIB exceedences for any of the three tests. *Vibrio cholerae* was less likely to be detected in water samples that exceeded the FIB cutoffs, in contrast with *V. parahaemolyticus*, which was more likely to be detected in water samples with elevated FIB levels.

Multivariate models were used to determine if accounting for the individual effects of multiple pathogens at the same time would alter the significance of pathogen detection at elevated FIB concentrations (Table 39). When multiple pathogens were included in each FIB model using a backward stepping approach, only *Cryptosporidium* detection was significantly associated with total coliform levels that exceeded water quality criteria cutoffs, while associations between *V. cholerae* detection and elevated FIB was no longer significant. Across all three indicator bacteria, *Cryptosporidium* was detected three to four times more often in surface water samples that exceeded FIB cutoff values. Other pathogens that remained significant along with *Cryptosporidium* in the fecal coliform model were *Giardia, Salmonella*, and *V. parahaemolyticus,* whereas only *Giardia* and *V. parahaemolyticus* remained significant in the *Enterococci* model. Collectively these findings of increased pathogen detection at elevated FIB concentrations support the continued use of FIB cutoffs for predicting health risks

during recreational water contact uses, although our data confirmed that pathogens could be present in recreational waters at acceptable FIB levels. Adding *Bacteroidales* enumeration and typing as an alternative FIB method could provide quantitative data for further refinement of water quality criteria cutoffs, as well as complementary information on the range of animal or human hosts that are contributing to fecal pollution in a given watershed and their relative degree of fecal contribution.

Table 38. Univariate analysis of associations between fecal indicator bacterial counts exceeding standard water quality criteria for recreational contact and enteric pathogen detection in surface water samples collected along the central California coast (2008-2010).

Indicator Bacteria:	Total coliform >10,000/100ml		Fecal coliform >400/100 ml		Enterococci >104/100 ml	
Pathogen	Odds Ratio	P value	Odds Ratio	P value	Odds Ratio	P valued
Cryptosporidium	2.9	0.010**	3.2	<0.001***	4.5	<0.001***
Giardia	2.1	0.079	2.1	0.016*	2.5	0.002**
Salmonella	1.1	0.917	3.6	0.010**	2.8	0.038*
Campylobacter	ND****	ND	0.15	0.072	0.42	0.187
Vibrio cholerae	0.24	0.025*	0.62	0.156	0.51	0.039*
Vibrio parahaemolyticus	1.2	0.847	3.1	0.026*	3.1	0.024*

*P<0.05; **P<0.01; ***P<0.001; ****ND=not done because of perfect correlation for pathogens detected at high FIB levels.

Table 39. Multivariate model of associations between fecal indicator bacterial counts exceeding standard water quality criteria for recreational contact and enteric pathogen detection in surface water samples collected along the central California coast (2008-2010).

Indicator Bacteria:	Total coliform >10,000/100ml		Fecal coliform >400/100 ml		Enterococci >104/100 ml	
Pathogen	Odds Ratio	P value	Odds Ratio	P value	Odds Ratio	P valued
Cryptosporidium	2.9	0.010**	3.2	<0.001***	4.1	<0.001***
Giardia			2.1	0.020*	2.1	0.015*
Salmonella			3.2	0.021*		
Campylobacter						
Vibrio cholerae						
Vibrio parahaemolyticus			3.7	0.013*	3.6	0.019*

*P<0.05; **P<0.01; ***P<0.001

4.4 Are mussels better indicators of ocean bacterial water quality than water samples?

An objective of the present study was to compare indicator and pathogen detection in paired shellfish and seawater samples collected from the same sites and timepoints. Bivalves are filter-feeding organisms that can concentrate a wide range of bacteria, viruses, and parasites; bioaccumulation may vary by bivalve and pathogen type (Hood et al., 1983; Burkhardt et al., 1992; Gillespie et al., 2001; Graczyk et al., 2003; Marino et al., 2005). In addition to their potential usefulness for monitoring pathogens and pollutants present in surrounding water, mussels and other bivalves are also important as vehicles for food-borne pathogens, as outbreaks of human illness have been associated with consumption of raw and undercooked shellfish (Gillespie et al., 2001).

To determine whether mussels or seawater were better matrices for detecting the presence of pathogens in this study, both enteric pathogen prevalence and concentrations were considered. The prevalence of *Giardia* and *Vibrio* species (*V. cholerae, V. parahaemolyticus, V. alginolyticus*) were similar in seawater and mussel samples, with ≤2% difference, as shown in Table 24 above. However, *Campylobacter* and *Salmonella* were detected in time and location-matched seawater samples when they were not detected in mussels, with 10% and 5% prevalence, respectively, in seawater. Similarly, *Cryptosporidium* oocysts were detected in 26% of seawater samples, but only 6% of mussel batches. Overall, the McNemar's test for paired positive/negative data showed no significant difference ($P > 0.05$) in pathogen detection between seawater and mussels.

When the same dataset for pathogen concentration, the Wilcoxan signed-rank test for bacteria counts revealed significant differences in mean TC concentrations ($P = 0.007$) between seawater and mussels, with a mean TC of 537cfu/100g in mussels, compared to 189cfu/100ml in seawater. Significant Spearman's rank correlations were identified between all three measures of FIB presence and *V. cholerae* detection in seawater, with $r_s = 0.44$ for TC ($P = 0.005$) and with $r_s = 0.42$ for FC ($P = 0.007$), and $r_s = 0.44$ for EN ($P = 0.004$). Similar results were obtained for mussels, and all 3 FIB measures were highly correlated with *V. alginolyticus* concentrations (TC ($r_s = 0.41$, $P = 0.008$), FC ($r_s = 0.61$, $P < 0.001$), and EN ($r_s = 0.68$, $P < 0.001$).

Shellfish, including mussels, clams and oysters have been proposed as bioindicators of water quality in aquatic ecosystems (Hood et al., 1983; Miller et al., 2006; Marino et al., 2005; Lhafi et al., 2007). Seawater temperatures may affect both pathogen survival and depuration from mussel tissues (Burkhardt et al., 1992; Louis et al., 2003; Marino et al., 2005). In the present studies, *Cryptosporidium, Giardia,* and *Vibrio* spp. were the most common pathogens detected in mussels, similar to the findings of Ripabelli et al. (1999). In some previous studies, bacterial accumulation was higher in shellfish than in the ocean (Burkhardt et al., 1992; Marino et al., 2005). However, in the current study, no significant differences were found for pathogen detection in mussels, when compared to time and location-matched seawater. This could reflect pathogen purging by shellfish; prior studies demonstrate that while bivalves concentrate pathogens from surrounding water, they also depurate these pathogens over the following days or weeks (Miller et al., 2005). It is also possible that our bivalve sampling may not have occurred during periods following storm events when bivalves would be most likely to retain higher pathogen concentrations, or that differences in detection methods affected recovery. Bivalves may be most useful as bioindicators during times of high fecal contamination of aquatic ecosystems, such as during or after storm events. Differences in the bivalve type or species selected for testing may also affect results, as prior studies demonstrate significant differences

between invertebrate species with respect to ability to bioaccumulate biotoxins and anthropogenic pollutants (Graczyk et al., 2003; Marie et al., 2006; Murray et al., 2009).

4.5. Which of the three microbial source tracking methods evaluated appears most promising as a tool for environmental surveillance, and what can we learn about trends in human versus animal sources of fecal pollution along the central coast of California?

***Bacteroidales* and microbial source tracking in surface waters**. Similar to FIB, the universal *Bacteroidales* marker was detected in all water samples (100%). The human *Bacteroidales* marker was detected in 37% of samples, while the cow (8%) and dog (6%) *Bacteroidales* markers were detected in less than 10% of samples. Median concentrations of universal *Bacteroidales* markers were greatest at the Salinas River (34,805 gene copies/mL [gc/mL]) and Watsonville Slough (19,037 gc/mL) sites. Universal *Bacteroidales* marker concentrations were lowest at the Scott Creek (1,126 gc/mL) and Big Sur River (937 gc/mL) sites, which are both located at the less developed northern and southern fringes of Monterey Bay, respectively (Figure 9). Overall, *Bacteroidales* concentrations ranged from 87-1.3 million gc/mL for universal markers, 45-17,268 gc/mL for human markers, 3-92 gc/mL for cow markers, and 12-575 gc/mL for dog markers. As with FIB, no marked seasonality for *Bacteroidales* markers was observed with respect to prevalence or concentration (Figure 9).

Human *Bacteroidales* markers were detected most frequently at the San Lorenzo River study site (57% of water samples), and this site also exhibited the greatest ratio of human to universal *Bacteroidales* concentrations (0.22), as shown in Table 40. Watsonville Slough was the site with the greatest enteric pathogen and FIB concentrations, and the second greatest median concentration of universal *Bacteroidales* markers among all tested sites. All three *Bacteroidales* host markers were detected at Watsonville Slough, which is consistent with known fecal inputs from human, cow, and dog sources in the area. All three *Bacteroidales* markers were also detected in water from Soquel Creek, a highly developed suburban site where *Cryptosporidium* oocysts were most frequently detected.

Table 40. Site characteristics with respect to land use, main upstream fecal source, and *Bacteroidales* marker detection in surface waters.

Site ID	Major land uses[a]	Major host fecal sources[b]	n[C]	Proportion of water samples positive (%)			Mean host *Bacteroidales* to universal *Bacteroidales* marker ratio		
				Human	Cow	Dog	Human	Cow	Dog
Waddell Creek	rr, rec	W	14	36	-	7	0.12	< 0.01	< 0.01
Scott Creek	rr, ag, rec	L, W	16	25	-	-	0.10	< 0.01	< 0.01
San Lorenzo River	urb, rr, rec	H, P, W	14	57	14	-	0.22	< 0.01	< 0.01
Soquel Creek	urb, rr, rec	H, P, L, W	14	21	7	21	0.11	< 0.01	< 0.01
Watsonville Slough	urb, rr, ag, rec	H, P, L, W	14	29	7	7	0.09	< 0.01	< 0.01

Site ID	Major land uses[a]	Major host fecal sources[b]	n[C]	Proportion of water samples positive (%)			Mean host *Bacteroidales* to universal *Bacteroidales* marker ratio		
				Human	Cow	Dog	Human	Cow	Dog
Pajaro River	urb, rr, ag, rec	H, P, L, W	14	21	-	7	0.10	< 0.01	< 0.01
Elkhorn Slough	rr, ag, rec	H, P, L, W	15	47	-	-	0.06	< 0.01	< 0.01
Salinas River	urb, rr, ag, rec	H, P, L, W	14	36	-	7	0.08	< 0.01	< 0.01
Carmel River	urb, rr, ag, rec	H, P, L, W	16	44	-	-	0.16	< 0.01	< 0.01
Big Sur River	rr, ag, rec	H, L, W	12	42	-	8	0.15	< 0.01	< 0.01
Overall % positive				36	3	6			

[a] urb = urban development; rr = rural residential development; ag = agriculture including crops, rangeland, livestock; rec = recreational areas including forest and trails.
[b] H = humans; P = pets such as dogs and cats; L = livestock; W = wildlife.
[C] number of samples taken at location

In this study, less than 1% of the universal *Bacteroidales* signal was made up of cow- or dog-specific markers. On average, the human-specific assay signal for each site was equal to 1-22% of the universal signal, which is still a small proportion of total *Bacteroidales* load in the surface water, suggesting that non-characterized fecal inputs such as wildlife or domestic animals predominate in these watersheds. The median sample-specific limits of detection (SLOD) were relatively low: 24 gc/mL (universal, cow, and dog) and 84 gc/mL (human). The SLOD ranges were relatively wide, with values from 3-3,155 gc/mL for universal, cow and dog markers and from 9-10,842 gc/mL for human markers, Recoveries of spiked surrogates were good for environmental samples (median 66%) and inhibition factors were relatively low (median of 3) (44), but for some samples recoveries below 10% were observed, which are reflected in higher SLOD values.

The ability to use host-specific fecal assays for microbial source tracking is strongly linked to assay specificity. Based on validation of host-specific assays using known fecal samples, the conditional probability, diagnostic sensitivity and specificity, and positive and negative predictive values that fecal contamination from a given source is present or absent in a water sample can be calculated (Table 41).

Table 41. Conditional probability, sensitivity, specificity, positive and negative predictive value, and prevailing rate for four 16S rRNA-based qPCR assays targeting universal, human-, cow-, and dog-specific *Bacteroidales* markers in surface waters.

Assay Target	Conditional probability	Sensitivity	Specificity	Predictive value		Prevailing rate
				Positive	Negative	
Universal	1.00	1.00	1.00	1.00	1.00	1.00
Human	0.75	0.84	0.87	0.75	0.92	0.32
Cow	0.19	0.73	0.63	0.19	0.95	0.11
Dog	0.15	0.74	0.78	0.15	0.98	0.05

Combining validation data we have previously obtained (Kildare et al., 2007) with our study-specific results, the conditional probability for the universal marker was determined to be 100%, which means that it was virtually certain that samples testing positive for the universal *Bacteroidales* assay actually contained traces of host feces. The probabilities for human- and dog-specific assays were 75% and 15%, respectively. This can be interpreted as a relatively high (75%) probability that water that test positive for the human-specific marker truly contained traces of human feces. In contrast, reliability for the dog-specific assay was lower, with only a 15% probability of dog feces truly being present in samples testing positive, based on previous specificity data. Likewise, for the cow-specific assay, the conditional probability was only 19%, due in part to cross-specificity with horse samples reported previously (Kildare et al., 2007), and the relatively low prevalence of detection in the current study (Table 41).

Negative *Bacteroidales* assay results were estimated to be 100% reliable for the universal marker, 92% for the human marker, 98% for the dog marker, and 95% for the cow *Bacteroidales* marker detection. Thus, in the current study, the likelihood of human, dog or cow fecal contamination being present in surface water samples when a negative test result was obtained was 8%, 2%, and 5% respectively. As additional specificity and prevailing host data are obtained, updated conditional probabilities can be calculated.

When considering the actual presence/absence observations for *Bacteroidales*-specific markers in this study, there was a very high likelihood that all of the universal assay results were true positives. For samples that tested positive with the the human-specific assay, some false-positive test results could have been obtained, but based on the test characteristics we estimate that at least 39 of the 53 positive test results were truly positive for human feces. We have reduced confidence in results for the dog- and cow- specific assays because these assays may cross-react with human and horse-derived fecal pollution, respectively. However, both the dog and cow assay exhibited excellent negative predictive values of 0.98 and 0.95, which means that the absence of detection provides high confidence that no dog or cow fecal pollution was present. An external demographic or chemical measure of host distribution at our study sites would allow for additional insights on contributing host sources.

This study was the first in California to compare detection of *Bacteroidales* markers and FIB in ambient water samples with concurrent isolation of specific bacterial and protozoal waterborne pathogens. A suite of laboratory screening tests were utilized to detect and quantify the presence of standard FIB, *Bacteroidales* as an alternative indicator for microbial source tracking, and the presence, absence and concentration of selected pathogens in impaired and more pristine surface waters of the central California coast over 14 months. Significant correlations were observed for some, but not all indicator and pathogen combinations. The calculation of a new parameter, Predictive Qualifier (PQ), facilitated evaluation of both conventional and alternative indicator bacteria as predictors for selected pathogens. This was possible because the PQ gives a pathogen presence-weighted percentage of how often indicator presence co-occurs with pathogen detection when certain indicator threshold concentrations are applied. In contrast to the *Bacteroidales* assays, the *Enterococcus esp* assay and the total: fecal coliform ratios did not display as much predictive power to facilitate microbial source tracking.

Universal *Bacteroidales*, total coliforms and fecal coliforms were detected in at least 99% of water samples, while enterococci were only detected in 75%, human *Bacteroidales* markers in 37%, and dog- or cow-specific markers in less than 10% of samples. These findings likely reflect that uncharacterized, non-human fecal sources such as wildlife or domestic animal species were contributing significantly to fecal loading of the various water bodies evaluated during this study.

Such a scenario is plausible given the large populations of wild birds and small mammals that reside along these waterways, and the significant number of domesticated small ruminants, poultry and cats that are known to be present in these areas. The ratio of host to universal *Bacteroidales* markers only provides a rough estimate of the proportion of total *Bacteroidales* signal attributable to each host, because ratios can vary within individual fecal samples within each species, although ratios are assumed to be constant over time. There is supporting evidence for the latter based on controlled decay studies in marine waters that employed the same range of *Bacteroidales* assays used in the present study (Bag et al., 2008). The change in ratio of a specific marker can be useful for comparisons between sites and time points. As development and validation of *Bacteroidales* assays progress, our ability to characterize contributing host sources will improve, and additional host-specific assays may be available to facilitate source-tracking efforts.

All of the rivers tested in the current study exhibited some degree of human activity based on both direct observation and results from *Bacteroidales* testing. Detection of both human and universal *Bacteroidales* markers at all sites suggests that current management of human fecal waste is not preventing contamination of ambient waterways. By using the prevailing (or background) detection rates for each marker in this study, we were able to calculate the conditional probabilities of correctly identifying contributing host fecal sources for each assay. For samples where the human *Bacteroidales* assay was positive, they were estimated to correctly identify human fecal sources 75% of the time, which is lower than the 98% probability calculated in a previous study (Kildare et al., 2007). However, when samples tested negative for human *Bacteroidales* markers, we can expect that the samples were truly negative 92% of the time. As additional studies are performed and background rates for detection are better understood, more accurate probability calculations will be possible.

The accuracy and efficacy of employing traditional fecal indicators to estimate health risks, FIB presence, and even pathogen presence in surface waters is being increasingly questioned. FIB presence and concentration may not always correlate with specific pathogen exposure and actual health risk from water contact recreation, given the wide variation in infectivity, exposure dose, pathogen virulence, and differential immunity that may affect disease expression. Different microbial species and strains may also vary in virulence and ability to cause disease in humans and animals (Colwell, 1996; Bang et al., 2001; Cooper et al., 2007; Till et al., 2008), so no definitive conclusions should be drawn regarding their pathogenic potential without further characterization. *Vibrio* were deliberately included in the suite of target bacteria, in order to explore their relationships with traditional FIB and *Bacteroidales*, in part because their niches in coastal ecosystems are more complex and less well characterized than some other potentially pathogenic organisms. However, the majority of environmental *V. cholerae* strains are non-pathogenic unless the cholera toxin gene has been acquired via phage transduction (Faruque et al., 1998), and the same may be true for other pathogens evaluated in this study. The proportion of virulent strains can also vary by water type, temperature and season, and different risk factors may exist for persons exposed to freshwater versus marine environments (Stricker et al., 2008). Epidemiological and QMRA studies that evaluate associations between environmental risk factors and health outcomes in a variety of settings are needed. Findings from the current study can help guide the combination of environmental screening assays, microbes and risk factors that are most important to consider for subsequent research efforts.

Our results demonstrate how, in combination with traditional fecal indicator bacteria, *Bacteroidales* markers can provide complementary information to facilitate microbial source

tracking efforts. In this study the detection frequencies of the host-specific *Bacteroidales* markers were relatively low, and so a more detailed discussion of these concentrations was not considered worthwhile (though we do present concentrations in Table 40 in the form of ratios of universal to host specific *Bacteroidales)*. However, some of our ongoing studies, especially those in urbanized areas, demonstrate a much different picture, so concentration data will be very important Assessment of the type-specific *Bacteroidales* concentrations facilitates the identification of hotspots, prioritization of treatment measures, and identification of the relative contribution of natural and anthropogenic sources to total fecal loading.

Bacteroidales cannot be cultured, and so molecular methods such as qPCR are required to provide the most useful suite of data. One benefit of these molecular approaches is that, in contrast with culture-based methods, the samples are all treated the same for *Bacteroidales* assays. Another benefit is that nucleic acid extracts can be stored long-term (under appropriate conditions to minimize degradation), thus facilitating repeat testing of the same sample set using additional or improved assays. Future studies that analyze assay performance in other settings, and employ complementary assays to characterize loading sources (e.g., aging fecal inputs), will further augment our understanding of potential uses for *Bacteroidales* in microbial source tracking and mitigation efforts. The fact that *Bacteroidales* is anaerobic and is less likely to multiply in the environment than traditional FIB may also make it a more useful indicator of recent fecal pollution, which is more likely to contain viable fecal pathogens than pollution events that occurred longer ago. Given that the probability of viable pathogen presence increases with the amount of recent fecal pollution in a watershed when numbers of contributing individuals are high, the ability to quantify recent fecal pollution is desirable.

***Enteroccocus esp* assay.** The *Enterococcus esp* PCR assay was established in our laboratories as described by Scott et al. (2005), but it never performed as expected using known positive isolates of *Enterococcus faecium*. Our original plan after reading the literature for promising MST approaches had been to use detection of the human *E. faecium* surface protein gene, esp_{fm}, as a method of detecting human fecal pollution in surface water samples (Scott et al., 2005). Briefly, the methods described in the Scott paper were to concentrate water samples by membrane filtration (0.45 um), with the membrane placed subsequently on agar media selective for *Enterococcus* (mEnterococcus agar) and incubated for 48 hr. Next, the colonies were washed from the filter by suspension and vortexing in tryptic soy broth (TSB) and incubated at 41^{o}C for 3 hours to further enrich for *Enterococci*. DNA was then extracted from TSB using a QIAamp DNA extraction kit according to the manufacturer's instructions. The PCR to detect the *E. faecium* esp_{fm} gene (presumed to be specific for human *E. faecium*) was performed on extracted DNA using the described primers and reaction conditions.

Initial applications of the *Enterococcus* PCR assay were to detect the esp_{fm} gene in wastewater samples (influent) collected from wastewater treatment plants located along the central California coast. We were able to detect the target gene in wastewater samples, as expected. However, in order to examine assay specificity, confirmed *Enterococcus* isolates from humans (hospitalized individuals), animals (dogs, cats, gulls), and environmental water samples were tested for the presence of this gene. We were unable to detect esp_{fm} in any individual *Enterococcus* isolate including human-origin isolates; indicating that this assay may not be very sensitive for detecting human fecal pollution.

Several studies have now questioned the utility of esp_{fm} detection as a microbial source tracking method (Whitman et al., 2007; Byappanahalli et al., 2008; Layton et al., 2009). Layton

et al. (2009) evaluated the specificity of esp_{fm} detection in samples from northern California, and found the assay to be non-specific, with 83% of human feces testing positive, but 64% of animal fecal samples also tested positive. In fact, 100% of dog and seal samples tested positive using the *esp* assay, confirming that it is not specific for detection of human fecal pollution in California. Whitman et al. (2007) also found that 8% of all animal sources in their study tested positive, confirming that the esp_{fm} gene assay was not specific for human fecal material. They also examined the occurrence of this gene in raw wastewater influent, septic waste, and active pit toilets. The prevalence of this gene was 93.1%, 30%, and 0% respectively. These data suggest that detection of the esp_{fm} gene can be inconsistent, depending on the environmental source(s) of human fecal contamination. This microbial source tracking method may be useful for detecting concentrated fecal matter that includes relatively recent input from multiple individuals, but has far less utility when examining sources with less diverse, longer term fecal input that is likely to exhibit a greater degree of microbial decomposition, such as septic tanks and pit toilets. A more recent paper from the same group (Byappanahalli et al., 2008) examined the presence of esp_{fm} in 452 water samples and found detection was not a good predictor of post-precipitation contamination of lake water, unlike more conventional methods involving detection of *E. coli* or *Enterococci*. Given these findings and the uncertainty of PCR detection of esp_{fm} for microbial source tracking across geographic regions, use of this testing method was discontinued in the current study, and is not recommended.

Total: fecal coliform ratios. Similar to Haile et al. (1999), we expected that detection of specific bacterial pathogens would be highest when the ratio of total to fecal coliforms was low, indicating a greater proportion of fecal contamination. Consistent with this hypothesis, Haile et al. (1999) reported an increased risk of specific bacterial pathogen detection with decreasing cutpoints, especially at a cutpoint of 2. However, when we examined associations between the ratio of total to fecal coliforms and fecal pathogen detection in river and ocean water samples, no significant patterns were observed at a cutpoint of 2, although *Giardia* was 3.4 times more likely to be detected when using a cutpoint of 5 (p=0.031) (Table 42). Haile et al. (1999) also observed that obtaining a ratio of 5 when total coliforms are low relative to fecal coliforms may not reflect increased risk, but this same ratio may reflect increased human health risk from exposure to fecal pathogens when the total coliform density exceeds 1,000 or 5,000 cfu. However, in the current study, where analysis of river and ocean water samples was restricted to sites of specific pathogen detection and total coliform densities >1,000 or 5,000 cfu, no significant differences in pathogen detection were observed (Tables 43 and 44). Also, no significant associations were observed for specific pathogen detection and total: fecal coliform ratios for stormwater samples collected during this study (Table 45). All wastewater influent and effluent samples contained *Cryptosporidium* and *Giardia*, regardless of total coliform: fecal coliform ratios. No significant patterns were observed when we examined associations between total: fecal coliform ratios and *Salmonella* detection in wastewater influent and effluent samples ($p > 0.9$). Similarly, when analysis of wastewater effluent samples was restricted to samples testing positive for specific pathogens, and with total coliform densities >1,000 or 5,000 cfu, no significant differences in *Salmonella* detection were observed ($p > 0.5$).

Table 42. Simple logistic regression of total coliform: fecal coliform ratios (cfu/100ml) associated with detection of enteric bacterial pathogens in river and ocean water samples from central California (2008-2010).

Total Coliform:Fecal Coliform Ratio (cfu/100ml)	n	*Salmonella*		*Cryptosporidium*		*Giardia*		≥1 Pathogen	
		OR[a]	P-value	OR	P-value	OR	P-value	OR	P-value
>2	81	1.0	---	1.0	---	1.0	---	1.0	---
<2	97	3.1	0.17	0.8	0.57	0.8	0.45	0.8	0.57
>5	33	1.0	---	1.0	---	1.0	---	1.0	---
<5	145	1.9	0.56	0.8	0.69	3.4	0.031[b]	1.6	0.26

[a]OR = odds ratio.
[b]Significantly different than reference category, based on P< 0.05.

Table 43. Associations between total coliform: fecal coliform ratios (cfu/100ml) and enteric bacterial pathogen detection for river and ocean water samples with total coliform counts >1000 (cfu/100ml).

Total Coliform: Fecal Coliform Ratio (cfu/100ml)	n	*Salmonella*		*Cryptosporidium*		*Giardia*		≥1 Pathogen	
		Prevalence (%)	Fisher's Exact P-value[a]	Prevalence (%)	Fisher's Exact P-value	Prevalence (%)	Fisher's Exact P-value	Prevalence (%)	Fisher's Exact P-value
>2	19	11	---	26	---	16	---	37	---
<2	7	0	0.53	43	0.36	14	0.71	43	0.56
>5	14	7	---	29	---	7	---	36	---
<5	12	8	0.72	33	0.56	25	0.29	42	0.54

[a]1-sided Fisher's Exact, P-value < 0.05.

Table 44. Associations between total coliform: fecal coliform ratios (cfu/100ml) and specific bacterial pathogen detection for river and ocean water samples with total coliform counts >5000 (cfu/100ml).

Total Coliform: Fecal Coliform Ratio (cfu/100ml)		*Cryptosporidium*		*Giardia*		≥1 Pathogen	
	n	Prevalence (%)	Fisher's Exact pvalue[a]	Prevalence (%)	Fisher's Exact pvalue	4.3. Prevalence (%)	4.4. Fisher's Exact pvalue
>2	8	25	---	13	---	25	---
<2	1	0	0.78	0	0.89	0	0.78
>5	6	17	---	0	---	17	---
<5	3	33	0.58	33	0.33	33	0.58

[a]1-sided Fisher's Exact., P < 0.05.

Table 45. Associations between total coliform:fecal coliform ratios (cfu/100ml) and specific bacterial pathogen detection in stormwater samples from the central California coast (Simple logistic regression).

Total Coliform: Fecal Coliform Ratio (cfu/100ml)		*Salmonella*		*Cryptosporidium*		*Giardia*		≥1 Pathogen	
	n	OR[a]	P-value[b]	OR	P-value	OR	P-value	OR	P-value
>2	21	1.0	---	1.0	---	1.0	---	1.0	---
<2	20	0.3	0.23	0.7	0.52	0.8	0.75	1.3	0.71
>5	33	1.0	---	1.0	---	1.0	---	1.0	---

[a]OR = odds ratio

[b]Significantly different than reference category, based on P < 0.05.

In conclusion, the *Enterococcus esp* assay and total:fecal coliform ratios did not perform as well as as *Bacteroidales* detection for future MST efforts, and neither assay should be used alone in future studies. The current study has highlighted the associations, or lack thereof, between findings for traditional FIB methods, detection of *Bacteroidales* host markers, and the presence or absence of specific waterborne pathogens in surface waters. We found that traditional indicator assays such as total coliforms and enterococci, correlated with detection of some, but not all bacterial and protozoal pathogens included in this study, suggesting that monitoring for FIB alone may not accurately predict the risk of contact with fecal pathogens in surface waters. Both *Bacteroidales* and FIB bacteria were detected in the majority of surface water samples, which demonstrates the high sensitivity of *Bacteroidales* as an alternative fecal indicator assay. This conclusion is supported by prior specificity testing that demonstrated discrimination between human and animal fecal sources (Schriewer et al., 2010).

One benefit of *Bacteroidales* detection over traditional FIB testing is that it provides information about contributing host fecal sources, in addition to providing quantitative indicator bacterial data. We have demonstrated that *Bacteroidales* performs similarly to traditional FIB when predicting pathogen presence in surface waters, providing resource managers with greater flexibility in choosing monitoring approaches to evaluate water quality, remediate pollution, and reduce health risks. Utilizing a combination of indicator and pathogen assays can provide useful information regarding the presence, relative abundance, and contributing sources of fecal contamination in environmental water samples. This approach may be increasingly important for future monitoring and mitigation efforts in shared watersheds

4.6. Is there a connection between fecal pathogens from terrestrial sources and those found in sea otters?

Bacterial pathogen prevalence

The main focus of this aspect of the study was to identify several enteric bacterial and protozoal pathogens infecting various domestic and wildlife species from coastal California, and to compare them with isolates from downstream marine mammals (sea otters). As part of this effort we determined the prevalence of bacterial and protozoal pathogens and associated risk factors for selected terrestrial and marine animal species; screened *V. cholerae* and *V. parahaemolyticus* isolates for the presence of virulence genes; completed side-by-side molecular comparisons of selected bacterial strains isolated from sympatric terrestrial and marine animals; and performed preliminary molecular characterization of protozoal pathogens isolated from terrestrial animals.

Fecal samples were collected from 808 animals from 2007 through 2010, and 28% were positive for at least one of the target bacteria. *Campylobacter* was the most commonly isolated bacterium (11%), followed by *V. cholerae* (8%), *Salmonella* spp. (6%), *V. parahaemolyticus* (5%), and *V. alginolyticus* (3%). All samples tested negative for *E. coli* O157:H7, therefore it was excluded from further statistical analyses.

The prevalence and number of culture-positive fecal samples by risk factor category is summarized in Table 46. All animal groups with the exception of opossums tested positive for *Campylobacter* spp. Dogs exhibited the greatest proportion of *Campylobacter*-positive samples (24%), followed by wild felids (17%) and sea otters (16%). Only cows (n = 3) and gulls (n = 6) tested positive for *C. jejuni*, whereas dogs (n = 1) and gulls (n = 1) tested positive for *C. coli*. All animal groups tested positive for *Salmonella* except cats, wild felids, and cows. The *Salmonella*

prevalence was greatest for opossums (39%) followed by wild canids (11%). *Vibrio cholerae* was only detected opossums (6%), gulls (23%), and otters (30%). Similarly, *V. parahaemolyticus* was detected in opossums (4%), gulls (1%), and otters (36%), and *V. alginolyticus* was cultured from gulls (11%), sea otters (8%) and a dog (0.5%).

Bacterial pathogen univariate analysis

When tested individually, all defined risk factors (animal group, gender, age, sample season, sample location, collection year and live or dead status at the time of sampling) demonstrated significant associations with detection of one or more target bacteria. Animal group was a significant risk factor for detection of all bacterial groups in univariate analyses (Table 47). Domestic cats, beef cattle, and wild canids were less likely to test positive for *Campylobacter* spp. than dogs ($P<0.001$), whereas no significant difference was observed between dogs and wild felids or sea otters. With respect to *Salmonella* shedding, feces from wild canids, opossums, gulls, and otters were far more likely to test positive than were domestic dogs ($P<0.001$). Among marine species, where *Vibrio* detection was most common, sea otters were forty times more likely to be positive for *V. parahaemolyticus* than gulls ($P=0.009$), while no significant differences between gulls and otters were observed for *V. cholerae* and *V. alginolyticus*. Overall, sea otters were five times more likely ($P<0.002$) and opossums were twice as likely ($P<0.001$) to test positive for $\geq$1 pathogen than dogs. In contrast, domestic cats and beef cattle were significantly less likely to test positive for $\geq$1 of the target bacteria ($P<0.001$) than were dogs.

Associations among the remaining risk factors and enteric bacterial detection were more varied (Table 47). For example, gender and live-dead status categories only were important for *Salmonella* detection. Females were 1.3 times more likely to test positive for *Salmonella* than males ($P<0.001$) and fecal samples from necropsied animals were 3.4 times more likely to test positive than those from live animals ($P<0.001$). Season was only significant for detection of *V. alginolyticus*; samples collected during low rainfall periods were 6.2 times more likely to test positive for *V. alginolyticus* than wet season samples ($P=0.03$). Year, however, was a significant risk factor for *Campylobacter*, *V. parahaemolyticus*, *V. alginolyticus*, and individuals testing positive for $\geq$ 1 target bacteria. Age, also was a significant risk factor for *V. parahaemolyticus* and for individuals testing positive for $\geq$ 1 target bacteria. Subadults and individuals $\leq$ 6 months were less likely to test positive for *V. parahaemolyticus* and $\geq$ 1 target bacteria, respectively than adults.

Table 46. Prevalence with 95% binomial confidence intervals (CI) for bacterial pathogens cultured from terrestrial and marine animal species in the Monterey Bay region of California, 2007-2010.

		Campylobacter		*Salmonella*		*V. cholerae*		*V. parahaemolyticus*		*V. alginolyticus*	
Variable	***n***	**Prevalence (%)**	**95% CI**	**Prevalence (%)**	**95% CI**	**Prevalence (%)**	**95% CI**	**Prevalence (%)**	**95% CI**	**Prevalence (%)**	**95% CI**
Animal Group											
Dogs	182	23.6	17.6-.30.5	1.1	0.1-3.9	0.0	0-2.0[a]	0.0	0-2.0[a]	0.6	0-3.0
Cats	75	4.0	0.8-11.2	0.0	0-4.8[a]	0.0	0-4.8[a]	0.0	0-4.8[a]	0.0	0-4.8[a]
Beef Cattle	201	4.5	2.1-8.3	0.0	0-1.8[a]	0.0	0-1.8[a]	0.0	0-1.8[a]	0.0	0-1.8[a]
Wild Canids	18	11.1	1.4-34.7	11.1	1.4-34.7	0.0	0-18.5[a]	0.0	0-18.5[a]	0.0	0-18.5[a]
Wild Felids	12	16.7	2.1-48.4	0.0	0-26.5[a]	0.0	0-26.5[a]	0.0	0-26.5[a]	0.0	0-26.5[a]
Opossums	70	0.0	0-5.1[a]	38.6	27.2-51.0	5.7	1.6-14.0	4.3	0.9-12.0	0.0	0-5.1[a]
Gulls	149	8.7	4.7-14.5	6.7	3.3-12.0	23.5	16.9-31.1	1.3	0.2-4.8	11.4	6.8-17.6
Sea Otters	101	15.8	9.3-24.4	5.0	1.6-11.2	29.7	21.0-39.6	35.6	26.4-45.8	7.9	3.5-15.0
Age											
≤ 6 months	24	4.2	0.1-21.1	0.0	0-14.2[a]	0.0	0-14.2[a]	0.0	0-14.2[a]	0.0	0-14.2[a]
Subadults	160	3.8	1.4-8.0	6.9	3.5-12.0	3.1	1.0-7.1	1.3	0.1-4.4	0.6	0-3.4
Adults	175	5.1	2.4-9.5	11.4	7.1-17.1	1.7	0.3-4.9	3.4	1.3-7.3	1.1	0.1-4.1
Unknown	449	16.0	12.8-19.8	3.3	1.9-5.4	13.6	10.5-17.1	7.4	5.1-10.2	5.1	3.3-7.6
Sex											
Males	108	5.6	2.1-11.7	10.2	5.2-17.5	2.8	0.6-7.9	3.7	1.0-9.2	0.0	0-3.4[a]
Females	152	4.6	1.9-9.3	13.2	8.2-19.6	3.3	1.1-7.5	2.6	0.7-6.6	2.0	0.4-5.7
Unknown	548	13.7	10.9-16.8	2.7	1.5-4.5	11.1	8.6-14.1	6.0	4.2-8.3	4.2	2.7-6.2
Watershed											
Elkhorn Slough	502	10.4	7.8-13.4	7.2	5.1-9.8	7.6	5.4-10.2	6.8	4.7-9.3	2.6	1.4-4.4
Carmel River	283	12.4	8.8-16.8	2.8	1.2-5.5	10.3	7.0-14.4	0.7	0.1-2.5	3.5	1.7-6.4
Monterey Bay	23	4.4	0.1-21.9	8.7	1.1-28.0	8.7	1.1-28.0	21.7	7.5-43.7	13.0	2.8-33.6

Variable	*n*	*Campylobacter* Prevalence (%)	*Campylobacter* 95% CI	*Salmonella* Prevalence (%)	*Salmonella* 95% CI	*V. cholerae* Prevalence (%)	*V. cholerae* 95% CI	*V. parahaemolyticus* Prevalence (%)	*V. parahaemolyticus* 95% CI	*V. alginolyticus* Prevalence (%)	*V. alginolyticus* 95% CI
Season											
Wet (November-April)	352	11.7	8.5-15.5	5.4	3.3-8.3	6.8	4.4-10.0	6.0	3.7-9.0	0.9	0.2-2.5
Dry (May-October)	456	10.3	7.7-13.5	5.9	3.9-8.5	9.9	7.3-13.0	4.4	2.7-6.7	5.0	3.2-7.5
Year											
2007	345	8.4	5.7-11.8	5.5	3.3-8.5	12.5	9.2-16.4	3.5	1.8-6.0	6.7	4.3-9.8
2008	447	12.1	9.2-15.5	5.8	3.8-8.4	5.8	3.8-8.4	6.0	4.0-8.7	0.7	0.1-1.9
2009-10	16	31.3	11.0-58.7	6.3	0.2-30.2	0.0	0-20.6[a]	12.5	1.5-38.3	0.0	0-20.6[a]
Live-Dead Status											
Live	697	11.6	9.3-14.2	4.5	3.0-6.2	9.2	7.1-11.6	5.2	3.6-7.1	3.6	2.3-5.2
Dead	111	6.3	2.6-12.6	13.5	7.8-21.3	4.5	1.5-10.2	4.5	1.5-10.2	0.9	0-4.9

[a]one-sided, 97.5% confidence intervals

Table 47. Univariate logistic regression of risk factors associated with detection of bacterial pathogens cultured from terrestrial and marine animal species in the Monterey Bay region of California, 2007-2010.

		Campylobacter		*Salmonella*		*V. cholerae*		*V. parahaemolyticus*		*V. alginolyticus*		≥1 Target Bacteria	
Variable	***n***	**OR[a]**	**P value**	**OR**	**P value**	**OR**	**P value**	**OR**	**P value**	**OR**	**P value**	**OR**	**P value**
Age													
Adults	175	1.0	---	1.0	---	1.0	---	1.0	---	1.0	---	1.0	---
≤ 6 months	24	0.8	0.825									0.2	<0.001[b]
Subadults	160	0.7	0.431	0.6	0.460	1.8	0.699	0.4	0.046[b]	0.5	0.335	0.6	0.442
Unknown	449	3.5	<0.001[b]	0.3	<0.001[b]	9.0	<0.001[b]	2.2	0.427	4.7	0.262	2.3	0.014[b]
Sex													
Males	108	1.0	---	1.0	---	1.0	---	1.0	---	1.0	---	1.0	---
Females	152	0.82	0.790	1.3	<0.001[b]	1.2	0.732	0.7	0.324	0.5	0.565	1.0	0.959
Unknown	548	2.7	0.036[b]	0.2	<0.001[b]	4.4	<0.001[b]	1.7	0.592			1.7	<0.001[b]
Season													
Wet (November-April)	352	1.0	---	1.0	---	1.0	---	1.0	---	1.0	---	1.0	---
Dry (May-October)	456	0.9	0.724	1.1	0.802	1.5	0.692	0.7	0.518	6.2	0.030[b]	1.4	0.590
Year													
2008	447	1.0	---	1.0	---	1.0	---	1.0	---	1.0	---	1.0	---
2007	345	0.7	0.358	0.9	0.375	2.3	0.261	0.6	<0.001[b]	10.6	<0.001[b]	1.5	0.381
2009-10	16	3.3	0.002[b]	1.1	0.647			2.2	0.017[b]			2.4	0.090[b]
Live-Dead Status													
Live	697	1.0	---	1.0	---	1.0	---	1.0	---	1.0	---	1.0	---
Dead	111	0.5	0.190	3.4	<0.001[b]	0.5	0.167	0.9	0.887	0.2	0.288	0.9	0.412

[a]OR = odds ratio. Odds ratios only shown for variables where bacteria were detected; [b]Significantly different than reference category based on P< 0.1.

Bacterial pathogen multivariate analysis

When all risk factors were evaluated together in regression models, animal group, age, and year were significantly associated with detection of ≥ 1 target bacteria. Animal group however, was an especially important risk factor when bacteria models were assessed individually (Table 48). For example, when animal group was included in models to assess risk factors for *Campylobacter* shedding, the remaining risk factors were not statistically significant. Dogs ($P<0.001$) were more likely to test positive for *Campylobacter* spp. than were domestic cats ($P<0.001$), beef cattle ($P<0.001$), and wild canids ($P<0.001$), whereas no significant difference in *Campylobacter* spp. detection was observed between dogs and wild felids ($P=0.424$) or sea otters ($P=0.226$). Similarly, only animal group remained a significant risk factor for fecal shedding of *Salmonella*, with local wildlife species posing the greatest risk of fecal spread. Feces from opossums, wild canids, gulls and sea otters were 56.5, 11.3, 6.5 and 4.7 times more likely, respectively, to test positive for *Salmonella* spp. ($P<0.001$) than dog feces.

Age was the only risk factor significantly associated with shedding of *V. cholerae*, whereas animal group, age, and year were significantly associated with *V. parahaemolyticus* detection. Interestingly opossums were 16 times more likely ($P=0.063$) and sea otters 63.6 times more likely ($P<0.001$) than gulls to test positive for *V. parahaemolyticus*. Additionally, subadult animals were less likely to test positive for *V. parahaemolyticus* than adults ($P<0.001$). The risk of *parahaemolyticus* detection was highest in 2009-10 and lowest in 2007. For *V. alginolyticus* shedding, animal group, season, and year were significant risk factors. Domestic dogs were less likely than gulls to test positive, and samples collected during the dry season were 3.7 times more likely to test positive than wet season samples ($P=0.012$). In contrast with *V. parahaemolyticus*, samples collected during 2007 were more likely to test positive for *V. alginolyticus* than samples collected during 2008 ($P<0.001$).

Virulence analysis

All 51 tested *V. cholerae* isolates were negative for the *ctx* toxin gene on PCR. In contrast, the *tdh* gene was detected in 22% of 18 *V. parahaemolyticus* isolates tested. All *tdh*-positive isolates were obtained from sea otters.

PFGE

All *Salmonella* serotypes can cause disease in humans and are often classified according to their adaptation to animal hosts. Most serotypes, however, have a broad host-spectrum, including *S.* Enteriditis and *S.* Typhimurium, the two most common serotypes for salmonellosis transmitted from animals to humans (CDC, 2008). During our study, opossums (n = 7), gulls (n = 9) and sea otters (n = 2) tested positive for *S.* Typhimurium and *S.* Typhimurium (Copenhagen), providing an opportunity to examine land-sea transfer of bacterial pathogens to sea otters in the Monterey Bay area through characterization of bacterial strains from terrestrial and marine animal species. *Salmonella* Typhimurium and *S.* Typhimurium (Copenhagen) serotypes are distinguished only by a single epitope (variant Copenhagen lacks O:5) (Frech et al., 2003), thus isolates recovered from different animal types can be genetically closely related. These serotypes also can be very diverse genetically. Zaho et al. (2005) reported a total of 311 pulsed field gel electrophoresis (PFGE a means of producing a genetic fingerprint) patterns generated among 588 *S.* Typhimurium and *S.* Typhimurium (Copenhagen) isolates tested from seven animal species. Our PFGE results also indicated a diverse *S.* Typhimurium and *S.*

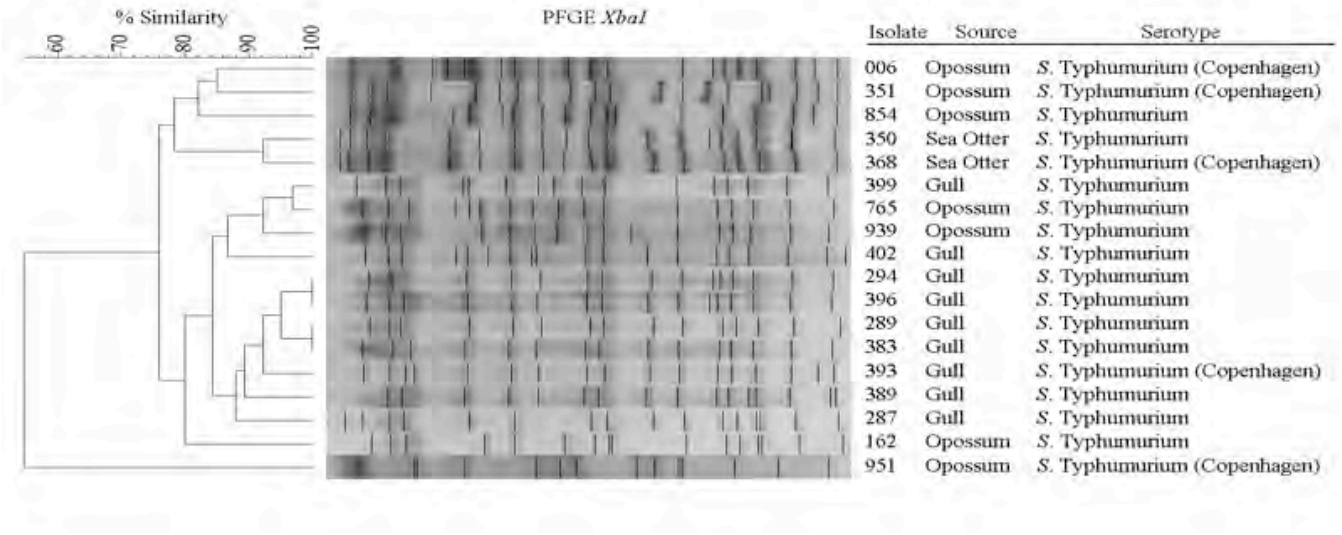
Table 48. Multivariate logistic regression of risk factors associated with detection of bacterial pathogens cultured from terrestrial and marine animal species in the Monterey Bay region of California, 2007-2010.

		Campylobacter		*Salmonella*		*V. cholerae*		*V. parahaemolyticus*		*V. alginolyticus*		≥1 Target Bacteria	
Variable	***n***	**OR[a]**	**P value**	**OR**	**P value**	**OR**	**P value**	**OR**	**P value**	**OR**	**P value**	**OR**	**P value**
Animal Group													
Dogs	182	1.0	---	1.0	---					0.1	<0.001[b]	1.0	---
Cats	75	0.1	<0.001[b]									0.3	<0.001[b]
Beef Cattle	201	0.1	<0.001[b]									0.3	<0.001[b]
Wild Canids	18	0.4	<0.001[b]	11.3	<0.001[b]							1.3	0.565
Wild Felids	12	0.6	0.424									1.6	0.292
Opossums	70			56.5	<0.001[b]			16.0	0.063[b]			7.2	<0.001[b]
Gulls	149	0.3	<0.001[b]	6.5	<0.001[b]			1.0	---	1.0	---	2.3	0.167
Sea Otters	101	0.6	0.226	4.7	<0.001[b]			63.6	0.001[b]	1.2	0.134	7.9	<0.001[b]
Age													
Adults	175					1.0	---	1.0	---			1.0	---
≤ 6 months	24											0.6	0.682
Subadults	160					1.8	0.699	0.9	0.001[b]			1.1	0.247
Unknown	449					9.0	<0.001[b]	4.9	0.009[b]			3.2	<0.001[b]
Season													
Wet (Nov-April)	352									1.0	---		
Dry (May-Oct)	456									3.7	0.012[b]		
Year													
2008	447							1.0	---	1.0	---	1.0	---
2007	345							0.8	0.044[b]	2.9	<0.001[b]	1.1	0.565
2009-10	16							6.4	0.006[b]			1.8	0.063[b]

[a]OR = odds ratio. [b]Significantly different than reference category, based on $P < 0.1$

typhimurium (Copenhagen) population with four PFGE patterns with an overall similarity of 55% observed among 18 isolates (Figure 18). Group I contained isolates from three opossums (006, 351, 854) that exhibited an 82% similarity. Group II contained two identical isolates (93% similarity), both from sea otters (350, 368). Group III was the largest cluster, containing 12 isolates with a similarity of 82%. The isolates in Group III were highly related with one group of three identical isolates (92% similarity) consisting of one gull (399) and two opossums (765 and 939) and a second group consisting of five identical (92% similarity) gull isolates (294, 396, 289, 383, and 393). Group IV had only 1 isolate from opossum 951. The PFGE patterns observed among opossum isolates were more diverse as compared with other animal isolates. Opossums exhibited patterns of three PFGE groups, including two that were unique to their species. Gulls however were only contained in group III, with the majority of isolates (56%) having identical fingerprints. Sea otters isolates were 78% similar to isolates in Group I and 76% similar to isolates in Group III indicating the possibility of a similar genetic lineage among PFGE patterns. However, a similar lineage does not always translate to an epidemiological link (Tenover et al., 1995). Further testing of a greater number of isolates is needed to confirm the possibility of land-sea transfer of *S.* Typhimurium to sea otters.

Figure 18. *Salmonella* Typhimurium dendrogram showing intra-species relatedness of Pulsed Field Gel Electrophoresis (PFGE) fingerprints after *Xba*I digestion.



Antimicrobial Susceptibility Testing

Results from antimicrobial susceptibility testing are summarized in Table 49. Among the 30 isolates of *Salmonella*, all exhibited resistance to the same three antimicrobials: erythromycin, penicillin, and rifampin (Table 49). This resistance is expected as enterobacteriaceae are resistant to these agents almost uniformly. Resistance also was observed, but to a lesser extent, to tetracycline (53%). All isolates were uniformly susceptible to imipenem; however, 19%, 27%, 12%, 8%, of wastewater isolates demonstrated resistance to other beta-lactam drugs: ampicillin, amoxicillin-clavulanic acid, cefazolin, and ceftazidime, respectively. Wastewater influent (n=8) and effluent (n=18) differed with 6% of influent wastewater isolates demonstrating resistance to enrofloxacin or gentamicin and 13% of effluent wastewater isolates demonstrating resistance to

trimethoprim/sulphamethoxazole (TMS). With sea otters (n=4) only exhibiting resistance to erythromycin, penicillin, rifampin, and tetracycline, wastewater isolates (n=26) were resistant to considerably more antimicrobials.

Among the 63 isolates of *V. cholerae*, all were resistant to penicillin and with the exception of one isolate, all were resistant to erythromycin. Resistance also was observed, but to a lesser extent, to rifampin (60%) and ampicillin (11%). All isolates were uniformly susceptible to ceftazidime, enrofloxacin, gentamicin, and imipenem, however, 3% of isolates demonstrated resistance to tetracycline and 2% of isolates demonstrated resistance to amoxicillin-clavulanic acid, cefazolin, or TMS. Wastewater (n=4), river (n=32), and sea otters (n=27) isolates all exhibited similar patterns with resistance to erythromycin, penicillin, and rifampin. However, one river isolate demonstrated additional resistance to amoxicillin-clavulanic acid, two wastewater isolates were resistant to tetracycline and one to TMS, and one sea otter isolate was resistant to cefazolin.

Among the 14 isolates of *V. parahaemolyticus*, all were resistant to erythromycin and penicillin; and with the exception of one isolate, all were resistant to rifampin. Resistance also was observed, but to a lesser extent, to ampicillin (86%), cefazolin (14%), tetracycline (14%), amoxicillin-clavulanic acid (7%), and gentamicin (7%). All isolates were uniformly susceptible to ceftazidime, enrofloxacin, imipenem, and TMS. Sea otter isolates (n=10) and isolates from river samples (n=4) exhibited similar resistance patterns with only a few exceptions. For example, 10% of sea otter isolates demonstrated resistance to amoxicillin-clavulanic acid and gentamicin and 20% of sea otter isolates demonstrated resistance to cefazolin and tetracycline, whereas, river isolates were susceptible to all four of these antimicrobials.

Protozoal pathogen prevalence

Fecal samples were collected from 802 animals from 2007 through 2010, and 6% were positive for *Cryptosporidium*, 15% were positive for *Giardia*, and 3% were positive for both organisms. The prevalence and number of culture-positive fecal samples by risk factor category is summarized in Table 50. All animal groups with the exception of wild felids and gulls tested positive for *Cryptosporidium* spp. Virginia opossums and wild canids exhibited the greatest proportion of positive samples (23%) and (22%) respectively. All animal groups tested positive for *Giardia* spp. with the greatest percentage of positive samples recovered from wild canids (39%) and beef cattle (34%). Subadult animals exhibited the greatest proportion of positive samples for *Cryptosporidium* spp. (15%) and *Giardia* spp. (42%). Males also appeared to have a greater percentage of positive samples for *Cryptosporidium* spp. (13%) and *Giardia* spp. (19%) than females.

Protozoal pathogen univariate analysis

When tested individually, all defined risk factors (animal group, gender, age, sample season, sample location, collection year and live or dead status at the time of sampling) demonstrated significant associations with detection of *Cryptosporidium* and/or *Giardia* spp. Animal group was a significant risk factor for detection of both protozoal pathogens in univariate analyses (Table 51). Domestic cats, beef cattle, wild canids and opossums were more likely to test positive for *Cryptosporidium* spp. than dogs ($P<0.001$), whereas no significant difference was observed between dogs and sea otters. With respect to *Giardia* shedding, feces from domestic cats, beef cattle, wild canids, wild felids and opossums were far more likely to test positive than were domestic dogs ($P=0.004$). In contrast, gulls were less likely to test positive for *Giardia* spp.

Table 49. Antimicrobial resistance patterns of *Salmonella, Vibrio cholerae, and Vibrio parahaemolyticus* isolated from wastewater, river, and sea otter fecal samples.

	% of Isolates with Resistance Profile								
	Salmonella			*V. cholerae*				*V. parahaemolyticus*	
Resistance Pattern to the Anitimicrobial Panel	**Wastewater Influent** (*n*=18)	**Wastewater Effluent** (*n*=8)	**Sea Otter** (*n*=4)	**Wastewater Influent** (*n*=3)	**Wastewater Effluent** (*n*=1)	**River** (*n*=32)	**Sea Otter** (*n*=27)	**River** (*n*=4)	**Sea Otter** (*n*=10)
AMC,AMP,CFZ,CAZ,ERY,PCN,RIF,TET	6	13							
AMC,AMP,CFZ,ERY,PCN,RIF,TET	6								10
AMC,AMP,ERY,PCN,RIF,TET	6	13							
AMP,ERY,PCN,RIF,TET		13		33					10
AMP,ERY,PCN,RIF,SXT		13							
AMP,CFZ,ERY,PCN,RIF									10
AMP,ERY,GEN,PCN,RIF									10
ERY,GEN,PCN,RIF,TET	6								
ERY,PCN,RIF,TET,SXT					100				
ERY,PCN,RIF,TET	22	38	50						
ENR,ERY,PCN,RIF	6								
AMP,ERY,PCN,RIF				67		9		75	40
CFZ,ERY,PCN,RIF							4		
ERY,PCN,RIF	50	13	50			41	59		20
AMC,PCN,RIF						3			
AMP,ERY,PCN						3		25	
ERY,PCN						44	37		

[a] Drug abbreviations: AMC: amoxicillin-clavulanic acid; AMP: ampicillin; CFZ:cefazolin; CAZ: ceftazidime; ENR: enrofloxicin; ERY: erythromycin; GEN: gentamicin; IPM: imipenem; PCN: penicillin; RIF: rifampin; TET: tetracycline; SXT: trimethoprim- sulphamethoxazole.

than dogs (P=0.004) and no significant difference between dogs and sea otters were observed. Overall, wild canids were 52 times more likely (P=0.002), domestic cats were 16 times more likely (P<0.001), opossums were 11 times more likely (P=0.026) and beef cattle were seven times more likely (P<0.001) to test positive for both protozoal pathogens than were dogs.

Associations among the remaining risk factors and protozoal pathogen detection were more varied (Table 51). For example, the live-dead status category only was important for *Cryptosporidium* detection whereas, gender only was important for *Giardia* detection. Fecal samples from necropsied animals were 3.4 times more likely to test positive for *Cryptosporidium* than those from live animals (P=0.01) and males were 1.6 times more likely to test positive for *Giardia* spp. (P=0.088). Season was only significant for detection of *Giardia*; samples collected during greater rainfall periods were 1.8 times more likely to test positive for *Giardia* than dry season samples (P=0.017). Age and year, however, were significant risk factors for *Cryptosporidium*, *Giardia*, and individuals testing positive for both pathogens. Subadults were 2.3 times more likely to test positive for *Cryptosporidium* (P=0.039), 5.2 times more likely to test positive for *Giardia* (P<0.001), and 3 times more likely to test positive for both pathogens (P=0.003) than were adults.

Table 50. Prevalence with 95% binomial confidence intervals (CI) of protozoal pathogens recovered from terrestrial and marine animal species from areas in or near Monterey Bay, California during 2007-2010.

		Cryptosporidium spp.			*Giardia spp.*		
Variable	**n**	**Count**	**Prevalence (%)**	**95% CI**	**Count**	**Prevalence (%)**	**95% CI**
Animal Group							
Dog	182	2	1.10	0.1-3.9	16	8.79	5.6-15.2
Cat	74	8	10.81	5.4-22.5	11	14.86	9.0-29.1
Beef Cattle	201	13	6.47	3.7-11.5	68	33.83	42.3-59.9
Wild Canids	18	4	22.22	1.4-34.7	7	38.89	30.8-89.1
Wild Felids	11	0	0.00	0-28.5*	2	18.18	2.8-60.0
Virginia Opossum	68	16	23.53	18.7-45.1	10	14.71	8.6-29.4
Gull spp.	145	0	0.00	0-2.5*	3	2.07	0.4-6.0
Sea otter	103	1	0.97	0.02-5.3	1	0.97	0.02-5.3
Age							
≤ 6 months	24	2	8.33	1.1-24.5	3	12.50	3.0-36.3
Subadult	158	23	14.56	11.1-24.5	67	42.41	63.3-82.3
Adult	175	12	6.86	3.9-12.5	21	12.00	8.6-20.1
Unknown	445	7	1.57	0.6-3.3	27	6.07	4.3-9.3
Sex							
Male	108	14	12.94	8.4-23.7	21	19.44	15.6-34.5
Female	149	14	9.40	5.8-16.8	19	12.75	9.0-21.9
Unknown	545	16	2.94	1.7-4.9	78	14.31	13.4-20.4
Watershed							
Elkhorn Slough	498	31	6.22	4.5-9.3	87	17.47	17.3-25.4
Carmel River	280	13	4.64	2.6-8.2	31	11.07	8.6-17.2

Variable	n	*Cryptosporidium spp.* Count	Prevalence (%)	95% CI	*Giardia spp.* Count	Prevalence (%)	95% CI
Monterey Bay	24	0	0.00	0-14.2*	0	0.00	0-14.2*
Season							
Dry (May-October)	453	25	5.52	3.8-8.5	52	11.48	9.8-16.6
Wet (November-April)	349	19	5.44	3.5-8.8	66	18.91	18.5-28.7
Year							
2007	342	11	3.22	1.7-5.9	35	10.23	8.1-15.5
2008	449	33	7.35	5.5-11.0	83	18.49	18.5-27.3
2009-10	11	0	0.00	0-28.5*	0	0.00	0-28.5*
Live-Dead Status							
Live	693	33	4.76	3.5-6.9	102	14.72	14.3-20.5
Dead	109	11	10.09	5.7-19.2	16	14.68	10.2-26.4

(*) one-sided, 97.5% confidence interval

Table 51. Univariate logistic regression of variables with detection of protozoal pathogens recovered from terrestrial and marine animal species from areas in or near Monterey Bay, California during 2007-2010.

Variable	n	*Cryptosporidium spp.* Odds Ratio	P-value	*Giardia spp.* Odds Ratio	P-value	Both Pathogens Odds Ratio	P-value
Animal Group							
Dog	182	1.0	---	1.0	---	1.0	---
Cat	74	10.9	<0.001*	1.8	<0.001*	15.9	<0.001*
Beef Cattle	201	6.2	<0.001*	5.3	0.061*	6.5	<0.001*
Wild Canids	18	25.7	<0.001*	6.6	<0.001*	51.7	0.002*
Wild Felids	11			2.3	0.03*		
Virginia Opossum	68	27.7	<0.001*	1.7	0.088*	11.3	0.026*
Gull spp.	145			0.2	0.004*		
Sea otter	103	0.9	0.932	0.1	0.120	1.8	0.805
Sex							
Female	149	1.0	---	1.0	---	1.0	---
Male	108	1.4	0.651	1.6	0.088*	0.3	0.004*
Unknown	545	0.3	0.005*	1.1	0.531		
Age							
Adult	175	1.0	---	1.0	---	1.0	---
≤ 6 months	24	1.2	0.868	1.0	0.970	3.1	0.321
Subadult	158	2.3	0.039*	5.4	<0.001*	3.0	0.003*
Unknown	445	0.2	<0.001*	0.5	0.096	0.2	0.036*
Season							

Variable	n	*Cryptosporidium spp.*		*Giardia spp.*		Both Pathogens	
		Odds Ratio	P-value	Odds Ratio	P-value	Odds Ratio	P-value
Dry (May-October)	456	1.0	---	1.0	---	1.0	---
Wet (November-April)	352	1.0	0.945	1.8	0.017*	1.7	0.33
Year							
2008	447	1.0	---	1.0	---	1.0	---
2007	345	0.4	<0.001*	0.5	<0.001*	1.5	0.381
2009-10	16					2.4	0.09*
Live-Dead Status							
Live	697	1.0	---	1.0	---	1.0	---
Dead	111	2.2	0.01*	1.0	0.993	3.6	0.019*

*Significantly different than reference category, based on P< 0.1.

Protozoal pathogen multivariate analysis

When all risk factors were included in the regression model, animal group, age, year and status were significantly associated with the detection of *Cryptosporidium* spp. (Table 52). Domestic cats, beef cattle, wild canids and opossums were significantly more likely to test positive for *Cryptosporidium* spp. than dogs (P<0.001), whereas no significant difference was observed between dogs and sea otters. Subadults were 2.8 times more likely to test positive for *Cryptosporidium* spp. (P<0.001) when compared to adults. Samples collected during 2007 were significantly less likely to test positive for *Cryptosporidium* than samples from 2008 (P<0.001). Fecal samples from necropsied animals also were significantly less likely to test positive for *Cryptosporidium* spp. than live-sampled animals (P<0.001). Animal group, gender, age and season were significantly associated with detection of *Giardia* spp. Domestic cats and wild canids were significantly more likely to test positive compared with domestic dogs. Gulls and sea otters were significantly less likely to test positive for *Giardia* spp. than dogs and no significant difference was observed between dogs and beef cattle, wild felids or opossums. Males were 1.2 times more likely to test positive for *Giardia* spp. than females (P<0.001). Subadults were 2.6 times more likely to test positive for *Giardia* spp. (P=0.003) when compared to adults. Samples collected in the wet season were 1.9 times more likely to test positive for *Giardia* spp. compared to samples collected in the dry season (P<0.001). Animal group, gender, age and year were significant risk factors associated with detection of both pathogens.

Protozoal pathogen molecular characterization

Sequence analysis revealed that all three opossum *Cryptosporidium* isolates shared 99% homology with opossum genotype 1040 (Xiao et al., 2002). Sequence analysis of *Giardia* isolated from domestic dogs indicated that 29% were assemblage C, 57% were assemblage D, and 14% were assemblage E, whereas wild canid isolates were comprised of assemblages A (33%), B (33%), and D (33%). Sequence analysis of domestic cat isolates indicated that 80% were assemblage B and 20% were assemblage E. One wild felid isolate also was positive for assemblage E. Sequence analysis of *Giardia* isolated from beef cattle revealed that all were comprised of assemblage E. Further characterization is being performed for publication.

Table 52. Multivariate logistic regression of variables with detection of protozoal pathogens recovered from terrestrial and marine animal species from areas in or near Monterey Bay, California during 2007-2010.

		Cryptosporidium spp.		*Giardia spp.*		Both Pathogens	
Variable	**n**	**Odds Ratio**	**P-value**	**Odds Ratio**	**P-value**	**Odds Ratio**	**P-value**
Animal Group							
Dog	182	1.0	---	1.0	---	1.0	---
Cat	74	10.4	<0.001*	1.7	<0.001*	6.9	0.012*
Beef Cattle	201	4.0	0.001*	1.6	0.206	2.0	0.042*
Wild Canids	18	27.5	<0.001*	5.9	0.001*	24.4	<0.001*
Wild Felids	11			1.0	0.989		
Virginia Opossum	68	24.1	<0.001*	1.3	0.444	4.5	<0.001*
Gull spp.	145			0.3	0.027*		
Sea otter	103	0.9	0.946	0.1	0.086*	1.4	0.833
Sex							
Female	149			1.0	---	1.0	---
Male	108			1.2	<0.001*	1.3	<0.001*
Unknown	545			3.4	<0.001*	1.7	0.158
Age							
Adult	175	1.0	---	1.0	---	1.0	---
≤ 6 months	24	1.4	0.807	0.4	0.318	2.1	0.651
Subadult	158	2.9	<0.001*	2.7	<0.001*	3.7	<0.001*
Unknown	445	1.3	0.648	0.3	<0.001*	0.6	0.718
Season							
Dry (May-October)	456			1.0	---		
Wet (November-April)	352			2.3	0.038*		
Year							
2008	447	1.0	---			1.0	---
2007	345	0.9	<0.001*			0.6	0.019*
2009-10	16						
Live-Dead Status							
Live	697	1.0	---				
Dead	111	0.7	<0.001*				

*Significantly different than reference category based on P< 0.1.

Discussion of fecal pathogens from terrestrial sources and sea otters

Animal group was the most important risk factor associated with shedding of the target bacteria and both protozoal parasites. *Campylobacter* spp. detection was greatest in domestic dogs, with an overall prevalence of 24%. Similar prevalences, ranging up to 41.5% have been reported previously in adult household dogs (Fleming, 1983; Torre and Tello, 1993; Sandberg et al., 2002; Engvall et al., 2003; Wieland et al., 2005; Acke et al., 2009). The *Campylobacter* species typically associated with enteric disease in humans include *C. jejuni* and *C. coli*,

accordingly *Campylobacter* isolates obtained during our study were characterized as either *C. jejuni*, *C. coli*, or *Campylobacter* spp. In previous studies of adult domestic dogs, prevalences of *C. jejuni* and *C. coli* were low (1.2%) (Parsons et al., 2010) and (3%) (Acke et al. 2009), respectively. Similarly, in the current study, no dogs tested positive for *C. jejuni* and only one tested positive for *C. coli*. In contrast, the prevalence of *Salmonella* in feces was lower for domestic dogs than for all other animals. In prior studies, fecal prevalence for *Salmonella* in dogs ranged from 2.4 to 30% (Morse et al., 1976; Jofee and Schlesinger, 2002), compared to 1% in the current study. Interestingly, one dog tested positive for *V. alginolyticus*, a bacterium typically associated with the marine and brackish water environments (Kristensen, 1974). This isolate was obtained from fresh feces collected at a beach that was open to recreational use by dogs. Coastal sand, beachwrack or sediments can act as reservoirs of marine bacteria (Boehm et al., 2009), thus serving as a source of potential infection.

Dogs also exhibited lower prevalences of *Cryptosporidium* and *Giardia* than most of the other terrestrial animal species. In the current study, 1% of dogs tested positive for *Cryptosporidium*, which is within range of prior studies of dogs (0 to 19.6%) (Simpson et al. 1988; el-Ahraf et al., 1991; Grimason et al., 1993; Johnston and Gasser, 1993; Milstein and Goldsmid, 1995; Bugg et al., 1999). *Giardia* appears to be more common and has been reported to affect 15 to 31% of dogs in previous studies (Itoh et al., 2001; Huber et al., 2005; Overgaauw et al., 2009), however during this study prevalence was only 9%. The difference in prevalence may be attributed to sampling design. Greater prevalences have been observed in younger individuals or shelter dogs (Huber et al., 2005) whereas the majority of samples collected during this study were from adult household pets.

Domestic cats and free-ranging beef cattle proved to have the lowest prevalence of the target bacteria. Although previous studies reported *Salmonella* in 0.8%-29% of cats (Fox and Beaucage, 1979; Spain et al., 2001) and 8.5% of beef cattle (Rodriguez et al., 2006), no domestic cats or beef cattle in the present study tested positive for *Salmonella*. Furthermore, neither species tested positive for *Vibrio* spp. and <5% of sampled animals shed *Campylobacter*. Feces from 4% of domestic cats tested *Campylobacter*-positive, which is within the range of prior *Campylobacter* studies in cats (0.8%-42.9%) (Fox et al., 1983; Moser et al., 2001; Spain et al., 2001; Sandberg et al., 2002; Bender et al., 2005; Wieland et al., 2005; Acke et al., 2009). Only 4.5% of fecal samples from beef cattle tested positive for *Campylobacter* in the current study; previous *Campylobacter* prevalences have been reported from 5-89.4% in free-ranging beef cattle (Stanley et al., 1998; Hoar et al., 2001; Inglis et al., 2003; Inglis et al., 2004; Besser et al., 2005). Of the cattle that tested *Campylobacter*-positive in the current study, 33% were positive for *C. jejuni*. This low bacterial prevalence could reflect differences in management practices or age structure of sampled animals.

In contrast, both domestic cats and beef cattle appeared to be potential reservoirs of *Cryptosporidium* and *Giardia*. Similar to a study of household pets in the Netherlands where 5% of cats tested positive for *Cryptosporidium* and 14% were positive for *Giardia* (Overgaauw et al., 2009), we observed that 11% of domestic cats shed *Cryptosporidium* and 15% shed *Giardia*. Previous studies of beef cattle have reported prevalence rates ranging from 1 to 66% for *Cryptosporidium* spp. (Scott et al., 1995; Olson et al., 1997; Atwill et al., 1999; Hoar et al., 2001) and from 0 to 11% for *Giardia* spp. (Buret et al., 1990; Olson et al., 1997; Hoar et al., 2001). During our study, *Cryptosporidium* prevalence rates (6%) were within the previously reported range, however, *Giardia* prevalence (34%) was much greater than values reported in previous studies of beef cattle. Similar to our bacterial results, these differences may depend on the type

and age-class of cattle and management practices such as length of calving season, age at weaning, pasture location, or stocking density (Atwill et al., 1999; Hoar et al., 2001). In the current study, all sampled cattle were maintained under more sparsely stocked, free-range conditions and the majority of samples were collected from adults.

Interestingly, the prevalence of bacterial and protozoal pathogens in feces from terrestrial wildlife differed from their domestic counterparts, possibly reflective of different food sources and habitat use. Wild canids were significantly less likely to test positive for *Campylobacter* than domestic dogs. In contrast, wild felids exhibited a much greater prevalence for *Campylobacter* infection than domestic cats. Wild canids were also 11.3 times more likely to test positive for *Salmonella* than domestic dogs. Wild canids were 27 times more likely to test positive for *Cryptosporidium* and six times more likely to test positive for *Giardia* than domestic dogs. Wild felids were negative for *Cryptosporidium* and did not significantly differ from domestic cats with respect to *Giardia* prevalence.

The importance of opossums as environmental reservoirs of *Salmonella enterica* was previously discussed by Thigpen et al. (1975), but bears close consideration. During our study, opossums tested positive for five *Salmonella enterica* serotypes that are commonly associated with clinical disease in humans and animals. Two of these serotypes (*S*. Newport and *S*. Typhimurium) have been isolated from sea otters. Although *Vibrio* spp. are typically found in marine environments, 10% of opossums tested positive for *V*. *cholerae* or *V*. *parahaemolyticus*. To our knowledge, neither of these bacteria have been reported in opossums. The Monterey Bay region offers a unique situation because terrestrial species such as opossums that inhabit coastal areas may live at the land-sea interface, and may scavenge and defecate along shorelines and in tidal wetlands (Oates pers. obs.). In the current study, opossums were commonly sampled along the land-sea interface, often within sight of the ocean. As well documented omnivores, opossums are prone to using the most abundant foods available and for those individuals living along the land-sea interface that may include consuming marine foods and trash left by beachgoers. In contrast, no opossums tested positive for *Campylobacter*, which may be explained by biological characteristics of opossums, compared to other animals sampled as part of this study. The optimum growth temperature of thermotolerant *Campylobacter* spp. lie between 37-42°C, whereas, the average body temp of opossums is lower, ranging between 35-35.5 °C (Higgenbotham and Koon, 1955; Morrison and Petajan, 1962; McManus, 1969). Opossums may commonly be exposed to *Campylobacter* spp. through their scavenging activity; however, gastrointestinal colonization may be inhibited by a lower body temperature, when compared with other animals. It is also possible that differences in diet and/or intestinal microbiota may explain the lack of *Campylobacter* spp. in opossum fecal samples.

Twenty-three percent of fecal samples recovered from opossums tested positive for *Cryptosporidium* and 15% were positive for *Giardia*. Opossums were also 24 times more likely to test positive for *Cryptosporidium* than domestic dogs. In addition to serving as a potential environmental reservoir for *Cryptosporidium* and *Giardia* infection, opossums are also the definitive host of another protozoan parasite, *Sarcocystis neurona* (Fenger et al., 1995). *Sarcocystis neurona* has become an important cause of morbidity and mortality for southern sea otters, pinnipeds and cetaceans in areas where opossums have become endemic (Rejmanek et al., 2010). The Virginia opossum is a non-native species that was introduced to California by humans during the early twentieth century (Grinnell, 1915). Population estimates for California are unknown and currently unmanaged. Fecal inputs from opossums to the coastal environment

may be quite high, exposing native wildlife (e.g., threatened southern sea otters) to these novel and potentially deadly pathogens.

Gulls are one of the most commonly documented carriers of *Campylobacter* and *Salmonella*, particularly because of their marked scavenging behavior (Greig et al., 1986). In the present study, gulls tested positive for *Campylobacter*, *Salmonella* and all three *Vibrio* species. Furthermore, 4% of gulls were positive for *C. jejuni* and 0.67% tested positive for *C. coli*. Our results are similar to Quessy and Messier (1992), where 15.9% and 8.7% of ring-billed gulls tested positive for *Campylobacter* and *Salmonella*, respectively.

Vibrio cholerae, *V. parahaemolyticus*, and *V. alginolyticus* infection have previously been reported in gulls (Ogg et al., 1989; Buck, 1990). In the current study there were no significant differences among gulls, opossums, and sea otters for risk of shedding *V. cholerae*. However, opossums were 16 times more likely and sea otters 64 times more likely to shed *V. parahaemolyticus*. We surmise that diet and feeding habits may play an important role in the prevalence of *Vibrio* infection in these species. As vibrios are commonly found in the marine environment and in the filter-feeding invertebrate prey of sea otters (Miller et al., 2006), the high prevalence of all three *Vibrio* species observed in sea otter fecal samples is not surprising.

Although fecal samples from gulls tested positive for all target bacteria during our study, none were positive for *Cryptosporidium* and only 2% were positive for *Giardia*. These low values were similar to prevalences reported in previous studies where 0 to 5% of gull feces tested positive for *Cryptosporidium* (Smith et al., 1993; Miller et al., 2002; Moore et al., 2002) and 4% were positive for *Giardia* (Gaydos et al., 2008).

Sea otters tested positive for *Campylobacter*, *Salmonella*, *V. cholerae, V. parahaemolyticus* and *V. alginolyticus*, similar to reports by Miller et al. (2009). The prevalence of *Campylobacter* and *Salmonella* infection of sea otters, however, was greater in the current study; 16% of sea otters tested positive for *Campylobacter,* compared with 6.5% for Miller et al. (2009) and *Salmonella* was cultured from 5% of animals, compared with 1.4% for this previous study of the same sea otter population. In the current study, the prevalence of *Vibrio* detection in sea otters was greater for *V. cholerae* and *V. parahaemolyticus*, but lower for *V. alginolyticus* (Miller et al., 2009). Differences in bacterial pathogen prevalence between the two studies could be due in part to improvements in bacterial detection methods over time, different sampling periods (e.g., seasonal or annual differences) and in the proportion of samples from necropsied otters, where the prevalence of *V. alginolyticus* detection can be lower (Miller et al., 2009). Although fecal samples from sea otters have been tested for protozoal pathogens for the past decade, none have tested positive for either *Cryptosporidium* or *Giardia*. During our study however, one sea otter, an adult female from the Pebble Beach area, was positive for small numbers of both *Cryptosporidium* and *Giardia*.

In the present study, vibrios were recovered only from species known to inhabit marine waters (sea otters and gulls) or the marine/terrestrial interface (opossums and dogs). *Vibrio* spp. are ubiquitous in the aquatic environment, thus our findings are consistent with data from past studies (DePaola, 1981). Non O1 *V. cholerae* are commonly isolated from shellfish, particularly filter feeders, and have been found in a variety of domestic and wild animals, including dogs (Sack, 1973), and aquatic birds (Ogg et al., 1989). Relative pathogenicity of *Vibrio* strains is often linked to toxin gene expression, including the *ctx* (Mekalanos et al., 1983) and *tdh* (Nishibuchi et al., 1989) genes. The majority of environmental *Vibrio* strains are non-pathogenic unless a toxin gene has been acquired via phage transduction (Faruque et al., 1998). In the present study, all *V. cholerae* strains tested negative for the virulence *ctx* gene, but several *V.*

parahaemolyticus isolates obtained from sea otters were positive for the *tdh* gene. The health of animals infected with these toxin-positive strains is unknown, however asymptomatic carriers have been reported in humans (Morris, 1990). In addition, *Vibrio* species that test negative for virulence genes can be opportunistic pathogens under some circumstances (Grimes, 1991).

Of the 16 *Salmonella enterica* serotypes identified in this study, 8 have been associated with clinical disease in human and non-human animals, including, *S*. Typhimurium, *S*. Heidelberg, *S*. Enteriditis, *S*. Newport, *S*. Mbandaka, *S*. Give, *S*. Montevideo, and serovar 4,5,12:i:- (CDC, 2008). Opossums exhibited 11 different *Salmonella* serotypes, including a possible monophasic variant of *S*. Typhimurium (serovar 4,5,12:i:-), which is a newly emerging serovar associated with foodborne infections in human and non-human animals (Spain et al., 2001; Agasan et al., 2002). The *Salmonella* serotypes isolated from wild canids were not detected in other species and are not well known causes of clinical disease in humans, suggesting that wild canids may not be an important reservoir for human *Salmonella* infection in the study area. However, dogs, gulls, and opossums all shed *S*. Newport, a serotype that appears to be especially prevalent among California wildlife (Smith et al., 2002), has been isolated previously from sea otters (Miller et al., 2009) and is an important serotype in human disease. Three different serotypes were isolated from sea otters, including *S*. Typhimurium, *S*. Enteriditis, and *S*. Heidelberg. This is the first report of *S*. Heidelberg detection in sea otters; it is more commonly reported from poultry, cattle, pigs, and wildlife (CDC, 2008), but it has been recovered from filter-feeding invertebrates, such as fat innkeeper worms (*Eurechis caupo*), that are consumed by sea otters in central California (Miller et al., 2006).

Direct pathogen exposure is not the only concern associated with fecal pollution of the marine environment. In a previous study, nearly one-third of fecal coliforms isolated from wastewater, surface waters, and seawater were found to be resistant to one or more antimicrobial drugs (Niemi et al., 1983). Antimicrobial-resistant bacteria are a concern for both humans and animals because they decrease efficacy of commonly used antimicrobials, limit the range of treatment options, increase the cost and duration of treatment, and lead to higher morbidity and mortality (Williams, 2001; Hosein et al., 2002; Travers and Barza, 2002). We examined *Salmonella* and *Vibrio* isolates collected from wastewater, river, and sea otter samples to determine whether they could serve as a potential source of antimicrobial-resistant bacteria exposure in sea otters. Antimicrobial resistance patterns were quite variable among isolate types, however, all wastewater, river and sea otters isolates were resistant to erythromycin and penicillin. This is not surprising because *Vibrio* and *Salmonella* are Gram negative bacteria and should be more resistant to β-lactam antibiotics (penicillins, cephalosporins, and carbapenems) and the macrolide antibiotics such as erythromycin have limited effectiveness to inhibit growth of Gram negative organisms. Brownstein et al. (2011) reported similar results and recommended fluoroquinolones or a third generation cephalosporin for treatment of suspected *Salmonella* spp. or *Vibrio* spp. infections in sea otters. Interestingly, wastewater isolates were resistant to considerably more antimicrobials than river samples or sea otters. Most municipal wastewater generated along coastal California is released into the ocean after treatment, but primary and secondary treatment fails to kill many pathogenic bacteria (Rao et al., 1986). Wastewater effluent contains antimicrobial residues that may drive emergence of antimicrobial-resistant strains (Murray et al., 1984). Our results demonstrate the variability of antimicrobial resistance of pathogenic and opportunistic bacteria and the possibility of the transfer of antimicrobial-resistant bacteria to sea otters in the nearshore marine environment.

Although wildlife species have been considered potential reservoirs of protozoal pathogens such as *Cryptosporidium* and *Giardia*, reports based solely on microscopy do not provide information on the potential risk of transmission to humans or domestic animals (Trout et al., 2006). Recently, molecular studies have shown that wildlife can harbor both host-adapted and zoonotic strains of both *Cryptosporidium* and *Giardia* spp. (Sulaiman et al., 2003; Zhou et al., 2004). In the case of *Cryptosporidium* spp., *C. hominis* generally exhibits a human-to-human transmission cycle, although animal infections have been reported (Morgan-Ryan et al., 2002). The primary zoonotic species is *C. parvum*, although *C. canis*, *C. felis*, *C. meleagridis*, *C. muris*, C. *suis*, and *Cryptosporidium* cervine genotype have zoonotic potential as well (Xiao et al., 2004). During this study, three *Cryptosporidium* isolates recovered from opossums could be sequenced. These sequences shared a 99% homology with the host-specific opossum genotype II (Xiao et al., 2002). The host-adapted nature of these *Cryptosporidium* parasites indicates that the parasites probably do not have a high infectivity to humans or other wildlife (Xiao et al., 2002). For *G. duodenalis*, assemblages A and B have the widest host ranges, infecting humans and a variety of other animals. The remaining assemblages have restricted host ranges: C and D infect dogs, E infects hoofed livestock, F infects cats, and G infects rats (Monis et al., 2003). Similar to previous studies, the majority (86%) of dogs tested positive for the host-specific assemblages C and D. The majority (80%) of domestic cats however tested positive for the non-host specific assemblage B. Trout et al. (2006) reported coyotes testing positive for assemblages B, C, and D; similarly during this study, wild canids were positive for assemblages A, B, and D. All cattle did test positive for the host-specific assemblage E (Monis et al., 2003). Interestingly, one domestic dog, one domestic cat, and one wild felid also were positive for assemblage E. Further analysis will be performed to confirm this finding, as they may be a first in California. In summary, our results demonstrate a possible connection between fecal pathogens from terrestrial sources and those found in sea otters, supporting the concept that southern sea otters may act as sentinels of land-sea fecal pathogen pollution. Our hypothesis that sea otter enteric pathogen flora should reflect that of adjacent terrestrial animals was supported by the fact that the otters tested positive for the same range of bacteria as sampled domestic and wildlife species; however *S. enterica* Typhimurium isolated from feces of terrestrial animals were only distantly related to isolates recovered from downstream sea otters. Interestingly, antimicrobial susceptibility suggested a more riverine rather than wastewater source. Unlike other protozoal species, *Cryptosporidium* and *Giardia* were rare in sea otters with only one individual testing positive for both pathogens. The majority of protozoa are host-adapted, however during this study many terrestrial animals tested positive for non-host specific assemblages. Furthermore, the potential for a host-adapted species to cross over to a different species was demonstrated by three carnivores testing positive for a *Giardia* assemblage typically associated with livestock.

4.7. Are wetlands effective for reducing fecal pathogen loads in polluted water and what wetland characteristics are most critical for pathogen reduction?

Pathogen Tank Exposure Experiments

Using three mesocosm tanks located at the MWVCRC in Santa Cruz, a range of hydrological variables (e.g. water flow rate and salinity) were evaluated to determine their effect on protozoal transport through surface waters (Table 53). *Cryptosporidium* counts obtained from water samples in 0.1% salinity tanks were significantly lower than counts obtained from water samples

in 3% salinity tanks. The odds ratio (OR) of high:low salinity on *Cryptosporidium* counts was 1.77 (P <0.0001), indicating that at higher salinity levels (Coralife ® Scientific Grade Marine Salt at 30 ppt compared to 1 ppt) more oocysts were detected in water samples than at lower salinity levels (Figure 19). There was no significant difference between salinity levels for *Giardia* counts (p =0.6899), and flow rate was not statistically significant for *Cryptosporidium* nor *Giardia* detection.

Table 53: Effect of water flow rate and salinity on *Cryptosporidium* oocyst and *Giardia* cyst counts in non-vegetated mesocosm tanks (Univariate analysis)

Condition	β estimate	Odds Ratio	P-value
Cryptosporidium			
Salinity Low	Reference	Reference	
Salinity High	0.57	1.77	<.0001***
Flow Rate Slow	Reference	Reference	
Flow Rate Fast	0.12	1.13	0.52
Giardia			
Salinity Low	Reference	Reference	
Salinity High	-0.37	0.69	0.29
Flow Rate Slow	Reference	Reference	
Flow Rate Fast	0.38	1.47	0.28

*P<0.05, **P<0.01, ***P<0.0001

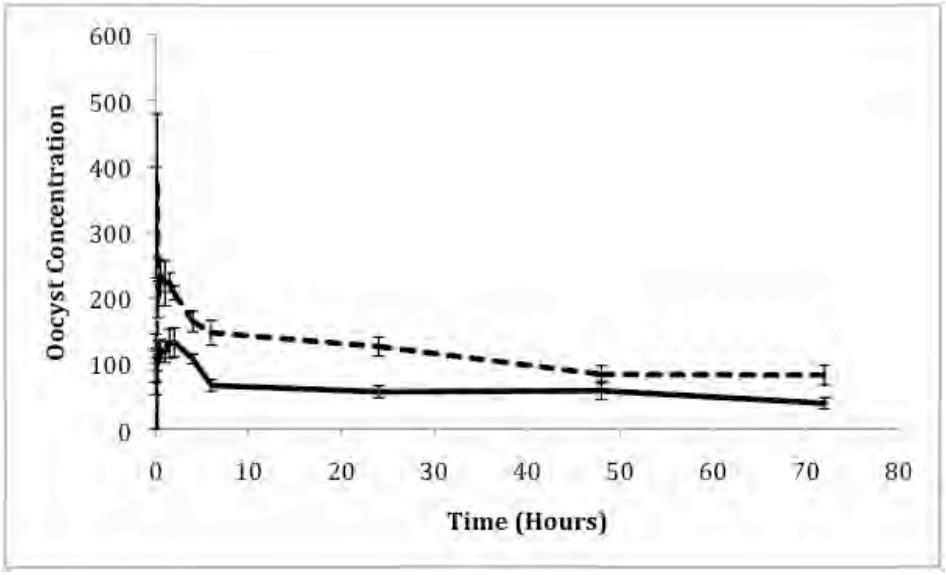


Figure 19. Effect of salinity on *Cryptosporidium* oocyst detection in 50 mL water sub-samples from non-vegetated mesocosm tanks following oocyst release. Overall, greater *Cryptosporidium* oocyst detection was observed with higher salinity. Solid line: 0.1% salinity, Dashed line: 3% salinity.

Common wetland restoration practices were also evaluated in the mesocosm tanks to determine the effects of vegetation type, vegetation amount, and planting configuration on protozoal reduction. The presence or absence of vegetation, as well as its type and configuration all had significant effects on *Cryptosporidium* and *Giardia* recovery, based on univariate analysis (Table 54). This second round of mesocosm experiments was conducted only at 0.1% salinity, while flow rate was varied as a potential effect modifier. Univariate analysis revealed that presence of vegetation, especially slough sedge, resulted in significantly lower *Cryptosporidium* counts, when compared to mesocosm tanks with no vegetation (Figure 20). Similarly, the presence of vegetation, especially California bulrush, resulted in significantly lower *Giardia* counts, when compared to mesocosm tanks with no vegetation (Figure 21). These findings support our hypothesis that artificial wetlands containing vegetation would more effectively reduce oocyst and cyst detection in wetlands water. Our study finding suggest that eroded and devegetated coastal areas will more effectively transport fecal protozoa from land to sea than areas with abundant vegetation like wetlands. These study findings lend strong support to the value of conserving coastal wetlands for improving fecally-impacted waters.

Table 54. Effect of defined vegetation parameters on counts of *Cryptosporidium* and *Giardia* in mesocosm tanks (univariate analysis)

Condition	β estimate	Odds Ratio	P-value
Cryptosporidium			
Vegetation (Buffer configuration only)			
Absent	Reference	Reference	
California Bulrush	0.25	1.28	0.08
Slough Sedge	0.46	1.59	0.001**
Configuration (California bulrush only)			
Absent	Reference	Reference	
Buffer	0.25	1.28	0.08
Channel	0.38	1.46	0.007**
Giardia			
Vegetation (Buffer configuration only)			
Absent	Reference	Reference	
California Bulrush	-0.58	0.56	0.007**
Slough Sedge	0.21	1.23	0.33
Configuration (California bulrush only)			
Absent	Reference	Reference	
Buffer	-0.58	0.56	0.007**
Channel	-0.37	0.69	0.09

*P<0.05, **P<0.01.

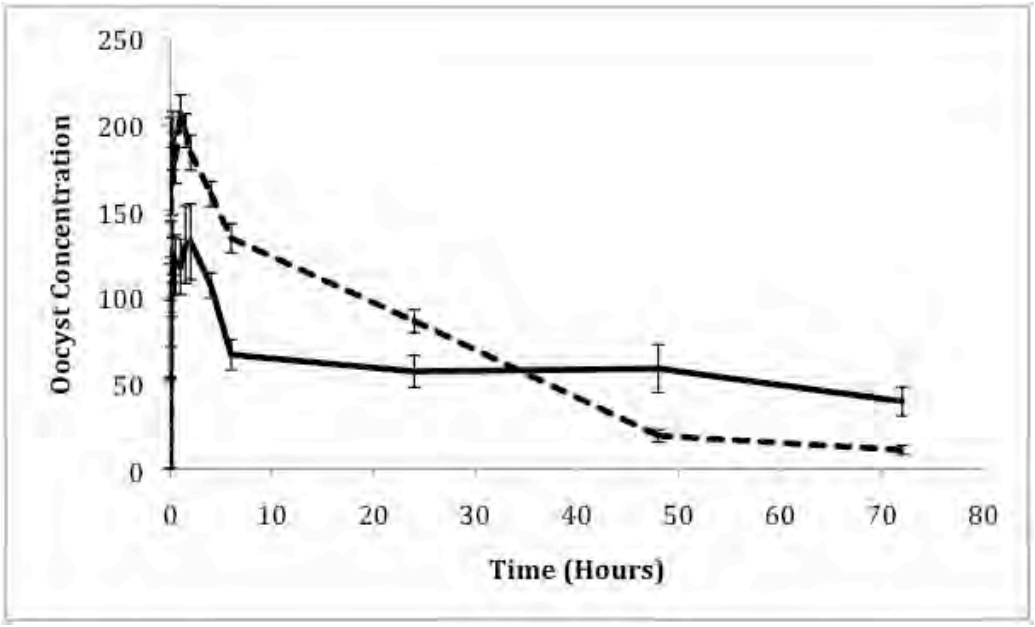


Figure 20. Effect of vegetation type and configuration on *Cryptosporidium* oocyst detection in 50 mL water sub-samples from mesocosm tanks following release of $4.5x10^6$ oocysts at time=0. The rate of *Cryptosporidium* oocyst reduction in water samples increased when vegetation was present. Solid line: vegetation absent. Dashed line: vegetation present

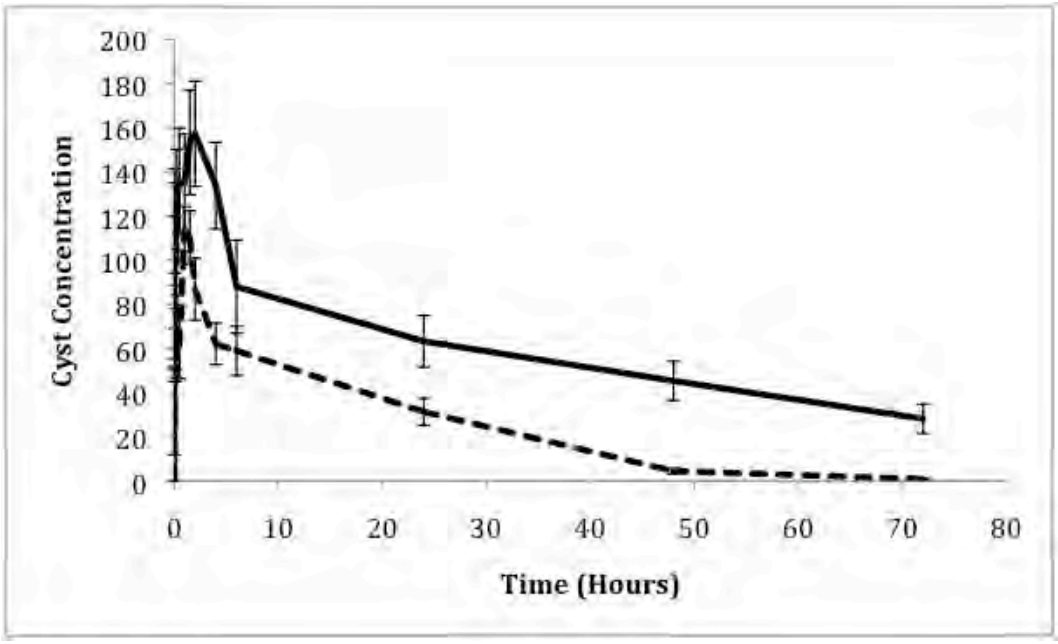


Figure 21. Effect of vegetation type and configuration on *Giardia* cyst detection per 50 mL water in relation to the presence or absence of vegetation in mesocosm tank studies. *Cryptosporidium* oocyst detection in 50 mL water sub-samples from mesocosm tanks following release of $4.5x10^6$ oocysts at time=0. The number of *Giardia* cysts detected in water samples was significantly lower when vegetation was present. Solid line: vegetation absent. Dashed line: vegetation present.

Next, the effects of vegetation type and configuration, water flow and other factors on *Cryptosporidium* and *Giardia* counts in the wetland mesocosm tanks was examined using multivariate analysis, where multiple predictor variables can be considered simultaneously. When vegetation type, configuration and sample period were analyzed together, *Cryptosporidium* oocyst counts varied significantly by vegetation type, configuration, and the presence or absence of vegetation (Table 55). For *Giardia cyst* detection, California bulrush, regardless of configuration, was significantly more effective at reducing protozoal counts, when compared to tanks where no vegetation was present (Figure 22). In contrast, the effect of slough sedge on *Giardia* cyst detection did not differ fromthat of mesocosm tanks where no vegetation was present (Table 56). When configuration of California bulrush was evaluated, no significant difference was detected between the buffer and channel configuration for either *Cryptosporidium* or *Giardia* detection, as evaluated by Wald tests.

Examinations of the effect of water flow rate (fast: 1.0 cm/sec, versus slow: 0.1 cm/sec) on protozoal detection revealed a slight reduction in *Giardia* cyst counts when vegetation was present. Faster flow rates were associated with higher *Giardia* counts for the same vegetation type and configuration, but this effect was not observed for the smaller parasitic oocyst, *Cryptosporidium*. Ultimately, for both protozoa, the presence of California bulrush significantly reduced the number of oocysts or cysts detected in any given sample, although placement of California bulrush in either the buffer or channel configuration did not result in differences in *Cryptosporidium* or *Giardia* detection. Presence of slough sedge significantly reduced counts of *Cryptosporidium* oocysts, but not *Giardia* cysts. Overall, these findings indicate that when protozoa are present in water that flows through vegetated areas, protozoa numbers are likely to be reduced.

Table 55. *Cryptosporidium* oocyst detection in relation to sample time and vegetation presence, type and configuration in mesocosm tanks (multivariate estimates)

Condition	β estimate	Odds Ratio	P-value
Individual Factors			
Vegetation Absent	Reference	Reference	
Vegetation-California bulrush, buffer	0.44	1.56	0.014*
Vegetation-California bulrush, channel	0.61	1.84	0.0006**
Vegetation-Slough sedge, buffer	0.58	1.79	0.0012**
Interaction with Time			
Vegetation-Time	Reference	Reference	
California bulrush, buffer-Time	-0.03	0.97	<0.0001***
California bulrush, channel-Time	-0.04	0.96	<0.0001***
Slough sedge, buffer-Time	-0.02	0.98	<0.0001***

*P <0.05, **P <0.01, ***P <0.0001

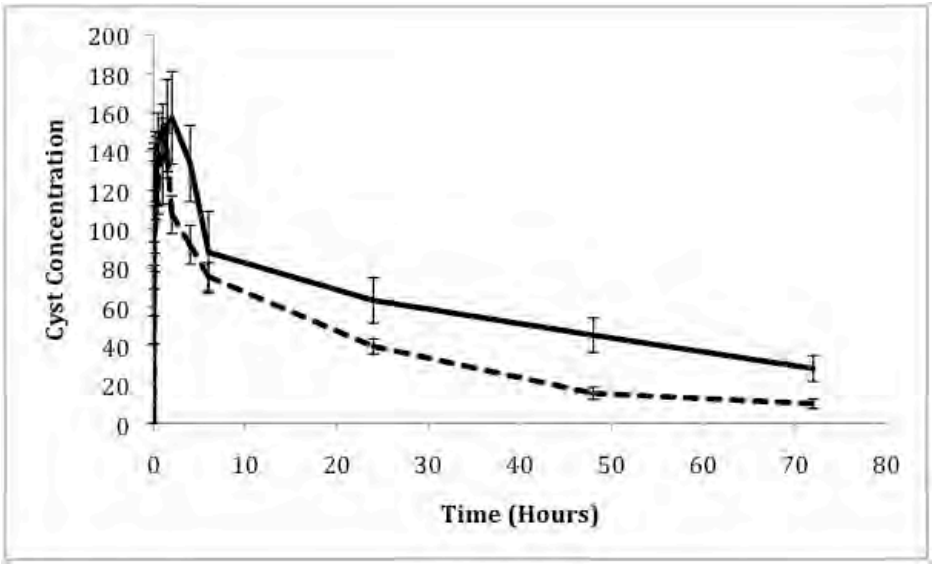


Figure 22. Effect of vegetation presence (California bulrush in buffer configuration-Dashed line) or absence (Solid line) on *Giardia* cyst detection in 50 mL water sub-samples from mesocosm tanks. *Giardia* counts were significantly lower when California bulrush was present.

Field Sampling at Tembladero Slough Constructed Wetland

Cryptosporidium and *Giardia* were both detected in water from the Tembladero Slough Constructed Wetland. A total of 92 samples were analyzed across 6 sample sites, including source water (Table 57). Each of the sampling sites was positive for at least one of the protozoa. *Cryptosporidium* oocysts were more prevalent (40/92, 44%) than *Giardia* cysts (11/92, 12%) among all samples. Seven samples contained both protozoa. As expected, protozoal prevalence for samples taken from the source water of Tembladero Slough was high, with 50% of samples positive for either *Cryptosporidium* or *Giardia*. For both *C. parvum* and *G. lamblia,* the first sampling site within the channel of the wetland, located at the upper inflow, had a high prevalence of protozoa (47% and 17%, respectively) within the constructed wetland, and the prevalence decreased over the next two sites within the channel. For *Cryptosporidium*, the prevalence increased in the last site within the flood plain section of the wetland, although this may be due to cross-contamination with Tembladero Slough water during flooding events. The mean concentrations of these protozoa was fairly low, with ranges of 0-148.3 oocysts/10 L and 0-33 cysts/10 L enumerated from any given sample.

Table 56. *Giardia* cyst detection in relation to flow rate, sample time and vegetation presence, type and configuration in mesocosm tanks (multivariate estimates)

Condition	β estimate	Odds Ratio	P-value
Giardia			
Individual Factors			
Vegetation absent	Reference	Reference	
Vegetation-California bulrush, buffer	-.74	0.48	<0.0001***
Vegetation-California bulrush, channel	-.67	0.51	<0.0001***
Vegetation-slough sedge, buffer	.011	0.99	0.95
Flow rate-slow	Reference	Reference	
Flow rate-fast	.27	1.31	0.03*
Interaction			
Flow rate slow-vegetation-time	Reference	Reference	
Flow rate fast-California bulrush, buffer-time	-0.029	0.97	<0.0001***
Flow rate fast-California bulrush, channel-time	0.0096	0.99	0.089
Flow rate fast-slough sedge, buffer-time	0.0087	0.99	0.12

*P<0.05, **P<0.01, ***P<0.0001

Table 57. Site-specific detection of *C. parvum* and *G. lamblia* in water from Tembladero Slough Constructed Wetland and source water.

Site		*Cryptosporidium*			*Giardia*		
	N	Prevalence (positive/total tested)	Mean Concentration (10 L^{-1})	Range (10 L^{-1})	Prevalence (positive/total tested)	Mean Concentration (10 L^{-1})	Range (10 L^{-1})
1 Upper Inflow	23	48% (11/23)	2.4	0-12	17% (4/23)	1.2	0-14
1.5 Lower Inflow	5	20% (1/5)	0.8	0-4.2	0% (0/5)	0	0
2 Mid Upper	18	22% (4/18)	2.6	0-28	11% (2/18)	0.1	0-1
3 Upper Outflow	22	50% (11/22)	3.6	0-35	4.5% (1/22)	0.2	0-4
4 Lower Outflow	15	60% (9/15)	22.8	0-148.3	13% (2/15)	0.6	0-6.9
5 Tembladero Slough Source Water	4	50% (2/4)	2.7	0-6	50% (2/4)	1.2	0-33
Total	92	44% (40/92)			12% (11/92)		

To determine the effectiveness of the Tembladero Slough Constructed Wetland on pathogen reduction, fecal indicator bacterial counts were also examined. All 78 samples were analyzed for the detection of total coliforms and *E. coli* using Colilert-18 tests (Table 58), and 48 samples were analyzed for the detection of total coliforms, fecal coliforms, and *Enterococcus* using membrane filtration (Table 59). Amongst all sample sites, the source waters of Tembladero

Slough had the highest total coliform and *E. coli* MPN. The first sample site within the wetland had the highest MPN or CFU for all pathogens tested, while all subsequent sample locations within the wetland channel exhibited lower counts. Similar to the trend observed with protozoal detection, the last sample site within the wetland floodplain consistently had higher MPN or CFU counts for all pathogens, except fecal coliforms.

Table 58. Detection of Total Coliforms and *E. coli* in water from Tembladero Slough Constructed Wetland and source water using Colilert-18 testing.

Site		Total coliforms		*E. coli*	
	N	Mean MPN ($100\ mL^{-1}$)	Range ($100\ mL^{-1}$)	Mean MPN ($100\ mL^{-1}$)	Range ($100\ mL^{-1}$)
1 Upper Inflow	20	176179	4870- 2420000	3038	100-22600
1.5 Lower Inflow	4	53365	25300-123300	NT	NT
2 Mid Upper	16	83677	5460- 517200	1298	100- 6300
3 Upper Outflow	19	50288	2790-224700	2059	4.3-16070
4 Lower Outflow	13	242574	1320- 2420000	4970	100-29500
5 Tembladero Slough Source Water	4	716508	92080- 2420000	11883	200-46400
Total	78				

NT = Not tested.

Table 59. Detection of total coliforms, fecal coliforms, and Enterococci in Tembladero Slough Constructed Wetland and source water using membrane filtration.

Site		Total coliforms		Fecal coliforms		Enterococci	
	N	Mean CFU ($100\ mL^{-1}$)	Range ($100\ mL^{-1}$)	Mean CFU ($100\ mL^{-1}$)	Range ($100\ mL^{-1}$)	Mean CFU ($100\ mL^{-1}$)	Range ($100\ mL^{-1}$)
1 Upper Inflow	12	1913	70-7200	1796	30-8300	576	75-3200
1.5 Lower Inflow	4	520	30-1600	283	200-500	NT	NT
2 Mid Upper	11	818	50-2400	580	15-1700	239	9-1100
3 Upper Outflow	12	901	100-2800	996	190-2600	450	35-2200
4 Lower Outflow	8	2214	40-16000	338	4-1540	1077	165-6000
5 Tembladero Slough Source Water	4	NT	NT	NT	NT	NT	NT
Total	48						

NT = Not tested.

Wilcoxon scores were calculated to evaluate associations between *Cryptosporidium* and *Giardia* counts, seasonality and rainfall events. *Cryptosporidium* counts were weakly associated with season (U=1520.5, P=0.0002) and rainfall within three days of sampling (U=1320.0,

P=0.0070, Table 60) while *Giardia* counts were not significantly associated with season or rainfall.

Numerous water quality variables were assessed at each sample date and site, as shown in Table 61. Using Spearman rank correlations, *Cryptosporidium* counts were weakly associated with salinity (r = -0.35985, P<0.01), total dissolved solids (r = -0.35274, P<0.01), percent dissolved oxygen (r = 0.3412, P<0.01), and wetland depth (r = 0.56196, P<0.01) whereas *Giardia* counts were correlated only with water depth (r = -0.46003, P<0.05). Higher water salinity was associated with lower *Cryptosporidium* counts, which contrasts with evidence gathered in the mesocosm tank experiments. A California Sea Grant that was funded based on preliminary data from the current study will help clarify effects of salinity and sediment on pathogen transport in tank, constructed, and tidal wetlands in central California. In the current project, *Cryptosporidium* and *Giardia* counts were also examined for correlations with total coliforms and *E. coli*, however, no statistical associations were found between indicator and pathogen concentrations.

Table 60. Associations between seasonality, rainfall events and *Cryptosporidium* or *Giardia* counts in the Tembladero Slough Constructed Wetland (Wilcoxon Score U)

Variable	*Cryptosporidium* (U)	*Giardia* (U)
Season	1521 **	1858
Rain event on sampling date	1274 *	1043.5
Rain event on sampling date or within 3 days prior	1320 **	1664.5

*P<0.05, **P<0.01, ***P<0.001.

Table 61. Associations between water quality parameters and *Cryptosporidium* or *Giardia* counts in the Tembladero Slough Constructed Wetland (Spearman's rho correlation)

Parameter	*Cryptosporidium* (rho)	*Giardia* (rho)
Temperature (*C)	-0.0313	0.1213
Turbidity (NTU)	0.0830	0.2022
Salinity (ppt)	-0.3599 **	-0.0936
Conductivity (um)	-0.2874 *	-0.1059
Total Dissolved Solids	-0.3527 **	-0.0884
Dissolved Oxygen (mg/L)	0.3122 **	0.0487
Dissolved Oxygen (%)	0.3412 **	0.0732
pH	-0.0605	0.0077
Wetland depth (cm)	-0.5620**	-0.4600 *

*P<0.05, **P<0.01, ***P<0.001.

Discussion-Effectiveness of Wetlands to Reduce Fecal Pathogen Loads

Based on studies of pathogen transport in mesocosm tanks and a constructed wetland, a number of hydrologic and ecological parameters were shown to reduce *Cryptosporidium* and *Giardia* counts in polluted water. The mesocosm tanks were used to model specific wetland

characteristics to provide a complementary approach to the wetland field studies, which were observational studies that relied on pre-existing protozoal contamination in source water. Under non-vegetated conditions in the mesocosm tanks, increased salinity was associated with increased *Cryptosporidium* detection in water, but this was not observed for *Giardia*; this contrast is a good reminder that not all pathogens share the same environmental transport characteristics, given the variability in microbe size and surface properties.

Similarly, when the effects of vegetation type and configuration were assessed, *Cryptosporidium* counts were reduced under all vegetation conditions (bulrush channel, bulrush buffer and slough sedge buffer), compared to non-vegetated tanks, while *Giardia* counts were reduced only in tanks containing California bulrush. It appears that different vegetation types vary in their efficiacy for trapping pathogens and improving water quality during wetland transit, possibly due to differences in vegetation height, structure, and surface properties. The formation of biofilms on vegetation surfaces may enhance pathogen removal if the pathogens adhere to this film. Lastly, some vegetation types require shorter time periods to grow and become established in coastal wetlands, possibly improving efficacy of pathogen removal; for example bulrush is sometimes called a 'workhorse' plant because it grows rapidly and can reach to significant heights, when compared to some native sedge grasses.

For *Giardia*, but not *Cryptosporidium*, increased wetland flow rates were associated with increased cyst detection in water samples, perhaps due to a reduction in settling time for cysts exposed to faster flow rates. However, faster flow rates were also associated with decreased cyst detection when bulrush vegetation was added in a buffer configuration; suggesting that water turbulence or other, as yet unrecognized factors induced by the presence of plant material arranged in a buffer configuration across the direction of flow substantially improves protozoal pathogen removal from polluted water. Collectively our study findings strongly support the use of vegetation buffers as a Beneficial Management Practice (BMP) to improve water quality in coastal ecosystems. As wetlands are reconstructed to facilitate pollution control, careful selection of specific wetland characteristics will be important to maximize the efficacy of pathogen removal. Incorporating vegetation into coastal wetlands near potential sources of fecal contamination could provide significant reduction of fecal pathogen loads in downstream waters.

During Tembladero Slough field wetland experiments, the location of water sampling within the wetland greatly affected both protozoal and indicator bacterial counts. Seasonality and rainfall also effected the amount of *Cryptosporidium* detected, but not *Giardia.* Other water quality parameters like salinity were also correlated with *Cryptosporidium* counts, but not *Giardia*.

Collectively our findings from the mesocosm and field wetland studies suggest that the efficacy of removal of pathogenic protozoa in polluted water may be affected by numerous hydrological and physical characteristics of wetland systems, including salinity, presence or absence of vegetation, and wetland length. Adhesion and settling dynamics of *Cryptosporidium* are affected by changes in salinity (Bradford and Schijven, 2002; Carey et al., 2004; this study). Salinity-mediated effects on *Cryptosporidium* oocysts may interact with other wetland characteristics accounting for the contrasting results observed in this study for the effect of salinity on *Cryptosporidium* counts in mesocosm tanks, when compared to the field wetland. Additional studies are underway to further clarify the effect of salinity, by evaluating changes in vertical settling velocity under varying salinity conditions, and examining associations between salinity, suspended sediment pathogen reduction in the mesocosm tanks.

While the mesocosm tank experiments examined *a priori* defined variables, the wetland field study evaluated the effect on parasite reduction under natural conditions, and including a larger range of variables. Progressive reduction of both protozoal and indicator bacterial counts was commonly observed water samples collected at increasing distances from the contaminated source water in the wetland channel, suggesting that efficient pathogen reduction was achieved for source water flowing through this portion of the wetland. In contrast, samples from the flood plain area of the Constructed Wetland exhibited higher and inconsistent pathogen loads, likely due to periodic flooding of this lower portion of the wetland by polluted source water. . Both seasonal and water quality parameters tended to be correlated more strongly with *Cryptosporidium* counts than with *Giardia*. However, this effect may be spurious, because the mean counts for both protozoa were extremely low.

Previous studies have examined the potential for wetlands to improve water quality, especially with regard to secondary and tertiary treatment of wastewater and stormwater (Quinonez-Diaz et al., 2001; Sundaravadivel and Vigneswaran, 2001; Vymazal, 2005). These studies emphasized a broad range of pathogens; more in-depth studies on the effects of coastal wetland characteristics on protozoal reduction are needed. Additionally, vegetation located along the side of a water channel may reduce pathogen loads for run-off (Atwill et al., 2006; Winkworth et al., 2008) entering water channels, thus augmenting the efficacy of wetlands for pathogen removal. Findings from this project suggest specific parameters to consider when designing or utilizing wetlands to mitigate fecal pathogen pollution. Most relevant for wetland reconstruction and rehabilitation managers may be the effect of vegetation on protozoal pathogen removal. Both California bulrush and slough sedge are widely used for wetland reconstruction, embankment stabilization, and erosion control (Stevens and Hoag, 2003; Stevens and Hoag, 2006). The clear benefit that these plants exhibit for *Cryptosporidium* and *Giardia* reduction supports their continued use. Comparison of California bulrush configuration patterns in the mesocosm tanks demonstrated no difference in the efficacy of protozoal removal between buffer and channel configurations, providing engineers and water quality managers with greater flexibility during wetland design efforts.

5. Conclusions

This study, funded by the Proposition 50 Coastal Management Program, has contributed important new insights regarding the efficacy of traditional fecal indicator assays to predict the risk of human exposure to enteric pathogens in surface water, storm runoff, sewage effluent and ocean water in coastal California. It has also clarified the utility of newer tests such as *Bacteroidales* detection as an alternative fecal indicator assay that provides information on the relative fecal contribution by different hosts within a given watershed. The efficacy of coastal wetlands for removal of fecal pollution was assessed in both the laboratory and field setting and specific wetland characteristics that improved pathogen removal were revealed. Comparison of the efficacy of FIB and specific pathogen detection from ocean water and location- and time-matched mussels revealed new perspectives on the utility and application of both samples for monitoring coastal water quality, and recognizing potential hazards associated with water contact recreation and shellfish consumption, especially following storm events or in watersheds with significant fecal impairment. Additionally, characterizing the potential source(s) and epidemiology of specific fecal pathogens infecting coastal terrestrial and marine animals has provided important new information of the role of livestock, pets, wildlife and introduced species as potential sources of contamination of watersheds, humans and sensitive marine species by zoonotic enteric pathogens.

Collectively, this data provides significant insights for water resource managers, biologists and public health managers to optimally focus mitigation efforts, reduce pathogen loads and improve water quality along the California coast. In addition, this study provided preliminary data needed to secure California Sea Grant funding to continue this important research, aimed at optimizing water quality in multi-use ecosystems. Finally, six PhD, MPVM and MS students at UC Davis and CSUMB conducted graduate research utilizing cutting edge surveillance techniques to assess fecal pathogen pollution in freshwater and marine aquatic systems as part of the Proposition 50 Coastal Management Program-funded research. These future scientists and wetland managers will now be better able to protect water quality in California and beyond.

The main findings from this study are summarized as follows:

1. Counts of standard indicator bacteria, including coliforms and enterococci were found to correlate with detection of some but not all bacterial and protozoal enteric pathogens, suggesting that monitoring for indicator bacteria alone may not accurately and consistently predict the risk of recreational, occupational, or food-borne contact with fecal pathogens in surface waters.
2. *Bacteroidales* and standard FIB bacteria were both detected in 99% of surface water samples, demonstrating the high sensitivity of this alternative indicator assay. The utility of *Bacteroidales* for microbial source tracking was demonstrated, and is supported by specificity testing in prior studies that observed excellent discrimination between human and animal fecal sources.
3. Bivalve shellfish, including mussels and clams, have been used in prior studies as bioindicators of water quality, and are also important prey items for threatened southern sea otters. In the current study we found no significant differences in the sensitivity of pathogen detection for mussels, when compared to time- and location-matched seawater, suggesting that bivalves may be most useful as bioindicators during periods of enhanced risk of fecal

contamination, such as during or after storm events.

4. Domestic and wild animals, as well as humans, contribute to fecal loading in multi-use watersheds. This study detected many of the same fecal pathogens and risk factors in sympatric terrestrial animals, sea otters and humans, highlighting the complexity and interconnectedness of coastal ecosystems.

5. Wetland mesocosm tank and field studies illustrated the potential benefits of wetland restoration for improving water quality, as well as evaluating the potential effects that environmental climate change factors may have on protozoal transport in wetland systems. The importance of wetland vegetation in fecal pathogen removal of from polluted surface waters indicates that conserving and restoring coastal wetlands should help improve overall ecosystem health and reduce health risks for downstream recreational users.

6. Utilizing a combination of indicator and pathogen assays can provide useful information regarding the presence, relative abundance, and contributing sources of fecal contamination in environmental samples. This approach may be important for future monitoring and mitigation efforts in multi-use watersheds.

In conclusion, this multi-year research effort strategically utilized a suite of laboratory and field approaches to identify factors that optimize water quality monitoring efforts and mitigation of fecal pathogen pollution along the central California coast. The study findings will help inform resource managers and stakeholder groups when seeking a balance between the full range of beneficial uses of coastal watersheds and their associated health risks. Although this work was completed in central California, our findings are relevant to other coastal watersheds in California, and to water policy stakeholders and scientists worldwide. Publications and outreach materials related to this project will be posted on the website www.pathogenpollution.org.

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8. APPENDIX A

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS:

2009 Miller, M.A., B.A. Byrne, S.S. Jang, E.M. Dodd, E. Dorfmeier, M.D. Harris, J. Ames, D. Paradies, K. Worcester, D.A. Jessup, and W.A. Miller. Enteric bacterial pathogen detection in southern sea otters (*Enhydra lutris nereis*) is associated with coastal urbanization and freshwater runoff. *Veterinary Research* 41:1.

2010 Schriewer, A., W.A. Miller, B.A. Byrne, M.A. Miller, P.A. Conrad, D. Hardin, H. Yang, S.C. Oates, N. Chouicha, A. Melli, D. Jessup, and S. Wuertz. *Bacteroidales* as a predictor of pathogens in surface waters of the central California coast. *Applied and Environmental Microbiology* 76:5802-5814.

2011 Oates, S.C., M.A. Miller, B.A. Byrne, N. Chouicha, D. Hardin, D. Jessup, C. Dominik, A. Roug, A. Schreiwer, S. S. Jang, and W.A. Miller. Epidemiology and potential land-sea transfer of enteric bacterial pathogens from terrestrial to marine species in the Monterey Bay region of California. *Veterinary Research*, Submitted.

PRESENTATIONS:

2007 Miller, W.A., D. Hardin, B. Byrne, P. Conrad, M. Miller, S. Wuertz, S. Oates, C. Dominik, A. Melli, A. Packham, E. Owens, N. Yang, and D. Jessup. Pathogen pollution project: sea otter habitat quality update. Sea Otter Research Conference, Santa Cruz, California.

2008 Hogan, J., W.A. Miller, and P.A. Conrad. *Cryptosporidium* and *Giardia* in wetland systems. Northern California Parasitologists Conference, Tomales, California.

2008 Chouicha, N., W.A. Miller, M. Miller, C. Hastey, A. Melli, S. Jang, and B. Byrne. Characterization of *Clostridium perfringens* toxinotypes isolated from sea otters and mussels from the central California coast. Anaerobe Conference, Long Beach, California.

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