FINAL REPORT

SOURCE TRACKING PROTOCOL DEVELOPMENT PROJECT

Submitted to the City of Santa Barbara, Creeks Division

Prepared by:
Patricia A. Holden[1,2], Bram Sercu[1,2], Laurie Van De Werfhorst[1,2], Jessie Golman[1,2], Eric Dubinsky [3], and Gary Andersen[3]

[1] ERI, University of California, Santa Barbara, CA

[2] Bren School of Environmental Science & Management, University of California, Santa Barbara, CA

[2] Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

June 1, 2011

Contents

Title Page 1

Acknowledgements 6

I. *Executive Summary* 7
- **1.1 Background, Goal, and Objectives** 7
- **1.2 Project Approach** 7
- **1.3 Summary of Results** 8
- **1.4 Conclusions and Future Recommendations** 10

II. Introduction 12
- **2.1 Background to this Section and Relation to Other Sections** 12
- **2.2 Project Scope and History** 12
- **2.3 Problem Statement: Beach Water Quality** 12
- **2.4 Need for a Research Project and Focus on Storm Drains** 13
- **2.5 Project Overview** 14
- **2.6 Project Description** 15
- **2.7 Study Locations** 16
- **2.8 References** 17

III. Literature Review 19
- **3.1 Background to this Section and Relation to Other Sections** 19
- **3.2 Beach Microbiological Water Quality** 19
- **3.3 Tracking FIB Sources in Coastal Waters and Watersheds** 19
- **3.4 Sewage as an FIB Source in Coastal Zones** 21
- **3.5 Potential Sources of Human Fecal Markers and Sewage in Surface Waters** 21
- **3.6 Sanitary Surveys and Microbial Source Tracking** 22
- **3.7 The Gap Filled by this Research** 24
- **3.8 References** 24

IV METHODS 30
- **4.1 Fecal indicator bacteria** 30
- **4.2 Nutrients and anionic surfactants by colorimetry** 31
  - *4.2.1 Introduction* 31
  - *4.2.2 Selection of equipment* 31
- **4.3 Nutrients by flow injection analysis** 32
- **4.4 Methanobrevibacter smithii PCR for the nifH gene (Mnif-PCR)** 33
- **4.5 Human-specific Bacteroidales qPCR (HBM-qPCR)** 34
- **4.6 Enterococcus spp. qPCR (ENT-qPCR)** 34
- **4.7 ELISA for caffeine and cotinine** 35
- **4.8 Automated sampling and flow measurement** 36
  - *4.8.1 Automated sampling* 36
  - *4.8.2 Continuous flow measurement* 37
- **4.9 Automated rhodamine WT monitoring** 38
  - *4.9.1 Method summary* 38
  - *4.9.2 Tips for using the 600 OMS V2 sonde* 38

V. Specificity of human-specific assays 46

5.1 Background **46**
5.2 Materials and Methods **46**
5.2.1 Fecal Source Sampling 46
5.2.2 DNA extraction of new fecal samples 47
5.2.3 Quantitative-PCR (qPCR) and conventional PCR analysis of all samples 47
5.3 Results **48**
5.3.1 Non-human sources 48
5.3.2 Human sources 48
5.4 Discussion **48**
5.5 References **50**

VI. Fecal Source Identification in the Mission Creek Area using PhyloChip Analysis **60**
6.1 Summary **60**
6.2 Introduction **61**
6.3 Methods **62**
6.3.1 Sample Description 62
6.3.2 Polymerase Chain Reaction 62
6.3.3 PhyloChip Assay Design 63
6.3.4 Preparation of Samples for PhyloChip Assays 63
6.3.5 PhyloChip Assay Analysis 64
6.3.6 Determination of source identifier taxa 64
6.4 Results and Discussion **64**
6.4.1 Fecal microbial communities and identifier taxa 64
6.4.2 Detection of fecal sources in water samples 68
6.5 Conclusion **73**
6.6 References **74**

VII. ALTERNATIVE INDICATORS FOR IDENTIFYING SEWAGE POLLUTION **75**
7.1 Summary **75**
7.2 Methods **75**
7.2.1 Sampling locations and experimental design 75
7.2.2 Preliminary testing of fluorometry for optical brighteners 75
7.2.3 Preliminary testing of colorimetry for nutrients and anionic surfactants 76
7.2.4 Nutrients by flow injection analysis 76
7.3 Results **76**
7.3.1 Preliminary testing of alternative sewage indicators 76
7.3.3 Spatial concentration patterns 78
7.3.5 Relationships between sewage-specific markers and alternative indicators 81
7.4 Discussion and conclusions **83**
7.5 References **84**

VIII. FLOW MEASUREMENT AND AUTOMATED STORM DRAIN SAMPLING **117**
8.1 Summary **117**
8.2 Experiments **117**
8.2.1 Location L15: Salsipuedes @ Cota 118
8.2.2 Location A5 – Hope drain diversion 119
8.3 Results **120**
8.3.1 Location L15 120
8.3.2 Location A5 121

IX. RHODAMINE WT DYE STUDIES **142**
9.1 Abstract **142**

9.2 Introduction **142**
9.3 Materials and Methods **143**
9.3.1 Study sites and infrastructure details 143
9.3.2 RWT dosing 144
9.3.3 RWT monitoring 144
9.3.4 Flow rate calculations 144
9.3.5 Water sampling and microbiological analyses 144
9.4 Results and Discussion **145**
9.4.1 Field study 1: leaking sanitary sewer due to surcharge conditions 145
9.4.2 Field study 2: storm drains below sanitary sewers 146
9.4.3 Field study 3: storm drains above sanitary sewers 147
9.4.4 Importance of exfiltration to storm drain water quality 147
9.4.5 RWT dye studies for localizing sewage exfiltration pollution 148
9.5 RWT testing at Chino/Micheltorena **148**
9.5.1 Introduction 148
9.5.2 Experiments and results 149
9.6 RWT testing at Nopal/Cota **149**
9.6.1 Introduction 149
9.6.2 Experiments and results 149
9.7 References **150**

X. GIS Analyses **161**
10.1 Background & Objectives **161**
10.2 Data Collection & Compilation **161**
10.3 Method & Results **161**

XI. Microbial Source Tracking Protocol **169**
11.1 Background to this Section and Relation to Other Sections **169**
11.2 Overall approach to "drain tracking" **169**
11.3 Recommended Steps in Source Tracking Human Waste in Urban Coastal Communities **170**
11.4 References **174**

APPENDIX I: Standard Operating Procedures (SOPs) **175**
SOP# H02 ..... **175**
SOP# H03 ..... **182**
SOP# H04 ..... **185**
SOP# H05 ..... **188**
SOP# H07 ..... **192**
SOP# H08 ..... **195**
SOP# H09 ..... **199**
SOP# H010 ..... **203**
SOP# H011 ..... **207**
SOP# H012 ..... **212**
SOP# H013 ..... **217**

APPENDIX II. UCSB Progress Reports to the City of Santa Barbara **222**
UCSB Mission Project –Progress Report October 14 – December 31, 2008 ..... **222**
UCSB Mission Project –Progress Report January 1 – May 31, 2009 ..... **222**
UCSB Mission Project –Progress Report June 1 – June 30, 2009 ..... **224**
UCSB Mission Project –Progress Report July 1 – July 31, 2009 ..... **224**
UCSB Mission Project –Progress Report August 1 – August 31, 2009 ..... **224**

UCSB Mission Project –Progress Report September 1 – September 30, 2009 225
UCSB Mission Project –Progress Report October 1 – October 31, 2009 226
UCSB Mission Project –Progress Report November 1 – November 30, 2009 227
UCSB Mission Project –Progress Report December 1 – December 31, 2009 228
UCSB Mission Project –Progress Report February 1 – February 28, 2010 229
UCSB Mission Project –Progress Report March 1 – March 31, 2010 229
UCSB Mission Project –Progress Report April 1 – April 30, 2010 230
UCSB Mission Project –Progress Report May 1 – May 31, 2010 231
UCSB Mission Project –Progress Report June 1 – June 30, 2010 232
UCSB Mission Project –Progress Report July 1 – July 31, 2010 233
UCSB Mission Project –Progress Report August 1 – August 31, 2010 233
UCSB Mission Project –Progress Report September 1 – September 30, 2010 234
UCSB Mission Project –Progress Report October 1 – December 31, 2010 235

Acknowledgements

This research was supported by State of California Proposition 50 funds to the City of Santa Barbara and subcontracted to the University of California, Santa Barbara (UCSB), administered by the Clean Beaches Initiative (CBI #516) within the State Water Resources Control Board (SWRCB; Grant Agreement 07-583-550). Staff members of SWQCB (Laura Peters, Tom Peltier, and Joan Weber) are acknowledged for their assistance to this project. The Earth Research Institute (ERI) at UCSB administered the project funding for UCSB researchers, and assisted with supplies procurements. Kilty Inafuku assisted with the research during a summer 2010 internship within the UC LEADS program. Ning Jiang and Ngoc Hoang assisted in the field and laboratory at UCSB. Professor Jim Frew of the Bren School, UCSB consulted regarding the development of the GIS database. City of Santa Barbara Creeks Division staff assisted with this project including: Dr. Jill Murray, Cameron Benson, Jim Rumbley, Vidal Gonzalez. Kenneth Ficklin from the Streets Division, City of Santa Barbara also assisted.

I. *Executive Summary*

1.1 Background, Goal, and Objectives

Santa Barbara, like many urbanized coastal communities in California, frequently determines that fecal indicator bacteria (FIB) concentrations in surf zone water exceed State of California AB411 criteria. The City of Santa Barbara Creeks Division, with funds from the State of California Water Resources Control Board, contracted with the University of California, Santa Barbara, to perform research in support of determining origins of FIB associated with human fecal pollution (sewage) in storm drains that discharge to coastal creeks flowing to Santa Barbara beaches. The overall goals are to determine origins of sewage contamination in storm drains during dry weather, and to compile a protocol for other communities to use for similar source tracking purposes, based on the results of this research. The specific tasks included:

- Select and purchase field and lab equipment.
- Develop detailed sampling plans for field investigation.
- Conduct field sampling over two (2) AB411 Seasons.
- Perform laboratory analysis of field samples
- Perform data analysis
- Develop GIS tools for visualizing and analyzing source tracking and sanitary survey investigation.
- Develop a "Source Tracking Protocol" for coastal managers.

1.2 Project Approach

Research was planned to occur over two AB411 field sampling seasons, and detailed field research planning was conducted collaboratively by the City and UCSB. During the course of the research, the City and UCSB had frequent meetings in person and over the telephone to coordinate and select field research. Equipment was researched, selected, and purchased in support of this project. Research was performed through field work, field sampling of water and fecal samples, and laboratory analysis of field samples. Methods for alternative sewage indicators were researched using the published literatures, and selected methods were then recruited and tested for their usefulness in assessing sewage amounts in storm drains. Approaches for studying exfiltration in the field were researched, and a novel approach was developed that involved dispensing rhodamine WT dye into sanitary sewers and detecting a fluorescence signal continuously, in real time, in storm drain manholes using a field-deployable, battery-driven submersible optical probe with data logger. The specificity of two similar, widely-used approaches for quantifying sewage contamination in water samples through quantitative polymerase chain reaction (qPCR) analysis of DNA markers in human waste was tested. This involved collecting fecal samples from various non-target (raccoon, gull, rat, dog, cat) hosts and target (human, septage, sewage) materials, then comparing the specificity of two popular qPCR-based approaches

for quantifying human markers. Equipment items for flow measurement in storm drains and automated continuous sampling were researched and selected, then deployed at two locations (Salsipuedes and Cota, and the Hope Diversion at La Cumbre Mall) for 72 hour campaigns to better understand temporal variation in human waste contamination and in storm drain flow. Samples from these campaigns were analyzed for FIB and markers of human waste, plus nutrients and chemical markers of human waste. A high-density microarray of DNA probes, the PhyloChip was used to assess differences in fecal source bacterial communities for the non-target (as above) and human-associated (as above) fecal samples. The fecal source bacterial community profiles by PhyloChip were then compared to water sample bacterial communities for samples collected in various sites of interest based on historical FIB and/or human DNA-marker contamination. This sub-study was intended to push the envelope on assessing the use of community-based fecal source tracking. A GIS database was assembled with microbiological data—both historical from recent studies conducted by UCSB for the City and also from this study—displayed along with storm drains, sanitary sewers and streets with the latter three types of data already contained in the City GIS database as separate information sets (layers). The GIS assemblage of spatial, physical infrastructure, metadata of physical infrastructure (i.e. sanitary sewer age and material of construction, and depths and diameters of sanitary sewers and storm drains) and scientific information (microbiological and chemical analysis results) was used to generate several products for visualizing information and for planning remediation and research. Taken together, the results of this project were co-evaluated and proposed for a stepwise decision tree approach to source tracking human waste in storm drains, generally.

1.3 Summary of Results

Of the various potential chemical markers tested for use in tracing sewage in storm drains, three appeared useful: ammonium, phosphate, and anionic surfactants (methylene blue active substances, or MBAS). These markers appeared to be most useful when sewage contamination was greater than 10%; at concentrations less than approximately 10% (by volume of water samples), other background sources of these constituents confounded their relationship to sewage as a contamination source. Other chemical markers previously employed and used here (caffeine and cotinine) were also found to be generally useful and more specific to human waste than ammonium, phosphate or surfactants. However, assays for both caffeine and cotinine are comparatively expensive and require laboratory expertise that is greater than assays for ammonium, phosphate and surfactants. Because ammonium and phosphate can be measured in the field easily, these approaches have additional advantages and were therefore folded into the source tracking protocol development.

The approach for tracing sewage exfiltration from sanitary sewers into storm drains was successfully demonstrated in two distinct scenarios: 1) an acute spill at State Street and Plaza (across from La Cumbre Mall) where a sewage lift station was over-pressurizing a sanitary sewer and forcing sewage into an adjacent storm drain, 2) a region (Nopal St. area) of the City where older sanitary sewers were confirmed as leaking chronically, and

apparently slowly, through soil separating the sanitary sewers from storm drains. In the latter, multiple locations were implicated as sources of exfiltration from sanitary sewers to storm drains, but the exact locations were not pinpointed within the area surveyed. However, considering the GIS database which was used to display where sanitary sewers were shallower than, and also crossed, storm drains, the result showing exfiltration from multiple locations is logical. In another location (Haley) that previously had been shown to have human waste contamination in storm drains, the tracer study did not reveal exfiltration from sanitary sewers into the storm drains, and thus the source of contamination remains unresolved. In light of the GIS-based display of sanitary sewer versus storm drain orientations (depths, and parallel or crossing), this result from the exfiltration study is logical: sanitary sewers and storm drains are quite spatially distant, at least vertically, in the region (Chapala from Canon Perdido to Haley Street) studied. Thus, this latter area served as a good negative control for dye tracing exfiltration from sanitary sewers to storm drains.

Of the two human-specific marker qPCR-based approaches for quantifying sewage contamination in field water samples, one approach—the approach already adopted and used previously for City-funded research by UCSB—was comparatively much more specific to human waste. This approach (qPCR HF183, or HBM for human *Bacteroides* marker, in this report) was used to analyze field samples for other studies (exfiltration tracer study samples, and temporal variation samples) in this research.

As above, the use of GIS for displaying and scrutinizing infrastructure data was beneficial in interpreting exfiltration results; GIS was also very useful for gaining overall perspectives of the spatial context of microbiological results. The methodology for building the GIS database was described, and examples of results (i.e. types of data displays) useful to planning research and remediation are demonstrated.

The PhyloChip, used previously in other studies conducted by UCSB including prior research with the City in the Mission Creek area, was assessed in this study for its utility in discerning fecal sources from one another and from background microbial communities in field water samples. Based on all valid probe hybridization events, gull and raccoon microbial communities appeared distinct from all other microbial communities. Water sample microbial communities by PhyloChip were indicative of sewage contamination in the Nopal/Canon Perdido area; cat and dog contamination was also present, which was concluded to arise with sewage due to how domestic pet feces may be disposed. PhyloChip analyses also indicated the presence of raccoon and possibly gull feces at Haley and Chapala. Other samples, where source-specific communities were not observed, may be contaminated by fecal indicator bacteria that are not related to fecal sources, i.e non-target bacteria that arise with FIB analysis.

Standard operating procedures (SOPs) were developed for all procedures and assays found to be most useful in tracking human waste in storm drains. A protocol for implementing these procedures was designed for others to use in similar studies.

1.4 Conclusions and Future Recommendations

The conclusions of this study regard how communities, based on our experiences in this research, should best approach source tracking human waste in storm drains. Taking into account the costs associated with implementing advanced DNA-based assays, we conclude that a progressive implementation of simple-to-complex approaches is most prudent. Specifically, we recommend that communities first compile all FIB monitoring data into a GIS-based format and display the data alongside relevant (sanitary sewer, storm drain, street, creek) system data. Metadata (pipe depths, diameters and materials of construction) should be included in the database, and all data should be verified for consistency (units of measure, completeness, and accuracy). Spatially displaying microbiological monitoring data with potential source information will aid in prioritizing more intensive spatial sampling in creeks and drains. For putative sources of human waste (i.e. sewage, septage, and/ or human feces), characteristics should be measured or acquired from the local wastewater treatment plant (WWTP), including nutrient (ammonia and phosphate), surfactant, and FIB concentrations. If samples are acquired specifically for characterizing sewage as a potential source of storm drain contamination, several replicate samples taken over different days should be filtered and filters archived frozen for DNA extraction and analysis; filtrate can be analyzed for the dissolved constituents and subsamples of unfiltered material are analyzed for FIB content. Standard operating procedures (SOPs) for all assays should be adopted and adhered to. Implementation of new SOPs, for example if a new DNA-based method is recruited, should be preceded by careful specificity testing to ensure that false positives are not generated when testing environmental waters for the presence of fecal sources. Source concentrations of caffeine and cotinine, or of any other potential waste assay, must also be acquired for multiple samples so that sources are characterized in advance of, or simultaneously with, source tracking field samples.

Based on GIS representation of FIB monitoring data, "hot spots" where FIB are frequently in high concentration in creeks or drains discharging into creeks should be identified. Field sampling should be planned at these hotspots, performed at multiple times for grab samples, and initially assessing nutrient and other water characteristics in the field, including field probe-based measures such as dissolved oxygen, temperature and conductivity. Where nutrients by colorimetry, and potentially probe-based measures, indicate concentrations that could occur in 10% strength or greater sewage, then follow-up samples for DNA-based analysis and/ or other chemical marker (caffeine or cotinine) analyses should be gathered. Sampling and analysis should be planned for spatial regions, at multiple storm drain manholes and drain discharges into creeks, that appear problematic based on high FIB concentrations. If waste marker analyses and the GIS-based display of infrastructure characteristics, including the vertical and lateral proximity of storm drains and sanitary sewers, indicate that a storm drain network in a defined region is contaminated at levels of sewage much below 10%, then dye studies should likely be performed to assess if exfiltration is occurring from the sanitary sewer to the storm drain. While drain and sewer televising was not performed by the researchers in this project, it is recommended that televising be used as an intermediate tool, i.e. to rule in or out the presence of illicit connections of sanitary sewer laterals to storm drains.

Microbial source tracking is an emerging endeavor. It is important to stay aware of new technologies, mainly DNA-based, that can improve the speed and specificity of source tracking. Technologies that can also indicate the presence of pathogens have the potential to enable water quality managers to most appropriately focus their remediation investments for safeguarding public health. While not tested here, assays for pathogenic and host-specific viruses are available. Other host-specific assays based on qPCR analyses are also available beyond those tested here. Community analysis, as per the PhyloChip, has the potential of enabling comprehensive source tracking, i.e. accounting for host markers and pathogens at once. This type of approach shows promise in this study, and should be considered in the future as methods for its use in source tracking become more developed.

II. Introduction

2.1 Background to this Section and Relation to Other Sections

This section introduces the problem and project that are the subjects of this report. Relevant published literature is reviewed in section III (Literature Review) as background. Subsequent sections of the report provide standard operating procedures (SOPs) for methods used in this research project, results from the research, and synthesis of results and findings into a recommended Microbial Source Tracking Protocol (section XI).

2.2 Project Scope and History

The City of Santa Barbara, Creeks Division, initiates and implements projects to improve and safeguard creek and coastal water quality in the City of Santa Barbara region. Funded by a State of California Proposition 50 grant through the CA Clean Beaches Initiative, the City of Santa Barbara undertook this project, entitled "Source Tracking Protocol Development Project", to develop and demonstrate a systematic approach to determining the origins of fecal indicator bacteria (FIB) that signal unhealthy waters. The main goal of this project was to develop a protocol for determining FIB origins and to communicate the protocol in a report that could be used by other coastal urban communities for similar purposes. Microbial Source Tracking (MST) is a broad endeavor that occurs in urban, suburban, and agricultural areas. Here, MST was focused within an urban area, and more specifically in storm drain networks that had previously been diagnosed in Santa Barbara as contaminated with sewage (2, 4).

The research described in this report, along with the Microbial Source Tracking Protocol (section XI) was substantially performed by the University of California, Santa Barbara, in collaboration with the City of Santa Barbara, Creeks Division. The research was originally proposed between UCSB and the City for the period July 1, 2007 through June 30, 2009 but was then contracted for the period October 14, 2008 through October 13, 2010. The project commenced in October, 2008. The City issued a "stop work" order on December 23, 2008 to UCSB due to a "Notice of Suspension of State Water Board General Obligation (GO) Bond Grants", dated December 18, 2008. The stop-work order was rescinded by the City on February 2, 2009, at which time UCSB began working part-, then full-, time to complete this project. A "no cost extension" was issued by the City, with approval by the State, for completing the laboratory and data analysis, plus report writing, through March 1, 2011. The research progressed as contracted (see UCSB monthly progress reports in the Appendix). Meetings were held between the City and UCSB at least quarterly, and more frequently during the field seasons. During the period May 2010 through August 2010, approximately weekly phone conferences were held between UCSB and the City to coordinate research activities and equipment ordering. This document overall reports the work performed for the contract between UCSB and the City.

2.3 Problem Statement: Beach Water Quality

The broad water quality problem motivating this project is frequent beach warnings at California beaches due to high levels of FIB. As required by law (e.g. Assembly Bill 411 in

CA), coastal water quality is assessed by quantifying cultured FIB: total coliform, either fecal coliform or *Escherichia coli*, and enterococci (1, 5), and beaches are posted where concentrations exceed standards. Beach advisories and closures are undesirable: they signal unhealthy waters and incur economic losses in beach communities. The research here is to have application to beaches throughout the State. While the specific project focuses on identifying sources of human waste to East Beach at Mission Creek, one of Santa Barbara's most popular beaches, lessons learned are applicable State-wide. Due to its proximity to downtown and beach hotels, East Beach receives in excess of one million visitors per year. Similar to other coastal communities, the City of Santa Barbara is committed to improving the quality of creek and beach water for public health and recreational access. The reduction of FIB pollution will improve the quality of life, protect human health, improve habitat, and expand recreational uses of Santa Barbara beaches.

Both Mission Lagoon and the Laguna Lagoon discharge into the Pacific Ocean at East Beach. Mission Lagoon is fed by Mission Creek and the upstream Mission watershed, and Laguna Lagoon is fed by Laguna Channel and the upstream Laguna watershed. Since ocean surf zone testing began in 1999, East Beach was posted an average of 9.5% of the beach days. Table 2.1 summarizes historical exceedances of bacterial indicators and number of days of AB411 postings and closures from 1999 – 2009.

Table 2.1. Bacterial indicator exceedances and AB411(April 1 – October 31) postings at Mission Creek at East Beach.

Year	Beach Warnings	Beach Days Posted	% Beach Days Posted	Number of Beach Days Closed
1999	5	25	12	0
2000	5	8	4	0
2001	6	16	7	0
2002	7	26	12	0
2003	1	2	1	0
2004	6	15	7	0
2005	13	42	20	0
2006	16	60	28	0
2007	5	19	9	0
2008	3	6	3	0
2009	1	2	1	0
2010	8	26	12	0

2.4 Need for a Research Project and Focus on Storm Drains

The first step in solving FIB contamination is to identify the biological origins involved. As discussed in the Literature Review (III), FIB can come from many different sources in addition to human waste/sewage, including animal waste, soil, plants, and even

growth in storm drains, on kelp, and in sand. The research community has made significant progress in developing and testing techniques for source identification.

The potential sources of indicator bacteria at California's AB411 beaches, including East Beach at Mission Creek, include human waste and/or sewage, animal waste, soil and sediment, and even growth in the environment. In terms of human pathogens and risk to human health, by far the greatest risk comes from the presence of raw human waste/untreated sewage. The City has conducted extensive monitoring of indicator bacteria in Mission Creek and Laguna watersheds to complement the weekly beach data collected by the County of Santa Barbara at East Beach at Mission Creek. Until recently, the source of indicator bacteria in the creeks had not been identified. Work by UCSB and the City previously confirmed the presence of human waste in creek samples, storm drain samples, and the surf zone (2, 4). Once human waste has been identified in recreational waters, it is imperative to track contamination upstream in order to eliminate or reduce the input. Possible physical sources of human waste that were of interest in this research included illicit cross connections between sewer and storm drains, diffuse connections between sewer and storm drains, and dynamic flow from contaminated sumps.

It is not enough to attribute sources of FIB to different groups of animal sources in order to solve contamination problems. Most creeks and large storm drain networks in Southern California have dozens to hundreds of inputs. Armed with the knowledge that key storm drains discharge consistent, relatively high levels of human waste and/or sewage into Santa Barbara creeks (2, 4), this project focused on tracking sources up storm drain networks. Stymied by high variability in flow and concentrations, source tracking protocols should include flow and load assessment tools in addition to the molecular (DNA-based) techniques (as per the Literature Review, III) to understand contamination. The research pursued here combines DNA-based and chemical indicator techniques to characterize sources, tests the importance of flow variation, and develops a tracer approach for discovering contamination origins in storm drains.

2.5 Project Overview

This project was conducted as a partnership between the City and UCSB. Complementing the City's capital program that has installed end-of-pipe projects in three locations, the City has worked with Dr. Holden's group at UCSB to conduct source tracking research to identify the sources and routes of contamination. The research has confirmed and quantified markers for human waste in creeks, lagoons, and the surf zone. Unfortunately, the physical sources of human contamination remain elusive. The research here was to identify where, when and how human waste is transported to storm drains, creeks, and beaches in Santa Barbara. The value of the research is that it provides protocols for coastal managers throughout California to use for conducting source investigations of storm drains that produce exceedances of AB411 indicator bacteria standards.

Original research, as described in this report (i.e. sections V through X) was performed in support of the protocols described in section XI. The research complied with the funding contract for this project and other related documents, including the Monitoring Plan (MP) and Quality Assurance Project Plan (QAPP) submitted by the City to, and approved by, the CA SWRCB. Most assays that were intended for use were described in the MP and QAPP documents (see Appendix), with the exceptions of: 1) not using either

terminal restriction fragment length polymorphism (TRFLP) analysis or qPCR *Enterococcus*, and 2) using the PhyloChip microarray method, defining and testing alternative sewage indicators (as reported on in section VII), and testing the BacHum-UCD method of Kildare *et* al.(3) as per section V. Also not described in detail in either the MP or QAPP were the final methods for testing exfiltration of sewage from sanitary sewers into storm drains. The latter methods were developed and applied as a course of performing this research.

2.6 Project Description

This project involved the following tasks:

- Purchase Equipment. Install and test field and lab equipment, including: toolkit for investigating connections between sanitary sewers and storm drains (flow meter(s), sensors, and autosamplers, field GPS, and/or smoke, televising, and dye testing equipment), screening toolkit for determination of sewage pollution in storm sewers (i.e. fluorescence/colorometric assay equipment and reagents for chemical indicators) and quantitative polymerase chain reaction (PCR) instrument.
- Develop detailed sampling plans for field investigation.
    - Prepare detailed sampling plans for each sampling season, as defined in Health & Safety Code section 115880 (AB411 Season) of the contract period by March of each year.
    - Conduct meetings with City staff and Dr. Holden's laboratory group to finalize sampling plans.
- Conduct field sampling over two (2) AB411 Seasons.
    - Investigate diffuse flow and illicit connections as sources of storm drain contamination.
    - Deploy automatic in-channel flow monitors and autosamplers. Measure flow continuously over the course of twenty-four (24) hours, and recorded in one to two -minute intervals using Doppler technology based on velocity and area of flow.
    - Repeat experiment on three (3) or four (4) days (non-consecutive) during the AB411 Season.
    - Use tracer (dual tracer technique or similar) and other techniques (e.g. smoke testing, televising) to look for transport between sewer pipe and storm drains.
- Laboratory Analysis
    - Process samples for indicator bacteria (using IDEXX methods), human-specific *Bacteroidales* (using quantitative PCR methods), *Methanobrevibacter smithii nifH* gene (using PCR), sewage indicator chemicals (using ELISA) according to QAPP.
    - Analyze select samples for chemical wastewater indicators, including caffeine, anionic surfactants (methylene blue active substances), and/or fluorescent optical brighteners, and Phylochip analysis (by Lawrence Berkeley National Laboratory, LBNL).
- Data Analysis

- Modeling. Develop GIS tools for visualizing and analyzing source tracking and sanitary survey investigation.
- Source Tracking Protocol Report for Coastal Managers. Prepare a report for coastal managers that summarizes protocols tested, their efficacy, and provide case studies of the research conducted here.

2.7 Study Locations

This research project mainly focuses on solving contamination problems at the Haley Street Storm Drain in the Mission Creek watershed and the Nopal/Cota street area in the Laguna Watershed. The Haley Street Drain has been chosen as a pilot study area because of the extensive background data collected on the drainage area and preliminary research (4). Previous work by UCSB has identified consistent contamination at the drain, but the sources have been uncertain. With funding from CBI-Proposition 40, the flow in the drain is now diverted to the sanitary sewer during the summer months. Even with the diversion, the City and residents are committed to solving contamination sources so that flow can one day be restored to the creek. The Nopal/Cota area is selected based on results from the Laguna Watershed Study (2), which was funded by an additional Clean Beaches Proposition 50 grant. The goal of that study was to identify problem areas and choose a treatment option. A map of the two areas is provided below (Fig. 2.2). Specific sampling sites were nominated in the Monitoring Plan (Appendix) and are shown in subsequent sections of this report.

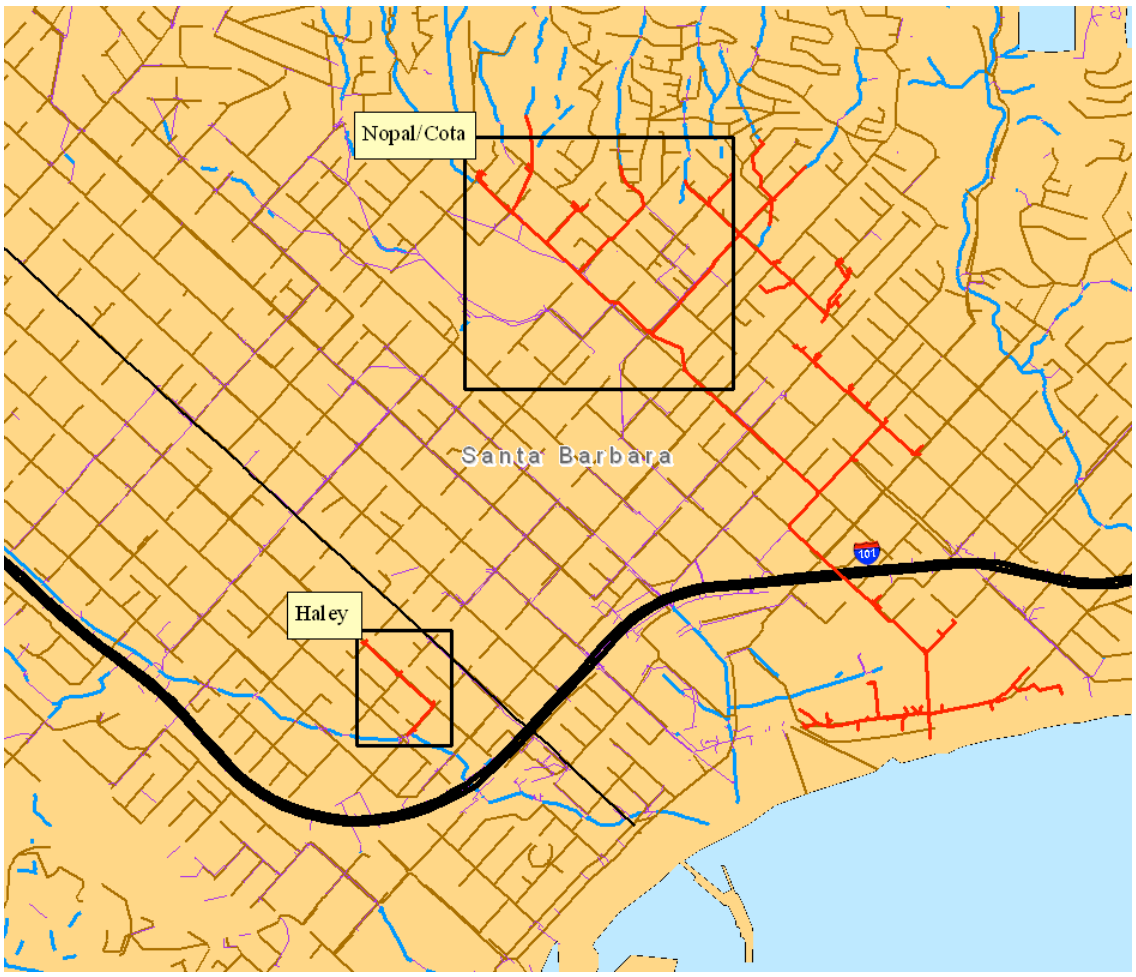


Fig. 2.2. Map of Santa Barbara, showing the Haley and Nopal/Cota study areas. Lines identify major roads (black), creeks (blue), sanitary sewers (brown), storm sewers (purple), storm sewer networks sampled during the proposed project (red).

2.8 References

1. **Eaton, A. D., L. S. Clesceri, A. E. Greenberg, M. A. H. Franson, American Public Health Association, American Water Works Association, and Water Environment Federation.** 1998. Standard Methods for The Examination of Water and Wastewater, 20th ed. American Public Health Association, Washington, DC.
2. **Geosyntec Consultants.** 2009. Laguna Watershed Study and Water Quality Improvement Feasibility Analysis, Laguna Channel Project Number LA0178. City of Santa Barbara, Creeks Division.
3. **Kildare, B. J., C. M. Leutenegger, B. S. McSwain, D. G. Bambic, V. B. Rajal, and S. Wuertz.** 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: A Bayesian approach. Water Research **41:**3701-3715.

4. **Sercu, B., L. C. Van De Werfhorst, J. Murray, and P. A. Holden.** 2009. Storm drains are sources of human fecal pollution during dry weather in three urban southern California watersheds. Environmental Science & Technology **43:**293-298.
5. **U.S. Environmental Protection Agency.** 2000. Improved enumeration methods for the recreational water quality indicators: Enterococci and *Escherichia coli* EPA 821/R-97/004.

III. Literature Review

3.1 Background to this Section and Relation to Other Sections

As described in the Introduction, the rationale for this study is that a protocol is needed for California urban beach communities to track the origins of high FIB with an emphasis on human waste, especially sewage, in separated storm drains discharging into coastal creeks. This study and report begins to fill this gap, and provides (see section XI Microbial Source Tracking Protocol) a methodology that other small communities can implement for tracking waste sources in their urban watersheds.

For the research reported herein, assay and method selection, field study designs, and results interpretation were substantially informed by published scientific and government report literature. Thus, some of the cited published literature was influential in this research. Other literature is introduced to provide context for the section XI Protocol.

3.2 Beach Microbiological Water Quality

Based on local and regional monitoring data that are compiled annually across the U. S., coastal creeks and beach waters appear chronically-polluted with fecal indicator bacteria (FIB) [1]. In recent years, the situation worsened along increasingly urbanized coastlines [2-4]: in 2006, fecal indicator bacterial levels in the U.S. were the worst in 17 years, with over 25,000 advisories and closures; ca. 5000 closures were in CA—a number 30% higher than in 2004 [5]. The lower rainfall year of 2007 was still problematic, with "22,571 days of closings and advisories at U.S. ocean, bay, and Great Lakes beaches"[6]. In 2009, the number of closures or advisories in CA (2,904 days) decreased from 2008 (4,133 days) and 2007 (4,736 days) [1]. These trends are concerning, as high FIB concentrations indicate unhealthy waters with economic consequences to coastal communities: based on water quality data and epidemiological models using FIB, the cost of beach-related gastrointestinal illness for urban Orange and Los Angeles Co. beaches, alone, is estimated in the $10's of million annually [7].

3.3 Tracking FIB Sources in Coastal Waters and Watersheds

FIB-based beach water quality criteria were originally adopted because epidemiological research linked increased swimmer illness to raw sewage discharges [8, 9]. Nowadays, most wastewater in the U.S. is treated to secondary standards [10]. Still, FIB, including DNA-based *Enterococcus* measurements [11], appear to signal unhealthy waters as per epidemiological relationships [12-18]. However, while FIB are legitimately the "gold standard" in food and potable water safety, FIB are imperfect tracers for the main threats to public health, i.e. pathogen–containing fecal material. First, culture-based assays require ca. 24 hrs during which contamination and field conditions change. To address, a newer culture-independent method for quantitative PCR (qPCR) of *Enterococcus* sp.[19, 20] can enable rapid FIB measurements. However, *Enterococcus* by qPCR can arise from non-human sources and persist in the environment [21]. More broadly, FIB in coastal urban areas may arise from many sources [1] including near-shore leaking sewer lines [22, 23],

but also decaying wrack [24], algae [25], or coastal marshes with waterfowl [26]. Septic systems in unsewered areas are sometimes a source [27-29], as are livestock in agricultural areas [30-32], and pets in suburbia [27, 33]. Urban development generally correlates with high FIB in surface waters [34] arriving via runoff [35-37] or dry-weather drains [38]. But individual sources are mostly "unknown" [1]. Diagnosing coastal waters by FIB assays is further complicated because FIB survive [39-44] differently than pathogens [45], and they can become non-culturable [46, 47] leading to costly false positive-based beach closures, or the false illusion of safe water.

Clearly, with the myriad of potential FIB sources in coastal waters [1], effective water quality management requires more information than FIB monitoring data alone can provide. To differentiate various FIB sources, "microbial source tracking" (MST) uses sensitive and specific DNA-based methods [48], for example including polymerase chain reaction (PCR) for detecting [49] and quantifying low levels [50] of DNA encoding a 16S rRNA sequence of a human-specific (sewage, septage, feces) *Bacteroides* bacterium. Further, the human-specific *Bacteroides* gene marker (HBM) persists for days in sewage-contaminated water [50-52], making it a reliable indicator of spills. Molecular (DNA-based) techniques can also assay pathogens by their virulence genes [53, 54]. *E. coli* virulence gene studies, mainly conducted in controlled conditions, show extra-host expression [55-57] subject to temperature and aeration [57], although environmental *E. coli* virulence gene expression variations are mostly unknown which may still limit understanding the actual pathogenicity of contaminated waters.

As of the writing of this report, many DNA-based assays have been developed for microbial source tracking fecal contamination, and results of their use have been published. New assays, targeting human and other waste sources, are being introduced and continuously developed. Not all available assays are applicable for tracing human or other waste in storm drains because they are designed to target livestock, or other host, wastes of various types. Meanwhile, earlier assays are being re-evaluated for their specificity to human waste [58]. Thus, it is important to stay abreast of the scientific literature that publicizes new assays and their performance as they emerge. It is also important to recognize deficiencies in this area of research and practice [59], including: 1) no single molecular marker at once accounts for FIB, human waste, and pathogens, 2) most assays are insufficiently tested to know their absolute specificity to target fecal material, and 3) the environmental ecology of most DNA-based waste markers is unknown, precluding linking quantities (e.g. via fate and transport modeling) to far-upstream sources, or predicting the health consequences. These factors support that, while DNA-based assays for assessing the presence of human and other fecal sources are increasingly powerful tools for tracking fecal sources that contribute to high FIB, guidance is needed for when and how to employ them. The rest of this section focuses on the potential for sewage to contaminate coastal zones, evidence for sewage occurrence in separated coastal zone storm drains, existing guidance for determining the origins of sewage in storm drains, and how existing guidance can be expanded to make use of new, DNA-based and other, technologies for tracking human waste in storm drains to its origins.

3.4 Sewage as an FIB Source in Coastal Zones

Since half (nearly 150 million people) of the U.S. population is on coastlines [2, 3] with the percentage expected to increase dramatically in the next 15 to 20 years [4], and 8 of the world's 10 largest cities are coastal (2 in the U.S.—New York and LA) with at least 3 billion people living in or around the world's coastal cities [4], it is clear that managing human waste (a.k.a. wastewater, sewage, fecal material, septage) in coastal environments is an enormous and growing challenge.

As above, most wastewater in the U.S. is treated to secondary standards [10]. Still, many U.S. cities have combined sewer overflows (CSOs) that discharge untreated wastewater during wet weather [1]. DNA-based evidence for human waste markers is found in beaches where CSOs are used [60]. However, DNA-based evidence for human waste markers is also found when CSOs are not operating [60], and in areas where storm drains and sanitary sewers are separated [61, 62]. Where sanitary and storm sewers are separated, human waste in coastal zones can arise from near shore sewer pipes leaking into the surf zone [23]. However, storm sewers also discharge markers of human waste in coastal creeks [63, 64], implying that there are upstream sources of human waste entering such systems—i.e. storm drains should only convey freshwater and infiltrated water associated with irrigation or other similar sources during dry weather. According to the NRDC [1], "Human waste may [also] find its way into storm drain systems from adjacent sewage pipes that leak, or from businesses or residences that have illegally connected their sewage discharge to the storm drains. Illicit discharges also occur when people empty holding tanks from recreational vehicles and trailers into storm drains." Taken together, there are many routes for sewage to enter coastal creeks from watershed-related sources, including near shore sewer line breaks, and runoff. But how can runoff, particularly when storm drains are separate systems from sanitary sewers, become contaminated with DNA-based markers of human waste, i.e. sewage?

3.5 Potential Sources of Human Fecal Markers and Sewage in Surface Waters

As above, many potential sources of human waste (sewage or septage) to coastal waters have been nominated, including sewer line breaks near beaches, illicit connections to storm drains from sanitary sewers, and possible leaker sewers. However, very little is actually known about the relative importance of these sources in coastal urban landscapes. In 2007, experts convened for an EPA-led workshop on recreational water quality research needs listed "pathogen presence" related to "sources in urban landscapes such as broken / leaky sewer pipes, CSOs, stormwater and urban runoff" as among the highest priorities in the near term (2 to 3 years) [65]. Over the longer term (8 to 10 years from the workshop), another high priority research need was described as "Research on GIS layers relevant to modeling" such that "GIS data" are "readily available and useable for models (e.g. POTW locations, recent land use categories, storm sewer locations)" [65]. However, as of the writing of this report, GIS does not appear to have been advanced for systematically differentiating between the physically very different origins when comparing sewer line breaks to sanitary sewers chronically leaking. Further, other than this report, there does not appear to be much additional insight into which urban sources are important for introducing sewage into the coastal watershed environment.

In a report to the State of California in 2001 regarding "Source Investigations of Storm Drain Discharges Causing Exceedances of Bacteriological Standards" [66], "broken sewer lines" are among the listed possible sewage sources to the environment. Sanitary sewers are described as potentially "leaking" but from the "outside to the inside", i.e. the process of infiltration which is relatively known. However, exfiltration—the process by which sanitary sewers leak from the inside to the outside—also is a serious problem, and of concern enough to warrant estimating the magnitude and thus the threat to groundwater quality [67]. In the State of California's "Draft Guidance for Salt Water Beaches" [68], potential sources of microbiological contamination in recreational waters are also nominated as "failures in human sewage treatment facilities" and "leaking sewer lines". The potential for flow of sewage from sanitary sewers into adjacent storm drains is mentioned as a potential outcome of exfiltration [67], but the magnitude of this process or proof of its occurrence was not described. Much of the U.S. wastewater infrastructure, i.e. sanitary sewers and WWTPs, is beyond its design life [69]. Yet it appears that no systematic investigation has been performed regarding the poor integrity of sanitary infrastructure as a vehicle for contaminating storm drains with human waste.

3.6 Sanitary Surveys and Microbial Source Tracking

A "Report to the Legislature" was made in December 2001 by Dr. Michael Stenstrom (UCLA) regarding "Source Investigations of Storm Drain Discharges Causing Exceedances of Bacteriological Standards" [66]. As stated in the Executive Summary of this report

> *"Assembly Bill (AB) 538 (Chapter 488, Statutes of 1999) enacted Water Code section 13178, which requires the State Water Resources Control Board (SWRCB), in conjunction with the California Department of Health Services (DHS) and a panel of experts, to develop source investigation protocols for identifying the sources of discharges from storm drains that exceed the State's bacteriological standards (Appendix 1). The protocols must include methods for identifying the location and biological origins of sources of bacteriological contamination and require source investigations if (1) bacteriological standards are exceeded in any three weeks of a four-week period, or (2) bacteriological standards are exceeded 75 percent of testing days in areas where testing is done more than once a week. Section 13178 also requires the SWRCB, in conjunction with the DHS, to report to the Legislature on the methods by which the SWRCB intends to conduct source investigations of storm drains that produce exceedances of bacteriological standards. The report must also include: (1) the approximate number of public beaches expected to be affected by the exceedance of standards, (2) the estimated costs for source investigation of the storm drains affecting those public beaches, and (3) a timeline for completion of source investigation."*

As of the writing of this report, source identification protocols have not been codified by the State of California, and are thus not available for any of the conditions described above. In the 2001 Report [66], recommendations were made regarding how to approach identifying sources of fecal bacteria in storm drains. However, best technologies for differentiating human from other fecal sources have changed substantially, as described in

the US EPA Microbial Source Tracking Guide Document [48] and more recently in peer-reviewed journals [59, 70]. Further, the above 2001 report did not discuss exfiltration as a potential source of contamination [66]. While the methods for tracing sewage were potentially appropriate when the State commissioned the 2001 report [66], more tools are now available and thus an improved and more systematic approach is possible.

In the "Draft Guidance for Salt Water Beaches" for California [68], it is recommended that a "sanitary survey" be performed to determine "actual or potential sources of microbiological contamination of recreational waters and beach areas". The recommended elements of a sanitary survey are not defined in this guidance document, but such elements are defined by the State of Maine in their "Municipal Guide to Clean Water: Conducting Sanitary Surveys to Improve Coastal Water Quality" [71]. The latter guidance stresses that a sanitary survey, defined as a process "to identify and document sources of bacterial contamination affecting water resources", is not linear but should be thorough. For example, the report of a sanitary survey would include: a description of beach and watershed areas, results of special studies or surveys to characterize conditions, delineation of potential and known bacterial contamination sources, description of strategies and successes in controlling contamination, recommendations for management actions, and descriptions of missing information [71]. Several indicators are discussed for determining if illicit sewage discharges are contaminating stormwater, with those deemed most likely to be useful including ammonia and surfactants [71]. Other water testing approaches mentioned include caffeine and molecular (DNA-based) methods in MST, but how and when to use such methods was not defined [71]. More details are provided regarding when and how to use dye testing, for example to assess if septic tank leach fields are directing microbial contamination into surface waters [71]. Smoke testing and video surveillance are both discussed with regards to assessing illicit connections. With smoke testing, cross connections from sanitary sewers into storm drains can be assessed, and damaged storm drains can be discovered [71]. Video surveillance of storm drains is not recommended where discharges might be discontinuous [71].

The U.S. EPA provides guidance for Illicit Discharge Detection and Elimination (IDDE; http://cfpub.epa.gov/npdes/stormwater/idde.cfm) which is mainly focused upon detecting and eliminating cross connections, i.e. sanitary sewer laterals illicitly connected to storm drains [72]. A prior study that is referenced in the IDDE manual [72] is of bacterial contamination in storm drainage in Canada were culture-based approaches—appropriate at the time—revealed many human pathogens in dry and wet weather [73]. The latter report refers to studies of exfiltration, but does not suggest that it is a major process by which storm drains become contaminated by sanitary sewers [73]. A later report provides more detailed guidance for tracing contamination in storm drains during wet and dry weather [74], but this guidance is more focused on chemical markers that may be useful for concluding that sewage is contaminating storm drains. While exfiltration is mentioned as a possible source of sewage entry into storm drains, no methods are proposed for directly assessing the phenomenon.

3.7 The Gap Filled by this Research

While there are many areas of related research, there is an apparent gap that exists in procedures for determining the exact origins of human waste contamination in coastal urban watersheds. Summarizing the above, currently available guidance can be described as providing:

- General recommendations for, and approaches to, performing a sanitary survey for coastal recreational waters, but with little emphasis on specific analytical methods to be used, where to use particular methods, and in what sequence
- Recommendations regarding how to determine if there are illicit connections to storm drains from sanitary sewers, but with no explicit direction on how to differentiate between illicit connections and insidious exfiltration from aged sanitary sewers to nearby storm drains
- Guidance on state-of-the-art DNA-based methods for detecting and quantifying the presence of human waste in drainage and surface waters, but without explicit direction as to where to sample and when to invest in these expensive assays within the context of fully executing a sanitary survey.
- Recommendations for incorporating GIS databases into sanitary surveys and into microbial source tracking, but little definition as to exactly how this should be performed and to what end.

A conclusion of this literature review is that, while DNA-based methods can be used to show that human waste can be present in storm drains during dry weather, a gap exists in available guidance for systematically, and comprehensively, determining the origins of human waste markers and thus sewage in storm drains during dry weather conditions. Thus, the need for the research described in this report is obviated.

3.8 References

1. Dorfman, M. and K.S. Rosselot, *Testing the Waters: A Guide to Water Quality at Vacation Beaches*. 2010, Natural Resources Defense Council: Washington, D. C.
2. Beach, D., *Coastal Sprawl: The Effects of Urban Design on Aquatic Ecosystems in the United States*. 2002, Pew Oceans Commission: Arlington, VA. p. 40.
3. Culliton, T.J., *Population: Distribution, Density and Growth*, in *NOAA's State of the Coast Report*. 1998, U.S. National Oceanic and Atmospheric Administration: Silver Spring, MD.
4. United Nations. *Atlas of the Oceans*. 2006.
5. Dorfman, M. and N. Stoner, *Testing the Waters: A Guide to Water Quality at Vacation Beaches*. 2007, Natural Resources Defense Council: Washington, D. C. p. 377.
6. Dorfman, M. and K.S. Rosselot, *Testing the Waters: A Guide to Water Quality at Vacation Beaches*. 2008, Natural Resources Defense Council: New York. p. 447.
7. Given, S., L.H. Pendleton, and A.B. Boehm, *Regional public health cost estimates of contaminated coastal waters: A case study of gastroenteritis at southern California beaches.* Environmental Science & Technology, 2006. **40**(16): p. 4851-4858.

8. Cabelli, V.J., et al., *Swimming-associated gastroenteritis and water quality.* American Journal of Epidemiology, 1982. **115**(4): p. 606-616.
9. Cabelli, V.J., *Indicators of recreational water quality*, in *Bacterial Indicators/Health Hazards Associated with Water, ASTM STP 635*, A.W. Hoadley and B.J. Dutka, Editors. 1977, America Society for Testing and Materials: Philadelphia.
10. National Research Council, *Managing Wastewater in Coastal Urban Areas*. 1993, Washington, D. C.: National Academy Press. 477.
11. Wade, T.J., et al., *Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestional illness.* Environmental Health Perspectives, 2006. **114**(1): p. 24-28.
12. Haile, R.W., et al., *The health effects of swimming in ocean water contaminated by storm drain runoff.* Epidemiology, 1999. **10**(4): p. 355-363.
13. Fleisher, J.M., et al., *Marine waters contaminated with domestic sewage: nonenteric illnesses associated with bather exposure in the United Kingdom.* Amer. J. Pub. Health, 1996. **86**(9): p. 1228-1234.
14. Seyfried, P.L., et al., *A prospective study of swimming-related illness II. Morbidity and the microbiological quality of water.* Am. J. Pub. Health, 1985. **75**(9): p. 1071-1075.
15. Fleisher, J., et al., *Estimates of the severity of illnesses associated with bathing in marine recreational waters contaminated with domestic sewage.* Intl. J. Epidem., 1998. **27**: p. 73.
16. Cabelli, V.J., *Swimming-associated illness and recreational water-quality criteria.* Water Science and Technology, 1989. **21**(2): p. 13-21.
17. Crabtree, K.D., et al., *Waterborne adenovirus: A risk assessment.* Water Science and Technology, 1997. **35**(11-12): p. 1-6.
18. Turbow, D.J., N.D. Osgood, and S.C. Jiang, *Evaluation of recreational health risk in coastal waters based on enterococcus densities and bathing patterns.* Environmental Health Perspectives, 2003. **111**(4): p. 598-603.
19. He, J.W. and S. Jiang, *Quantification of enterococci and human adenoviruses in environmental samples by real-time PCR.* Applied And Environmental Microbiology, 2005. **71**(5): p. 2250-2255.
20. Haugland, R.A., et al., *Comparison of Enterococcus measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis.* Water Research, 2005. **39**: p. 559-568.
21. Litton, R.M., et al., *Evaluation of chemical, molecular, and traditional markers of fecal contamination in an effluent dominated urban stream.* Environmental Science & Technology, 2010. **44**(19): p. 7369-7375.
22. Orange County Sanitation District, *Huntington Beach Closure Investigation: Phase I Final Report*. 1999: Fountain Valley. p. 45.
23. Boehm, A.B., et al., *Tiered approach for identification of a human fecal pollution source at a recreational beach: Case study at Avalon Cay, Catalina Island, California.* Environmental Science & Technology, 2003. **37**(4): p. 673-680.
24. Weiskel, P.K., B.L. Howes, and G.R. Heufelder, *Coliform contamination of a coastal embayment: sources and transport pathways.* Environ. Sci. Technol., 1996. **30**: p. 1872-1881.

25. Whitman, R.L., et al., *Occurrence of Escherichia coli and enterococci in Cladophora (Chlorophyta) in nearshore water and beach sand of Lake Michigan.* Applied and Environmental Microbiology, 2003. **69**(8): p. 4714-4719.
26. Grant, S.B., et al., *Generation of enterococci bacteria in a coastal saltwater marsh and its impact on surf zone water quality.* Environmental Science & Technology, 2001. **35**(12): p. 2407-2416.
27. Whitlock, J.E., D.T. Jones, and V.J. Harwood, *Identification of the sources of fecal coliforms in an urban watershed using antibiotic resistance analysis.* Water Research, 2002. **36**(17): p. 4273-4282.
28. Scandura, J.E. and M.D. Sobsey, *Viral and bacterial contamination of groundwater from on-site sewage treatment systems.* Water Science and Technology, 1997. **35**(11-12): p. 141-146.
29. Duda, A.M. and K.D. Cromartie, *Coastal pollution from septic tank drainfields.* J. Environ. Engr. Div. ASCE, 1982. **108**: p. 1265-1279.
30. Crowther, J., D. Kay, and M.D. Wyer, *Faecal-indicator concentrations in waters draining lowland pastoral catchments in the UK: relationships with land use and farming practices.* Water Research, 2002. **36**(7): p. 1725-1734.
31. Mallin, M.A., et al., *Demographic, landscape, and meteorological factors controlling the microbial pollution of coastal waters.* Hydrobiologia, 2001. **460**: p. 185-193.
32. Fisher, D.S., et al., *The relationship of land use practices to surface water quality in the Upper Oconee watershed of Georgia.* Forest Ecology and Management, 2000. **128**(1-2): p. 39-48.
33. Burnes, B.S., *Antibiotic resistance analysis of fecal coliforms to determine fecal pollution sources in a mixed-use watershed.* Environmental Monitoring and Assessment, 2003. **85**(1): p. 87-98.
34. Clinton, B.D. and J.M. Vose, *Variation in stream water quality in an urban headwater stream in the southern Appalachians.* Water Air and Soil Pollution, 2006. **169**(1-4): p. 331-353.
35. Dwight, R.H., et al., *Association of urban runoff with coastal water quality in Orange County, California.* Water Environment Research, 2002. **74**(1): p. 82-90.
36. Reeves, R.L., et al., *Scaling and management of fecal indicator bacteria in runoff from a coastal urban watershed in southern California.* Environmental Science & Technology, 2004. **38**(9): p. 2637-2648.
37. Jiang, S., R. Noble, and W.P. Chui, *Human adenoviruses and coliphages in urban runoff-impacted coastal waters of Southern California.* Applied and Environmental Microbiology, 2001. **67**(1): p. 179-184.
38. Petersen, T.M., et al., *Bacteria loads from point and nonpoint sources in an urban watershed.* Journal of Environmental Engineering-ASCE, 2005. **131**(10): p. 1414-1425.
39. Hazen, T.C., *Fecal coliforms as indicators in tropical waters--a review.* Toxicity Assessment, 1988. **3**(5): p. 461-477.
40. Davies, C.M., et al., *Survival of fecal microorganisms in marine and freshwater sediments.* Appl. Environ. Microbiol., 1995. **61**(5): p. 1888-1896.
41. Marino, R.P. and J.J. Gannon, *Survival of fecal coliforms and fecal streptococci in storm drain sediment.* Wat. Res., 1991. **25**(9): p. 1089-1098.

42. Rivera, S.C., T.C. Hazen, and G.A. Toranzos, *Isolation of fecal coliforms from pristine sites in a tropical rain-forest.* Applied and Environmental Microbiology, 1988. **54**(2): p. 513-517.
43. Hazen, T.C., et al. *Survival and activity of fecal coliform bacteria in near-shore coastal waters*. in *8th Annual Natural Resources Symposium*. 1982. San Juan, Puerto Rico: Department of Natural Resources, Commonwealth of Puerto Rico. 44. Anderson, M.L., J.E. Whitlock, and V.J. Harwood, *Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments.* Applied and Environmental Microbiology, 2005. **71**(6): p. 3041-3048.
45. Wyer, M.D., et al., *An investigation into parametric relationships between enterovirus and faecal indicator organisms in the coastal waters of England and Wales.* Wat. Res., 1995. **29**(8): p. 1863-1868.
46. Leadbetter, E.R., *Prokaryotic diversity: form, ecophysiology, and habitat*, in *Manual of Environmental Microbiology*, C.J. Hurst, et al., Editors. 1997, American Society for Microbiology: Washington, D. C. p. 14-24.
47. Statham, J.A. and T.A. Mc Meekin, *Survival of faecal bacteria in Antarctic coastal waters.* Antar. Sci., 1994. **6**: p. 333-338.
48. U.S. Environmental Protection Agency, *Microbial Source Tracking Guide Document*. 2005, Office of Research and Development: Cincinnatti, OH. p. 150.
49. Bernhard, A.E. and K.G. Field, *A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA.* Appl Environ Microbiol, 2000. **66**(10): p. 4571-4574.
50. Seurinck, S., et al., *Detection and quantification of the human-specific HF183 Bacteroides 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater.* Environmental Microbiology, 2005. **7**(2): p. 249-259.
51. Betancourt, W.Q. and R.S. Fujioka, *Bacteroides spp. as reliable marker of sewage contamination in Hawaii's environmental waters using molecular techniques.* Water Science and Technology, 2006. **54**(3): p. 101-107.
52. Walters, S.P. and K.G. Field, *Persistence and growth of fecal Bacteroidales assessed by bromodeoxyuridine immunocapture.* Appl Environ Microbiol, 2006. **72**(7): p. 4532-4539.
53. Chern, E.C., Y.L. Tsai, and B.H. Olson, *Occurrence of genes associated with enterotoxigenic and enterohemorrhagic Escherichia coli in agricultural waste lagoons.* Appl Environ Microbiol, 2004. **70**(1): p. 356-62.
54. Hamelin, K., et al., *A virulence and antimicrobial resistance DNA microarray detects a high frequency of virulence genes in Escherichia coli isolates from Great Lakes recreational waters.* Appl Environ Microbiol, 2006. **72**(6): p. 4200-6.
55. Fitzmaurice, J., et al., *Application of real-time PCR and RT-PCR assays for the detection and quantitation of VT 1 and VT 2 toxin genes in E-coli O157 : H7.* Molecular and Cellular Probes, 2004. **18**(2): p. 123-132.
56. Sharma, V.K. and E.A. Dean-Nystrom, *Detection of enterohemorrhagic Escherichia coli O157 : H7 by using a multiplex real-time PCR assay for genes encoding intimin and Shiga toxins.* Veterinary Microbiology, 2003. **93**(3): p. 247-260.
57. McIngvale, S.C., D. Elhanafi, and M.A. Drake, *Optimization of reverse transcriptase PCR to detect viable Shiga-toxin-producing Escherichia coli.* Appl Environ Microbiol, 2002. **68**(2): p. 799-806.

58. Layton, B.A., S.P. Walters, and A.B. Boehm, *Distribution and diversity of the enterococcal surface protein (esp) gene in animal hosts and the Pacific coast environment.* Journal of Applied Microbiology, 2009. **106**(5): p. 1521-1531.
59. Girones, R., et al., *Molecular detection of pathogens in water - The pros and cons of molecular techniques.* Water Research, 2010. **44**(15): p. 4325-4339.
60. Bower, P.A., et al., *Detection of genetic markers of fecal indicator bacteria in Lake Michigan and determination of their relationship to Escherichia coli densities using standard microbiological methods.* Applied and Environmental Microbiology, 2005. **71**(12): p. 8305-8313.
61. Sercu, B., et al., *Storm drains are sources of human fecal pollution during dry weather in three urban southern California watersheds.* Environmental Science & Technology, 2009. **43**(2): p. 293-298.
62. Geosyntec Consultants, *Laguna Watershed Study and Water Quality Improvement Feasibility Analysis, Laguna Channel*. 2009, City of Santa Barbara, Creeks Division: Santa Barbara, California. p. 264.
63. Noble, R.T., et al., *Multitiered approach using quantitative PCR to track sources of fecal pollution affecting Santa Monica Bay, California.* Applied and Environmental Microbiology, 2006. **72**(2): p. 1604-1612.
64. Smith, S. *An investigation into the water quality of the Charleston pennisula storm drains*. in *WEFTEC*. 2006.
65. U. S. Environmental Protection Agency, *Report of the Experts Scientific Workshop on Critical Research Needs for the Development of New or Revised Recreational Water Quality Criteria*, O.o.R.a.D. Office of Water, Editor. 2007, U. S. EPA: Washington, D. C. p. 199.
66. State Water Resources Control Board, *Source investigations of storm drain discharges causing exceedances of bacteriological standards: report to the legislature*, C.E.P. Agency, Editor. 2001: Sacramento. p. 74.
67. Amick, R.S. and E.H. Burgess, *Exfiltration in Sewer Systems*, O.o.R.a.D. National Risk Management Research Laboratory, Editor. 2000, U. S. Environmental Protection Agency: Cincinnati. p. 34.
68. California Department of Public Health, *Draft Guidance for Salt Water Beaches*, D.o.D.W.a.E. Management, Editor. 2006: Sacramento.
69. American Society for Civil Engineers. *Wastewater [D-]*. Report Card for America's Infrastructure 2005.
70. Stewart, J.R., et al., *The coastal environment and human health: microbial indicators, pathogens, sentinels and reservoirs.* Environmental Health, 2008. **7**.
71. Lindberg, K., *Municipal Guide To Clean Water: Conducting Sanitary Surveys to Improve Coastal Water Quality*, M.S. Grant, Editor. 2010, University of Maine Cooperative Extension Waldoboro, ME. p. 65.
72. Brown, E., D. Caraco, and R. Pitt, *Illicit Discharge Detection and Elimination: A Guidance Manual for Program Development and Technical Assessments*. 2004, Department of Civil and Environmental Engineering, University of Alabama, Tuscaloosa, AL. p. 378.
73. Pitt, R., *Rideau River Stormwater Management Study Technical Report: Urban Bacteria Sources and Control by Street Cleaning in the Lower Rideau River Watershed*. 1983, Ontario Ministry of the Environment: Blue Mounds, Wisconsin. p. 179.

74. Pitt, R., et al., *Investigation of inappropriate pollutant entries into storm drain systems: a user's guide*, U.S.E.P. Agency, Editor. 1993: Cincinnati, OH. p. 98.

IV METHODS

4.1 Fecal indicator bacteria

Indicator organisms, such as total coliforms, *Escherichia coli* and *Enterococcus* spp. are used to determine whether or not water is contaminated. These organisms are commonly found in the human or animal gut, and when detected in a sample suggest the presence of fecal pollution. The US Environmental Protection Agency (EPA) uses *Enterococcus* as a standard indicator, while the State of California also uses total coliform and fecal coliform (or *E. coli*) bacteria for assessing beach water quality. California AB411 standards state that a warning must be posted for a California beach if the total coliform exceeds 10,000 per 100 mL (if the fecal/total ratio is < 0.1) or 1,000 per 100 mL (if fecal/total ratio is > 0.1), fecal coliform exceeds 400 per 100 mL or *Enterococcus* exceeds 104 per 100 mL.
There are multiple methods for measuring FIB in water samples, including Multiple Tube Fermentation (MTF), Membrane Filtration (MF), and Defined Substrate Technology (IDEXX). For MTF, each sample is inoculated with sterile growth medium, followed by multiple serial dilutions to produce 50 tubes, with dilutions ranging from 1:10 through 1:10000. The tubes are then incubated at a pre-set temperature for a specified time, and afterwards the number of tubes with growth present is counted for each dilution. Statistical tables are used to calculate the concentration of organisms in the sample. In contrast, samples for MF are vacuum filtered through membrane filters which are placed onto plates with nutrient medium and incubated. Each membrane has a printed grid which is used to count the number of colonies formed at the end of incubation. Samples for IDEXX are added to 100 mL bottles, along with reagent packs, then poured into welled-trays and sealed. The sealed trays are incubated for 24 hours, and the number of positive wells is counted and converted to MPN/100 mL (most probable number).

The IDEXX method is perhaps one of the more convenient methods to measure FIB, and the method used in this project. Only 2 bottles are needed for each sample (1 for Colilert reagent to measure total coliform and *E. coli*; 1 for Enterolert reagent to measure *Enterococci*), and little equipment is needed (2 incubators, IDEXX plate sealer, UV lamp & enclosure). A free software program is available to calculate the MPN values with 95% confidence limits. Cost information is included in Table 4.4.

The advantages to measuring FIB are that the methods are relatively rapid compared to some analyses (results generally within 24 hours), do not require expensive equipment, and have standard, easy to follow protocols. Since FIB have been required to be measured in many municipalities for years, there can also be historical data that can be used to help determine trends and target locations for further analysis. However, since FIB is not specific to human-waste sources, high FIB levels do not necessarily indicate high amounts of human-waste pollution or human health hazards. Furthermore, FIB is a culture-based method, and as such can only detect organisms that are capable of growing in the specified media and incubation conditions.

SOP #H02 in the Appendix contains the details for FIB sample collection, preparation and quantification.

4.2 Nutrients and anionic surfactants by colorimetry

4.2.1 Introduction

Colorimetric methods can be used to analyze a wide variety of chemicals, and are generally easy to perform, inexpensive, fast, and portable. However, sensitivity, accuracy and precision may be lower compared to standard methods or other more sophisticated methods.
The procedure consists of adding reagents to a sample, usually in 2-3 steps, and some time is allowed for chemical reactions during each step. Reagents react with the analyte, and produce a color with intensity proportional to the analyte concentration. Visible light with a specific wavelength (complementary to the color developed in the sample vial) is passed through the sample, and the measure light absorption is proportional to the analyte concentration. Colorimetry can be used for quantification of a wide range of chemicals, and systems including colorimeter(s), reagents and glassware, are available from various suppliers (e.g. Lamotte, Hach, Hanna Instruments).
Colorimetry was used in this study to quantify alternative indicators for sewage, including nutrients and anionic surfactants.

4.2.2 Selection of equipment

Colorimetric methods were chosen for analyzing the chemicals in Table 4.1, because they are versatile, practical for field use and require a low investment. Ion specific electrodes (ISE) are available for some of the chemicals (NH_3, NO_3 and SO_4) and are a good alternative if only one chemical needs to be analyzed (e.g. NH_3), especially when samples area analyzed in a lab. Field use ISE is difficult because frequent calibration and magnetic stirring are required.
Several suppliers provide colorimeters, reagents and glassware for quantification of the selected chemicals. Purchase of sufficient vials (~20) is recommended when batches of samples will be analyzed. Reagents kits for various concentrations ranges are often available. An overview of the suggested concentration range and approximate time required to analyze a sample is presented in Table 4.2, for multiple suppliers. Lamotte and Hach also provide spectrophotometers for the same analyses, usually with a lower limit of detection, but with more expensive and non-portable equipment.

The **Lamotte Smart2 colorimeter** can quantify all sewage chemicals, has good sensitivity and is easy to carry around in the field. Different reagents/protocols are required depending on the concentrations of the chemicals. The instrument is an EPA approved instrument for NPDES compliance monitoring programs.
The **Hach colorimeter** is slightly more sensitive compared to the Lamotte equipment for all chemicals. However, anionic detergents, potassium and boron cannot be quantified using the colorimeter.

The **Hanna Instruments single parameter photometers** are available for all sewage chemicals, except B. They are more sensitive compared to the Lamotte instruments for NH_3, K, SO_4 and anionic detergents, and often have a wider analytical range. However, purchase of separate instruments for each chemical is required.
Other test kits using **test strips** are available from Hach and Hanna Instruments, and can also be used for rapid but approximate quantification of several sewage chemicals, with detection limits in the same range as the other colorimetric assays.

In this study, the Lamotte Smart2 colorimeter (Fig. 4.1) was selected for quantifying nutrients. All assays were straightforward to perform, and it took between 7-17 minutes for quantifying one sample. For analyzing batches of samples, it is recommended to prepare sample blanks and samples with reagents in separate bottles, so that sample analysis can be stacked for multiple samples. This procedure allows analysis of 5-10 samples in 30 minutes, depending on the assay. Reagent blanks should be analyzed for each batch of reagents. Note that some assays produce toxic waste that needs to be disposed of according to regulations.
A Hanna Instruments photometer was used for quantification of anionic detergents. This assay requires handling and disposal of toxic chloroform. The assay should be run using appropriate safety precautions: in chemical fume hood, wearing lab coat, safety goggles and appropriate safety gloves (e.g. 8mm ChemTek™, Ansell Healthcare). Latex, neoprene and nitrile gloves do not provide adequate protection. Problems occurred on multiple occasions ordering reagents from Hanna Instruments. Reagents were frequently backordered, and bottles with chloroform were not properly sealed, leading to leakage of some or all of the chloroform during transport.

Cost information for all assays is included in Table 4.4.

4.3 Nutrients by flow injection analysis

Flow injection analysis (FIA) provides a more accurate alternative for quantifying the nutrients NH_4^+, NO_2+NO_3 and PO_4. Concentrations ranges and precision for each method are shown in Table 4.2. Flow injection analysis was performed by the Marine Science Institute (MSI) analytical lab, at UCSB. Advantages of FIA include more accurate and precise quantification, and samples can be frozen and analyzed as one batch. The disadvantages of FIA include potential long turnover times (4-6 weeks at MSI) and higher cost per sample. Cost information for outsourcing the analysis is included in Table 4.4.

Samples for nutrient analysis should be filtered through a 0.45 µm filter, although unfiltered or turbid samples may be accepted for an additional fee. Samples should be collected in clean, pre-rinsed glass or plastic HDPE 20 ml scintillation vials. Some adsorption of PO_4 during storage in plastic containers has been noted, so glass may be preferred if PO_4 is to be determined. Caps should have a plastic liner. All foil lined lids should be avoided. Storage blanks are required if NH_4 is to be measured. Storage blanks are recommended for all other analytes to detect possible contamination. A minimum of 5mL of sample is required for the determination of a single nutrient species. 15-17 mL of sample

is necessary for simultaneous determination of all NH_4^+, NO_2+NO_3 and PO_4. Samples should be frozen immediately after collection and stored at -20°C or below. Sufficient headspace should be allowed when freezing for expansion of the sample without breaking the container. Frozen samples should be sent for analysis within one month of collection.

4.4 Methanobrevibacter smithii PCR for the nifH gene (Mnif-PCR)

In contrast to FIB (Section 4.1), DNA-based methods, such as Polymerase Chain Reaction (PCR), are culture-independent, meaning that they are not limited to measuring only the organisms capable of growing in certain conditions. PCR is a molecular technique used to amplify a specific target region of DNA. It uses thermal cycling (repeated cycles of heating and cooling) to melt the DNA and enable enzymatic replication, generating thousands to millions of copies of the target DNA sequence. The presence or absence of a PCR amplification product can be detected after thermal cycling.

Methanobrevibacter smithii is a methanogen, a microorganism that produces methane under anoxic conditions. It is abundant in the human intestine and is found in human feces and sewage, making it a useful indicator of human-waste pollution in the environment. This assay targets the *nifH* gene of *M. smithii*.

Prior to PCR, DNA must be extracted from the samples. Commercial kits are available that contain filters for capturing cells from water samples, as well as all the reagents and tubes necessary to extract DNA from those cells (e.g. MoBio PowerWater® DNA Isolation Kit) . A basic thermocycler is needed to perform PCR, and supplies include pipettes, pipette tips, and PCR tubes. Reagents include PCR kits, primers, Bovine Serum Albumin, and Molecular Biology Grade Water. Also, large equipment such as a biological safety cabinet or PCR workstation (to avoid cross-contamination) and autoclave are recommended for performing molecular analyses.

Gel electrophoresis is used to visualize PCR products and determine if a target (i.e. *M. smithii nifH* gene) is present. The equipment needed consists of a gel electrophoresis box and power supply, UV transilluminator, and a digital imaging set up or Polaroid camera. Note that digital imaging systems are very sensitive to weak band detection on gels, and are highly recommended over using a traditional Polaroid camera. Reagents include agarose powder and TBE buffer to create the gel, loading dye, ladder(s) for size reference, and Ethidium Bromide or SYBR Safe™ dye to stain the gel. Special care needs to be taken with toxic stains like Ethidium Bromide and with exposure to UV light sources. An alternative to purchasing all of the equipment separately is to use a complete system that has the gel box, power supply, and transilluminator integrated in one unit, and uses pre-made gel cassettes (e.g. E-Gel® from Invitrogen, FlashGel® from Lonza). These units can be more convenient since the gels are purchased pre-made, allow live migration viewing, and results are available within 5-10 minutes (vs. 30 minutes or longer with conventional gel electrophoresis systems). This type of unit might be a good choice for a lab just starting PCR analysis.

SOP #H11 in the Appendix contains the details for sample analysis for Mnif-PCR.

4.5 Human-specific Bacteroidales qPCR (HBM-qPCR)

Quantitative Polymerase Chain Reaction (qPCR) works in the same manner as PCR (See Section 4.4), with the added benefit that the number of targets formed at the end of each thermal cycle is quantified. QPCR is faster and more sensitive than PCR, and allows for simultaneous target quantification without the need for gel electrophoresis.

Bacteroidales is an order of microorganisms that are anaerobes, found in warm-blooded animals, and make up a significant portion of fecal bacteria. Researchers have identified a human-specific *Bacteroidales* 16S rRNA genetic marker that has been shown to be widely distributed and specific to humans, and have developed PCR and qPCR assays to detect the target in environmental samples.

QPCR requires a real-time PCR detection system, consisting of an optical unit combined with a thermal cycler. These units are more expensive than conventional PCR thermal cyclers, but can also run PCR as well (e.g. Bio-Rad CFX96). Recently, more affordable qPCR systems have become available as well (e.g. Eco Real Time PCR System, Illumina). Similar supplies are needed as with PCR (pipettes, tips), but 96-well (or 384-well) plates and sealing film are used instead of tubes. Reagents include master mix kits, primers, Bovine Serum Albumin, and Molecular Biology Grade Water.

At least one company in the U.S. offers PCR and qPCR analysis of DNA markers specific for human/sewage pollution and other sources of fecal pollution (Source Molecular Corporation, Miami, FL, www.sourcemolecular.com). Water samples can be shipped overnight (200-300 mL), and results are available in approximately 7-11 days. Pricing information is included in Table 4.4.

SOP #H05 in the Appendix contains the details for sample analysis for HBM-qPCR via SYBR® Green I chemistry.

4.6 Enterococcus spp. qPCR (ENT-qPCR)

Section 4.5 provides background information on Quantitative Polymerase Chain Reaction (qPCR). Section 4.4 explains the main advantage of using a DNA-based, culture-independent method such as qPCR.

As mentioned in Section 4.1, *Enterococcus* spp. (ENT) is used by the EPA and State of California as an indicator organism for fecal pollution in water sources. Quantifying ENT via a qPCR assay instead of traditional FIB methods allows for potentially faster results, and will not be limited to detecting only those ENT that will grow under specific conditions. The main disadvantage to using ENT as an indicator of human-specific waste pollution is that ENT are present in the intestines of most animals, and are able to persist in sediments and vegetation.

Equipment requirements for ENT-qPCR are the same as detailed in Section 4.5. Reagent costs are similar, with the addition of a TaqMan probe when running qPCR with TaqMan chemistry.

SOP #H10 in the Appendix contains the details for sample analysis for ENT-qPCR via TaqMan chemistry.

4.7 ELISA for caffeine and cotinine

Some organic chemicals found in sanitary sewage are relatively specific for sewage, and can be quantified in relatively short times, compared to FIB (see Section 4.1) and HBM-qPCR (see Section 4.4). Two such wastewater chemicals are caffeine and cotinine. Caffeine is widely consumed (coffee, tea, soft drinks, chocolate, pharmaceuticals, etc.), but can also originate from some vegetation. Cotinine is a metabolite of nicotine. A significant fraction of both chemicals is excreted from the body through urine.

Enzyme-linked Immunosorbent assays, known more commonly as ELISA, are biochemical assays to detect the presence of an antibody or antigen in a sample. ELISA kits to measure caffeine and cotinine are available from Abraxis (Warminster, PA). Their kits use a 96-well plate format, and are direct competitive ELISA tests. For each kit, when the target is present in a sample, it competes with target-HRP analogues for the binding sites of specific antibodies in the test solution. The target antibodies are then bound by a second antibody that is immobilized in the 96-well plate. After multiple washing steps, a substrate solution is added to the plate which enables color signal generation when the target is present. After a specified period of time, the color reaction is stopped and the absorbance of each well is read at 450 nm. Target concentration in each sample is then determined by standard curve interpolation.

Water samples for ELISA tests need some special handling to avoid loss of analytes. Extended contact with plastic containers should be avoided, and the water should not be filtered through Miracloth prior to pulling off aliquots for ELISA. Samples can be grab sampled in a plastic beaker, but aliquots for ELISA should be pulled off as soon as possible, using a sterile plastic syringe. The aliquots are then passed through a 0.2 μm Teflon syringe filter into amber glass vials. If analysis will be performed within a few days, the samples may be refrigerated, otherwise they should be frozen. As the Teflon filters are hydrophobic, it can take a great deal of pressure to filter the sample with a syringe. However, only a small volume of sample is needed (150 μL to run one ELISA in triplicate) so only a few milliliters of sample should be adequate, depending on the number of different ELISA tests to be performed.

The ELISA kits are available per 96-well plate for a single analyte (e.g. caffeine or cotinine). When running standards and samples in triplicate, which we highly recommend, 26 samples can be analyzed at once on a single plate. If samples are over-range, they can either be reported as over-range or diluted and run again in another kit. Due to the expense of the

kit, and the need to run a full standard curve each time, we do not recommend running partial plates. We also suggest using the lowest and highest standard replicates as cutoffs for quantification of samples.

In addition to the kits, a multi-channel pipette is required, preferably an electronic model. While an 8-channel pipette can be used, a 12-channel pipette allows for optimal pipetting when running the assay in triplicates, as the reagents can be added to all 12 wells in a row at once (e.g. Rainin EDP3, 20 µL to 300 µL). A microplate reader is also required to measure the absorbance. Nearly any plate reader can be used, but it is highly recommended that it also has software capable of calculating 4-parameter standard curves. Abraxis offers a plate reader with the necessary software, but you must provide the computer or laptop.

SOP #H12 in the Appendix contains the details for caffeine analysis via ELISA, and SOP #H13 for cotinine.

4.8 Automated sampling and flow measurement

4.8.1 Automated sampling

Using automated sampling equipment allows flexibility in sampling times and frequency, as well as conditional sampling (e.g. rain or flow triggered sampling). It can be a practical alternative to positioning personnel in the field for interval sampling. The most important factor to consider when selecting equipment is where it will be used. If the unit will be mainly used above ground, then size is not as critical as if will be installed inside manholes. If the unit will be installed inside manholes, then the diameter and length is critical and the equipment specifications should be compared with manhole sizes in the study area. Another important factor is the sample size and capacity of the unit, and whether or not sterile sample containers are an option.

We decided on the Isco 6712 Full Size Portable Sampler for our project based on a number of reasons (unit size, capacity, sterile sample container options, plug-in module options (e.g. flow), SDI-12 inputs, remote control and programming abilities). The 6712 Full Size can hold 24x 1L sampling containers, while the 6712 Compact Size unit can only hold 24x 500 mL. Since we routinely collect 2L of water per sample, the full size unit made more sense. Also, the 1 L bottles are made from polypropylene and can be autoclaved (< 145°F, pressure < 66 psi), while the 500 mL bottles are polyethylene and therefore must not be autoclaved. The full size unit can also use disposable ProPak 1 L sampling bags with special holders, enabling users without access to an autoclave the option to use sterile sampling containers. Note that certified sterile ProPak bags must be specifically requested (P/N 605304999), or else the sterility of the bags can not be guaranteed. The same caps can be used with both the polypropylene bottles and the ProPak bags, and must not be autoclaved. One way to sterilize them is to rinse with 70% ethanol, allow the liquid to evaporate in a biological safety cabinet, and then expose to UV light for 15-20 minutes. If using the ProPak bags, you also have the option to simply use a twist-tie or rubber band to seal the bag closed.

To put together a portable sampling system, we recommend purchasing extra battery packs and chargers so that a battery can also be charging, as well as extra bottle kits to allow easy bottle swapping in the field. Battery selection should be based on how long the sampler will be in the field, the number and frequency of measurements recorded, as well as how long the batteries will sit unused on the shelf. Isco makes a ProHanger bracket that spans manhole covers 18-24" in diameter, and allows the lid to lay nearly flush with the street level. The Sampler Suspension Harness is then used to attach the sampler to the bracket. Note that we highly recommend using duct tape to secure the harness attachments to the sampler, as there is the possibility of them coming loose if bumped against a ladder or other structure inside the manhole opening. Isco can make custom ProHanger brackets in other sizes, or you can also try enlisting a machine shop to build one for you. A complete sampling system includes the sampler, 2 batteries, charger, tubing, sample strainer, 2 bottle kits, ProHanger (stock size), and Suspension Harness. Cost information is included in Table 4.4.

4.8.2 Continuous flow measurement

4.8.2.1 Method summary

Flow in pipes can be continuously monitored using the area-velocity method, by multiplying the average velocity and the cross-sectional area of flow at a certain location. Cross-sectional area is often calculated from depth measurements, which can be measured by various methods (e.g. pressure sensors, ultrasonic sensors). Average velocity is often measured using ultrasonic Doppler sensor technology, where a sensor measures the Doppler shift in wavelengths of reflections from particles and bubbles moving with the flow. Flow in pipes can also be measured using primary structures (weirs and flumes), but this approach is not practical for measuring flow in various storm drains. An excellent overview of technologies and vendors providing flow equipment, and selection of the appropriate flow equipment for various applications was presented by Krajewski (2009).

4.8.2.2 Equipment selection

Since this project focuses on dry weather flow, flow measurement equipment and configuration had to be suitable for measuring level and velocity during low flow depths. Flow depths in storm drains were often in the range of 0.02-0.05 m. Based on the recommendations of Krajewski (2009), continuous wave Doppler technology was most appropriate, and flow measurement equipment available form Teledyne-Isco (Isco 2150 or 750 are velocity flow module), Hach (Sigma 900 series flow meters), and ADS (FlowShark).

For this study, two area velocity flow modules were selected. First, the Isco 750 area velocity flow module was selected because of its easy integration with the Isco 6712 autosampler. A low-profile area velocity sensor was selected instead of a standard area velocity sensor to allow for better measurement of low flows. Second, the Sigma 920 area velocity flow meter was selected, because it provides the option of combining a "wafer-thin" velocity sensor with ultrasonic depth sensor for greater accuracy in low flows (versus the standard pressure transducer/velocity sensor). Specifications for both flow meters are

shown in Table 4.3. Note that velocity in water depths > 0.5 m (20”) is underestimated due to limited range of Doppler velocity sensor (important for wet weather flow and large pipes). Cost information is included in Table 4.4.

4.9 Automated rhodamine WT monitoring

4.9.1 Method summary

Automated monitoring of rhodamine WT (RWT) in storm drains was used for identifying transport of sewage from sewer mains to storm drains (Chapter 9). The requirements for monitoring equipment included high sensitivity and specificity, continuous automated monitoring capability, sufficiently long battery life at short measurement intervals (~7 days at 2 min interval). Given that travel times of RWT from sanitary sewers to storm drain was unknown, and RWT peak width in storm drains was expected to be small, manual RWT measurements were not an option. Other tracers were considered, but did not provide unattended monitoring capability (bacteriophage PRD-1), or did not have sufficiently low detection limits (bromide).

A 600 OMS V2 sonde equipped with temperature and conductivity sensors and a rhodamine WT optical probe (YSI Incorporated, Yellow Springs, OH) was selected for automated RWT monitoring (Fig. 4.2). To the best of our knowledge, the 600 OMS V2 sonde was the only with the required specifications. The sonde was programmed for unattended monitoring, at 1 or 2 minute intervals. The sonde was calibrated using a 2-point calibration curve (0 and 100 ppb), has a detection limit of 1 ppb and a linear range of 1-200 ppb. RWT was purchased as Keyacid rhodamine WT liquid, and consisted of 20 % true dye concentration (Keystone Aniline Corporation, Chicago, IL). While temperature and conductivity sensors were not required for the RWT dye experiments, they are potentially useful for identifying inflow of industrial effluents in storm drains. The 600 OMS sonde can be equipped with a depth sensor, which could be useful for detecting flow changes in the storm drains. Cost information is included in Table 4.4.
A field laptop is recommended for programming and downloading data in the field. In addition, because approximately weekly battery replacement is required, rechargeable batteries are recommended.

4.9.2 Tips for using the 600 OMS V2 sonde

The 600 OMS V2 sonde was easy to program and use in the field. Submersion of optical sensor and temperature and conductivity sensors was sometimes challenging, as at least 0.04-0.06 m (1.5”-2.5”) of water depth is required. However, as the optical RWT sensor is located at the end of the probe, submersion of RWT sensor only can be achieved at lower water depths if the sonde is positioned at a slight angle with the bottom of the pipe. Sand bags can also be used to dam flow and increase water depth in the storm drain. Sand bags and sonde should be attached to manhole structures, to avoid loss of equipment. Also, cloth bags are preferable over plastic bags, as the latter provide less resistance against flow in smooth storm drain pipes.

The 600 OMS V2 sonde includes a wiper to clean the optical sensor during long-term deployments. The cleaning frequency greatly affects battery life, and should be set to at least 10 minutes to avoid frequent battery changes.

A defect wiper caused erroneous readings in some cases. The wiper positioned itself in the path of the optical sensor, causing erroneous high readings. The problem was detected during collection of background signal, and confirmed in the lab. Erroneous high readings typically occurred for one or more data points, but did not show a typical Gaussian concentration pattern (Fig. 4.3). The company renting the sonde (Fondriest Environmental, Beavercreek, OH) acknowledged that the problems with the wiper are known, and replaced the complete sensor promptly (at no cost for rental equipment). Therefore, when unexpected high RWT readings occur, or when RWT peaks do not display a typical Gaussian shape, the position of the wiper should be checked. If the wiper positions itself next to optics after a wiping cycle, instead of at 180 degrees, RWT sensor should be replaced.

Table 4.1. Optimal assay detection ranges (mg/L) and estimated analysis time per sample (min) for quantification of nutrients and anionic surfactants in urban storm drains by colorimetry.

	Lamotte Smart2		Hach DR/850		Hanna Instr. photometer	
	Range	Time	Range	Time	Range	Time
NH_3-N	0.05 - 4	7	0.08 – 2.5	5	0.01 – 10[a]	5
PO_4	0.05 – 3	7	0.14 – 30	15	0.1 – 30[a]	7
Anionic detergents.	0.75 - 8	7	-	-	0.01 – 3.5[a]	12
SO_4	4 - 100	7	5 - 70	7	1 – 150[b]	7
NO_3-N	0.1 - 3	17	0.3 - 30	7	0.1 – 30[b]	7
B	0.05 – 0.8	40	-	30	-	-
K	0.8 - 10	7	-	5	0.05 – 10[b]	5

[a]CalCheck photometer, waterproof
[b]Basic photometer

Table 4.2. Concentration range and precision for NH_4, NO_2+NO_3 and PO_4 by flow injection analysis.

Analyte	Concentration range (mg/L)	Precision
NH_4-N	0.0014-2.8	±0.00042 or 5%
(NO_2+NO_3)-N	0.0028-4.2	±0.00042 or 5%
PO_4	0.0095-19	±0.0019 or 5%

Table 4.3. Specifications of Isco 750 and Sigma 920 area velocity flow meters for measuring low flows.

Series Configuration	Isco 750 Standard AV	Isco 750 Low-profile AV	Sigma 920 Standard AV	Sigma 920 Wafer-thin velocity In-pipe ultrasonic depth
Min. depth for level	0.015 m	0.01-0.015 m	0.01 m	0.003 m
Min. depth for velocity	0.05 to 0.075 m	0.025 m	0.02 m	0.01 m
Velocity range	-1.5 to 6.2 m/s	-1.5 to 6.2 m/s	-1.5 to 6.2 m/s	-1.5 to 6.2 m/s
Power	Isco 6712	Isco 6712	battery	battery
Battery life[a]	24-36 hours	24-36 hours	90 days	90 days

[a]2 min recording intervals

Table 4.4. Pricing information for equipment and reagents used in this study. All prices are approximate.

Instrument/reagents	Price ($)
1. Fecal indicator bacteria (IDEXX)	
Incubators (requires 2)	675
IDEXX plate sealer	4000
UV lamp and enclosure	332
Colilert reagents	120/20 rxn
Enterolert reagents	145/20 rxn
Bottles	110/200
Quanti-Trays	150/100
2. Colorimetry for nutrients	
Lamotte Smart2 colorimeter	899
NH_3-N reagents	50/50 rxn
NO_3-N reagents	40/20 rxn
SO_4 reagents	30/50 rxn
PO_4 reagents	30/50rxn
3. Colorimetry for anionic detergents	
HI Cal Check waterproof photometer	350
Anionic detergents reagents	62/40 rxn
4. Flow injection analysis (MSI-UCSB) for NH_4, NO_2+NO_3 and PO_4	5.48/sample/analyte
5. Mnif-PCR	
MoBio PowerWater® DNA Isolation Kit	1424/100 samples
PCR thermocycler (basic)	<5000
PCR kit (Qiagen Core PCR)	158/68 samples
PCR primers	20[a]
BSA	14[a]
Molecular biology grade water	23/200 ml[a]
Gel electrophoresis box	1100
UV transilluminator	1800-2500
Digital imaging setup	2000-8000
Agarose	157/25 g[a]
10X TBE buffer	34/1 L[a]
Gel loading dye	38[a]
DNA size ladder	50[a]
E-gel® electrophoresis system (Invitrogen)	824
E-gel® gel cassette	10-12/each
FlashGel® electrophoresis system + camera (Lonza)	974
FlashGel® gel cassette	10-12
6. HBM-qPCR and ENT-qPCR	
MoBio PowerWater® DNA Isolation Kit	1424/100 samples
qPCR thermocycler	14,000-40,000
qPCR Master Mix Plus for SybrGreen (Eurogentec)	525/600rxn
PCR primers	20[a]
BSA	14[a]
Molecular biology grade water	23/200 ml[a]
TaqMan probe (Ent-qPCR only)	250/10+ plates
Human Bacteroidetes ID (Source Molecular Corporation)	595/sample
7. ELISA for caffeine and cotinine	
ELISA kit (Abraxis)	500/plate
12ch electronic pipette (ergonomic)	1000
96 well plate reader (need own computer) (Abraxis)	4200
8. Autosampling	

Isco 6712 Full Size sampling system (autosampler, 2 batteries, charger, tubing, sample strainer, 2 24x 1L bottle kits, ProHanger and suspension harness)	5000
9. Continuous flow measurement	
Sigma 920 with thin-wafer velocity sensor and ultrasonic in-pipe sensor	7900
Sigma 920 mounting bracket and ladder hanger	380
Sigma 920 support bracket 18-26” + cable	290
Sigma mounting bands for 48” + 54” pipe	1200
Isco 750 flow module set up, low-profile AV sensor	3500
Isco 581 RTD (data transfer)	645
Isco mounting bands for 48” + 54” pipe	1565
10. General equipment/supplies	
Autoclave	4,000-15,000
Biological safety cabinet/PCR workstation	3000-10,000
Set of pipettes	800-1600

[a]Usually enough for 6-12 months

Fig. 4.1. Lamotte Smart2 colorimeter for quantification of NH_3-N, NO_3-N, PO_4 and SO_4.

Fig. 4.2. Image of YSI 600 OMS V2 sonde with optical sensor for rhodamine WT. The length of the sonde is 54.1 cm (21.3”). The orange optical wiper can be seen at end of optical sensor on the right.

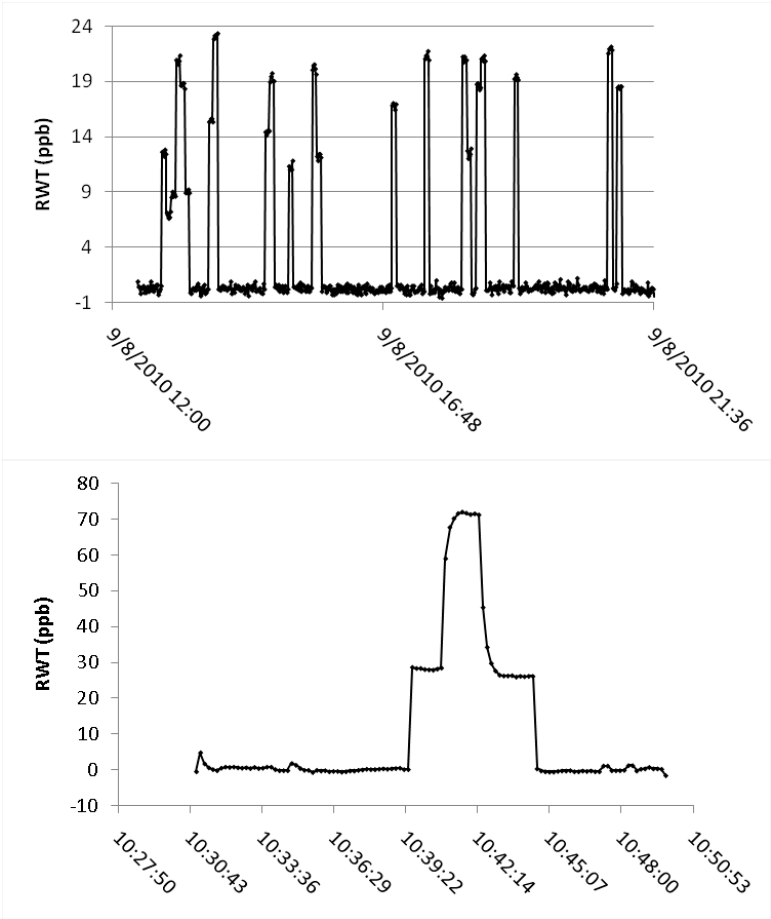


Fig. 4.3. Examples of erroneous RWT sensor signal due to wiper defects, short peaks in field (top) and long peaks in lab (bottom). No RWT was present during collection of the signal.

V. Specificity of human-specific assays

5.1 Background

When starting a new project involving the detection of human waste, it is important to verify the specificity of the assay(s) planned. While most methods have peer reviewed publications on their source specificity, it can be useful to evaluate their performance on local animal and human waste sources of interest to the current project. This is especially important when adopting a new method. Evaluating the performance of the new assay in comparison with those in current use will help determine whether or not the new assay is worth adding to the project toolbox.

One of the most common ways to quantify human waste is via a qPCR assay for human-specific *Bacteroidales* (See Section 4.5). We have successfully used the HF183 SYBR® Green I qPCR assay for human-specific *Bacteroidales* (HBM-qPCR) (Seurinck et al., 2005) for 5 years, and its specificity has been well documented (e.g. Ahmed et al., 2009; Shanks et al., 2010). A more recent human-specific *Bacteroidales* qPCR assay has been developed using TaqMan chemistry (BacHum-qPCR) (Kildare et al, 2007). The specificity of this assay has not been as widely documented as with HBM-qPCR, but looks promising (Ahmed et al., 2009). In addition to the *Baceroidales* assays, another method used to indicate human waste is conventional PCR targeting the *nifH* gene of *Methanobrevibacter smithii* (Mnif-PCR) (See Section 4.4; Ufnar et al., 2006).

For this project, these three assays were evaluated for specificity against cat, dog, gull, raccoon, and rat feces, as well as human feces, sewage, and septage samples. When performing PCR or qPCR assays, determining the presence of inhibition in each sample is critical to obtain accurate results. In this study, we used a separate qPCR assay (spiking samples with salmon testes DNA) to determine the dilution needed to remove inhibition for each sample. To standardize evaluation, this determined dilution for each sample was used in all three assays.

5.2 Materials and Methods

5.2.1 Fecal Source Sampling

Archived fecal samples
To increase the number and source types evaluated in this study, archived DNA from animal fecal samples that were collected from other projects was included (2 individual cats, 2 individual dogs, 1 composite gull, and 1 composite raccoon) (Tables 5.1 and 5.2). Archived DNA from human waste samples collected from other projects and sub-studies from this project were also analyzed in this study (1 composite and 7 individual human fecal samples, 3 septage samples, 4 raw sewage, and 6 WWTP sewage confluent samples) (Table 5.3).

New animal fecal samples
To supplement the archived fecal sources, new animal fecal samples were collected specifically for this study (10 individual cats, 10 individual dogs, 2 composite gull samples, 1 individual and 3 composite raccoon samples, and 1 individual and 3 composite rat samples) (Tables 5.1 and 5.2). All fecal samples were collected using Sterileware® Samplit™ Scoop/Containers, which have a scoop integrated into the lid.

Individual cat and dog fecal samples were collected fresh by their owners, stored at 4°C, and processed within 24 hours.

Composite gull fecal samples were collected on two dates (2/23/10 & 2/25/10) at East Beach, Santa Barbara, CA. Samples were obtained by baiting gulls onto new plastic tarps, and collecting feces as soon as they were spotted. Samples were stored on ice until transport to the lab and immediate processing. The actual number of gulls sampled is unknown; however, approximately 22 feces were collected from the tarp on the first date, and 13 on the second.

Raccoon samples were obtained from Nancy Callahan, working with the W.I.L.D.E. Service/Santa Barbara Wildlife Care Network. Feces were scooped from the litter box in each cage. If more than one individual was present in a cage, a portion from each feces found in the litter box was scooped and composited. Samples were stored on ice until transport to the lab and immediate processing.

The three composite rat fecal samples were obtained from Dale Schreve at Lenz Pest Control, who used the scoop systems to grab any samples he could positively identify as rat feces. The actual number of individuals represented in each sample is unknown. The single individual rat fecal sample was from a recently caught, baby wild rat in care of Nancy Callahan, of the W.I.L.D.E. Service/Santa Barbara Wildlife Care Network. Using sterile gloves, feces were picked from the bottom of the cage and deposited into the scoop systems. Samples were stored on ice until transport to the lab and immediate processing.

5.2.2 DNA extraction of new fecal samples

The PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) was used to extract DNA from all solid fecal samples, following SOP #H07 (See Appendix). For low yield DNA samples (e.g. gull feces), multiple separate DNA extractions were performed and then combined by ethanol precipitation.

Total DNA from the new fecal samples was quantified using the Quant-iT™ dsDNA Broad-Range Assay Kit (Invitrogen, Carlsbad, CA) on a BioTek Synergy 2 plate reader, following SOP #H04 (See Appendix). Standard curve and sample concentrations were calculated using BioTek's Gen5™ Reader Control and Data Analysis Software.

5.2.3 Quantitative-PCR (qPCR) and conventional PCR analysis of all samples

The qPCR assay for salmon testes DNA was performed prior to all qPCR and PCR assays in order to determine the lowest template dilution without inhibition. SOP #H08 contains the details for the salmon testes DNA qPCR, #H09 for Human-specific *Bacteroidales* qPCR via TaqMan chemistry (BacHum-qPCR), #H05 for Human-specific *Bacteroidales* qPCR via SYBR® Green I chemistry (HBM-qPCR), and #H11 for *Methanobrevibacter smithii* PCR for the *nifH* gene (See Appendix).

5.3 Results

5.3.1 Non-human sources

Table 5.4 displays the results for the animal sources. The BacHum-qPCR assay amplified 83% of cat fecal samples (10/12), 75% of dog samples (9/12), 33% of gull samples (1/3), and 40% of raccoon samples (2/5) within quantification range of the assay. It did not detect the BacHum marker in any of the 4 rat fecal samples.

In contrast, the HBM-qPCR assay amplified 8% of cat fecal samples (1/12) within quantification range of the assay. It did not detect the HBM in any of the 12 dog, 3 gull, 5 raccoon, or 4 rat fecal samples.

The Mnif-PCR target was not detected in any of the animal fecal samples.

5.3.2 Human sources

The human waste source results are presented in Table 5.5. The BacHum-qPCR assay detected 100% of human fecal (8/8), septage (3/3), and sewage (10/10) samples within quantification range of the assay.

The HBM-qPCR assay detected 63% of human fecal samples (5/8), 67% of septage samples (2/3), and 100% of sewage samples (10/10) within quantification range of the assay.

In total, 17 human waste samples were quantified by both qPCR methods. The BacHum assay reported higher average copy numbers for 14 of these samples (Fig. 5.1). When using all analytical replicates in calculations, 12 samples were significantly higher for BacHum, as evaluated via paired t-tests in Microsoft Excel ($p < 0.05$) (Fig. 5.2). However, the majority were at least within the same order of magnitude (Table 5.6).

The Mnif-PCR target was not detected in any of human fecal samples, but was positive for 100% of septage (3/3) and sewage (10/10) samples.

5.4 Discussion

- Although the number of targets detected from human feces might seem low compared to the sewage samples, the values are reasonable. Using the higher values from the human fecal results (E+08 copies/g), and assuming each person contributed an average of 100 to 250 g of feces (= E+10 to E+11 copies) and 400 L of

wastewater to the sanitary system each day, the resulting value (E+08 copies/L) is well within the range of our sewage samples (E+07 to E+09).

- The Bac-Hum assay reporting more copy numbers per sample compared to the HBM assay is not surprising, as each method uses very different chemistry and different target sequences. As the majority of human waste samples were within the same order of magnitude for both assays, the same degree of contamination would be indicated regardless of which assay was used. To maximize environmental sample comparison, selecting one human-specific *Bacteroidales* qPCR assay and using it consistently on all samples within a project is highly recommended.

- While the BacHum-qPCR assay had perfect detection to all our human waste samples, it is not sufficiently source specific when evaluated against animal fecal sources in our study area.

  The amplification of the BacHum marker in dog feces has been shown previously (Kildare et al., 2007; Ahmed et al., 2009), but in lower proportions of samples (13%, 1/8 and 6%, 2/33, respectively) than our 75%. This assay has also been previously tested with cat (7 samples) and gull (10 samples) feces (Kildare et al., 2007), with no BacHum markers detected. This is in contrast to our 85% cat and 33% gull sample detection, although we were only able to use 3 composite gull samples for this study. This is the first known instance of this assay being evaluated on raccoon or rat feces, so there is no data to compare our 40% raccoon sample detection. The rat fecal samples were the only non-human source to have no BacHum markers detected.

  To further illustrate the non-specificity of the Bac-Hum assay, the lower range of values from each source in this study were used to determine the amount of waste material that could cause the same quantity of targets. An environmental sample with 10^4 Bac-Hum copies/L detected could have been caused by 1 mL of raw sewage, 1L of septage, 1 g of human feces, 1 g of cat feces, 1 g of dog feces or 1 g of raccoon feces.

- The HBM-qPCR assay continues to be an excellent choice for detecting human waste. The assay had perfect detection for the sewage samples, and 63-67% for human feces and septage, respectively, and displayed very little non-human source detection (only 1 cat, and no dog, gull, raccoon, rat fecal samples).

  As there can be variation in microbial gut composition from individual to individual, it is not necessarily expected that HBM would be detected in every human fecal sample. Our results are similar to other reports (e.g. 5/7 human fecal samples in Seurinck et al., 2005; 11/18 samples in Kildare et al., 2007). This could also play a part in why HBM were not detected in one of the septage samples (sep03), which was from a single residential unit. That particular sample was also from an advanced treatment system, so it's possible the improved treatment removed or

reduced the HBM below detection limit. Of the 3 human waste source sample types evaluated here, it can be argued that sewage is the best indicator of human waste detection as these samples are composites of multiple individuals in a geographic area. This assay has also been previously evaluated against cat (14%, 1/7), dog (25%, 2/8), and gull (0%, 0/10) feces (Kildare et al., 2007). Our results are similar for cat (8%, 1/12) and gull (0%), but we did not detect any HBM in our 12 dog samples.

- The Mnif-PCR assay is another useful assay to use in human waste detection. The assay had perfect detection for the sewage and septage samples, but no detection in any of the human fecal samples. There was also no target amplification for any of the non-human sources.

  As mentioned above, the lack of target detection in individual human fecal samples is not necessarily unexpected. Ufnar et al. (2006) reported that of the 70 human fecal samples they tested, only 20 detected the target (=29%). In light of our much smaller sample size, our non-detects seem reasonable.

  One of the biggest advantages of adding this method is that since the assay is based on a different organism than the *Bacteroidales* assays, it can serve as a second, independent measure of human waste contamination. When environmental samples are both positive for Mnif-PCR and have quantifiable levels of HBM detected, there can be extra confidence in the presence human waste.

➢ Based on the results of this sub-study, all environmental samples for this project were analyzed via HBM-qPCR and Mnif-PCR. Due to its non-specificity, the BacHum-qPCR assay was not added to this project's toolbox.

5.5 References

Ahmed, W.; Goonetilleke, A.; Powell, D.; Gardner, T. Evaluation of multiple sewage-associated *Bacteroides* PCR markers for sewage pollution tracking. *Water Res.* **2009**, 43, 4872-4877.

Haugland, R. A.; Siefring, S. C.; Wymer, L. J.; Brenner, K. P.; Dufour, A. P. Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. *Water Res.* **2005**, 39, 559-568.

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

Sercu, B.; Van De Werfhorst, L. C.; Murray, J.; Holden, P. A. Storm drains are sources of human fecal pollution during dry weather in three urban southern California watersheds. *Environ. Sci. Technol.* **2009**, 43, 293-298.

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

Shanks, O. C.; White, K.; Kelty, C. A.; Sivaganesan, M.; Blannon, J.; Meckes, M.; Varma, M.; Haugland, R. A. Performance of PCR-based assays targeting *Bacteroidales* genetic markers of human fecal pollution in sewage and fecal samples. *Environ. Sci. Technol.* **2010**, 44(16), 6281-6288.

Ufnar, J. A.; Wang, S. Y.; Christiansen, J. M.; Yampara-Iquise, H.; Carson, C. A., Ellender, R. D. Detection of the *nifH* gene of *Methanobrevibacter smithii*: a potential tool to identify sewage pollution in recreational waters. *J. Appl. Microbiol.* **2005**, 101, 44-52.

Table 5.1. Cat and Dog Fecal Samples

ID	Source	Sample Date	Sex	Breed	Age	Notes
c01	cat	12/16/2004	M	unknown	unknown	Archived DNA; sample was from 1 or 2 cats
c02	cat	12/16/2004	M	Maine coon	8 yrs	Archived DNA
c03	cat	2/26/2010	F	unknown	10 months	
c04	cat	2/26/2010	F	Domestic short hair	5 years	same household as c05
c05	cat	2/21/2010	F	Tabby	12 years	same household as c04
c06	cat	2/22/2010	M	Domestic short hair	18 years	
c07	cat	2/24/2010	M	Domestic short hair	19 years	
c08	cat	2/26/2010	F	Calico	6 years	
c09	cat	3/4/2010	F	unknown	1 year, 9 months	
c10	cat	2/22/2010	M	Domestic short hair	1 year, 8 months	
c11	cat	2/22/2010	F	Shorthair tabby	8 months	
c12	cat	2/25/2010	M	American long hair (mutt)	9 months	
d01	dog	12/17/2004	F	Retriever	unknown	Archived DNA
d02	dog	12/17/2004	M	Shepard mix	unknown	Archived DNA
d03	dog	2/25/2010	F	Terrier mix	8 years	
d04	dog	2/26/2010	M	Chihuahua	12 years	same household as d05 & d06
d05	dog	2/26/2010	M	Terrier mix	12+ years	same household as d04 & d06
d06	dog	2/25/2010	F	Pomeranian	12 years	same household as d04 & d05
d07	dog	2/28/2010	M	Australian Shephard	12 years	
d08	dog	2/23/2010	M	Boston Terrier	10 weeks	
d09	dog	2/25/2010	M	Golden Retriever	12+ years	same household as d10 & d11
d10	dog	2/25/2010	M	Rottweiler	12+ years	same household as d09 & d11
d11	dog	2/26/2010	M	mutt	7-8 years	same household as d09 & d10
d12	dog	2/21/2010	M	Pug	7 years	

Table 5.2. Gull, Raccoon, and Rat Fecal Samples

ID	Source	Sample Date	Notes
g01	gull	3/28/2006	Archived DNA; from Goleta Beach, near slough outfall, composite of >3
g02	gull	2/23/2010	from East Beach, composite of >3
g03	gull	2/25/2010	from East Beach, composite of >3
rac01	raccoon	12/14/2004	Archived DNA; composite from 3 individuals
rac02	raccoon	2/24/2010	single raccoon in cage, wildfire victim
rac03	raccoon	2/24/2010	2 raccoons share cage, took composite sample
rac04	raccoon	2/24/2010	2 raccoons share cage, took composite sample
rac05	raccoon	2/24/2010	2 raccoons share cage, took composite sample
rat01	rat	2/22/2010	Santa Barbara, residential, droppings found in attic of home in foothills
rat02	rat	2/22/2010	Montecito, commercial property, droppings collected from exterior perimeter of office complex
rat03	rat	2/22/2010	Montecito, residential, droppings found in utility closet on side of house facing creek, ~15' from creek
rat04	rat	2/24/2010	droppings from bottom of cage of recently captured baby wild rat

Table 5.3. Human Waste Samples

ID	Source	Sample Date	Notes
h01	human feces	12/14/2004	from Cottage Hospital, composite of 3 individuals
h02	human feces	2002	from 1 individual (from SCCWRP)
h03	human feces	2002	from 1 individual (from SCCWRP)
h04	human feces	2002	from 1 individual (from SCCWRP)
h05	human feces	2002	from 1 individual (from SCCWRP)
h06	human feces	2002	from 1 individual (from SCCWRP)
h07	human feces	2002	from 1 individual (from SCCWRP)
h08	human feces	2002	from 1 individual (from SCCWRP)
sep01	septage	9/8/2005	from Santa Barbara Botanical Gardens
sep02	septage	10/1/2009	single residential conventional septic system (Malibu)
sep03	septage	10/1/2009	single residential advanced septic system (Malibu)
sew01	sewage	4/10/2006	El Estero WWTP confluent
sew02	sewage	10/24/2005	El Estero WWTP confluent
sew03	sewage	6/2/2006	El Estero WWTP confluent
sew04	sewage	4/17/2007	El Estero WWTP confluent
sew05	sewage	4/6/2009	El Estero WWTP confluent
sew06	sewage	7/8/2009	El Estero WWTP confluent
sew07	sewage	9/9/2009	North sewer @ Haley & Chapala - (Mission site 5)
sew08	sewage	9/9/2009	Sewer @ Nopal & Cota - (Mission site 7)
sew09	sewage	9/10/2009	North sewer @ Haley & Chapala - (Mission site 5)
sew10	sewage	9/10/2009	Sewer @ Nopal & Cota - (Mission site 7)

Table 5.4. Non-human Source Results

Animal fecal source results (dilution as determined by salmon testes DNA qPCR, average number of BacHum or HBM copies per liter filtered (copies/L) and standard error (SE), presence (+) or absence (-) of Mnif target. ND indicates that no targets were detected within the quantification range of the qPCR assay.

		TaqMan - Kildare		SYBR® Green I - Seurinck		
		BacHum-qPCR		**HBM-qPCR**		
ID	**Dilution (1/x)**	**Copies/g wet**	**SE**	**Copies/g wet**	**SE**	**Mnif-PCR**
c01	5	6.0E+04	3.9E+03	ND		-
c02	5	1.0E+05	1.8E+04	2.6E+03	1.7E+02	-
c03	10	7.1E+04	5.1E+03	ND		-
c04	10	9.7E+03	4.5E+02	ND		-
c05	20	2.2E+04	1.6E+03	ND		-
c06	40	3.9E+05	9.9E+03	ND		-
c07	5	ND		ND		-
c08	5	2.3E+03	1.8E+01	ND		-
c09	10	2.9E+05	2.6E+03	ND		-
c10	10	1.2E+04	1.3E+03	ND		-
c11	5	2.0E+03	9.8E+01	ND		-
c12	5	ND		ND		-
d01	5	ND		ND		-
d02	5	1.4E+04	6.3E+02	ND		-
d03	5	ND		ND		-
d04	5	8.9E+05	4.5E+04	ND		-
d05	5	7.7E+04	8.9E+03	ND		-
d06	5	4.0E+04	1.5E+03	ND		-
d07	5	4.2E+04	2.0E+03	ND		-
d08	5	ND		ND		-
d09	5	8.4E+05	5.4E+04	ND		-
d10	5	8.2E+04	1.0E+03	ND		-
d11	5	3.3E+05	3.9E+04	ND		-
d12	5	1.6E+05	2.5E+03	ND		-
g01	5	4.4E+02	1.8E+01	ND		-
g02	40	ND		ND		-
g03	10	ND		ND		-
rac01	5	ND		ND		-
rac02	5	ND		ND		-
rac03	5	ND		ND		-
rac04	5	1.1E+04	4.7E+02	ND		-
rac05	5	1.5E+05	7.9E+03	ND		-
rat01	5	ND		ND		-

rat02	5	ND	ND	-
rat03	5	ND	ND	-
rat04	5	ND	ND	-

Table 5.5. Human Source Results

Human waste source results (dilution as determined by salmon testes DNA qPCR, average number of BacHum or HBM copies per liter filtered (copies/L) and standard error (SE), presence (+) or absence (-) of Mnif target. ND indicates that no targets were detected within the quantification range of the qPCR assay.

		TaqMan - Kildare		SYBR® Green I - Seurinck		
		BacHum-qPCR		**HBM-qPCR**		
ID	**Dilution (1/x)**	**Copies/g wet or L**	**SE**	**Copies/g wet or L**	**SE**	**Mnif-PCR**
h01	10	9.2E+06	4.0E+05	5.0E+06	4.4E+04	-
h02	10	2.9E+05	6.2E+03	ND		-
h03	10	7.5E+06	4.3E+05	ND		-
h04	10	9.3E+04	6.0E+03	ND		-
h05	5	6.4E+04	2.4E+03	4.9E+03	1.0E+03	-
h06	10	5.1E+08	1.4E+07	5.3E+08	7.8E+06	-
h07	5	2.3E+06	6.6E+04	1.7E+06	7.0E+04	-
h08	10	7.2E+04	5.6E+03	1.3E+04	3.9E+03	-
sep01	20	6.5E+08	1.2E+07	4.9E+08	7.4E+06	+
sep02	10	7.6E+07	1.3E+06	9.8E+07	2.8E+06	+
sep03	5	4.2E+04	3.7E+03	ND		+
sew01	20	8.6E+08	4.1E+07	3.2E+08	6.0E+06	+
sew02	5	1.5E+09	3.4E+07	8.8E+08	2.6E+07	+
sew03	10	1.2E+09	2.3E+08	1.2E+09	6.5E+07	+
sew04	10	2.2E+09	7.9E+07	1.7E+09	2.0E+07	+
sew05	10	1.1E+09	3.5E+07	6.5E+08	5.9E+07	+
sew06	10	8.5E+09	7.8E+07	2.5E+09	9.3E+08	+
sew07	5	1.2E+09	4.1E+07	4.7E+08	1.7E+07	+
sew08	5	1.7E+08	1.3E+06	7.0E+07	4.4E+05	+
sew09	10	7.4E+08	6.3E+07	6.9E+08	9.1E+07	+
sew10	5	6.0E+07	7.7E+05	4.0E+07	1.5E+07	+

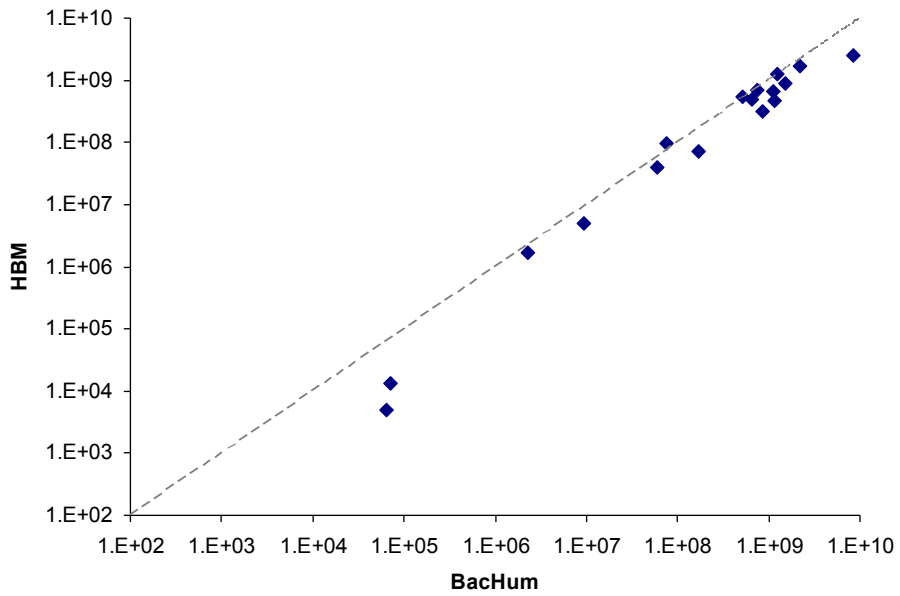


Figure 5.1. Average values of human waste source samples that were quantified in both assays, plotted against each other in log scale ($y = 0.062x^{1.1143}$, $R^2 = 0.9783$). Dashed line indicates 1:1 ratio. The BacHum assay reported higher average copy numbers for 14 of the 17 samples.

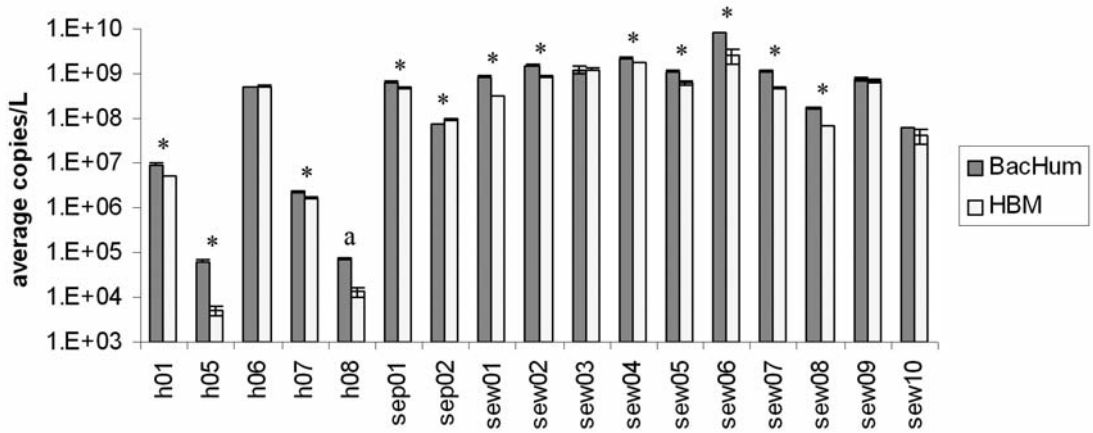


Figure 5.2. Comparison of BacHum and HBM human waste source samples that were quantified in both assays (average number of BacHum or HBM copies per liter filtered and standard error). * indicates significant difference between BacHum and HBM results using all analytical replicates for that sample ($p < 0.05$, paired t-test). [a] indicates sample could not be analyzed via paired t-test due to unequal number of replicates amplifying in both assays.

VI. Fecal Source Identification in the Mission Creek Area using PhyloChip Analysis

6.1 Summary

The objective of this project was to use the Lawrence Berkeley National Laboratory (LBL) PhyloChip to identify sources of fecal contamination around Mission Creek in Santa Barbara, CA. We used the PhyloChip to define which types of bacteria can be used to identify and distinguish suspected sources of fecal contamination in this area. We then applied this information to determine the potential contribution of these fecal sources to high fecal indicator bacteria counts in Mission Creek waters.

Fecal sources analyzed in this study were sewage, septage, and feces from humans, cats, dogs, gulls, raccoons and rats. Each fecal source contained an average of 4000 to 15,000 different bacterial taxa. To identify which of these taxa could be used to distinguish sources we grouped samples into five source types based on similarities in bacterial community composition: 1) human feces and sewage/septage, 2) cats and dogs, 3) gulls, 4) raccoons 5) rats. We identified 25 to 230 taxa per source type that can be used to identify or exclude each one as a source of fecal contamination in water samples

The occurrence and abundance of these source identifier taxa were then used to detect the influence of each source on the microbial community profiles measured in water monitoring samples. The results showed a large increase in the occurrence and abundance of human fecal identifier taxa in Nopal/Canon Perdido samples, with up to 80% detection and a significant increase in abundance of human fecal identifier taxa in these waters. No human fecal influences were detected at any other locations, including a sample taken from AB Lagoon in 2005 where human bacteroides marker was detected. Cat and dog fecal bacteria were detected only at Nopal/Canon Perdido, and followed similar detection patterns as the human fecal identifier taxa. These results suggest fecal material from these animals is input by sewage at this site. The detection rate of gull and raccoon identifier taxa was generally low at all sites, however their occurrence in one Haley and Chapala sample suggests inputs from these animals may contribute to high FIB counts at this location. No rat fecal influences were detected in any samples.

Results of this study indicate that analysis of the entire bacterial community with PhyloChip is a powerful method for identifying or excluding sources of fecal contamination. Human fecal sources and to a lesser extent cat and dog feces are responsible for high concentrations of fecal indicator bacteria at Nopal/Canon Perdido. At other sites the fecal sources measured in this study appear to have a minor or insignificant influence on fecal indicator bacteria. Identification of potential sources at these sites should consider other potential sources, including possible non-fecal inputs of fecal indicator bacteria

6.2 Introduction

The Lawrence Berkeley National Laboratory PhyloChip is a rapid method of microbial community analysis that detects over 50,000 different types of bacteria and archaea in a single test. The test probes for specific sequences of microbial DNA that allow taxonomic identification of nearly all bacteria and archaea. Each PhyloChip analysis yields a comprehensive inventory of most bacteria and archaea that occur in a sample, and quantifies the relative amounts of each type of microbe across samples. This method differs from other source tracking methods in that it measures thousands as opposed to one or a few types bacteria, and provides high-resolution taxonomic detail about each detected organism using a standardized measurement platform. If sufficient differences in community composition exist among different suspected sources of fecal contamination, the PhyloChip may be an extremely useful tool for detecting or excluding these sources in water samples that have unknown sources of fecal contamination.

We expected that microbial communities in both fecal source communities and waters impacted by fecal pollution would be highly complex and variable from sample to sample. With the current state of the technology, the only other viable option for such a broad level community analysis with the potential for identification of multiple types of bacteria for source-specific fecal identification was to use next-generation sequencing technologies, such as pyrosequencing. Hybridization based technologies, such as the PhyloChip, use the entire 16S rRNA gene amplified product for identification and determination of relative abundance. In contrast, next-generation sequencing uses a serial identification of a small fraction of the amplified products to examine thousands of individual fragments that is still only a small fraction of the total amplified product. This serial amplicon identification creates a number of sampling artifacts in complex samples, such as under-sampling, unequal sampling, random sampling, and taxonomic lumping (Zhou et al. 2008). Given the complexity of the fecal and watershed communities, random sampling process could have more dramatic influence on estimating the difference of microbial communities across different samples (i.e., β-diversity). PhyloChip analysis can reproducibly identify low abundance members of a community, which is essential when trying to identify sources that will be considerably diluted in receiving waters (DeSantis et al. 2007).

While the PhyloChip is an established platform for microbial community analysis and research, its application to microbial source tracking in water is only now under development. The problem of fecal source tracking in the Mission Creek area is a good test case for this method because of the multitude of sources that potentially impact water quality in this area. The approach of this study was to use the PhyloChip to define the characteristic microbiome of suspected sources of fecal contamination to Mission Creek. Differences in the microbial community composition among these sources were than used to define groups of bacteria that could be used to identify each source in waters impacted by high counts of fecal indicator bacteria. The method was applied to problematic water samples collected from the Mission Creek area.

6.3 Methods

6.3.1 Sample Description

LBL analyzed DNA extracts from 24 fecal source samples and 16 water samples provided by UC Santa Barbara. Fecal source samples included sewage, septage, and feces from humans, cats, dogs, gulls, raccoons and rats. Each fecal sample was pooled from multiple individuals, and three biological replicate samples were analyzed for each source type. Two gull samples (Gull 2 and Gull 3) could not be PCR amplified, likely due to inhibition and/or insufficient DNA concentration. To compensate for the lack of gull data, LBL contributed gull feces data collected previously with SCCWRP for two gull populations located at Huntington Beach and Long Beach (Cherry Beach).

Table 6.1 describes the analyzed water samples. Water sample M0719-21 (City Creeks Well groundwater) could not be analyzed by PhyloChip due to insufficient DNA template.

Table 6.1. Water samples analyzed by PhyloChip

Sample	Date	Location
M0729-30	7/29/10	Rattlesnake Canyon, bridge near Skofield Park
M0729-29	7/29/10	Mission Creek at botanical garden
M0719-22	7/19/10	Mission Creek at Rocky Nook Park
L0904-17	9/4/09	City Annex Yard
L0911-17	9/11/09	City Annex Yard
M0729-23	7/29/10	City Annex Yard, MH closest to outlet
M0820-9	8/20/09	Nopal & Canon Perdido
M0701-9	7/1/10	Nopal & Canon Perdido
M0709-9	7/9/10	Nopal & Canon Perdido
M0729-24	7/29/10	Laguna Lagoon in front of wall
M0729-26	7/29/10	AB Lagoon mouth, near rock outcropping
823-02	8/23/05	AB Lagoon mouth (2005)
M0819-5	8/19/09	Haley & Chapala (same as 2006 sampling)
M0909-5	9/9/09	Haley & Chapala (same as 2006 sampling)
M0910-5	9/10/09	Haley & Chapala (same as 2006 sampling)

6.3.2 Polymerase Chain Reaction

The 16S rRNA gene was amplified from each sample using PCR with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') for bacteria and 4Fa (5'- TCCGGTTGATCCTGCCRG-3') and 1492R for archaea. Each PCR reaction contained 1× Ex Taq buffer (Takara Bio Inc., Japan), 0.025 units/µl Ex Taq polymerase, 0.8 mM dNTP mixture, 1.0 µg/µl BSA, and 200 pM each primer and 1 ng genomic DNA (gDNA) as template for fecal samples and 10 ng gDNA for water samples. For the PhyloChip assay each sample was amplified in 8 replicate 25 µl reactions spanning a range of annealing

temperatures. PCR conditions were 95°C (3 min), followed by 30 cycles 95°C (30 s), 48-58°C (25 s), 72°C (2 min), followed by a final extension 72°C (10 min). Amplicons from each reaction were pooled for each sample, purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA), and eluted in 50 µL elution buffer.

6.3.3 PhyloChip Assay Design

A complete description of the PhyloChip design and analysis is described in the supplementary methods of Hazen et al. (2010). The PhyloChip was designed to detect most 16S rRNA gene sequences that identify bacteria and archaea. Aligned sequences were retrieved from the 16S rRNA gene database, greengenes.lbl.gov (DeSantis et al. 2006). The sequences were clustered to enable selection of perfectly complementary probes representing each sequence of a cluster. Putative amplicons containing 17-mers with sequence identity to a cluster were included in that cluster. The resulting 59,959 clusters, each encapsulating an average of 0.5% sequence divergence, were considered operational taxonomic units (OTUs). The OTUs represented 2 domains, 147 phyla, 1,123 classes, and 1, 219 orders demarcated within the archaea and bacteria. Each OTU was assigned to one of 1,464 families according to the placement of its member organisms in the taxonomic outline as maintained by Philip Hugenholtz (Hugenholtz 2002).

For each OTU, multiple specific 25-mer targets were sought for prevalence in members of a given OTU but dissimilar from sequences outside the given OTU. Probes complementary to target sequences that were selected for fabrication are termed perfectly matching (PM) probes. As each PM probe was chosen, it was paired with a control 25-mer (mismatching probe, MM), identical in all positions except the thirteenth base. The MM probe did not contain a central 17-mer complimentary to sequences in any OTU. The probe complementing the target PM and MM probes constitute a probe pair analyzed together. The average number of probe pairs assigned to each OTU was 37 (s.d. 9.6).

The chosen oligonucleotides were synthesized by a photolithographic method at Affymetrix Inc. (Santa Clara, CA) directly onto a glass surface at an approximate density of 10,000 molecules per µm2 and placed into "midi 100 format" hybridization cartridges. The entire array of 1,016,064 probe features was arranged as a grid of 1,008 rows and columns. Of these features, the majority represents publicly available 16S rRNA genes, as described above. Additional probes are for quality management, processing controls, image orientation, normalization controls, hierarchical taxonomic identification, or for pathogen-specific signature detection and some implement additional targeted regions of the chromosome.

6.3.4 Preparation of Samples for PhyloChip Assays

For PhyloChip hybridization, 500 ng of bacterial PCR product were prepared for PhyloChip hybridization. PCR products were fragmented with DNAse I to a range of 50-200 bp as verified by agarose gels. Commercial kits were utilized for DNA preparation: Affymetrix (Santa Clara, CA) WT Double Stranded DNA Terminal Labeling, and Affymetrix GeneChip Hybridization, Wash, and Stain kits were used for PhyloChip analysis. Briefly, fragmented

16S amplicons and non-16S quantitative amplicon reference controls were labeled with biotin in 40 µL reactions containing: 8 µL of 5X TDF buffer, 40 units of TDF, 3.32 nanomoles of GeneChip labeling reagent. After incubating at 37°C for 60 min, 2 µL of 0.5M EDTA was added to terminate the reaction. Labeled DNA was combined with 65µL of 2X MES hybridization buffer, 20.4 µL of DMSO, 2 µL of Affymetrix control oligo B2, and 0.4 µL nuclease free water. Each reaction mixture was injected into the hybridization chamber of an array cartridge and incubated for 16 h in an Affymetrix hybridization oven at 48°C and 60 RPM. Hybridization solution was t removed and the microarrays were stained and scanned according to the manufacturers instructions.

6.3.5 PhyloChip Assay Analysis

Fluorescent images were captured with the GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA). An individual array feature occupied approximately 8x8 pixels in the image file corresponding to a single probe 25mer on the surface. The central 9 pixels were ranked by intensity and the 75% percentile was used as the summary intensity for the feature. Probe intensities were background-subtracted and scaled to the Quantitative Standards (non-16S spike-ins) and outliers were identified as previously described (DeSantis et al. 2007). The hybridization score (HybScore) for an OTU was calculated as the mean intensity of the perfectly matching probes exclusive of the maximum and minimum. Procedures for presence/absence scoring are described in Hazen et al. (2010).

6.3.6 Determination of source identifier taxa

We determined which bacterial taxa were unique to each source. Source identifier taxa were defined as individual OTUs that were detected in samples of a single source type, but never detected in any samples from other sources. Once lists of identifier taxa were established for each source, the presence of those taxa was determined in individual water samples. Results are reported as the percent of source-specific identifier taxa that were detected in each sample. In addition, array intensities of all identifier taxa were compared among samples to determine trends in the abundance of these taxa for each source. An increase in array intensity for an identifier OTU signals that it is increasing in dominance from one sample to the next. Thus, a simultaneous increase in array intensity for many identifier OTUs for a given source type should signify increased contribution of that source to the overall composition of the microbial community.

6.4 Results and Discussion

6.4.1 Fecal microbial communities and identifier taxa

We detected 31,559 different bacterial OTUs in 2873 subfamilies (minimum 94-97% similarity among taxa) that occurred in at least one of the 24 fecal samples. In water samples, 26019 OTUs in 2259 subfamilies were detected in at least one of the 15 samples. Each source type contains bacterial OTUs in approximately 300-400 subfamilies that are candidate identifier taxa (Table 6.2). Up to 844 subfamilies were detected in individual water samples. In addition to bacteria, archaeal 16S rRNA gene amplification was

attempted on the fecal samples. We found archaea readily amplified for only some samples (0/3 cat, 1/3 dog, 1/3 gull, 1/3 rat, 3/3 human, 3/3 sewage, 3/3 septage) and did not have enough DNA template to completely troubleshoot archaeal PCR for all samples. To maintain consistency no archaea were analyzed by PhyloChip for any samples. All reported results are for bacteria only.

Table 6.2. Taxonomic richness of bacteria in fecal sources. Values in parentheses are standard errors.

Source	Subfamily richness	OTU richness
Cat	377 (27)	7022 (691)
Dog	390 (21)	6630 (575)
Gull	291 (24)	3868 (906)
Human	317 (11)	8265 (570)
Raccoon	318 (11)	4917 (225)
Rat	411 (61)	7701 (2624)
Septage	392 (36)	9479 (641)
Sewage	432 (43)	14745 (1696)

Community structure was analyzed using multivariate ordination techniques to determine the relative similarities among bacterial communities. Ordinations of community structure were conducted using both principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) with both Bray-Curtis and Unifrac (phylogenetic) distance measures.

Fecal sources could be discriminated by their community structure (Figures 6.1 and 6.2). Generally, bacterial communities from a particular source type clustered more closely together than with bacterial communities from other source types, meaning different sources have distinctive bacterial composition. Different ordination methods and distance measures yielded similar results. Most variation among samples (74.5%) could be projected in three dimensions (Figures 6.1 and 6.2). Fecal bacterial communities in gulls and raccoons were the most distinct and accounted for a large portion of the variance in the dataset represented by the first principal component (Figure 6.1). Principal components 2 and 3 showed discrimination among other waste types (Figures 6.1 and 6.2).

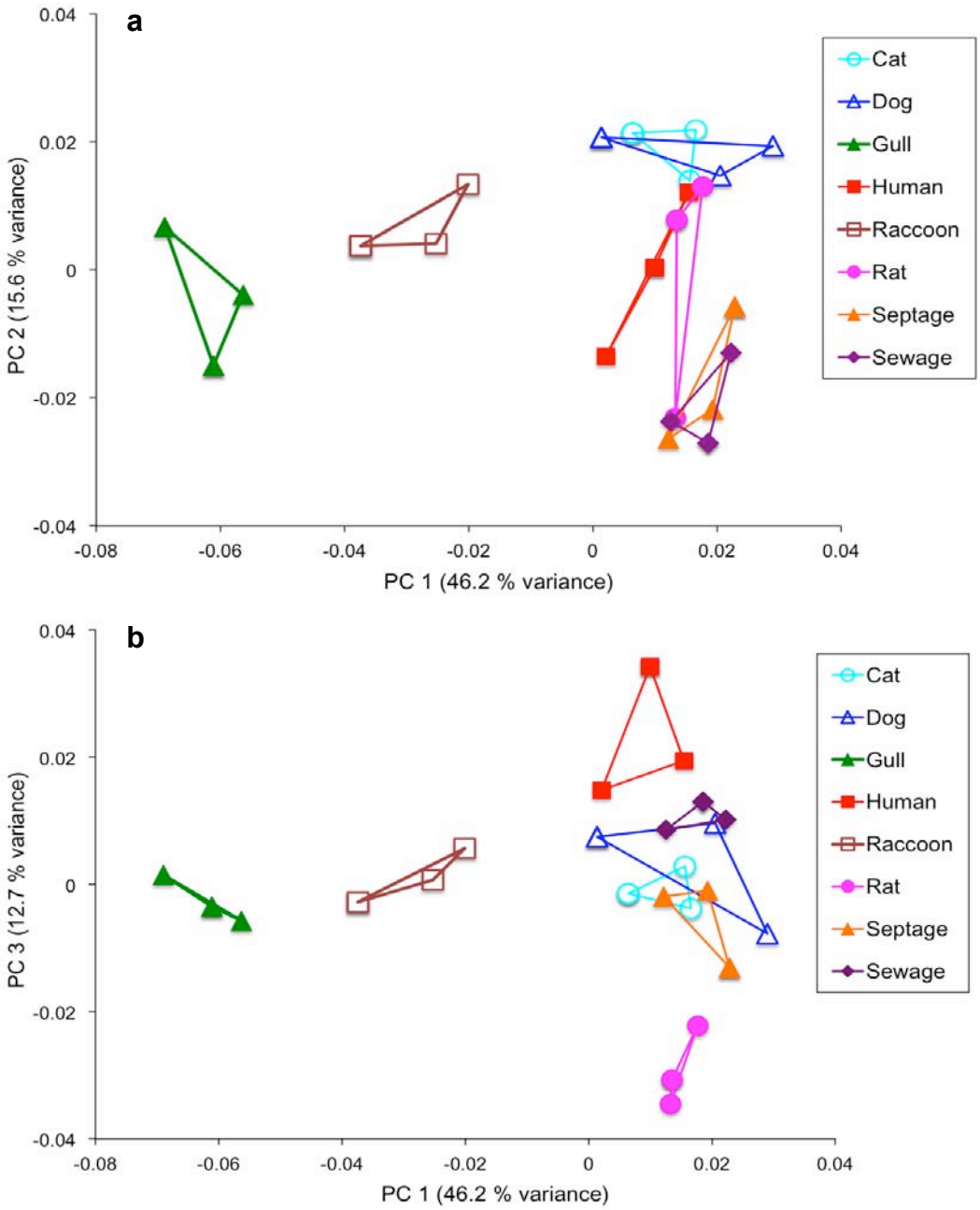


Figure 6.1. Analysis of bacterial community structure of fecal samples. Similarity among samples calculated using the Bray-Curtis similarity measure and analyzed using principal components analysis. The first three principal components (PC) project most of the variance among communities (74.5%). Shown are biplots of PC 1 versus PC 2 (1a) and PC1 versus PC 3 (1b).

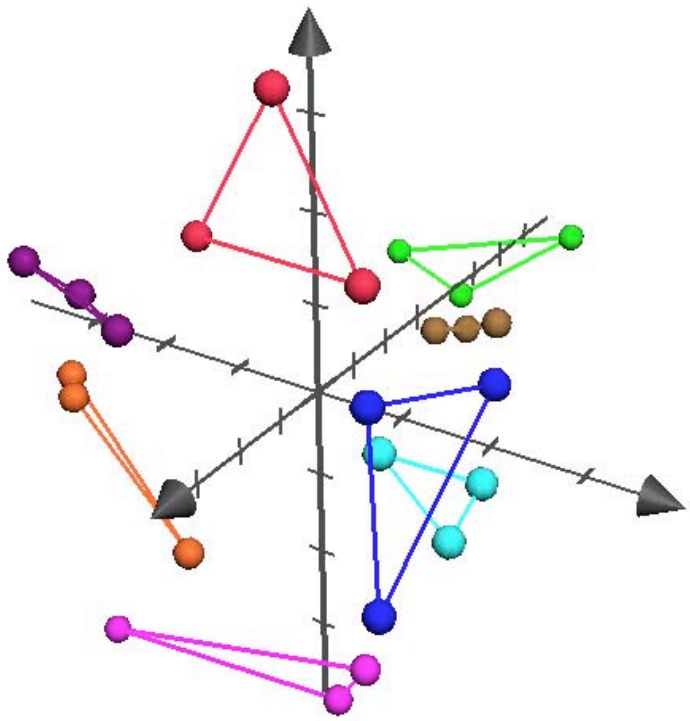


Figure 6.2. Three-dimensional biplot of principal components analysis of fecal bacterial communities. Cat (light blue), Dog (dark blue), Gull (green), Human (red), Raccoon (brown), Rat (light purple), Septage (orange), Sewage (dark purple).

To identify source identifier taxa we grouped the fecal samples into five types of fecal sources: human-derived wastes (human feces, sewage, septage), small domestic animals (cats, dogs), gulls, raccoons and rats. Identifier taxa were identified as those that occurred in the majority or all of samples in only one source type and never in any other source type (Table 6.3). For human sources and rats, identifier taxa were recruited if they occurred in every single sample of each respective source (9/9 human sources, 3/3 rats). For other source types, identifier taxa were recruited if they occurred in a majority of samples of each respective source (4/6 cats and dogs, 2/3 gulls, 2/3 raccoons). The PhyloChip detected far more unique taxa for human sources and rats, so recruitment criteria for these source types were made more stringent than other animal sources.

Most fecal identifier taxa that distinguished source types are different Firmicutes OTUs (Table 6.3). Many unique types of Clostridia and Bacilli occur in each fecal type, and thus serve as the best taxa to identify each waste type. Human wastes (feces, sewage, septage) share 230 different taxa that are never detected in other animal sources but reliably detected in human sources, and all but one of these taxa are classified as Firmicutes (Clostridia). In addition to Clostridia, several Actinobacteria were unique to cats and dogs, and many Bacteroidetes were unique to rats. A variety of other taxa were unique to gulls and raccoons including several types of Proteobacteria.

Table 6.3. Composition of identifier taxa for each fecal source type.

	Cat+Dog	Gull	Human	Raccoon	Rat
ABY1_OD1	0	1	0	0	0
Acidobacteria	0	1	0	0	0
Actinobacteria	8	1	0	1	1
Bacteroidetes	0	0	1	1	57
Caldithrix	0	0	0	1	0
Chloroflexi	0	0	0	1	0
Elusimicrobia	0	0	0	1	0
Firmicutes	16	40	229	47	53
Fusobacteria	1	2	0	1	0
Kazan-3B-22	0	0	0	1	0
Alphaproteobacteria	0	1	0	2	1
Deltaproteobacteria	0	2	0	1	0
Epsilonproteobacteria	0	2	0	0	0
Gammaproteobacteria	0	7	0	7	0
SC4	0	0	0	1	0
Thermosulfidobacterium	0	1	0	0	0
TM7	0	1	0	0	1
ZB2	0	1	0	0	0
TOTAL	**25**	**60**	**230**	**65**	**113**

6.4.2 Detection of fecal sources in water samples

Water samples contained between 2768 and 12345 different bacterial taxa (Table 6.4) that were analyzed for differences in community composition and the occurrence of fecal source identifier taxa.

Table 6.4. Bacteria taxonomic richness and fecal indicator counts in water samples.

Location	Sample ID	Subfamily richness	OTU richness	*E. coli* (MPN)	Enterococcus (MPN)	HBM (copies/L)
Rattlesnake Cyn	M0729-30	796	9472	122	389	ND
Mission Ck-botanical	M0729-29	479	5290	86	393	ND
Mission Ck-Rocky	M0719-22	593	5142	355	2481	ND
City Annex	L0904-17	670	10393	173289	37844	ND
City Annex	L0911-17	473	5681	20110	7450	ND
City Annex-outlet	M0729-23	696	8116	960	2755	ND
Nopal & Canon	M0820-9	793	12345	24196	14000	3.60E+07
Nopal & Canon	M0701-9	844	11711	959	52	1.30E+05
Nopal & Canon	M0709-9	774	10521	3448	545	5.30E+04
Laguna Lagoon	M0729-24	511	4319	502	375	ND
AB Lagoon-outcrop	M0729-26	714	11844	3076	10	ND
AB Lagoon-2005	823-02	520	7841	213	233	1.30E+04
Haley & Chapala	M0819-5	705	11472	3873	6488	ND
Haley & Chapala	M0909-5	519	6034	350	2046	ND
Haley & Chapala	M0910-5	508	2768	420	1421	ND

Community analysis of water samples shows variation in bacterial structure exists among different samples (Figure 6.3). Nopal/Canon Perdido samples that were distinct from other samples were also the samples in the dataset with the high fecal indicator counts and detectable human bacteroides (Table 6.4).

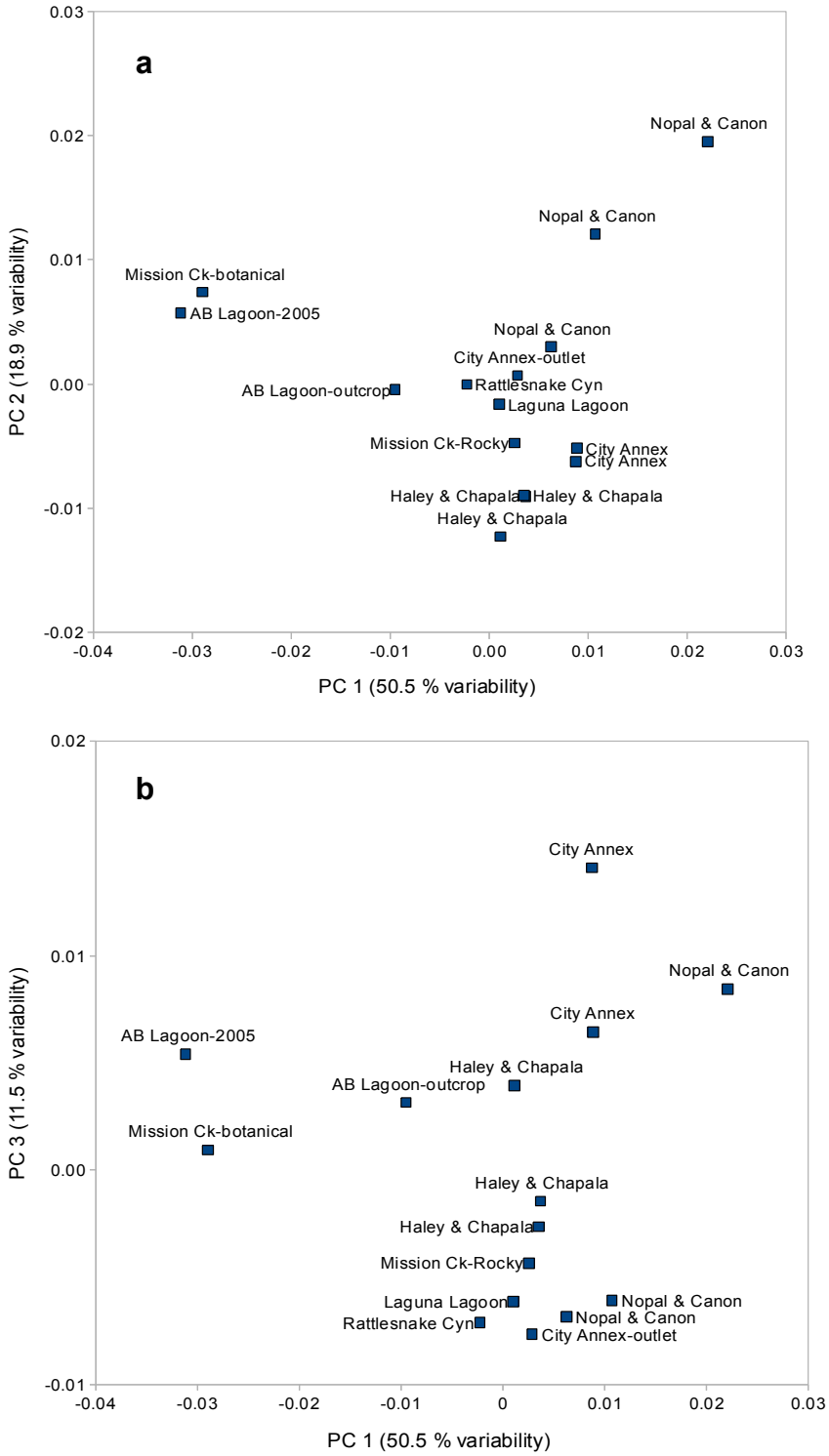


Figure 6.3. Analysis of bacterial community structure of water samples. The first three principal components (PC) project 80.9% of the variance among communities. Shown are biplots of PC 1 versus PC 2 (a) and PC1 versus PC 3 (b).

The results of the source tracking analysis show both elevated occurrence and abundance of human-specific identifiers and Nopal/Canon Perdido for all three sampling dates (Figure 6.4). In one sample from this site 80% of human-specific identifier taxa were detected. Changes in the magnitude of occurrence and abundance are consistent with patterns in the abundance of fecal indicator bacteria and human bacteroides marker, supporting the conclusion that human feces is the primary source of high fecal indicator counts at this site. In the sample collected on 8/20/09, the occurrence and abundance of cat/dog identifier taxa was elevated relative to other samples. These animals appear to be a secondary contributor to high counts of fecal indicator bacteria at this site and their source input may be the same as the detected human identifier taxa, probably sewage.

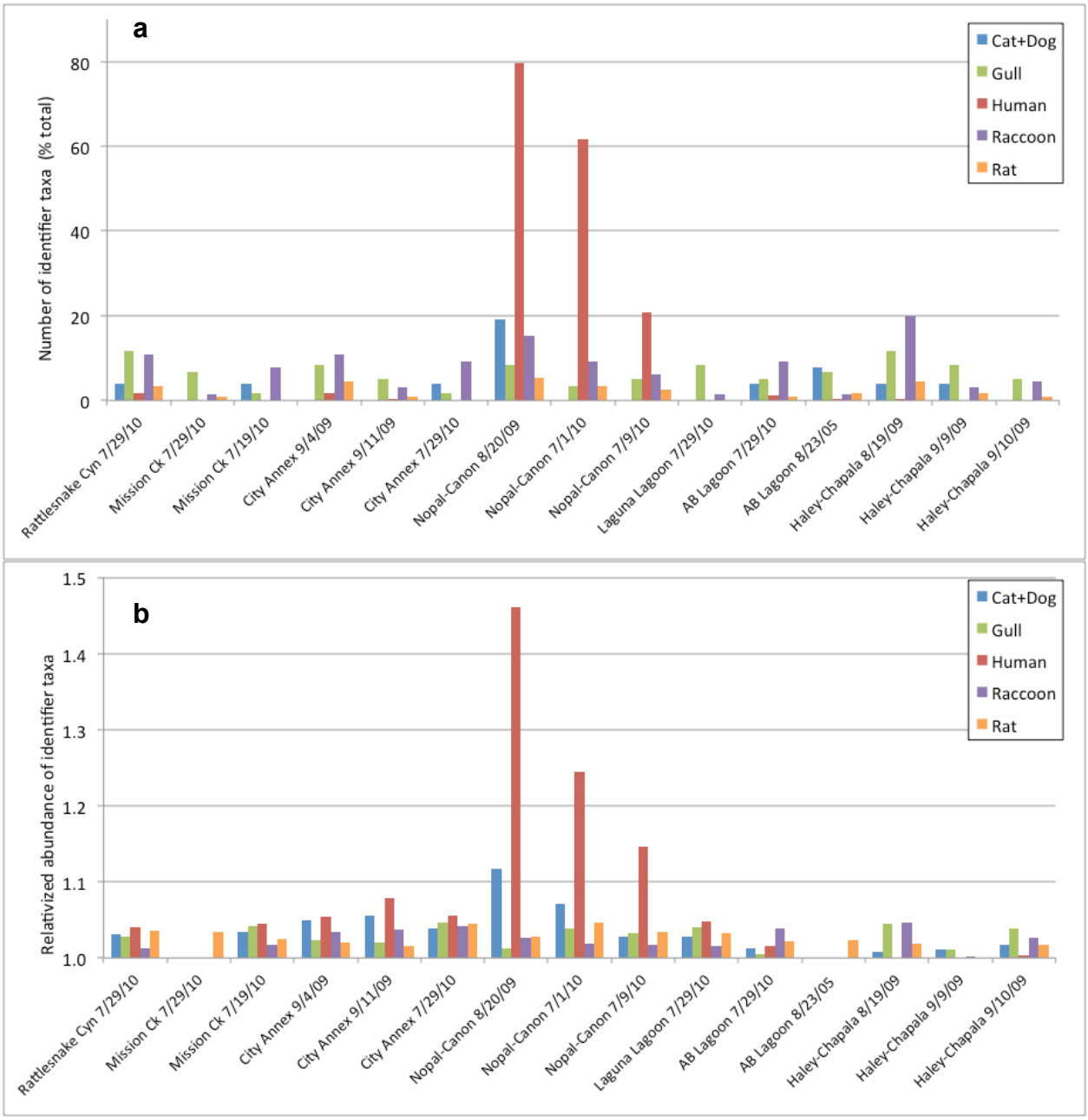


Figure 6.4. Occurrence (a) and abundance (b) of source identifier taxa in water samples. Occurrence is measured as the percent of all source-specific identifier taxa that were detected in the water microbial community (a). Relativized abundance of identifier taxa is measured as the mean microarray intensity of each set of source identifier taxa divided by the mean first quartile (Q1) value of each set of source identifier taxa for all samples (b). Relativized abundance is calculated using the complete set of identifier taxa for all samples, therefore it is possible that a source type may have a reported abundance value even if there were no identifier taxa detected in a sample.

The absence of high numbers of human identifier taxa at all other sites in this study indicates that human fecal sources are not important contributors to fecal indicator bacteria measured at locations other than Nopal/Canon Perdido (Figure 6.4). This includes the sample taken at AB Lagoon in 2005 that showed detectable human bacteroides (Table 6.4).

PhyloChip analysis suggests there may be some contribution of fecal bacteria from gulls and raccoons in some samples. In Rattlesnake Canyon and the highest FIB samples from both City Annex Yard and Haley-Chapala, 11-20% of gull and raccoon identifier taxa were detected. These numbers are higher than other sites or animals. However, this relatively low rate of detection and minor increase in relative abundance does not offer convincing evidence that these sources are the primary cause of high fecal indicator counts (Table 6.4) and differences in overall microbial community structure (Figure 6.4).

Cats and dogs are not responsible for high fecal indicator counts at sites other than Nopal/Canon Perdido. Rat fecal inputs appear negligible at all sites (Figure6.4).

In the analysis presented here, spikes in both occurrence and abundance of identifier taxa is the most reliable signal that a fecal source is present. The results offer convincing evidence that human fecal inputs are a concern at Nopal/Canon Perdido but not important at other locations. There is little evidence that other sources measured in this study are primarily responsible for high fecal indicator counts at other locations. It should be noted, however, that the detection rate of fecal taxa in water is a function of the various fates of each source identifier as it is diluted and acted upon by the environment. At this time there are no defined thresholds of occurrence and abundance for source identifier taxa that signify the importance of a particular source. Ongoing research at Lawrence Berkeley Lab will improve data interpretation.

Another challenge in this analysis is that the expected microbial composition in waters with no water quality problems remains undefined. Additional research needs to establish the background levels of source identifier taxa expected in surface waters with low fecal indicator counts in order to define statistical aberrations in the microbial community that are associated with elevated concentrations of fecal indicator bacteria. The samples in the surface waters analyzed in this study all exceeded FIB limits. Because the analysis of array intensity (abundance) relies on comparison among chips, if a particular fecal source is common to all samples then it may not stand out in the current analysis. Nonetheless, samples with relatively low abundance of identifier taxa for a specific source also had low numbers of detected identifier taxa (Figure 6.4), giving us confidence in these results.

6.5 Conclusion

Results of this study indicate that analysis of the entire bacterial community with PhyloChip is a powerful method for identifying or excluding sources of fecal contamination. Human fecal sources and to a lesser extent cat and dog feces are responsible for high concentrations of fecal indicator bacteria at Nopal/Canon Perdido. At other sites the fecal

sources measured in this study appear to have a minor or insignificant influence on fecal indicator bacteria. PhyloChip characterization of additional FIB source types (both fecal and non-fecal) and water samples (both pristine and contaminated) will help pinpoint additional causes of high fecal indicator bacteria in the Mission Creek area.

6.6 References

DeSantis, T. Z., P. Hugenholtz, P., N. Larsen, M. Rojas, E.L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, G.L.Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 72, 5069-5072.

DeSantis, T. Z., E.L. Brodie, J.P. Moberg, I.X. Zubieta, Y.M. Piceno, G.L. Andersen. 2007. High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. *Microb Ecol* 53, 371-383.

Hazen, T.C., E. A. Dubinsky, T. Z. DeSantis, G. L. Andersen, Y. M. Piceno, N. Singh, et al. 2010. Deep-Sea Oil Plume Enriches Indigenous Oil-Degrading Bacteria. *Science* 330, 204-208.

Hugenholtz, P. and T. Huber. 2002. Exploring prokaryotic diversity in the genomic era. *Genome Biol* **3**, 289-293.

Zhou JZ, Kang S, Schadt CW, Garten CT (2008). Spatial scaling of functional gene diversity across various microbial taxa. *Proc Natl Acad Sci U S A* **105:** 7768-7773.

VII. ALTERNATIVE INDICATORS FOR IDENTIFYING SEWAGE POLLUTION

7.1 Summary

Various sewage-specific DNA markers are available for sensitive and specific identification of sewage pollution in water samples. However, monitoring of sewage-specific DNA markers is not readily available for most communities, and outsourcing is expensive. Therefore, this study investigates the usefulness of alternative indicators, including nutrients, fecal indicator bacteria, anionic surfactants, caffeine and cotinine, and optical brighteners. These alternative indicators vary in their specificity and sensitivity to sewage, but may be useful for preliminary screening of urban storm drains.

Accuracy and precision of assays using colorimetry (NH_3-N, PO_4, NO_3-N, SO_4, and anionic surfactants) were determined. Fecal indicator bacteria were quantified by culture-based methods (IDEXX) and caffeine and cotinine by ELISA. Quantification of optical brighteners using fluorometry was tested, but was too insensitive to be useful. The concentrations of human-specific indicators, FIB and all alternative indicators in sewage and other potential source water samples were compared with concentrations in a variety of environmental samples (storm drains, creeks, lagoons, ocean). The usefulness of alternative indicators for identifying sewage pollution is discussed.

7.2 Methods

7.2.1 Sampling locations and experimental design

Ninety-six samples were collected in the period May-October 2010 in different watersheds in Santa Barbara, CA : Arroyo Burro watershed (A-samples), Haley drain (H-samples), Mission Creek watershed (M-samples), Laguna watershed (L-samples), and Nopal watershed (N-samples) (Fig. 7.1, Table 7.1). In addition, sewage (SEW), reclaimed water (REC) and groundwater (GW) were collected.

A subset of the samples collected during 06/10 (Table 7.1), was used for preliminary testing of fluorometry for optical brighteners (see section 7.2.2) and colorimetry for quantifying nutrients and anionic surfactants (see section 7.2.3). Subsequently, more samples were taken and analyzed for FIB (SOP #H02), HBM concentrations (SOP #H05), Mnif presence/absence (SOP #H11), caffeine (SOP #H12), cotinine (SOP #H13), anionic surfactants, NH_3-N, PO_4, NO_3-N, and SO_4.

7.2.2 Preliminary testing of fluorometry for optical brighteners

Presence or absence of optical brighteners was determined using fluorometry, following the protocol of Cao et al. (2009). In summary, the protocol consists of measuring fluorescence of a water sample before and after exposure to ultraviolet light. The decrease in fluorescence upon UV exposure is proportional to the concentration of optical brighteners. The exposure to UV light causes photodegradation of labile optical

brighteners, while most other dissolved organic matter do not degrade and therefore do not contribute to the fluorescence decrease. Fluorescence is measured using excitation and emission wavelengths of 375 nm and 445 nm, respectively, with an Aquafluor handheld fluorometer (Turner designs, Sunnyvale, CA). Samples are measured in 2 ml methacrylate cuvets, and fluorescence was measured before UV exposure, after 5 minutes UV exposure and after 10 minutes UV exposure in a cabinet equipped with one 6W 365 nm UV lamp. The fluorometer was calibrated to µl/L Tide equivalents. The decision criteria for determining if a sample is positive or negative for optical brighteners are shown in Fig. 7.2 (from Cao et al., 2009).

7.2.3 Preliminary testing of colorimetry for nutrients and anionic surfactants

The nutrients NH_3-N, PO_4, NO_3-N, SO_4, P, K, and anionic surfactants, were initially selected for testing in this study, based on their high expected concentrations in sewage (Table 7.2), or literature indicating their usefulness for identifying sewage pollution (Brown et al., 2004). Boron and potassium can potentially be used to distinguish between sewage contamination and other types of contamination (Brown et al., 2004) (Fig. 7.3), while NO_3 was selected because it can be formed by biological oxidation of NH_3 and may therefore be indirectly indicative of sewage contamination. Colorimetric quantification of NH_3-N, PO_4, NO_3-N, SO_4, P, K and anionic surfactants was performed using a Lamotte Smart2 colorimeter, according to the manufacturer's protocols. All samples were filtered through 0.22 µm filters and saved at 4 ºC prior to analysis.

First, water samples were taken on 6/3/2010 and analyzed at different dilutions to determine the occurrence of matrix effects interfering with accurate quantification. Analytes for which concentrations varied with sample dilutions were excluded as alternative indicators. Second, accuracy of all remaining assays was determined by analyzing standard curves. Standard curves included blanks and 3 concentration levels, and were prepared in triplicate, by adding known quantities of NH_4Cl, KNO_3, K_3PO_4, K_2SO_4 and the anionic surfactant sodium dodecyl sulfate (SDS) to deionized water. Third, matrix interference was tested by analyzing spiked samples, as recommended by the manufacturer. For spiking experiments, 3 storm drain samples (N4, L4 and L8) were collected and approximately 1 mg/L of NH_4Cl, KNO_3, K_3PO_4, K_2SO_4 and SDS were added, in triplicate. The measured and theoretical concentrations of each chemical after spiking were compared to determine the occurrence of sample matrix interference during quantification.

7.2.4 Nutrients by flow injection analysis

The nutrients NH_4^+, NO_2+NO_3 and PO_4 were also quantified using flow injection analysis. Water samples were filtered through 0.22 µm filters and stored at -20 ºC in HDPE bottles. Samples were sent to the Marine Science Institute analytical lab at UCSB for analysis.

7.3 Results

7.3.1 Preliminary testing of alternative sewage indicators

7.3.1.1 Optical brighteners

In total, 16 samples were analyzed for optical brighteners. Although all samples were positive based on the first criterion (sample fluorescence > 5 µl/L), none were positive based on the second criterion (fluorescence decrease after 5 min > 8%) (Table 7.3). Even samples A5 and A6, with significant amounts of sewage pollution based on HBM concentrations (see Section 7.3.2.1), were negative. Therefore, it was concluded that the protocol for detecting optical brighteners was not sensitive enough, and it was not further tested.

7.3.1.2 Accuracy of colorimetric assays based on sample dilution

Preliminary testing was performed to determine possible matrix interference for all colorimetric assays listed in Table 7.1, by analyzing various sample dilutions (Fig. 7.3). The grouped bars in Fig. 7.3 indicate sample concentrations for the same sample at different dilutions. The x-axis lists the dilutions, e.g. analyzed undiluted and 1:4 diluted is indicated by 1:4. Samples that are over-range are indicated by * (bar indicates minimum estimate). The concentrations of NH_3-N, NO_3-N, PO_4 and SO_4 are fairly consistent when using different dilutions. However, concentrations of K are up to tenfold lower when samples are diluted, indicating positive interference in less dilute samples. Also, concentrations of B often increase, up to fivefold, when samples are diluted, indicating negative interference in less dilute samples. Finally, surfactants concentrations vary significantly, at least twofold, when analyzing dilutions, with no consistent pattern of positive or negative interference. Therefore, colorimetry using the Lamotte Smart2 colorimeter appears not suitable for quantification of B, K and anionic surfactants.

7.3.1.3 Accuracy and precision of selected chemical indicator assays

Based on the results presented in Section 7.3.1.2, NH_3-N, NO_3-N, PO_4 and SO_4 were selected as alternative chemical indicators for comparison with sewage-specific indicators. Also, a new assay for quantifying anionic surfactants was added at this stage, using colorimetry but with a Hanna Instruments photometer.

Standard curves showed that the expected and measured chemical concentrations were slightly different in most cases (Table 7.4). For NH_3-N and PO_4, measured concentrations were within 17% of expected concentrations. For NO_3-N and SO_4, measured concentrations were within 10% of the expected concentrations for the mid to high ranges, but between 37-54% different for the lowest range. For SDS accuracy was lower, and measured and expected concentrations differed greatly (47-360%). Coefficients of variation (CVs) were maximally 5% for NH_3-N, PO_4 and SO_4, for the whole range of the assays (not including blanks). CVs for NO_3-N and SDS were slightly higher, between 4-11% and 7-17% respectively. CVs for the blanks were usually higher, but did not affect measurements greatly because absolute values were mostly low (< 5% of assay range). However, for SDS, variability in blank concentrations greatly influenced measured concentrations in the assay range below 1 mg/L, since blank concentrations were between 0.2-0.6 mg/L.

The standard curves (Fig. 7.5) all had high correlation coefficients ($R^2 > 0.98$), and regression curve slopes were close to 1.

Spiking experiments (Fig. 7.6) revealed no significant matrix effects for NH_3-N, PO_4, as the difference between expected and measured concentrations (< 7%) was less than the inaccuracy observed with the standard curves. Low but measurable matrix effects were observed for NO_3-N and SO_4 (< 19% difference between expected and measured concentrations). For SDS, inaccuracy due to matrix effects appeared low, and was less than inaccuracy based on standard curve analysis and variability of blank signal.

In conclusion, based on standard curves and spiking experiments, colorimetric assays are able to quantify the chemicals NH_3-N and PO_4 with an accuracy within 20% in storm drain samples. A similar accuracy can be obtained for NO_3-N and SO_4 in the mid to high ranges of the assays. However, in the lower ranges of the assays accuracy decreased (within 60% of true value). Calibration of the colorimeters is recommended to improve the accuracy of the assays. Sample matrix effects were absent or low for all analytes. Precision of all assays was less than 20%. Quantification of SDS was inaccurate, due to inaccuracy in the whole range of the test kit and the variation in the blank signal. The indicators boron and potassium were excluded as potential sewage indicators because of the inconsistent results when analyzing sample dilutions.

7.3.3 Spatial concentration patterns

For plotting spatial variations of concentrations of FIB, sewage-specific and alternative indicators, samples were grouped in the following categories:
- Source water (S, n = 5). These include possible sources of water into the storm drains: sewage (SEW), reclaimed water (REC), groundwater (GW), pristine creek water (M3, M4).
- Ocean, lagoon and creek samples (OLC, n = 11): A1-A4, M1-M2, L1-L4.
- Storm drain spatial samples (n = 41). Samples were collected in Haley storm drain (n = 8), Laguna storm drain (n = 18), Nopal storm drain (n = 10), and a few other storm drain (SD) locations (Hope drain, Westside drain, San Pascual drain, n = 5).
- Var1: storm drain samples collected using automated sampling at L15 (n = 24).
- Var2: storm drain samples collected using automated sampling at A5 (n = 18).

In order not to bias the spatial analysis towards samples L15 and A5, the samples grouped under Var1 and Var2 were not included in the analyses here, but were discussed separately in Chapter 8. We refer to the samples analyzed in this Chapter as the spatial samples.

7.3.4.1 Sewage-specific markers

Forty-five out of fifty-seven spatial samples were analyzed for HBM and MNIF. Thirteen were positive for HBM, and seventeen for MNIF (Fig. 7.7). HBM concentrations were high in SEW (~10^8 copies/L), but were below the detection limit in the other source water samples (groundwater, pristine creek water samples). HBM were detected once in the surf zone at Arroyo Burro beach (L1) and once in Laguna Channel (L3). In the storm drains, the highest HBM concentrations were measured at A5 and A6 (before the sewer leak was discovered and repaired, see Chapter 9). Other HBM-positive samples were detected in the Laguna and Nopal watersheds, but not in the Haley watershed. All samples collected at L15 (Cota and

Salsipuedes) using automated sampling were positive for HBM, but none of the samples collected at A5 (Hope drain, after discovery and repair of sewer leakage) using automated sampling were positive for HBM.

MNIF presence/absence corresponded fairly well with HBM concentrations (Fig. 7.8). Twelve samples were positive for MNIF and HBM, but five samples were positive for MNIF but not HBM (M9, N5, L11, A1, A3). One sample was positive for HBM but not MNIF (N5). The remaining 27 samples were negative for both human-specific markers.

7.3.4.2 Fecal indicator bacteria concentrations

A detailed overview of FIB concentrations for all samples is presented in Table 7.5, spatial trends are shown in Fig. 7.9. FIB concentrations were highest in SEW (~10^7 MPN/100 ml), and below the detection limit in GW and a few other storm drain samples. FIB levels were low but detectable in the pristine creek samples (M3, M4), ranging from 86-122 MPN/100 ml for EC and 389-393 MPN/100 ml for ENT. FIB concentrations in surf zone, lagoon and creek samples ranged between 31-3076 MPN/100 ml for EC and 10-2481 MPN/100 ml for ENT. FIB concentrations in storm drains ranged from below 10 MPN/100 ml to over 250,000 MPN/100 ml for EC and ENT.

7.3.4.3 Nutrients and anionic surfactants by colorimetry

Spatial trends of NH_3-N concentrations are shown in Fig. 7.10. Note that NH_3-N concentrations include NH_3-N and NH_4^+-N, and the relative fractions are determined by salinity and pH. However, at pH values below 7.5 and temperatures below 20 ºC, the fraction of NH_3-N is less than 1%. Concentrations of NH_3-N were 65 mg/L in SEW, 3.1 mg/L in REC, and < 0.1 mg/L in pristine creek samples. Two storm drain samples with known sewage contamination (A5 and A6, see Chapter 9) had the highest NH_3-N concentrations of all environmental samples (26-33 mg/L). Other samples with comparatively high NH_3-N concentrations (> 1 mg/L) were M9 (San Pascual drain), L8, L13 and L11 in Laguna watershed, and a number of samples collected using automated sampling.

Spatial trends of PO_4 concentrations are shown in Fig. 7.11. Concentrations of PO_4 were 12 mg/L in SEW, and 0.2-0.3 mg/L in the groundwater and pristine creek samples. Two storm drain samples with known sewage contamination (A5 and A6, see Chapter 9) had the highest PO_4 concentrations of all environmental samples (9-11 mg/L). Other storm drain samples with comparatively high PO_4 concentrations were M9 (San Pascual drain); M6 and M7 in Haley drain; L10, L11-L13 and L16 in Laguna watershed; and a number of samples collected at L15 and A5 using automated sampling.

Spatial trends of anionic surfactants concentrations are shown in Fig. 7.12. Concentrations were not available for sewage, but were low for all other source water samples (< 0.1 mg/L). Two storm drain samples with known sewage contamination (A5 and A6, see Chapter 9) had the highest anionic surfactants concentrations of all environmental samples (2.4-3.8 mg/L). Other storm drain samples with comparatively high anionic surfactant concentrations were M9 (San Pascual drain) and L13. Also a number of samples collected

at L15 and A5 using automated sampling showed elevated anionic surfactants concentrations.

Spatial trends of NO_3-N concentrations are shown in Fig. 7.13. Concentrations of NO_3-N were 2.9 mg/L in REC, 0.6 mg/L in GW and below 0.2 mg/L in SEW and pristine creek samples. Elevated NO_3-N concentrations (> GW) were observed in Haley drain samples M6 and M7, and some Laguna and Nopal watershed samples. NO_3-N-rich water could be traced, originating from L9 and flowing downstream to L7, L6, and L5.

Spatial trends of SO_4 concentrations are shown in Fig. 7.14. Concentrations of SO_4 were low in all source water samples, but high in the two surf zone samples L1 and A1 (2100-2300 mg/L, not shown). Storm drain samples with comparatively high SO_4 concentrations were L11 and L15 in Laguna watershed and N1-N4 in Nopal watershed. Storm drain samples with comparatively low SO_4 concentrations occurred in Laguna watershed, and a source of water with low SO_4 concentrations was observed flowing from L8 to L5.

The relations between NH_3-N, PO_4, anionic surfactants, NO_3-N, and SO_4 concentrations are presented in Fig. 7.15. In order not to bias the correlation analysis, only spatial samples were included in the correlation analysis, i.e. not the samples L15 and A5 analyzed during the variability study. The scatter plots show that NH_3-N, PO_4, and anionic surfactants are correlated in the highest concentration range (samples M9, A5, A6 and SEW), but not in the lower concentration ranges. SO_4 and NO_3-N concentrations did not correlate to NH_3-N, PO_4, and anionic surfactants.

7.3.4.4 Nutrients by FIA

Concentrations of NH_4^+-N, PO_4 and (NO_2+NO_3)-N for most samples collected from 7/1/2010 were also determined by FIA (Table 7.6). Scatter plots showing consistency between concentrations determined by FIA and colorimetry are shown in Figures 7.16 and 7.17.

Correlation for NH_4-N was low (Fig. 7.16, top), especially because of the outlier sample M9 with high NH_4-N concentrations by colorimetry. However, excluding outlier M9 increased the correlation coefficient (R^2) to 0.69. Most of the samples for which colorimetry overestimated NH_4-N concentrations were taken at the same location (A5), during the temporal variability study (Fig. 7.16, middle). Deleting all temporal variability study locations (A5 and L15) to avoid bias by a few locations greatly improved the correlation between both methods (Fig. 7.16, bottom).

Correlation for PO_4 was good (R^2 = 0.69), despite the outlier M9 that was overestimated by colorimetry (Fig. 7.17, top). When excluding M9, correlation increased to 0.81. Correlation was excellent for NO_3-N, but colorimetry slightly underestimated concentrations by FIA, likely because FIA also includes NO_2-N (Fig. 7.17, bottom).

Overall, colorimetry appears to agree well with FIA, and is therefore potentially useful for preliminary determination of nutrient concentrations. However, one outlier sample (M9) was identified, for which colorimetry greatly overestimated NH_4-N and PO_4 concentrations.

7.3.4.5 Caffeine and cotinine by ELISA

Spatial trends of caffeine concentrations are shown in Fig. 7.18 (top figure). Concentrations were highest in sewage (92 ppb), and in the storm drain samples with known sewage contamination (A5 and A6, see Chapter 9). Most environmental samples had non-detectable levels of caffeine (< 0.18 ppb). Caffeine was detected in at medium concentrations (3-12 ppb) in most Haley watershed samples (M6 and M7), and M9 (San Pascual drain). Caffeine was detected in low concentrations (< 0.5 ppb) at L11, L13, M1, and A1. Caffeine was also detected in some of the L15 samples, and all of the A5 samples, collected using automated sampling.

Spatial trends of cotinine concentrations are shown in Fig. 7.18 (bottom figure). Concentrations were highest in sewage (5.9 ppb), in the storm drain samples with known sewage contamination (A5 and A6, see Chapter 9), in L13 and M9. Cotinine was detected at low concentrations (0.1-0.2 ppb) in most Haley watershed samples (M6 and M7), L11 (City Annex Yard), and some of the samples collected using automated sampling (L15 and A5).

7.3.5 Relationships between sewage-specific markers and alternative indicators

7.3.5.1 Univariate relationships

To determine the usefulness of alternative indicators for identifying sewage pollution, we looked for relations between the concentrations of alternative indicators and sewage-specific indicators. Univariate relations were assessed using scatter plots for each alternative indicator versus HBM concentrations and MNIF presence/absence.

Samples A5 and A6, with the highest concentrations of NH_3-N , PO_4 and anionic surfactants (> 10 mg/L NH_3-N, > 6 mg/L PO_4, and > 2 mg/L anionic surfactants), had high HBM concentrations and were positive for MNIF (Fig. 7.19). Sample M9 also had high concentrations of NH_3-N (7.8 mg/L), PO_4 (5.2 mg/L) and anionic surfactants (1.5 mg/L), and was positive for MNIF but negative for HBM. However, the high nutrient concentrations may be caused by a false-positive colorimetric signal, as FIA indicated low NH_4-N and PO_4 concentrations. For concentrations of NH_3-N, PO_4 and anionic surfactants below 7 mg/L, 5 mg/L, and 1.5 mg/L, respectively, there was no correlation with sewage-specific markers. The latter cutoff values represent 10% sewage for NH_3-N and 40% sewage for PO_4. Therefore, colorimetry for NH_3 may be slightly more sensitive for detecting sewage pollution. Concentrations of anionic surfactants in sewage were not available, but were expected to be < 8 mg/L based on concentrations at A6. Therefore, the anionic surfactants assay is also less sensitive compared to NH_3-N.

Using the NH_4-N and PO_4 concentrations by FIA did not improve the consistency with HBM concentrations and MNIF presence/absence (Fig. 7.20). Note that samples A5 and A6 with high concentrations of both nutrients were not analyzed by FIA.

Sample L9 had the highest NO_3-N concentration and was positive for HBM and MNIF (Fig. 7.21). Otherwise, no correlation between NO_3-N concentrations and human-specific markers was observed. Similarly, the samples with the highest SO_4 concentrations (> 490 mg/L) all were positive for at least one of the sewage-specific markers (Fig. 7.21). In the

lower concentrations ranges of NO_3-N (< 10 mg/L) and SO_4 (< 490 mg/L), no correlations with sewage-specific markers were observed. While there appeared to be some relation between high concentrations of these nutrients and sewage contamination, this may be due to chance since neither NO_3-N nor SO_4 concentrations are high in fresh sewage. In addition, the relation was only based on one data point for NO_3-N, and the highest SO_4 concentrations were very similar to the average SO_4 concentrations. Therefore, based on the current data we do not recommend using NO_3-N and SO_4 for identifying sewage pollution.

Caffeine and cotinine correlated with HBM concentrations and MNIF presence/absence in the highest concentrations range (> 90 ppb caffeine and > 1.9 ppb cotinine), i.e. for samples A5 and A6 (Fig. 7.22). In the lower concentrations ranges, no correlations were observed.

There appeared to be some correlation between high FIB concentrations (over-range, > 25,000 MPN/ 100 ml) and high HBM concentrations (A5 and A6) (Fig. 7.23). However, a number of samples also had over-range FIB concentrations (> 25,000 MPN/100 ml) but no detectable HBM (L14, M7 for EC and M6, M7, M9 for ENT). Due to the over-range FIB concentrations, the exact correlation between FIB and HBM in the high concentration ranges could not be determined. No correlations were observed between FIB and sewage-specific markers for the samples with FIB concentrations within range.

7.3.5.2 Multivariate relationships

Mutivariate statistical analyses can display similarity of samples based on multiple parameters, i.e. FIB, nutrients and anionic surfactants. When analyzing multiple variables for a set of samples, they can be used as a first tier to identify major patterns and relationships between variables, and e.g. location.

Fig. 7.24 shows the results of PCA analysis, i.e. the relative similarity of samples based on the combined patterns of the variables NH_3, PO_4, anionic surfactants, NO_3-N, SO_4, caffeine, cotinine, EC and ENT. The variable vectors are superimposed on the plot, and indicate the direction in the plot for which the most significant changes occur for each variable, and the magnitude of the changes. Therefore, the top plot in Fig. 7.24 shows that samples A5, A6 and M9 have the highest NH_3, PO_4, anionic surfactants, caffeine and cotinine concentrations. It can also be seen that L9 has the highest NO_3 concentration. The relationships between variables are also clearly displayed: NH_3, PO_4, anionic surfactants, caffeine and cotinine concentrations are related, log-transformed EC and ENT concentrations are related, SO_4 concentrations are inversely related to EC and ENT concentrations. These relations were largely confirmed using the univariate relations. In the bottom graph of Fig. 7.24, outliers A5 and A6 were excluded for a better view of the relations between the remaining samples and variables. Several sample groups could be identified, such as L5-L7 and L9 with high NO_3-N concentrations, M6 and M7 with high FIB concentrations, and A5, M9 and L13 with high concentrations of all alternative indicators except SO_4 and NO_3-N.

Superimposing bubble plots, representing HBM concentrations, on the PCA plots, showed that samples with detectable HBM concentrations did not group together. This implies that detection of sewage-specific markers did not correlate with a certain combination of multiple alternative indicator concentrations (Fig. 7.25). The PCA plot confirmed the findings based on univariate correlations, i.e. the most contaminated samples A5 and A6 had high NH_3-N, PO_4, anionic surfactants, caffeine and cotinine concentrations, and samples were positive for HBM for the highest concentration ranges of NO_3-N and SO_4.

MDS analysis based using Bray-Curtis and Euclidian distances confirmed the findings by PCA, i.e. HBM-positive samples were not grouped separately (Fig. 7.26).

7.4 Discussion and conclusions

The suitability of colorimetric assays and caffeine and cotinine as alternative indicators for sewage pollution was assessed by (i) determining accuracy and precision of colorimetric assays, (ii) comparing concentrations in sewage, potential source waters to urban storm drains and urban storm drain samples, and (iii) correlating alternative indicator concentrations with HBM concentrations and MNIF presence/absence.

NH_3-N, PO_4, anionic surfactants, caffeine, cotinine and FIB appeared useful indicators for fresh sewage, based on high concentrations in sewage and low concentrations in groundwater, reclaimed water and pristine creek water samples. The theoretical sewage detection limit for each of the alternative indicators, calculated by dividing the concentration in sewage by the highest other source water sample concentration or the method detection limit, is shown in Table 7.7. Theoretical detection limits range from 1-5% for NH_3-N, PO_4, anionic surfactants, 0.2-0.8% for caffeine and cotinine, and 0.001-0.006% for FIB. However, the latter detection limits do not take into account other sources of alternative indicators in the watersheds, or growth of FIB, resulting in elevated background concentrations. Sources that could locally increase alternative indicator concentrations are fertilizers (NH_3 and PO_4), influent from cleaning operations (anionic detergents), coffee spills (caffeine), and likely other inputs from commercial or industrial sources. Certain plants could be sources of caffeine, although this is not likely in urban storm drains.

To account for unidentified inputs of alternative indicators, not related to sewage pollution, the correlations between alternative indicators and sewage-specific indicators were assessed. The HBM and MNIF markers are among the most sensitive and specific markers available, although it is still possible that samples with lower amounts of sewage contamination, or human fecal contamination from individual sources or households remain undetected. This study shows that NH_3-N, PO_4, anionic surfactants, caffeine and cotinine performed similarly in predicting locations with significant sewage pollution (> ~10% sewage). While high SO_4 and NO_3-N concentrations also seemed related to sewage pollution, these nutrients are not indicative of fresh sewage pollution, and the observed relations may have occurred by chance or locally elevated levels of nutrients.

The indicators NH_3-N and PO_4 can be rapidly quantified in the field, with limited cost. Colorimetry for NH_3-N appeared slightly more sensitive for detecting sewage pollution based on higher concentrations in sewage relative to background in urban storm drains. Therefore, using colorimetry for quantifying NH_3-N alone, or in combination with PO_4 for additional confirmation, is a useful approach for preliminary identification of severe sewage contamination (> 10%) in storm drains. While anionic detergents could be useful as well, the assay is not very practical because it cannot be performed in the field, and requires more time and handling of toxic chemicals.
Caffeine and cotinine are more specific to sewage pollution compared to nutrients and FIB. Their quantification requires some specialized equipment (plate reader), but quantification can be performed within a few hours for batches of archived samples. Therefore, they can be useful as a screening tool, but also for confirmation of sewage contamination in samples identified by colorimetry.

FIB do not appear superior to nutrients, anionic surfactants, caffeine and cotinine for identification of sewage pollution in the samples in this study. However, they may still be useful, as water quality standards are expressed as FIB concentrations, and a database of historical FIB data may be available for certain watersheds. The latter can be helpful when preparing a sewage identification study, as low FIB concentrations (less than ~ 100 MPN/100 ml) are usually not associated with sewage pollution. Also, FIB analysis can be performed within 24 hours without excessive cost or time investment.

Detection of human-specific markers still appears the most sensitive and specific method for identification of sewage pollution. Therefore, using one or more of these markers is recommended for confirmation of sewage pollution identified by other methods. However, using the alternative methods in a screening field study may limit the number of samples that require testing for sewage-specific markers.

7.5 References

Bracklow, U., Drew, A., Vocks, M. and Kraume, M., 2007. Comparison of nutrients degradation in small scale membrane bioreactors fed with synthetic/domestic wastewater. Journal of Hazardous Materials 144(3), 620-626.

Brown, E., Caraco, D. and Pitt, R., 2004. Illicit discharge detection and elimination. A guidance manual for program development and technical assessments, Office of Water and Wastewater, U.S. Environmental Protection Agency, Washington, D.C.

Cao, Y.P., Griffith, J.F. and Weisberg, S.B., 2009. Evaluation of optical brighteners photodecay characteristics for detection of human fecal contamination. Water Research 43(8), 2273-2279.

Carey, R.O. and Migliaccio, K.W., 2009. Contribution of wastewater treatment plant effluents to nutrient dynamics in aquatic systems: a review. Environmental Management 44(2), 205-217.

Holt, M.S., Waters, J., Comber, M.H.I., Armitage, R., Morris, G. and Newberry, C., 1995. AIS/CESIO environmental surfactant monitoring programme. Water Research 29(9), 2063-2070.

Tchobanoglous, G., Burton, F.L. and Stensel, H.D., 2003. Wastewater engineering: treatment and reuse/Metcalf & Eddy, Inc., McGraw-Hill, New York.

Wang, H.T., Li, F.T., Keller, A.A. and Xu, R., 2009. Chemically enhanced primary treatment (CEPT) for removal of carbon and nutrients from municipal wastewater treatment plants: a case study of Shanghai. Water Science and Technology 60(7), 1803-1809.

Weber, B. and Juanico, M., 2004. Salt reduction in municipal sewage allocated for reuse: the outcome of a new policy in Israel. Water Science and Technology 50(2), 17-22.

Table 7.1. Sampling dates, locations, UCSB IDs (for sample archiving), IDs (used in this report), human-specific *Bacteroidales* marker (HBM) concentrations and SE, and *Methanobrevibacter smithii nifH* gene (MNIF) presence (1) or absence (0). NA = not analyzed; ND = not detected.

Date	UCSB_ID	ID	HBM		MNIF	Location
			copies/L	SE		
6/11/2010	SEW	SEW	8.8E+07	4.2E+06	1	El Estereo WWTP confluent
6/11/2010	REC	REC	NA		NA	El Estero WWTP reclaimed effluent
7/19/2010	M21	GW	ND		0	City Corp Yard
7/29/2010	M27	A1	ND		1	AB surf
7/29/2010	M26	A2	ND		0	AB Lagoon mouth, near rock outcropping
7/29/2010	M32	A3	ND		1	Mesa Creek under bridge
7/29/2010	M28	A4	ND		0	Las Positas Creek, us of Veronica Spr. Rd.
6/9/2010	S20	A5	1.5E+07	1.8E+05	1	Hope Drain Diversion
7/29/2010	M31	A5	ND		0	Hope Drain Diversion
6/9/2010	S21	A6	6.3E+06	6.0E+05	1	State & Plaza
7/19/2010	M20	M1	ND		0	MC at Gutierrez, ds bridge left bank
7/19/2010	M22	M2	ND		0	MC at Rocky Nook Park
7/29/2010	M29	M3	ND		0	MC in botanical gardens
7/29/2010	M30	M4	ND		0	Rattlesnake Cyn
6/3/2010	SC-1	M5	NA		NA	Haley - MH upstream diversion
9/8/2010	M5	M6	ND		0	Haley/Chapala
9/13/2010	M5	M6	ND		0	Haley/Chapala
9/20/2010	M5	M6	ND		0	Haley/Chapala
9/8/2010	M6	M7	ND		0	Chapala/Ortega
9/13/2010	M6	M7	ND		0	Chapala/Ortega
9/20/2010	M6	M7	ND		0	Chapala/Ortega
6/3/2010	SC-2	M7	NA		NA	Chapala/Ortega
7/19/2010	M19	M8	ND		0	West side drain, at grate into OMC
7/19/2010	M2	M9	ND		1	San Pascual Drain, end of San Pascual St.
7/29/2010	M25	L1	9.82E+02	4.99E+02	1	Laguna surf
7/29/2010	M24	L2	ND		0	Laguna Lagoon in front of wall
7/14/2010	M18	L3	1.82E+03	4.28E+02	1	Laguna Channel US pump station
6/3/2010	SC-9	L4	NA		NA	Laguna Channel upstream RR
7/9/2010	SC-9	L4	NA		NA	Laguna Channel upstream RR
6/8/2010	S9	L5	ND		0	Laguna under 101
6/8/2010	S11	L6	ND		0	Laguna & Gutierrez
6/8/2010	S12	L7	ND		0	Laguna & Cota
6/3/2010	SC-3	L8	NA		NA	Laguna 702
6/8/2010	S13	L8	ND		0	Laguna 702
7/9/2010	SC-3	L8	NA		NA	Laguna 702
7/14/2010	M17	L9	6.95E+05	2.53E+04	1	Laguna/De La Guerra
6/3/2010	SC-4	L10	NA		NA	Laguna/Canon Perdido
6/3/2010	SC-8	L11	NA		NA	City Annex Yard, MH closest to outlet
7/29/2010	M23	L11	ND		1	City Annex Yard, MH closest to outlet
6/8/2010	S10	L12	ND		0	Montecito & Olive
6/3/2010	SC-7	L13	NA		NA	Quarantina/Gutierrez
7/14/2010	M16	L13	ND		0	Quarantina/Gutierrez
6/7/2010	S1	L14	4.7E+03	2.6E+02	1	Salsipuedes & Haley
6/7/2010	S2	L15	4.9E+04	4.4E+02	1	Cota @ Salsipuedes (sidewalk)
6/7/2010	S4	L16	ND		0	Milpas & Ortega
6/3/2010	SC-6	L17	NA		NA	Nopal/De La Guerra
6/7/2010	S3	L17	1.5E+04	1.1E+03	1	Nopal/De La Guerra
7/14/2010	M14	N1	ND		0	Nopal/Yanonali
7/14/2010	M15	N2	ND		0	Yanonali/Alisos

6/7/2010	S5	N3	ND		0	Nopal & Cota
6/3/2010	SC-5	N4	NA		NA	Nopal/Canon Perdido
7/1/2010	M9	N4	1.30E+05	6.84E+03	1	Nopal/Canon Perdido
7/6/2010	M9	N4	1.00E+02		0	Nopal/Canon Perdido
7/9/2010	M9	N4	5.30E+04	3.43E+03	1	Nopal/Canon Perdido
7/1/2010	M13	N5	ND		1	Nopal/Figueroa
7/6/2010	M13	N5	3.88E+03	5.91E+02	0	Nopal/Figueroa
7/9/2010	M13	N5	1.68E+04	1.09E+03	1	Nopal/Figueroa
9/28/2010	C1	L15	8.98E+03	8.10E+02	0	Cota @ Salsipuedes (sidewalk)
9/28/2010	C2	L15	2.65E+04	1.77E+03	0	Cota @ Salsipuedes (sidewalk)
9/28/2010	C3	L15	4.13E+03	3.11E+02	0	Cota @ Salsipuedes (sidewalk)
9/28/2010	C4	L15	4.45E+03	4.85E+02	0	Cota @ Salsipuedes (sidewalk)
9/28/2010	C5	L15	3.53E+04	1.63E+03	0	Cota @ Salsipuedes (sidewalk)
9/29/2010	C6	L15	2.99E+04	2.28E+03	0	Cota @ Salsipuedes (sidewalk)
9/29/2010	C7	L15	3.78E+04	7.49E+02	0	Cota @ Salsipuedes (sidewalk)
9/29/2010	C8	L15	1.27E+04	1.67E+03	0	Cota @ Salsipuedes (sidewalk)
9/29/2010	C9	L15	1.17E+04	1.77E+03	0	Cota @ Salsipuedes (sidewalk)
9/29/2010	C10	L15	3.20E+04	1.99E+03	0	Cota @ Salsipuedes (sidewalk)
9/29/2010	C11	L15	5.87E+03	8.84E+02	0	Cota @ Salsipuedes (sidewalk)
9/29/2010	C12	L15	2.84E+04	9.56E+02	0	Cota @ Salsipuedes (sidewalk)
9/29/2010	C13	L15	6.74E+04	2.58E+03	0	Cota @ Salsipuedes (sidewalk)
9/30/2010	C14	L15	3.09E+04	2.52E+02	0	Cota @ Salsipuedes (sidewalk)
9/30/2010	C15	L15	6.31E+04	2.16E+03	0	Cota @ Salsipuedes (sidewalk)
9/30/2010	C16	L15	3.14E+04	2.00E+03	0	Cota @ Salsipuedes (sidewalk)
9/30/2010	C17	L15	2.57E+04	2.41E+03	0	Cota @ Salsipuedes (sidewalk)
9/30/2010	C18	L15	6.84E+04	1.29E+03	0	Cota @ Salsipuedes (sidewalk)
9/30/2010	C19	L15	1.74E+04	5.54E+02	0	Cota @ Salsipuedes (sidewalk)
9/30/2010	C20	L15	2.13E+04	3.62E+03	0	Cota @ Salsipuedes (sidewalk)
9/30/2010	C21	L15	2.41E+04	5.80E+03	0	Cota @ Salsipuedes (sidewalk)
9/31/10	C22	L15	1.13E+04	8.10E+02	0	Cota @ Salsipuedes (sidewalk)
9/31/10	C23	L15	1.69E+04	5.08E+02	0	Cota @ Salsipuedes (sidewalk)
9/31/10	C24	L15	6.77E+03	3.13E+02	0	Cota @ Salsipuedes (sidewalk)
10/5/2010	H2	A5	ND		0	Hope Drain Diversion
10/5/2010	H3	A5	ND		0	Hope Drain Diversion
10/5/2010	H4	A5	ND		0	Hope Drain Diversion
10/6/2010	H5	A5	ND		0	Hope Drain Diversion
10/6/2010	H6	A5	ND		0	Hope Drain Diversion
10/6/2010	H7	A5	ND		0	Hope Drain Diversion
10/6/2010	H8	A5	ND		0	Hope Drain Diversion
10/7/2010	H9	A5	ND		0	Hope Drain Diversion
10/7/2010	H10	A5	ND		0	Hope Drain Diversion
10/7/2010	H11	A5	ND		0	Hope Drain Diversion
10/7/2010	H12	A5	ND		0	Hope Drain Diversion
10/7/2010	H13	A5	ND		0	Hope Drain Diversion
10/7/2010	H14	A5	ND		0	Hope Drain Diversion
10/8/2010	H15	A5	ND		0	Hope Drain Diversion
10/8/2010	H16	A5	ND		0	Hope Drain Diversion
10/8/2010	H17	A5	ND		0	Hope Drain Diversion
10/8/2010	H18	A5	ND		0	Hope Drain Diversion
10/8/2010	H19	A5	ND		0	Hope Drain Diversion

Table 7.2. Concentrations of potential alternative sewage indicators in raw municipal sewage.

Parameter	Units	Raw sewage	References
Fecal indicator bacteria (IDEXX)	MPN/100ml	10^5 – 10^7	
Ammonia	mg/L NH_3-N	12-82	1-4
Phosphate	mg/L PO_4	5-39	1-3
Anionic surfactants	mg/L LAS	11.8-18.2	5
Sulfate	mg/L SO_4	20-50	4
Nitrate	mg/L NO_3-N	0	
Boron	mg/L B	0.1-0.6	4,6
Potassium	mg/L K	7-15	4

[1](Wang et al., 2009)
[2](Carey and Migliaccio, 2009)
[3](Bracklow et al., 2007)
[4](Tchobanoglous et al., 2003)
[5](Holt et al., 1995)
[6](Weber and Juanico, 2004)

Table 7.3. Detection of optical brighteners: fluorescence before (F0) and after 5 minutes of UV exposure (F5), and percentage fluorescence decrease after 5 minutes of UV exposure (F5%).

Date	Sample	F0	F5	F5%
6/3/2010	M5	6.67	6.33	4.95
6/3/2010	M7	5.63	5.55	2.07
6/3/2010	L8	85.04	83.75	1.52
6/3/2010	L10	69.29	67.58	2.47
6/3/2010	N4	26.50	27.05	-2.39
6/3/2010	L17	102.70	99.66	2.96
6/3/2010	L13	24.06	23.42	3.02
6/3/2010	L11	121.20	118.03	2.61
6/3/2010	L4	32.34	30.87	4.38
6/7/2010	L14	31.76	31.29	1.48
6/7/2010	L15	80.14	79.54	0.75
6/7/2010	L17	148.8	145.9	1.95
6/7/2010	L16	28.89	28.74	0.52
6/7/2010	N3	44.83	44.1	1.63
6/9/2010	A5	59.45	56.36	5.18
6/9/2010	A6	75.64	76.78	-1.51

Table 7.4. Standard curves for NH_3-N, PO_4, SDS, NO_3-N, and SO_4: theoretical concentrations, measured average concentrations, coefficient of variation (CV) for measured concentrations (percent), measured average concentrations minus average blank, percent difference between measured concentrations minus blank and theoretical concentrations. All concentrations were in mg/L, and prepared in triplicate.

Chemical	Theoretical	Measured	CV	Measured - blank	% difference
NH_3-N	0	0.08	62	0	na
	0.36	0.36	2	0.27	-1
	1.8	1.62	4	1.54	-10
	3.6	3.13	0	3.04	-13
PO_4	0	0.01	87	0	na
	0.27	0.22	5	0.22	-17
	1.35	1.13	3	1.12	-17
	2.70	2.26	3	2.26	-16
SDS	0	0.60	17	0	na
	0.26	1.20	17	0.60	360
	1.31	2.17	7	1.57	66
	2.61	3.83	9	3.23	47
NO_3-N	0	0.15	17	0	na
	0.27	0.37	11	0.22	37
	1.35	1.41	7	1.26	4
	2.70	2.78	4	2.63	3
SO_4	0	0.67	87	0	na
	9.00	4.00	0	3.33	-56
	45.00	41.00	0	40.33	-9
	90.00	96.67	1	96.00	7

Table 7.5. Sampling dates, IDs, and concentrations of *E. coli* (EC), *Enterococcus* spp. (ENT), caffeine and cotinine. NA = not analyzed; ND = not detected.

Date	ID	EC	ENT	Caffeine		Cotinine	
		MPN/100 ml	MPN/100 ml	ppb	SE	ppb	SE
6/11/2010	SEW	14136100	6131400	91.853	2.277	5.889	0.160
6/11/2010	REC	NA	NA	NA		NA	
7/19/2010	GW	5	5	ND		ND	
7/29/2010	A1	31	10	0.239	0.038	NA	NA
7/29/2010	A2	3076	10	ND		ND	
7/29/2010	A3	41	41	ND		ND	
7/29/2010	A4	187	2382	ND		ND	
6/9/2010	A5	25000	25000	124.114	9.085	2.396	0.141
7/29/2010	A5	11199	8164	6.600	0.256	0.250	0.021
6/9/2010	A6	25000	25000	100.825	3.518	1.947	0.051
7/19/2010	M1	1086	408	0.293	0.002	NA	NA
7/19/2010	M2	355	2481	ND		ND	
7/29/2010	M3	86	393	ND		ND	
7/29/2010	M4	122	389	ND		ND	
6/3/2010	M5	NA	NA	NA		NA	
9/8/2010	M6	6867	24196	11.887	0.249	0.203	0.015
9/13/2010	M6	597.5	542.5	ND		ND	
9/20/2010	M6	7932.5	25000	8.185	0.469	0.164	0.001
6/3/2010	M7	NA	NA	NA		NA	
9/8/2010	M7	2755	24196	4.339	0.496	0.131	0.008
9/13/2010	M7	265	855	ND		ND	
9/20/2010	M7	25000	25000	3.823	0.128	0.098	0.012
7/19/2010	M8	5	5	ND		ND	
7/19/2010	M9	173	25000	10.031	0.506	1.314	0.121
7/29/2010	L1	331	435	ND		ND	
7/29/2010	L2	502	175	ND		ND	
7/14/2010	L3	402	241	ND		ND	
6/3/2010	L4	NA	NA	NA		NA	
7/9/2010	L4	NA	NA	NA		NA	
6/8/2010	L5	291.7	553.9	ND		ND	
6/8/2010	L6	41.3	173.1	ND		ND	
6/8/2010	L7	10.0	30.6	ND		ND	
6/3/2010	L8	NA	NA	NA		NA	
6/8/2010	L8	5	20.2	ND		ND	
7/9/2010	L8	NA	NA	NA		NA	
7/14/2010	L9	5475	17329	ND		ND	
6/3/2010	L10	NA	NA	NA		NA	
6/3/2010	L11	NA	NA	NA		NA	
7/29/2010	L11	960	2755	0.404	0.006	0.093	0.014
6/8/2010	L12	2098.2	6488.2	ND		ND	
6/3/2010	L13	NA	NA	NA		NA	
7/14/2010	L13	25000	19863	0.396	0.055	2.227	0.099
6/7/2010	L14	1313.5	1934.9	ND		ND	
6/7/2010	L15	10.0	317.0	ND		ND	
6/7/2010	L16	17328.9	1968.3	ND		ND	
6/3/2010	L17	NA	NA	NA		NA	
6/7/2010	L17	159.6	169.4	ND		ND	
7/14/2010	N1	1130	988	ND		ND	
7/14/2010	N2	30	860	ND		ND	
6/7/2010	N3	5	20.2	ND		ND	
6/3/2010	N4	NA	NA	NA		NA	
7/1/2010	N4	959	52	ND		ND	
7/6/2010	N4	624	134	ND		ND	
7/9/2010	N4	3448	545	ND		ND	
7/1/2010	N5	146	148	ND		ND	

7/6/2010	N5	408	462	ND		ND	
7/9/2010	N5	1180	152	ND		ND	
9/28/2010	L15	3654	373.4	ND		ND	
9/28/2010	L15	933.8	157.9	ND		ND	
9/28/2010	L15	393.1	86	ND		ND	
9/28/2010	L15	419.5	933.1	ND		ND	
9/28/2010	L15	663.1	1259.1	ND		ND	
9/29/2010	L15	2223.6	465.4	ND		ND	
9/29/2010	L15	1376.1	159.6	ND		ND	
9/29/2010	L15	416.95	245.4	ND		ND	
9/29/2010	L15	1575.6	1274	ND		ND	
9/29/2010	L15	604.9	857.4	ND		ND	
9/29/2010	L15	133.6	932.6	ND		ND	
9/29/2010	L15	336.1	1955.9	ND		ND	
9/29/2010	L15	1989	2480.9	ND		ND	
9/30/2010	L15	594.8	265.3	ND		ND	
9/30/2010	L15	959.4	1789	ND		ND	
9/30/2010	L15	1558.15	1477.8	0.206	0.020	ND	
9/30/2010	L15	1624.2	1144.6	0.389	0.033	0.053	0.004
9/30/2010	L15	425.7	288.2	ND		ND	
9/30/2010	L15	312.9	132.3	ND		ND	
9/30/2010	L15	187.3	25000	ND		ND	
9/30/2010	L15	25000	25000	ND		ND	
9/31/10	L15	25000	25000	ND		ND	
9/31/10	L15	25000	25000	ND		ND	
9/31/10	L15	24195.7	25000	ND		ND	
10/5/2010	A5	16743	15152	6.084	0.299	0.519	0.047
10/5/2010	A5	15525	15967	4.528	0.554	0.482	0.020
10/5/2010	A5	6631	13327	2.857	0.052	0.122	0.012
10/6/2010	A5	92084	250000	24.329	1.374	0.132	0.009
10/6/2010	A5	29093	18719	10.732	1.374	0.091	0.007
10/6/2010	A5	34480	51721	16.636	2.234	0.118	0.006
10/6/2010	A5	86644	68667	17.734	1.949	0.153	0.001
10/7/2010	A5	7116	2182	2.466	0.047	0.078	0.005
10/7/2010	A5	6439.5	24377	2.081	0.140	0.079	0.009
10/7/2010	A5	7976	2307	1.552	0.055	0.072	0.003
10/7/2010	A5	4347	4250	1.098	0.060	ND	
10/7/2010	A5	5908	5204	1.151	0.056	0.060	0.005
10/7/2010	A5	2034	10807	1.598	0.032	0.172	0.010
10/8/2010	A5	8261	11685	1.647	0.107	0.229	0.013
10/8/2010	A5	1100	1187	0.690	0.054	0.065	0.001
10/8/2010	A5	250000	77010	1.840	0.054	0.141	0.027
10/8/2010	A5	11446	2109	25.461	1.498	0.213	0.022
10/8/2010	A5	51721	40879	18.674	3.393	0.369	0.020

Table 7.6. Sampling dates, IDs, and concentrations of nutrients by colorimetry and flow injection analysis (FIA). NA = not analyzed.

Date	ID	Colorimetry					FIA		
		NH_3-N (mg/L)	PO_4 (mg/L)	An. Surf. (mg/L)	NO_3-N (mg/L)	SO_4 (mg/L)	NH_4 -N (mg/L)	PO_4 (mg/L)	(NO_2+NO_3)-N (mg/L)
6/11/2010	SEW	65	12.2	NA	0.1	160	NA	NA	NA
6/11/2010	REC	3.14	0.2	-0.16	0.63	210	NA	NA	NA
7/19/2010	GW	0.02	0.32	0.04	2.91	55	0.02	5.37	0.13
7/29/2010	A1	-0.04	0.15	0.18	0.18	2125	0.09	0.04	0.15
7/29/2010	A2	0.04	0.19	0.03	0.36	465	0.05	0.34	0.03
7/29/2010	A3	0.04	0.13	0.01	0.48	575	0.02	0.36	0.09
7/29/2010	A4	0.25	0.45	0.18	1.23	85	0.26	1.21	0.09
6/9/2010	A5	25.6	9.2	2.41	0.1	120	NA	NA	NA
7/29/2010	A5	1.88	2.73	0.98	1.08	295	1.61	1.12	1.63
6/9/2010	A6	33.1	11	3.78	0.12	120	NA	NA	NA
7/19/2010	M1	0.12	0.18	0.07	0.67	255	0.11	0.81	0.06
7/19/2010	M2	0.11	0.36	0.03	0.17	135	0.04	0.13	0.19
7/29/2010	M3	0	0.17	-0.01	0.18	275	0.02	0.20	0.04
7/29/2010	M4	0.06	0.31	0	0.09	185	0.02	0.11	0.16
6/3/2010	M5	0.29	0.4	NA	2.55	250	NA	NA	NA
9/8/2010	M6	0.34	0.75	0.13	5.1	240	0.12	6.14	0.47
9/13/2010	M6	0.19	0.46	-0.12	4.9	260	0.14	6.14	0.23
9/20/2010	M6	0.48	1.1	-0.08	4.75	250	0.06	5.60	0.59
6/3/2010	M7	0.25	0.47	NA	1.88	260	NA	NA	NA
9/8/2010	M7	0.2	0.77	-0.21	4.4	220	0.15	5.07	0.45
9/13/2010	M7	0.13	0.46	0.01	4.65	230	0.13	6.63	0.18
9/20/2010	M7	0.28	1.26	-0.08	4.95	260	0.17	5.33	0.82
7/19/2010	M8	0.02	0.61	0.01	1.08	195	0.03	1.15	0.42
7/19/2010	M9	7.8	5.16	1.49	-0.02	218	0.30	0.19	1.84
7/29/2010	L1	0.03	0.06	-0.02	0.09	2325	0.12	0.02	0.04
7/29/2010	L2	0.02	0.11	-0.01	1.33	315	0.02	1.32	0.02
7/14/2010	L3	0.11	0.49	0.02	2.56	145	0.12	2.90	0.18
6/3/2010	L4	0.24	0.8	0	1.75	200	NA	NA	NA
7/9/2010	L4	0.14	0.54	-0.03	0.72	125	NA	NA	NA
6/8/2010	L5	0.55	0.17	-0.03	8.1	110	NA	NA	NA
6/8/2010	L6	0.77	0.13	0.1	8.4	60	NA	NA	NA
6/8/2010	L7	0.73	0.11	-0.17	5.82	90	NA	NA	NA
6/3/2010	L8	5.95	0.05	-0.18	0.38	36	NA	NA	NA
6/8/2010	L8	5.28	0.06	NA	0.52	18	NA	NA	NA
7/9/2010	L8	6.01	0.08	0.03	0.34	-5	NA	NA	NA
7/14/2010	L9	0.28	0.23	-0.13	11.6	175	0.22	13.54	0.10
6/3/2010	L10	0.86	1.96	NA	0	295	NA	NA	NA
6/3/2010	L11	1.24	2.72	NA	0.09	480	NA	NA	NA
7/29/2010	L11	1.98	1.16	0.34	4.56	645	1.93	4.63	0.74
6/8/2010	L12	0.12	2.04	NA	0.16	320	NA	NA	NA
6/3/2010	L13	0.21	0.54	-0.21	1.56	210	NA	NA	NA
7/14/2010	L13	1.61	1.15	1.16	0.92	235	0.05	1.04	0.76
6/7/2010	L14	0.22	0.48	-0.16	1.84	230	NA	NA	NA
6/7/2010	L15	0.1	0.57	-0.11	0.23	640	NA	NA	NA
6/7/2010	L16	0.16	1.63	NA	5.6	260	NA	NA	NA
6/3/2010	L17	0.68	0.32	NA	2.08	340	NA	NA	NA
6/7/2010	L17	0.62	0.26	-0.07	2.57	320	NA	NA	NA
7/14/2010	N1	-0.03	0.92	-0.21	2.96	375	0.07	4.15	0.56
7/14/2010	N2	0.06	0.87	-0.21	5.8	385	0.10	7.24	0.64
6/7/2010	N3	0.03	1.32	0.04	0.88	460	NA	NA	NA
6/3/2010	N4	0.23	1.03	-0.21	3.1	500	NA	NA	NA
7/1/2010	N4	0.1	1.17	0.01	2.71	515	0.07	2.84	0.93
7/6/2010	N4	0.07	1.2	0.01	2.9	435	0.11	3.18	1.10
7/9/2010	N4	0.04	1.18	-0.19	2.72	495	0.05	2.95	0.87

7/1/2010	N5	0.11	0.33	0.07	0.82	215	0.09	1.04	0.30
7/6/2010	N5	0.13	0.52	0.08	1.32	195	0.10	1.32	0.41
7/9/2010	N5	0.06	0.41	0.18	1.39	195	0.09	1.31	0.30
9/28/2010	L15	0.17	0.48	0.04	1.7	250	0.18	2.07	0.21
9/28/2010	L15	0.17	0.75	0.07	1.6	260	0.20	2.17	0.20
9/28/2010	L15	0.16	0.46	0.08	2	230	0.23	2.24	0.18
9/28/2010	L15	0.22	0.43	0.02	1.7	250	0.24	2.25	0.15
9/28/2010	L15	0.26	0.47	0.21	2.1	250	0.18	2.31	0.12
9/29/2010	L15	0.19	0.42	0.07	1.8	230	0.19	2.26	0.19
9/29/2010	L15	0.09	0.42	0.09	1.9	250	0.22	2.32	0.12
9/29/2010	L15	0.11	0.39	0.05	1.8	250	0.24	2.28	0.16
9/29/2010	L15	0.07	0.65	0.12	1.95	280	0.23	2.77	0.29
9/29/2010	L15	0.13	0.47	0.12	1.45	240	0.19	2.09	0.29
9/29/2010	L15	0.05	0.52	0.24	1.7	230	0.23	2.29	0.23
9/29/2010	L15	0.17	0.65	0.16	1.95	230	0.18	2.30	0.24
9/29/2010	L15	0.14	0.48	0.24	2	220	0.24	2.27	0.15
9/30/2010	L15	0.15	0.57	-0.17	1.75	240	0.20	2.14	0.22
9/30/2010	L15	0.13	0.61	0.04	1.9	240	0.23	2.22	0.18
9/30/2010	L15	0.14	0.59	-0.01	1.85	230	0.18	2.35	0.24
9/30/2010	L15	0.19	0.42	0.49	1.7	230	0.19	2.17	0.25
9/30/2010	L15	0.16	0.41	0.67	2.25	240	0.24	2.17	0.21
9/30/2010	L15	0.2	1.5	0.62	2.6	250	0.24	2.25	0.16
9/30/2010	L15	0.24	0.74	-0.15	2.2	250	0.23	2.25	0.20
9/30/2010	L15	0.22	0.45	1.29	1.75	210	0.19	2.30	0.21
9/31/10	L15	0.21	0.41	1.48	1.5	230	0.20	2.16	0.16
9/31/10	L15	0.14	0.47	0.56	1.45	220	0.20	2.27	0.18
9/31/10	L15	0.3	0.48	0.02	1.85	230	0.22	2.34	0.25
10/5/2010	A5	1.94	1.14	1.34	0.19	250	0.33	0.26	0.96
10/5/2010	A5	1.73	1.49	0.74	0.21	240	0.45	0.43	1.26
10/5/2010	A5	0.55	1.2	1.07	1.45	210	0.25	1.88	0.93
10/6/2010	A5	3.49	2.46	0.96	2.25	46	3.22	3.45	2.24
10/6/2010	A5	0.95	0.7	0.26	0.63	3	0.72	0.78	0.95
10/6/2010	A5	1.28	1.56	0.69	0.78	47	0.93	1.12	1.57
10/6/2010	A5	1.42	1.36	0.85	0.74	29	0.94	0.88	1.41
10/7/2010	A5	0.17	0.65	0.15	3.35	190	0.05	4.02	0.56
10/7/2010	A5	0.21	0.78	0.19	3.2	190	0.08	3.76	0.55
10/7/2010	A5	0.2	0.78	0.07	3.85	180	0.09	4.26	0.56
10/7/2010	A5	0.18	0.92	-0.01	4.5	190	0.13	5.81	0.66
10/7/2010	A5	0.21	1.03	0.08	5.65	190	0.16	6.23	0.70
10/7/2010	A5	0.45	1	1.2	2.05	190	0.02	2.24	0.79
10/8/2010	A5	0.47	1.27	1.86	4.3	210	0.03	3.73	1.01
10/8/2010	A5	0.12	0.69	0.17	1.45	140	0.06	1.53	0.51
10/8/2010	A5	0.86	0.26	0.61	0.45	140	0.02	0.02	0.16
10/8/2010	A5	0.15	0.75	0.07	0.75	210	0.05	0.69	0.53
10/8/2010	A5	0.74	0.56	0.65	0.65	230	0.03	0.40	0.39

Table 7.7. Theoretical sewage detection limit (SEW-LOD) for HBM and alternative indicators, based on concentrations in sewage (SEW) and other source water samples (Source) or method limit of detection (method-LOD).

	HBM (copies/L)	NH_3-N (mg/L)	PO_4 (mg/L)	Anionic surfactants (mg/L)	Caffeine (ppb)	Cotinine (ppb)	EC (MPN/100 ml)	ENT (MPN/100 ml)
SEW	~1e8	65	12.2	>3.8	92	5.9	1.4e7	6.1e6
Source	ND	3	0.3	0.04	ND	ND	122	393
Method-LOD	~1e3	0.1	0.1	0.02	0.18	0.05	10	10
SEW-LOD (%)	~0.001	5	2	<1	0.2	0.8	0.001	0.006

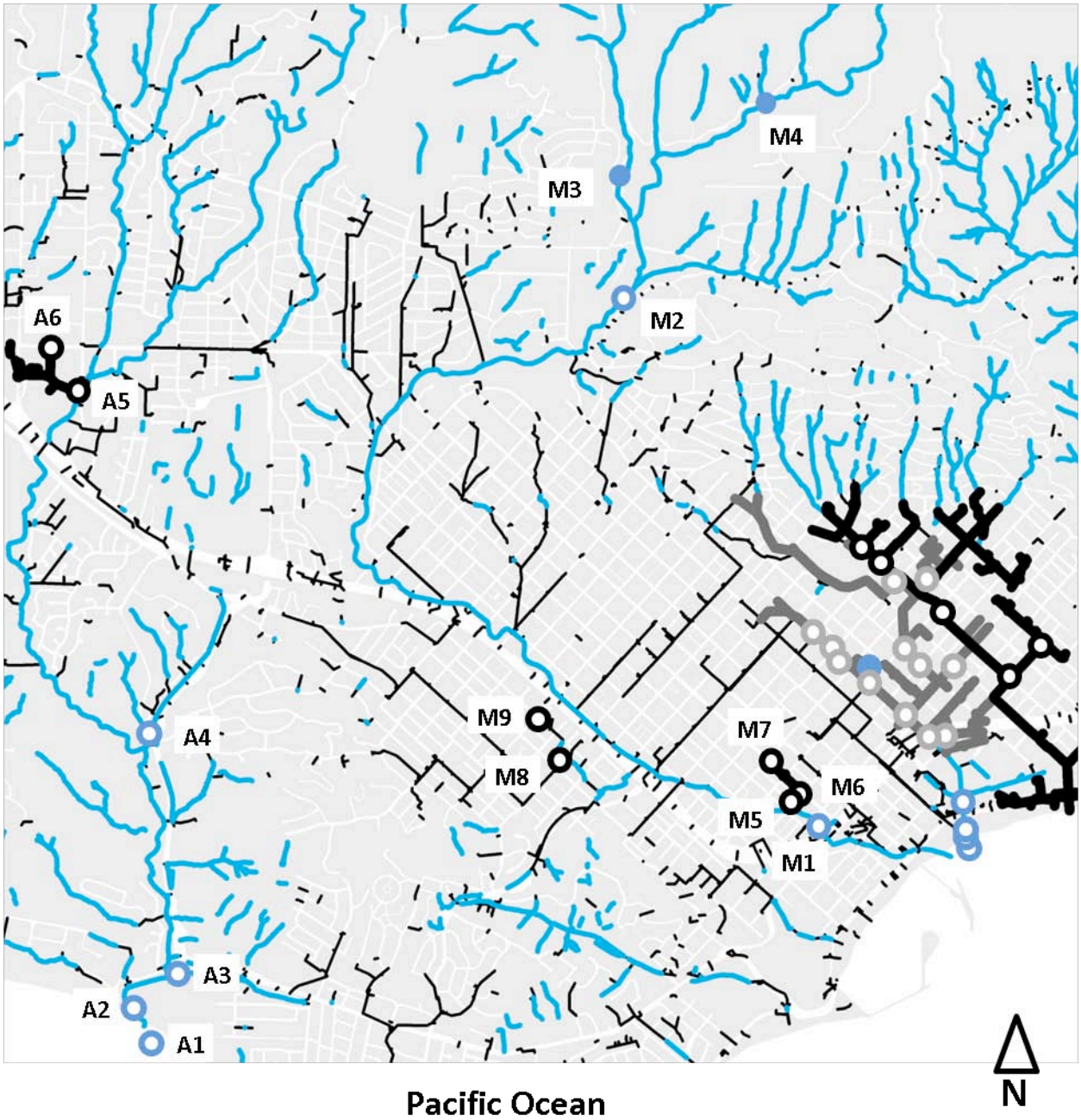


Figure 7.1. Location of samples in storm drains (open black and grey circles), ocean, creek and lagoon (open blue circles) and source water samples (full blue circles). Storm drains are shown in black and gray lines, open channels in blue lines. A detail of Laguna and Nopal watershed is shown separately.

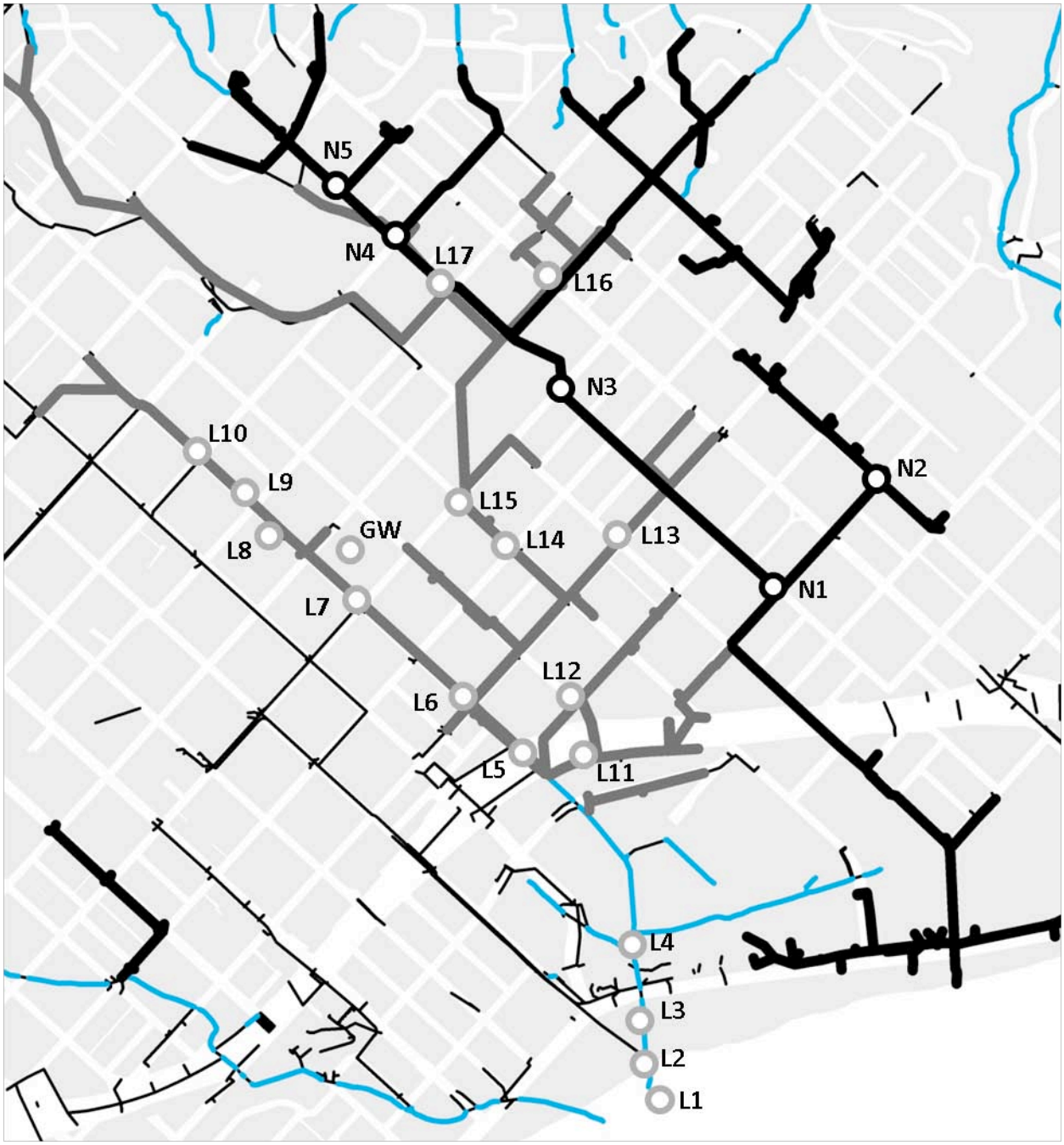


Figure 7.1. Continued

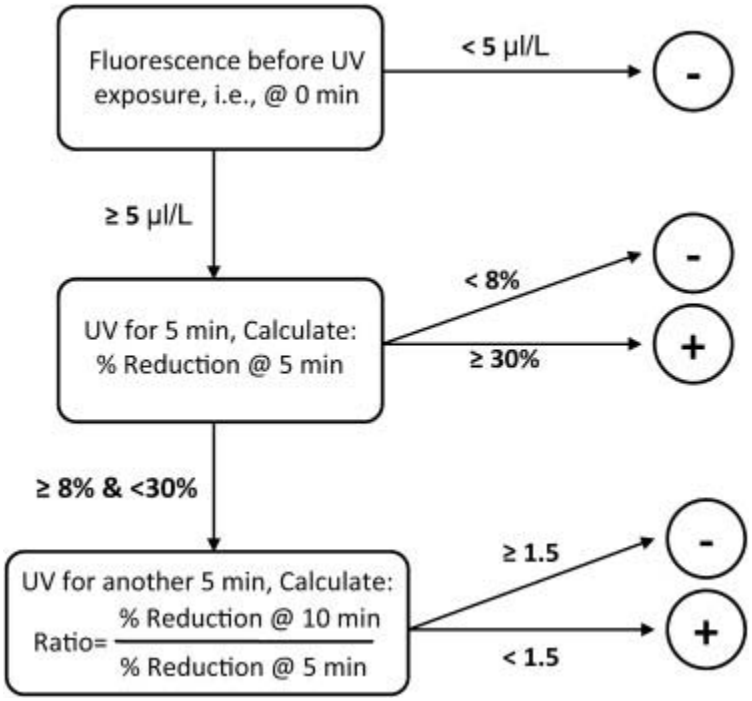


Figure 7.2. Decision criteria to determine presence or absence of optical brighteners in a water sample (from Cao et al., 2009).

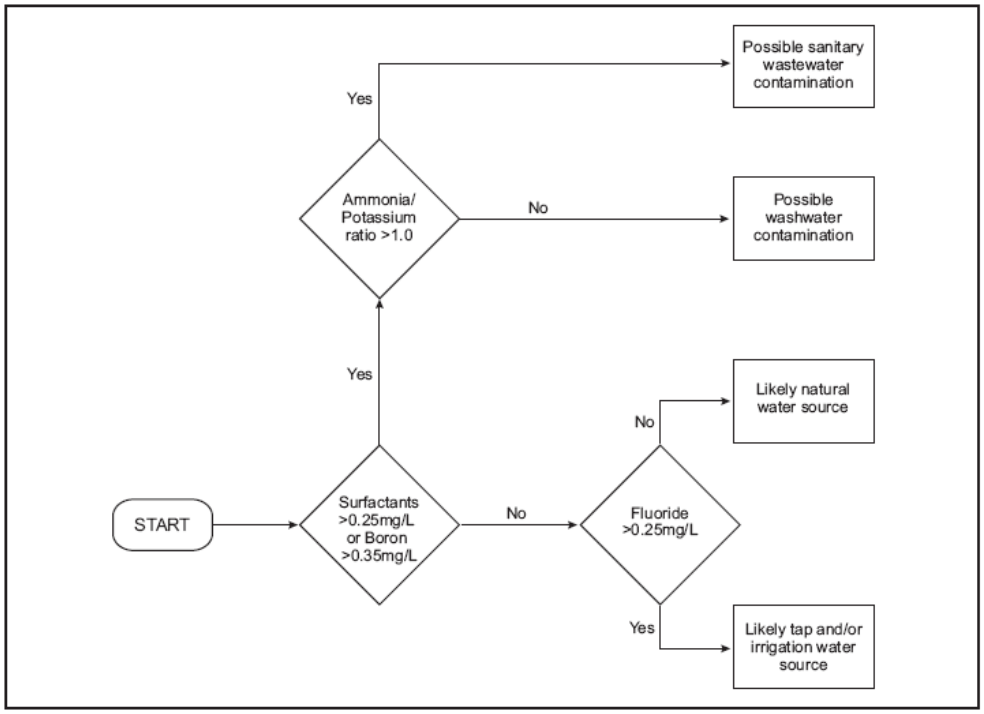


Figure 7.3. Proposed use of ammonia, boron, detergents and potassium for identification of sewage pollution in storm sewers (Brown et al., 2004).

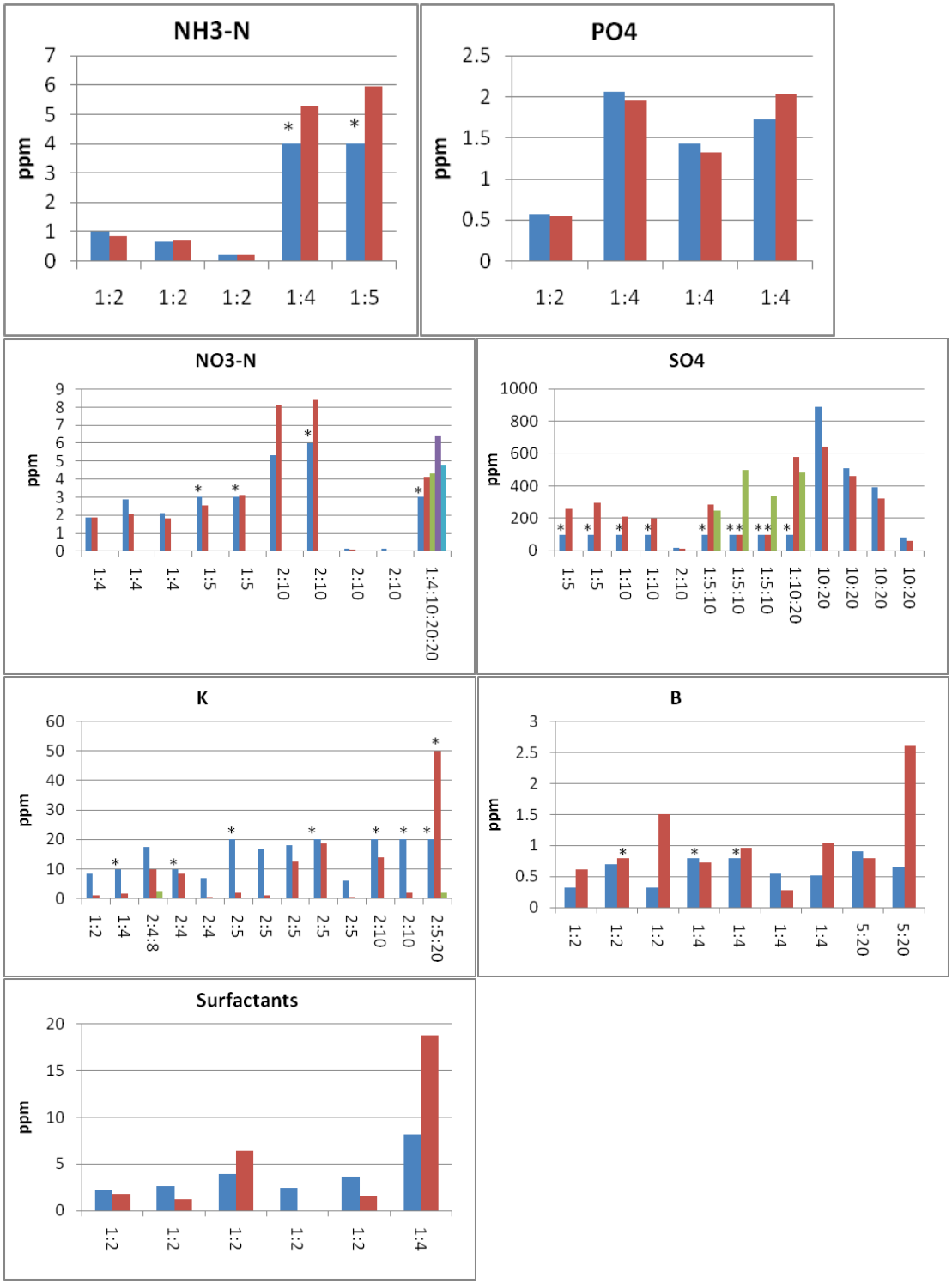


Figure 7.4. Chemical concentrations for various sample dilutions for selected storm drain samples. The grouped bars indicate sample concentrations for the same sample at different dilutions. The x-axis lists the dilutions, e.g. analyzed undiluted and 1:4 diluted is indicated by 1:4. Samples that are over-range are indicated by * (bar indicates minimum estimate).

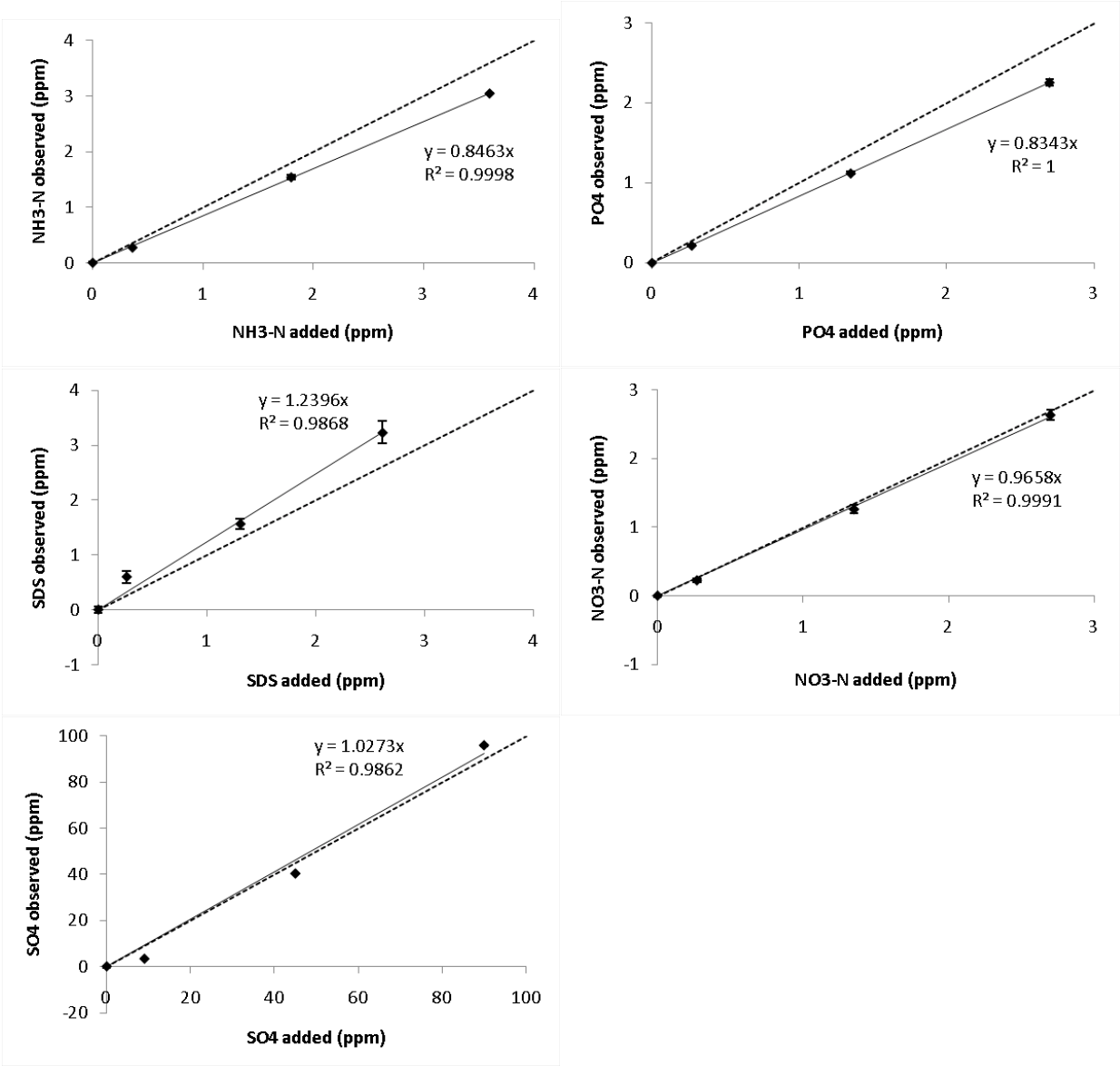


Figure 7.5. Standard curves comparing concentrations added and observed using colorimetry. Samples were analyzed in triplicate (error bars are shown) and average blank signal was subtracted. Standard curves regression lines through the origin are shown in black lines, and regression equations and R^2 are included. A line indicating 1:1 correlation is shown as a dashed line.

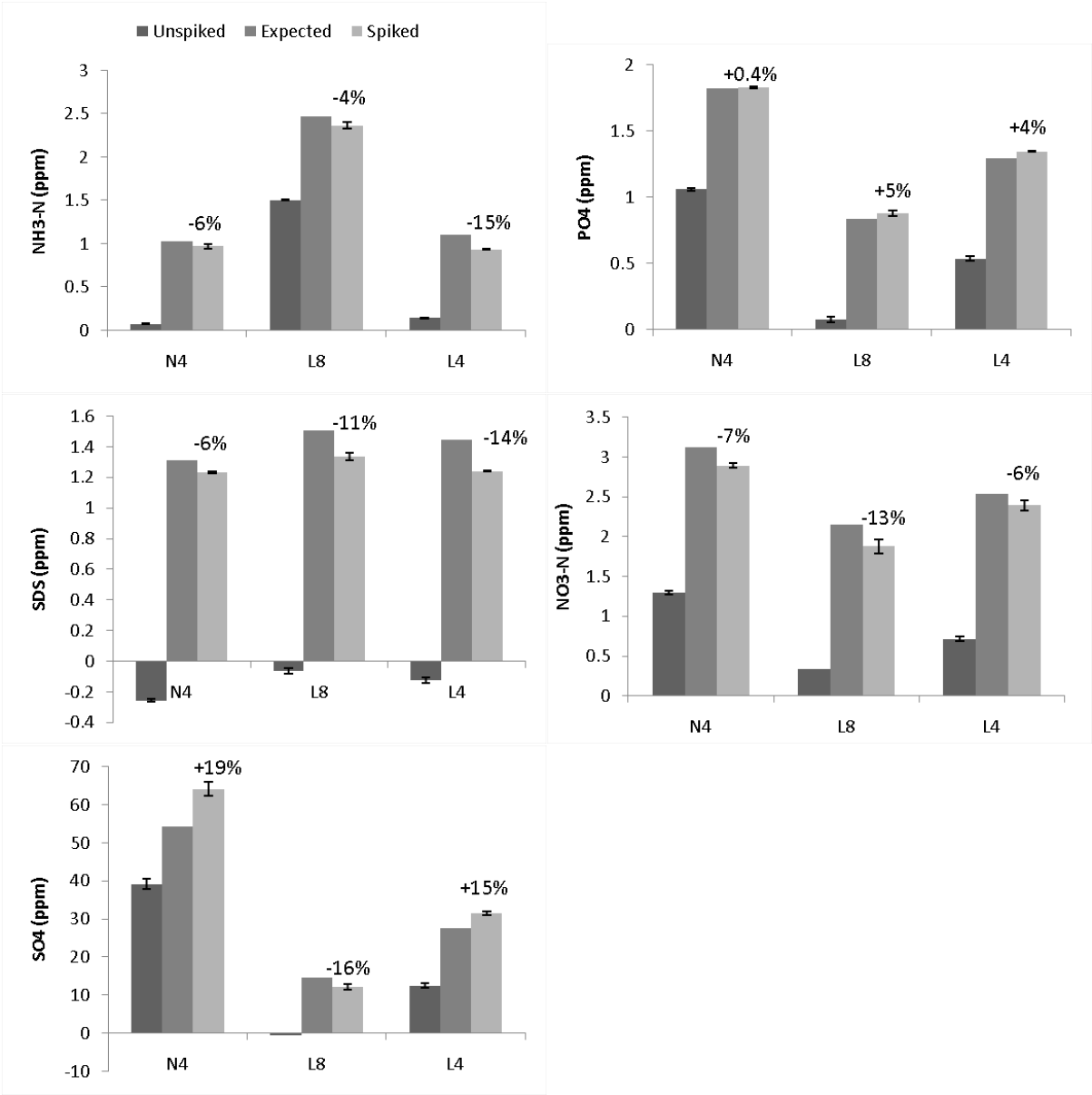


Figure 7.6. Nutrient and anionic surfactants (SDS) concentrations measured before (unspiked) and after spiking (spiked) samples N4, L8 and L4 (in triplicate, error bars shown). Theoretical concentrations after spiking are shown as well (expected), and percent difference between spiked and expected concentrations.

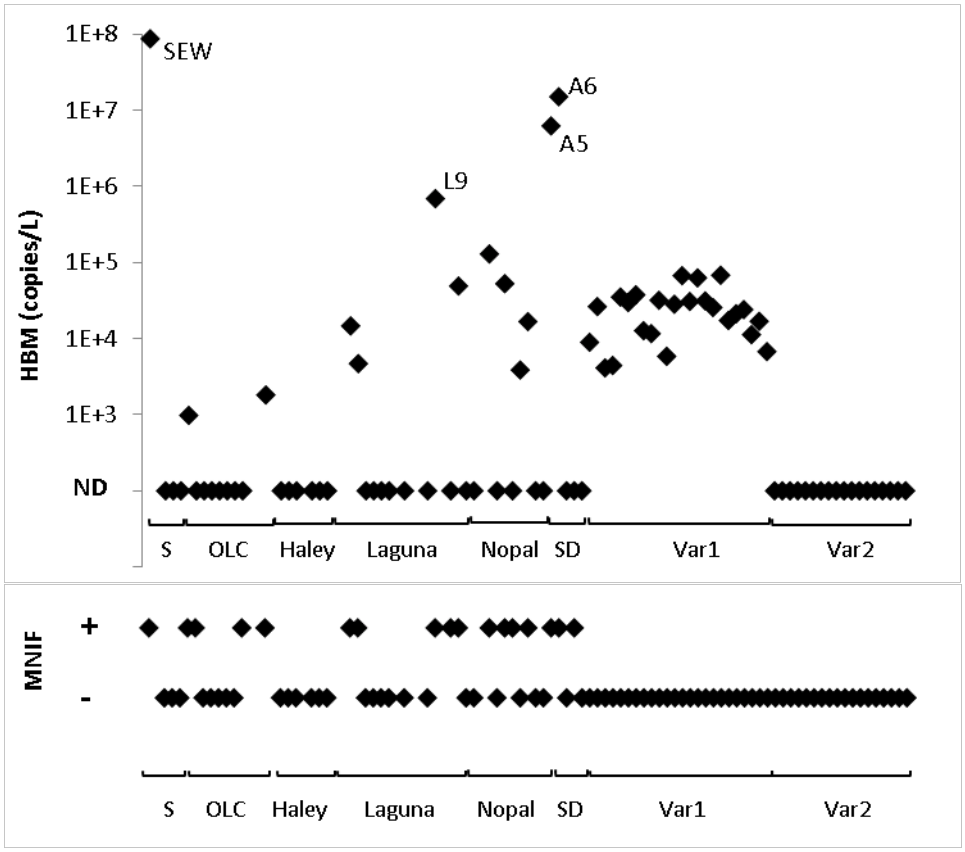


Figure 7.7. Concentrations of HBM (top), and MNIF presence/absence (bottom) for all samples. Samples are grouped according to sample type or location: S = source water samples; OLC = ocean, lagoon and creek samples; Haley = Haley drain storm drain samples; Laguna = Laguna watershed storm drain samples, Nopal = Nopal watershed storm drain samples, Var1 = samples at L15 collected using autosampler, Var2 = samples at A5 collected using autosampler. Samples with non-detectable HBM concentrations are indicated by "ND" on the y-axis.

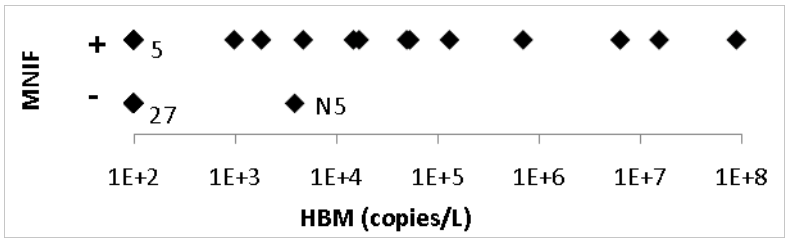


Figure 7.8. Consistency between HBM concentrations and MNIF presence/absence for all samples, excluding samples collected using automated sampling. Numbers on graph indicate number of observations positive and negative for MNIF with HBM below the limit of detection (set at 1E+2 copies/L).

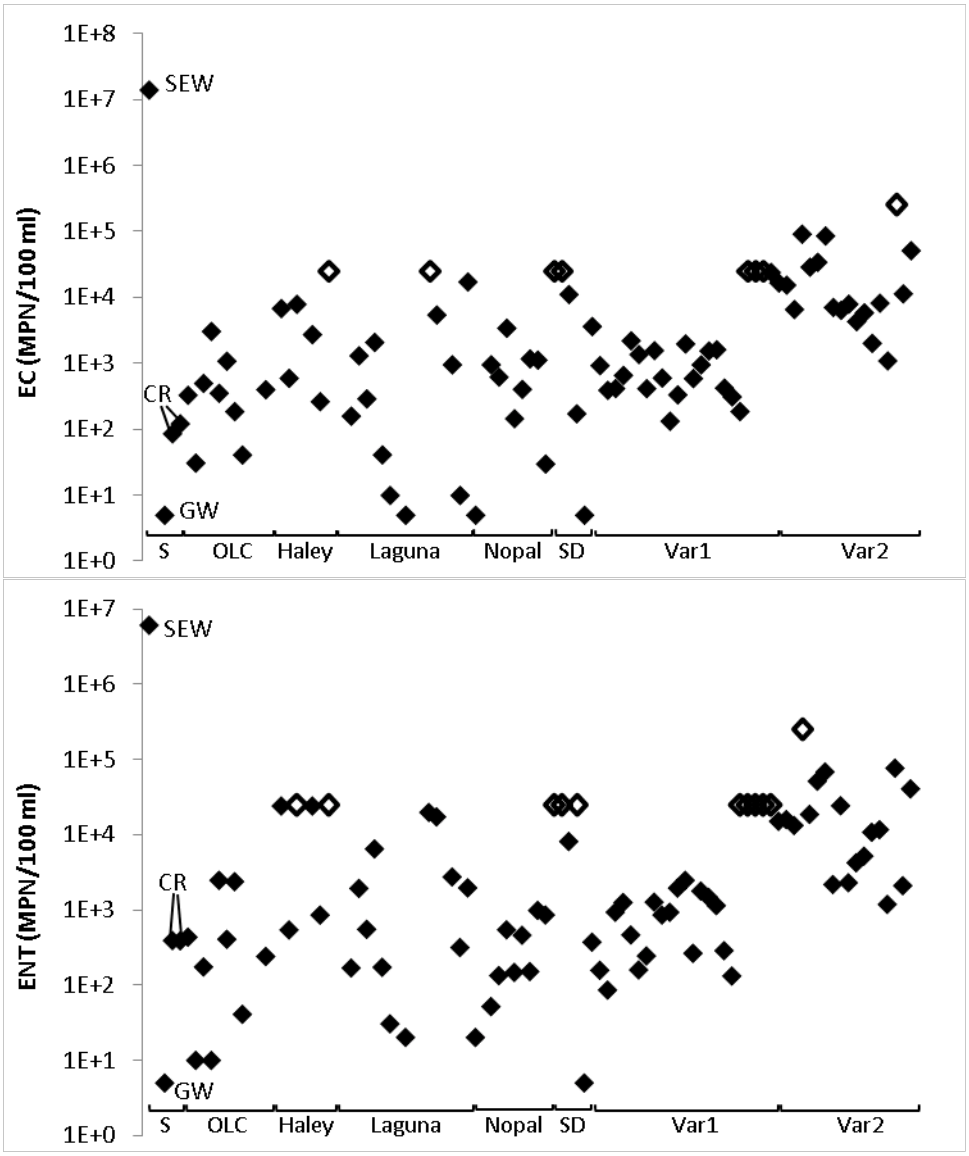


Figure 7.9. Concentrations of the fecal indicator bacteria *E. coli* (top) and *Enterococcus* (bottom) for all samples. Samples are grouped according to sample type or location: S = source water samples; OLC = ocean, lagoon and creek samples; Haley = Haley drain storm drain samples; Laguna = Laguna watershed storm drain samples, Nopal = Nopal watershed storm drain samples, Var1 = samples at L15 collected using autosampler, Var2 = samples at A5 collected using autosampler. Samples with over-range concentrations are indicated by open symbols.

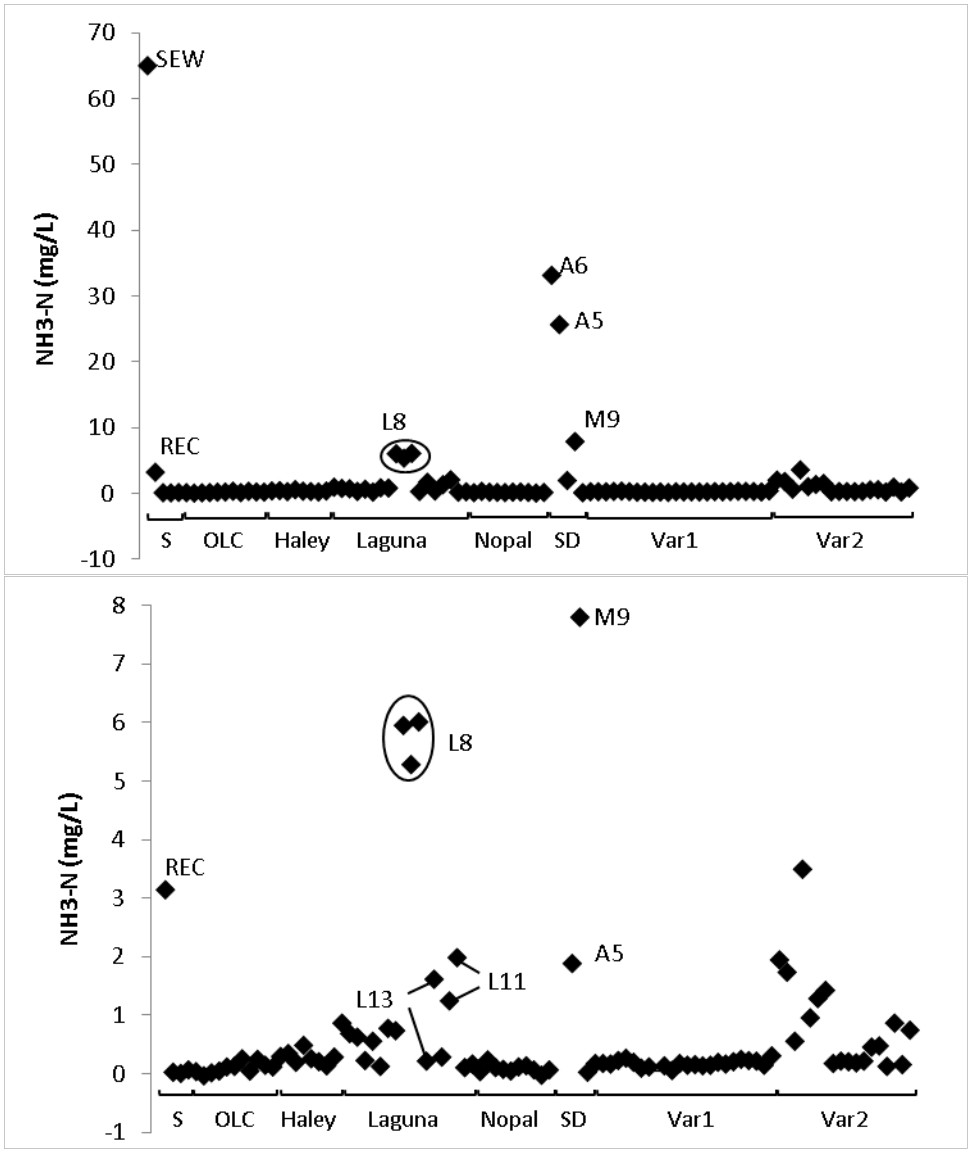


Figure 7.10. Concentrations of NH_3-N for all samples (top) and for samples below 8 mg/L (bottom). Samples are grouped according to sample type or location: S = source water samples; OLC = ocean, lagoon and creek samples; Haley = Haley drain storm drain samples; Laguna = Laguna watershed storm drain samples, Nopal = Nopal watershed storm drain samples, Var1 = samples at L15 collected using autosampler, Var2 = samples at A5 collected using autosampler. Samples from the same location are encircled.

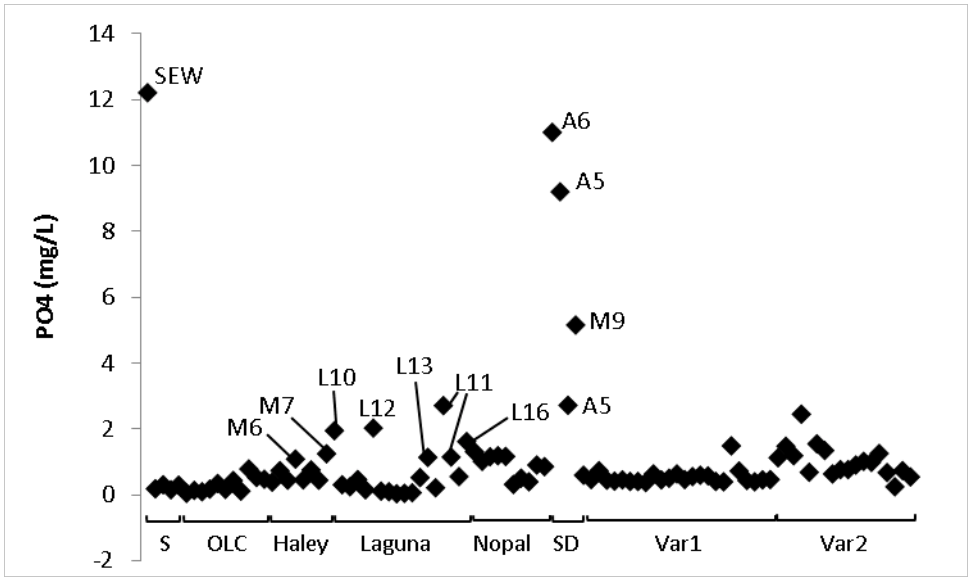


Figure 7.11. Concentrations of PO_4 for all samples. Samples are grouped according to sample type or location: S = source water samples; OLC = ocean, lagoon and creek samples; Haley = Haley drain storm drain samples; Laguna = Laguna watershed storm drain samples, Nopal = Nopal watershed storm drain samples, Var1 = samples at L15 collected using autosampler, Var2 = samples at A5 collected using autosampler.

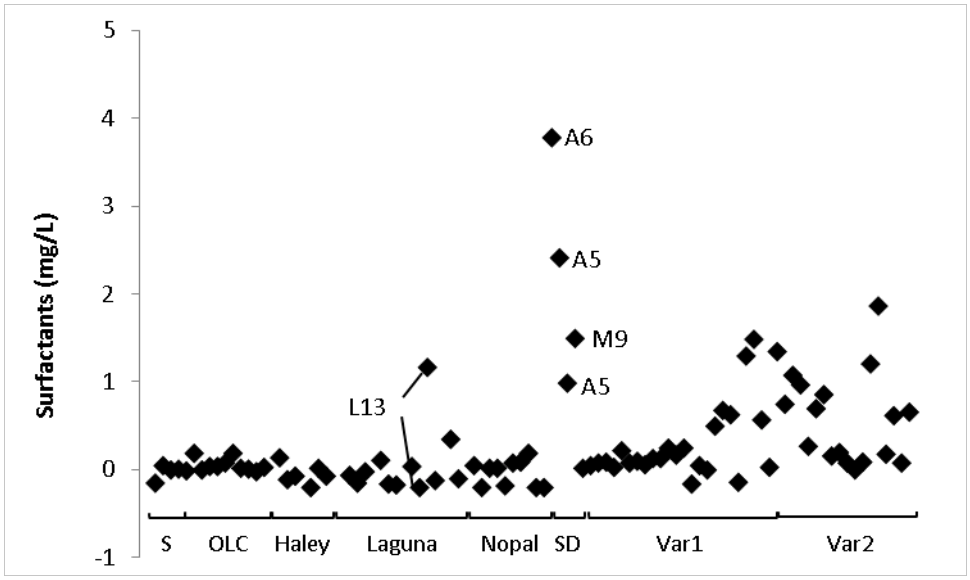


Figure 7.12. Concentrations of anionic surfactants for all samples. Samples are grouped according to sample type or location: S = source water samples; OLC = ocean, lagoon and creek samples; Haley = Haley drain storm drain samples; Laguna = Laguna watershed storm drain samples, Nopal = Nopal watershed storm drain samples, Var1 = samples at L15 collected using autosampler, Var2 = samples at A5 collected using autosampler.

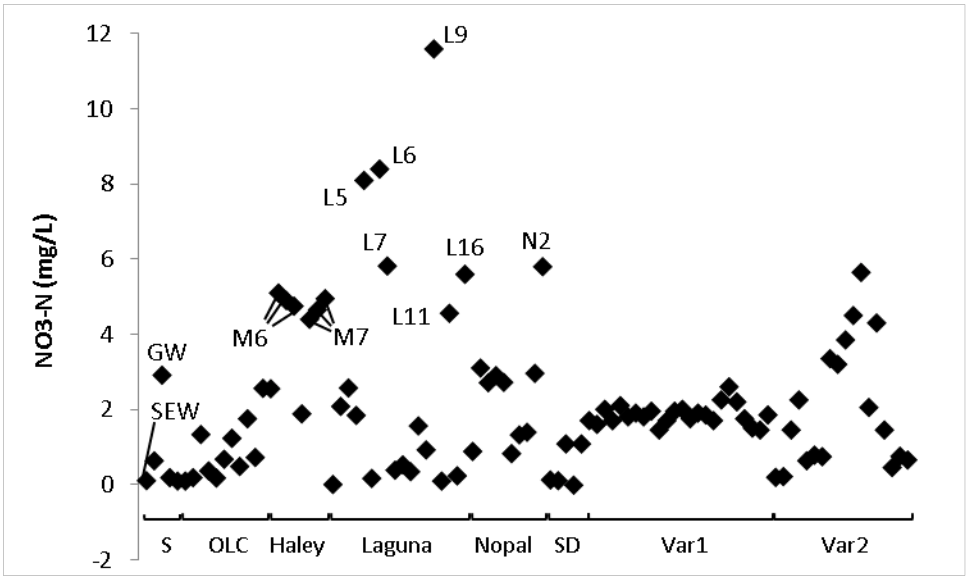


Figure 7.13. Concentrations of NO_3-N for all samples. Samples are grouped according to sample type or location: S = source water samples; OLC = ocean, lagoon and creek samples; Haley = Haley drain storm drain samples; Laguna = Laguna watershed storm drain samples, Nopal = Nopal watershed storm drain samples, Var1 = samples at L15 collected using autosampler, Var2 = samples at A5 collected using autosampler.

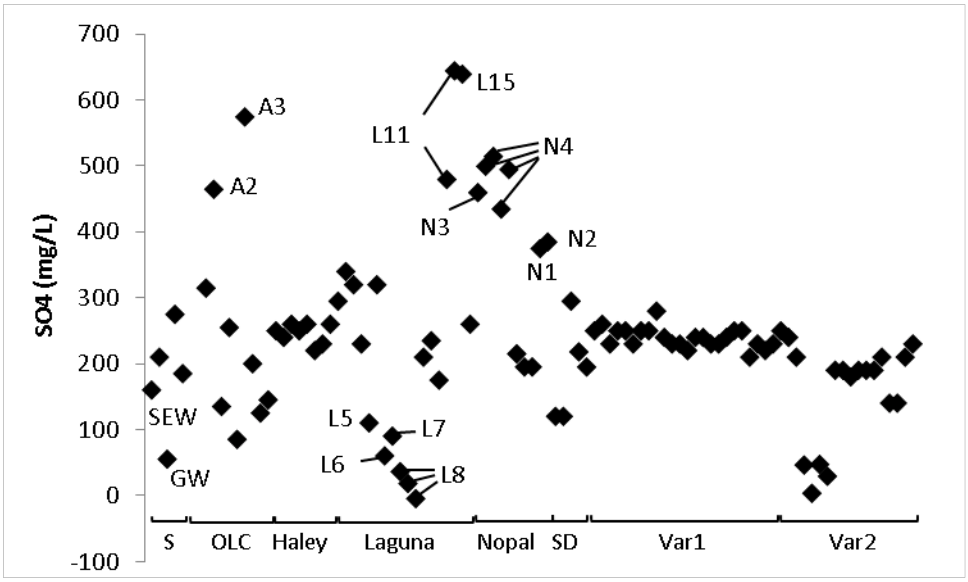


Figure 7.14. Concentrations of SO_4 for all samples except surf zone samples. Samples are grouped according to sample type or location: S = source water samples; OLC = ocean, lagoon and creek samples; Haley = Haley drain storm drain samples; Laguna = Laguna watershed storm drain samples, Nopal = Nopal watershed storm drain samples, Var1 = samples at L15 collected using autosampler, Var2 = samples at A5 collected using autosampler.

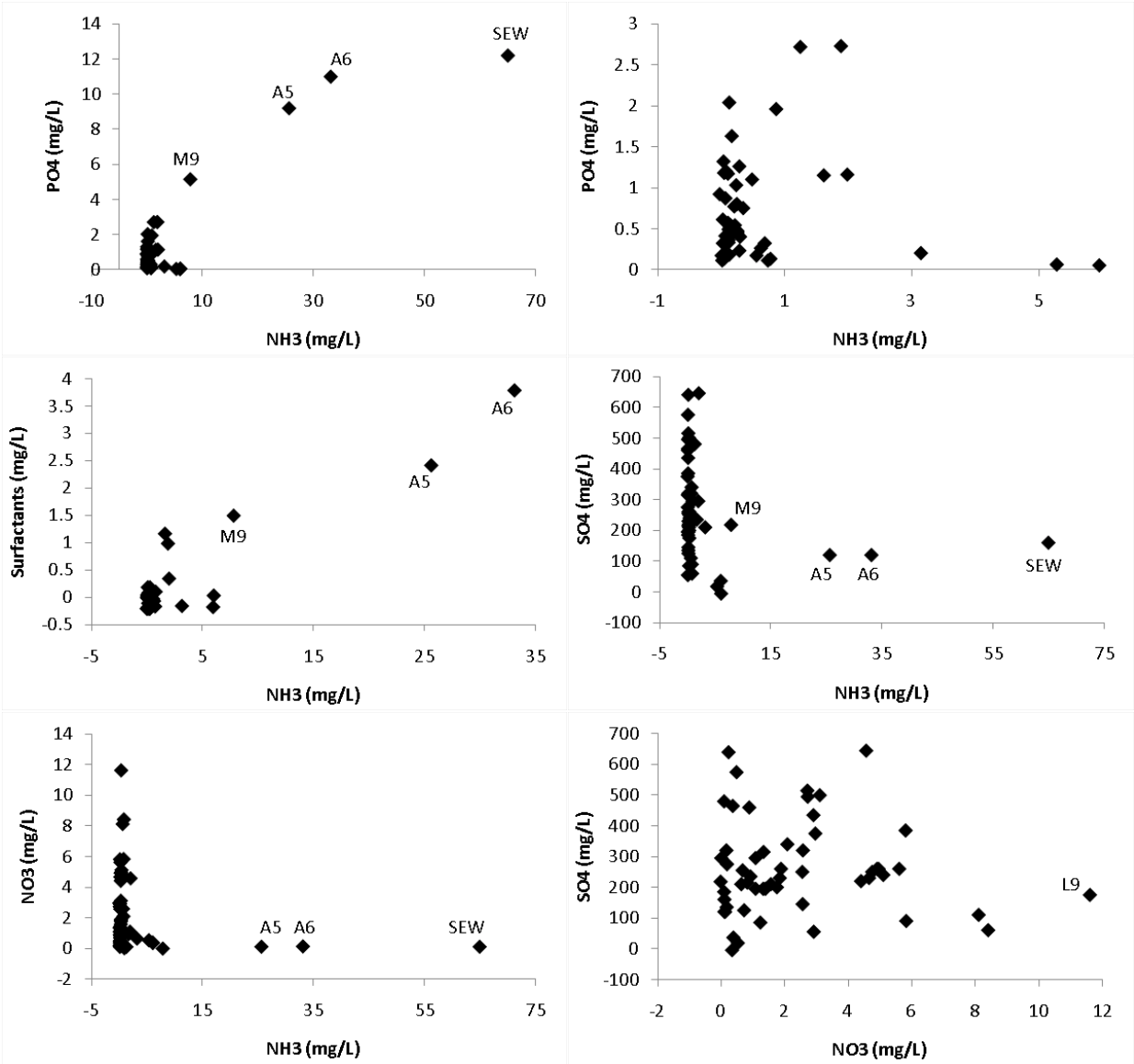


Figure 7.15. Scatter plots showing relationships between nutrients and anionic surfactants concentrations, by colorimetry, for all spatial samples (excluding variability studies at L15 and A5).

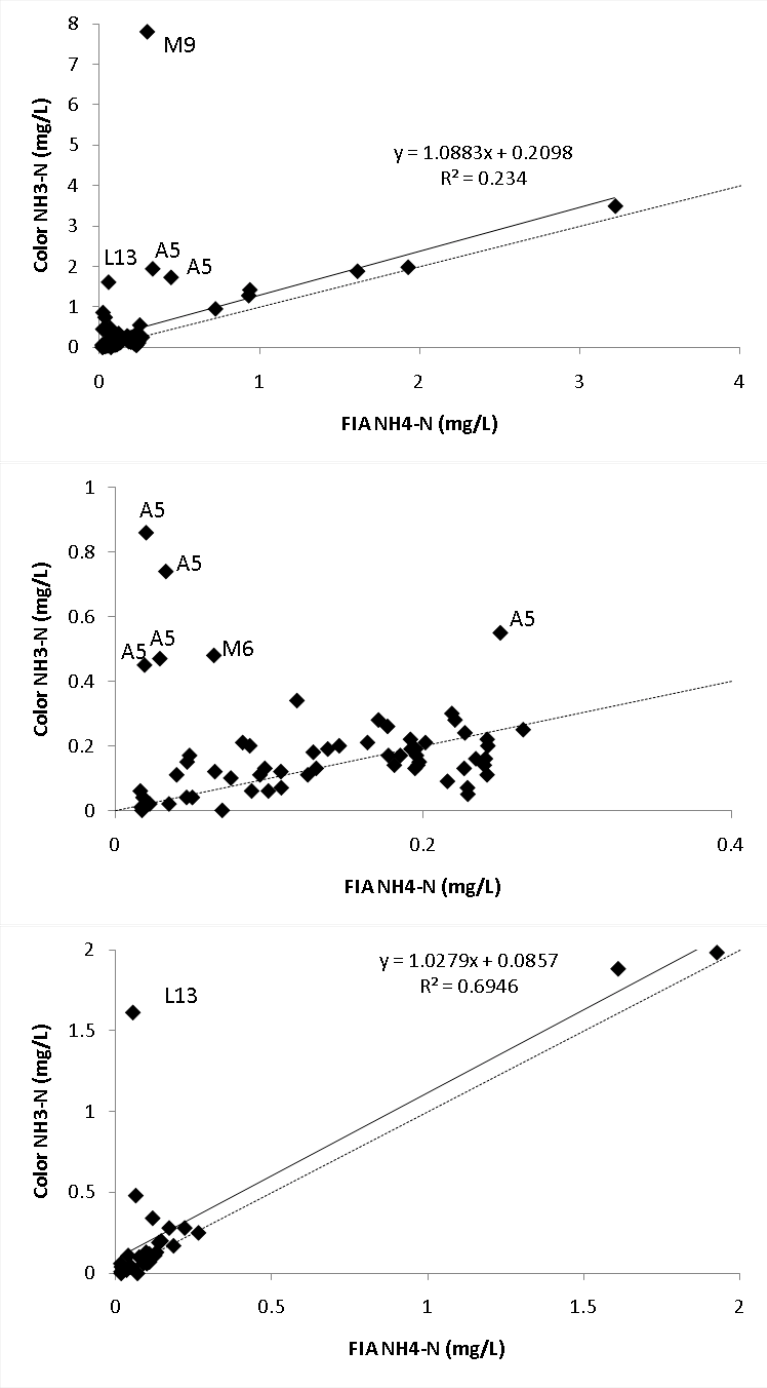


Figure 7.16. Scatter plots showing relations between NH_4-N determined by FIA and colorimetry for all samples (top), including a detail of the lowest concentration range (middle), and excluding variability study samples and outlier M9.

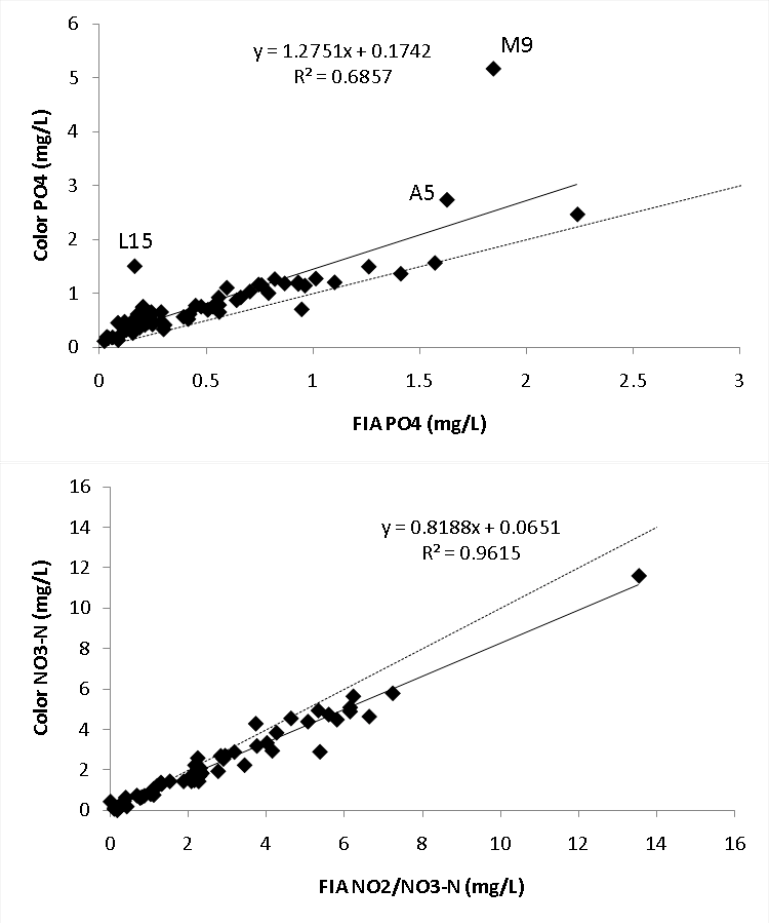


Figure 7.17. Scatter plots showing relations between PO_4 (top) and NO_3-N (bottom) determined by FIA and colorimetry for all samples.

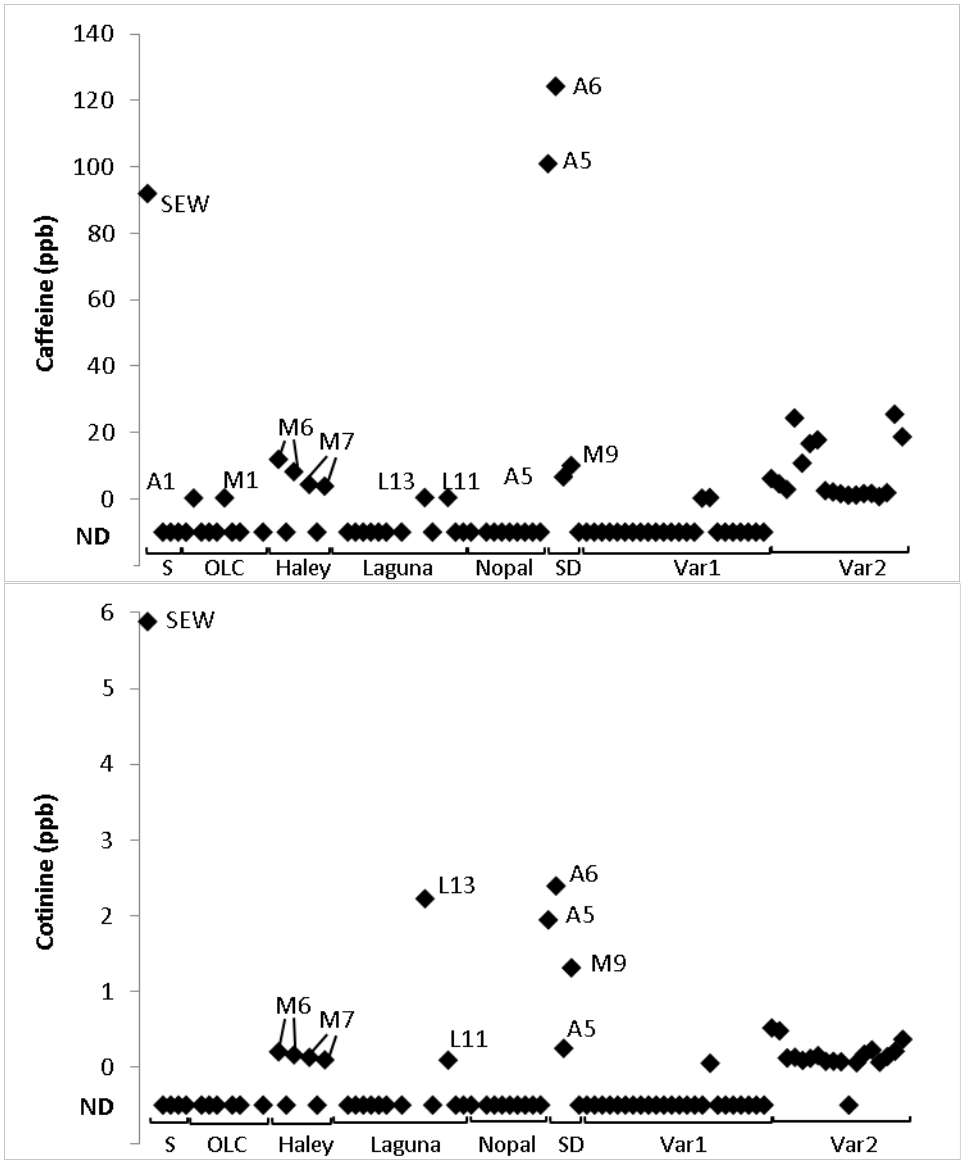


Figure 7.18. Concentrations of caffeine (top) and cotinine (bottom) for all samples. Samples are grouped according to sample type or location: S = source water samples; OLC = ocean, lagoon and creek samples; Haley = Haley drain storm drain samples; Laguna = Laguna watershed storm drain samples, Nopal = Nopal watershed storm drain samples, Var1 = samples at L15 collected using autosampler, Var2 = samples at A5 collected using autosampler.

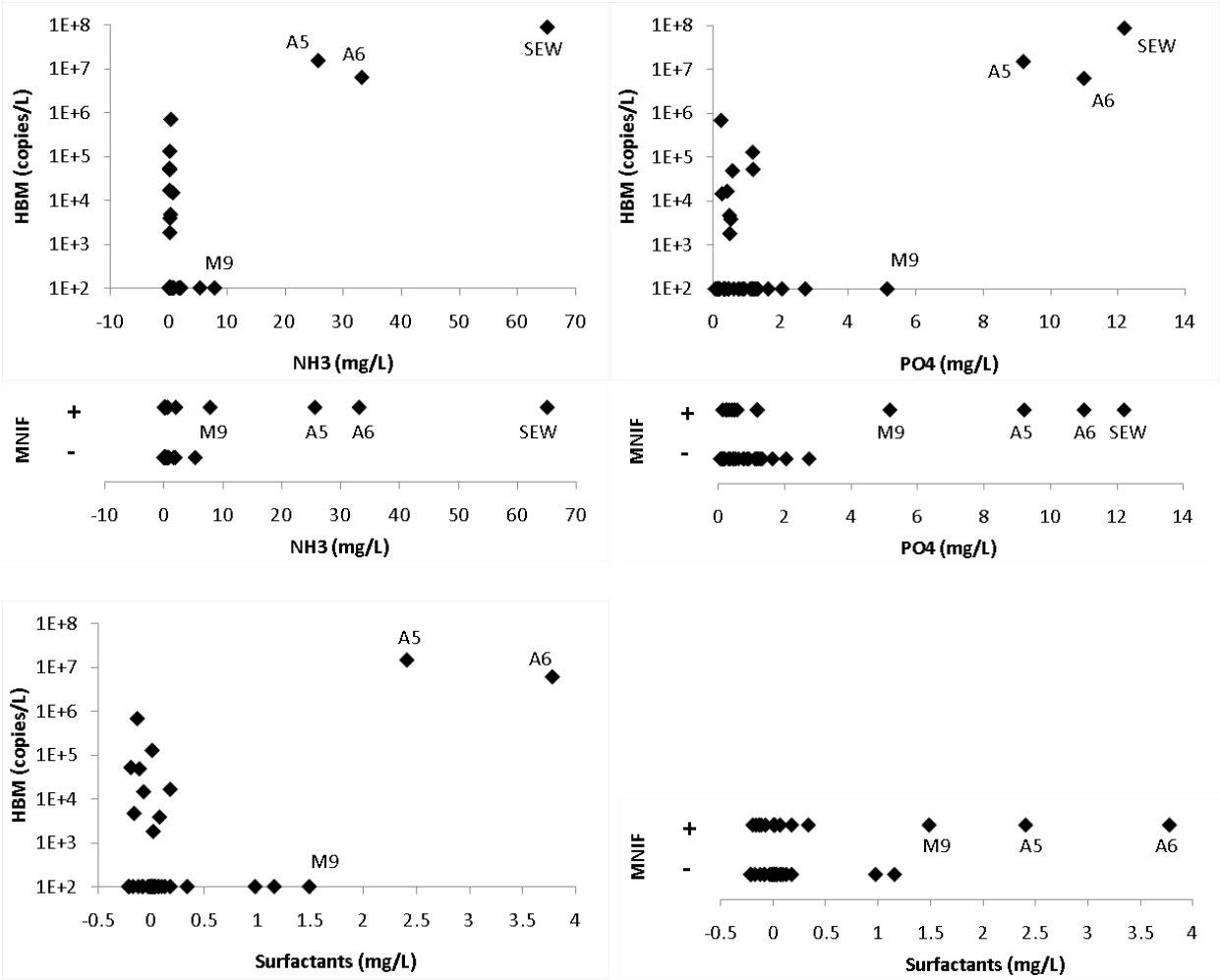


Figure 7.19. Consistency between NH_3-N, PO_4 and anionic surfactants concentrations and sewage-specific markers (HBM and MNIF) for all spatial samples (excluding L15 and A5 during variability study). HBM concentrations below the limit of detection are indicated at 1E+2 copies/L.

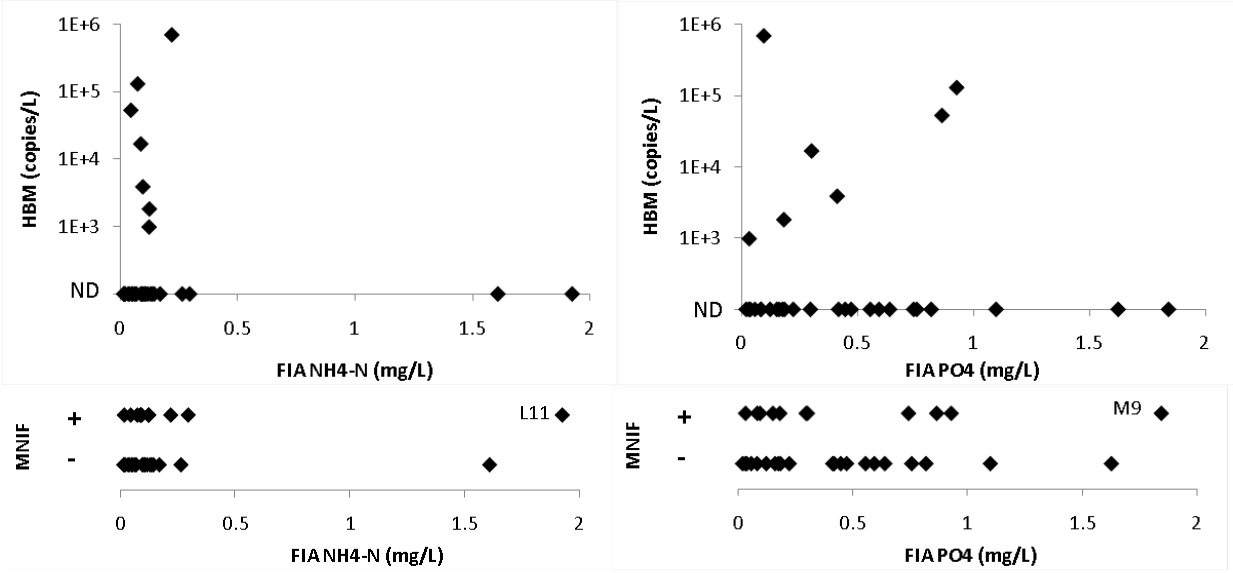


Figure 7.20. Consistency between NH_4-N and PO_4 by FIA, and sewage-specific markers (HBM and MNIF) for all samples (excluding L15 and A5 during variability study). HBM concentrations below the limit of detection are indicated by ND.

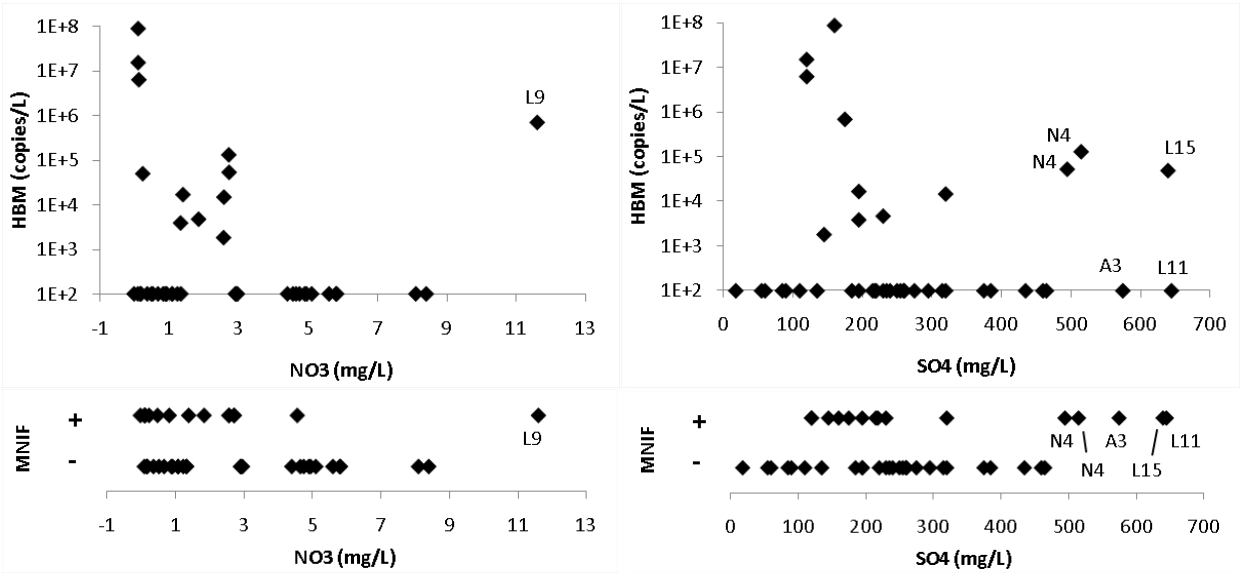


Figure 7.21. Consistency between NO_3-N and SO_4 concentrations and sewage-specific markers (HBM and MNIF) for all spatial samples (excluding L15 and A5 during variability study). HBM concentrations below the limit of detection are indicated at 1E+2 copies/L.

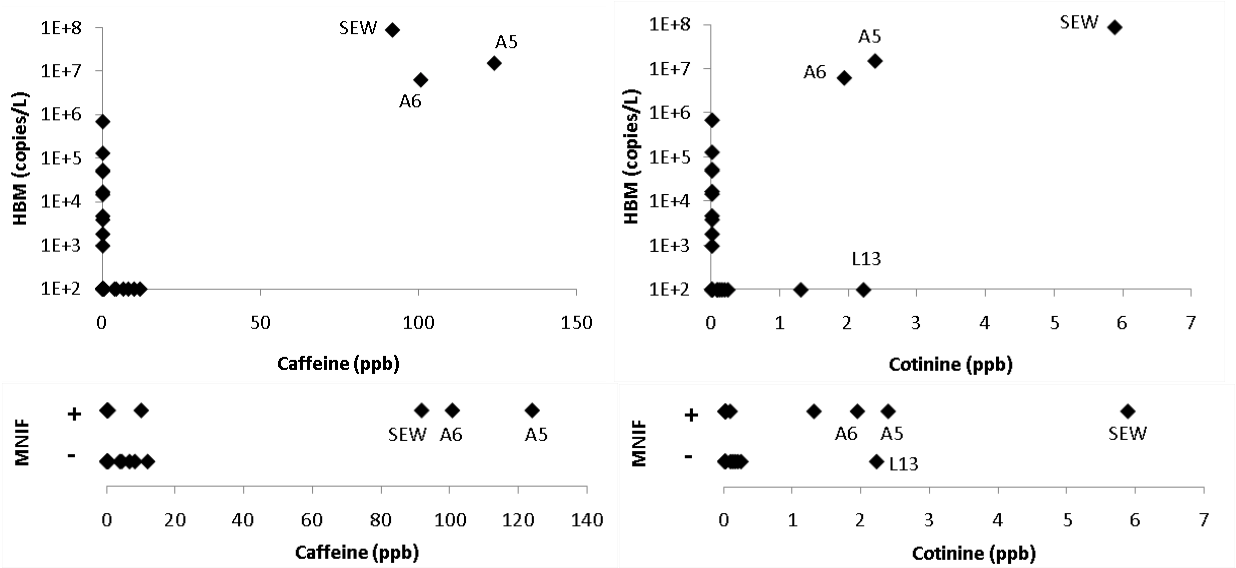


Figure 7.22. Consistency between caffeine and cotinine concentrations and sewage-specific markers (HBM and MNIF) for all spatial samples (excluding L15 and A5 during variability study). HBM concentrations below the limit of detection are indicated at 1E+2 copies/L.

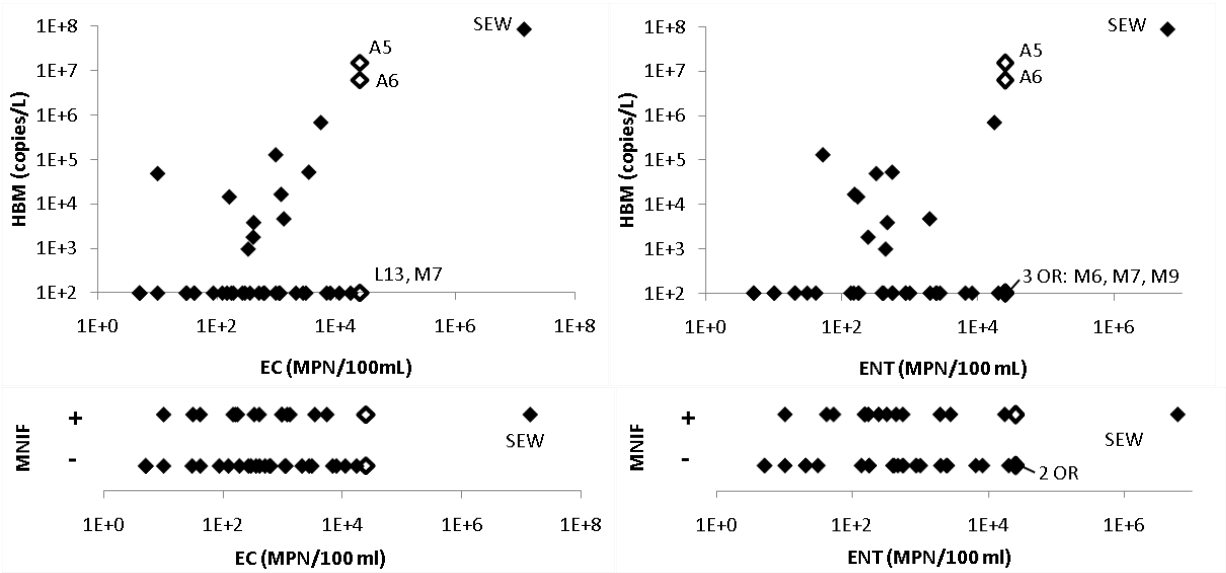


Figure 7.23. Consistency between *E. coli* and *Enterococcus* concentrations and sewage-specific markers (HBM and MNIF) for all spatial samples (excluding L15 and A5 during variability study). Over-range concentrations are indicated by open symbols, or by OR. HBM concentrations below the limit of detection are indicated at 1E+2 copies/L.

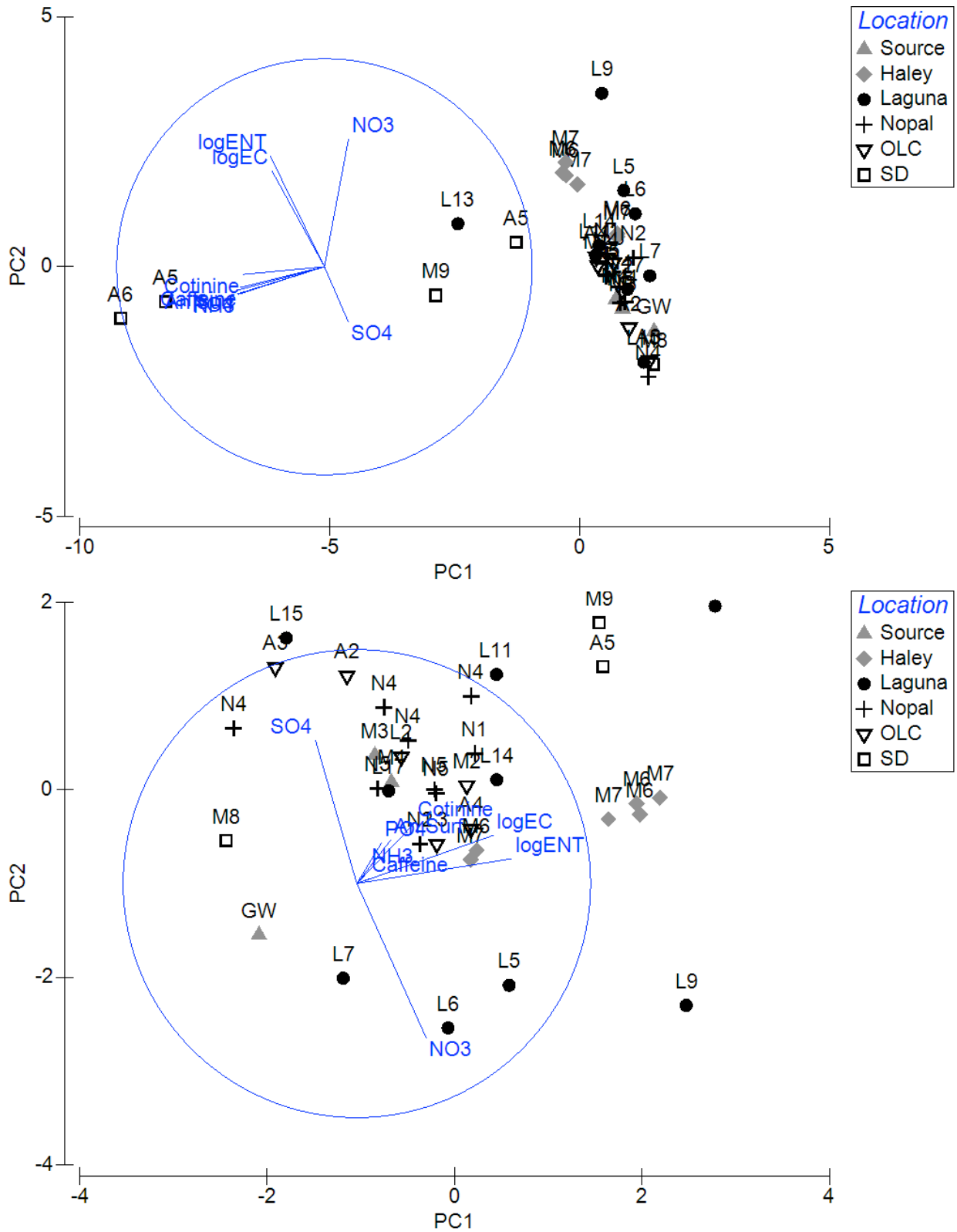


Figure 7.24. PCA plot showing all spatial samples (top) and spatial samples without A5 and A6, with symbols labeled according to sample type. Variable vectors are indicated on each graph.

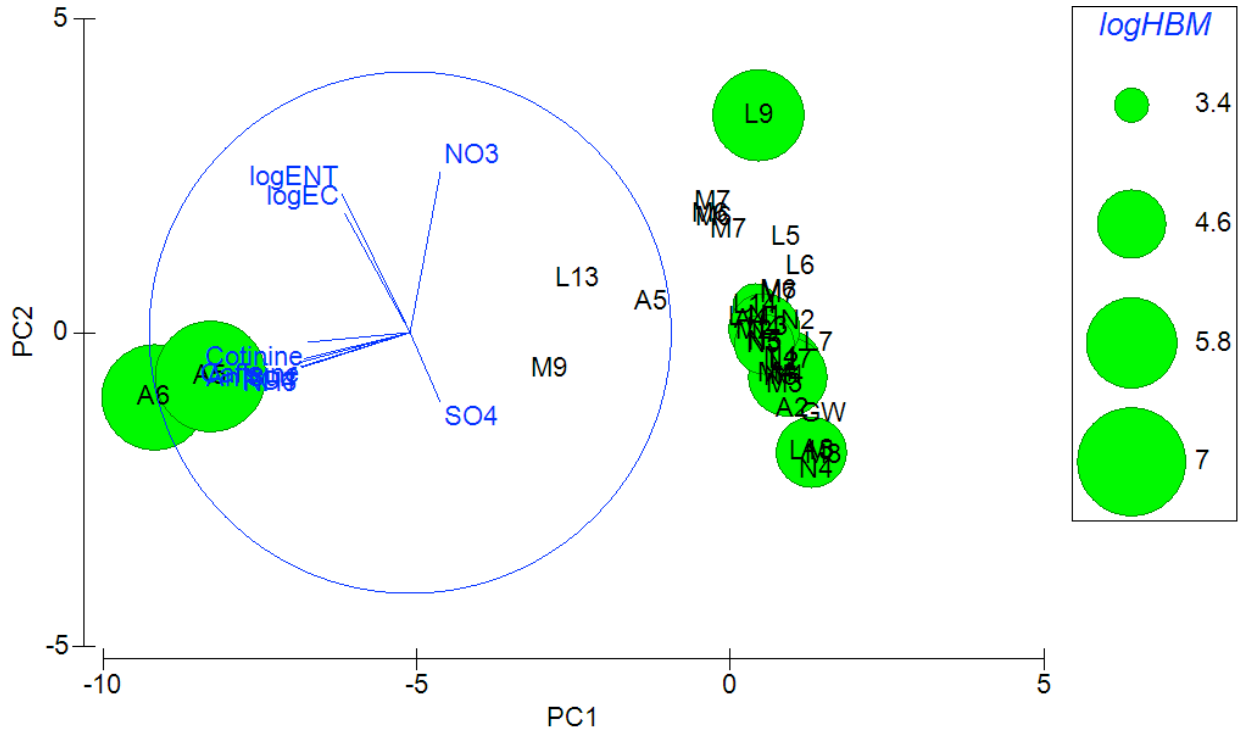


Figure 7.25. PCA plot showing all spatial samples, with symbols labeled according to sample type (top), and bubble plots showing HBM concentrations (bottom). Variable vectors are indicated on each graph.

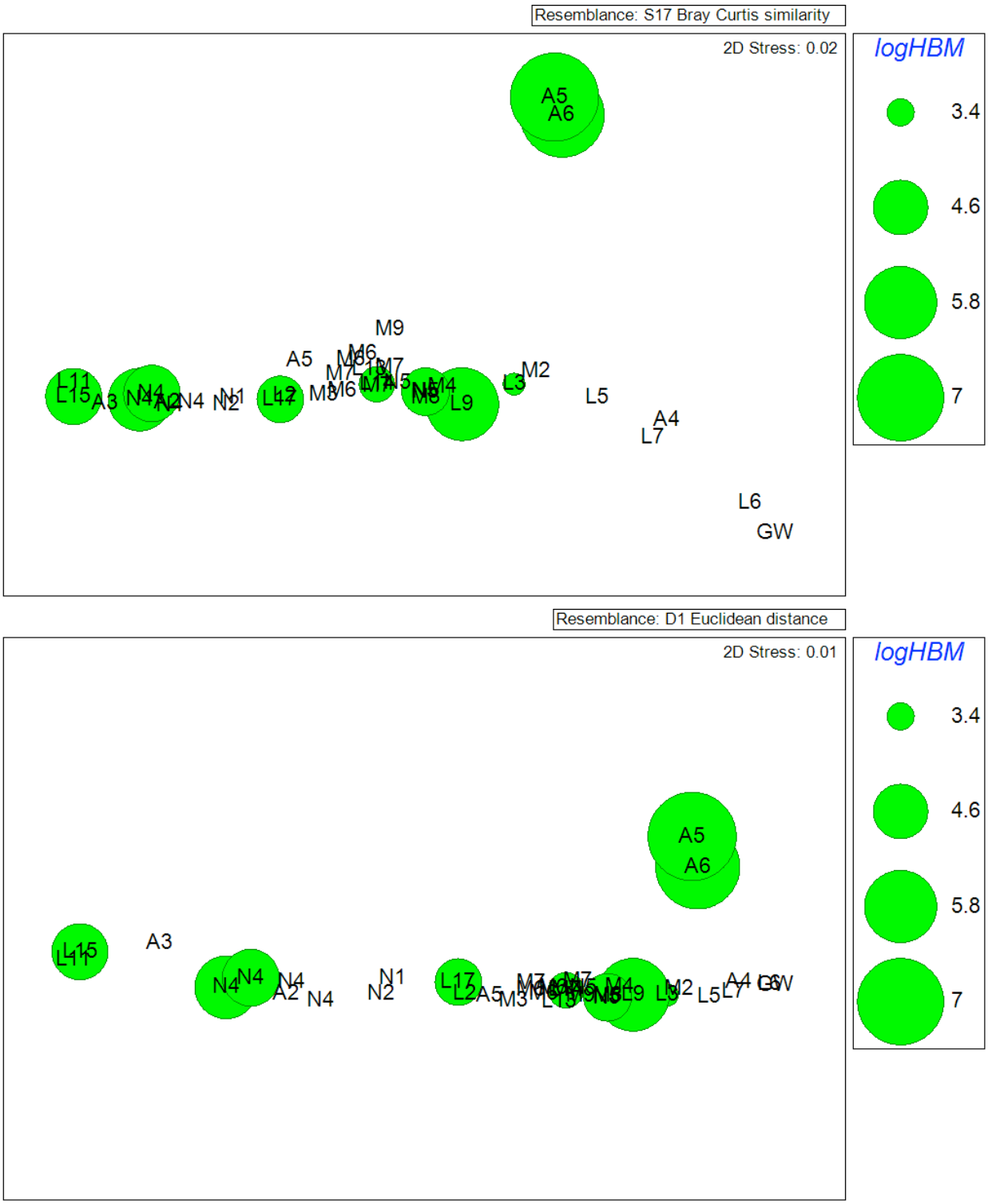


Figure 7.26. MDS plot for all spatial samples based on Bray-Curtis (top) and Euclidian (bottom) distances, with bubble plots representing HBM concentrations.

VIII. FLOW MEASUREMENT AND AUTOMATED STORM DRAIN SAMPLING

8.1 Summary

The goal of this study was to quantify diurnal and flow-dependent variations in concentrations of sewage-specific markers, fecal indicator bacteria and alternative indicators in storm drains during dry weather flow. Two flow meters configured for low-flow measurements were tested: an Isco 750 area-velocity flow module with low-profile AV sensor and a Sigma 920 flow module with low-profile velocity sensor and in-pipe ultrasonic level probe.

Flow at location L15 was fairly constant, but some of the contaminant concentrations varied greatly, independent of flow. Sewage contamination was consistently present based on HBM concentrations, but no correlations were observed between flow and contaminant concentrations, and between HBM and FIB or alternative indicators. The highest concentration variability was observed for FIB. In addition, a diurnal EC concentration pattern was observed, which has implications for monitoring FIB in storm drains. Temporal very high FIB concentrations were unrelated to sewage contamination.

Sampling at location A5 was not exclusively during dry weather, as intended. HBM and MNIF were not detected at this location. While flow in the storm drain was highly variable due to the rainfall, flow did not correlate with FIB or alternative indicator concentrations, except SO_4. As in L15, most contaminant concentrations showed high variability, even when only considering dry weather flow samples. Diurnal FIB concentration variations during dry weather were not observed at L15, based on the limited dry weather samples available.

Flow measurements using the Sigma 920 flow module set up were not successful, due to bad installation of the level sensor, although the velocity sensor provided useful information. The Isco 750 module performed satisfactorily, although velocity data collection was sometimes hindered by low flow depths and velocities, particularly at A5. Measurement of flow combined with automated sampling during dry weather flow was also challenging because of the limited number of locations where the equipment can be installed.

8.2 Experiments

The criteria for selecting storm drain locations for flow monitoring combined with automated sampling were the following:

- Known human fecal pollution based on previous HBM concentration measurements.
- Sufficient water depth (> 2.5 cm or 1”) for installing velocity and depth sensors.
- Dimensions of manhole and manhole cover. The Isco 6712 autosampler requires at least 0.54 m (21”) diameter clearance, and approximately 1.5 m (60”) depth (total depth after suspension of autosampler on manhole bracket). Ladders and other structures can also reduce practical manhole diameter or depth.
- Not located on busy road, as frequent replacement of sample bottles was required.

There were only few locations that matched all criteria, and location L15 and A5 were selected (Fig. 7.1).

Installation of flow equipment in storm drains was not trivial. Mounting rings and sensors were assembled above ground, based on the pipe diameters in the City storm drain database (48"and 54"). The mounting rings were lowered into the manholes using a tripod, and with the assistance of personnel certified for confined space entry. Street-level installation was not possible due to the large pipe diameters. It was fairly difficult to obtain a good fit of the mounting rings in the storm drains, and eventually the mounting rings had to be taken out of the manhole and re-assembled at street level for pipe diameters of approximately 45" and 51". Only with the smaller diameter, mounting rings fitted inside the pipes. In some cases debris had to be cleared before installation of sensors at the bottom of the pipe. Since a tripod and confined space entry certified staff was not available at the City of Santa Barbara Creeks Division and UCSB, the services of a contract plumber were used. Manhole entry was only required twice for each location, and automated sampling and sample collection could be done from street level.

During sampling, the Isco 6712 autosampler (approximately 45 kg with 24 full sample bottles) had to be retrieved twice a day, as mentioned below. Since a tripod was not available, but manhole entry was not required, a break down engine hoist was used to collect the sampler. The engine hoist could be easily transported in the back of a truck, and assembled in 10 minutes. Renting the engine hoist (rental at approximately $200/week) was a readily available and economical alternative to purchasing an appropriately rated tripod/winch system (approximately $7,000).

The temporal variability studies at each location consisted of 2 phases. First, flow was measured during 3-5 days to identify any temporal flow patterns. Second, based on initial flow measurements, automated samplers were programmed to collect storm drain samples, about 8 samples per day.

8.2.1 Location L15: Salsipuedes @ Cota

To measure baseline flow conditions, an Isco 6712 Portable Sampler with a 750 Area Velocity Flow Module with low-profile AV sensor was deployed from September 23 - 27, 2010. The unit was programmed to measure velocity and level every 1 minute. Due the frequency of measurements and the expected life of the 934 Nickel-Cadmium Battery, the sampler unit was retrieved once daily to download data using an Isco 581 RTD (Rapid Transfer Device) and exchange battery packs. Flow was calculated from the area and velocity measurements using Flowlink software.

No significant fluctuations or diurnal patterns were observed in the flow data, so the sampler was programmed to sample every 3 hours, from September 28 – October 1, 2010, collecting 24 samples in total. Velocity and level continued to be measured at 1 minute intervals during the sampling period. Prior to deployment for sampling, new sample, pump, and distribution tubing was installed in the unit. Isco 1L polypropylene bottles were sterilized by autoclaving. The lids were rinsed with 70% ethanol, air dried in a biological

safety cabinet, exposed to a UV lamp for 20 minutes, and placed into a sterile bag to bring in the field. The sampler was programmed to rinse the sampling line 3 times before collecting each sample, and to collect samples in 2x 1L bottles per sampling event. During the sample collection period, the sampler was retrieved twice daily to download data, exchange battery packs, collect samples, and replenish the sampler with fresh bottles and ice.

8.2.2 Location A5 – Hope drain diversion

To measure baseline flow conditions, a Sigma 920 flow meter with low-profile velocity sensor and ultrasonic level sensor was deployed from September 28 – October 1, 2010. The unit was programmed to measure velocity and level every 1 minute. Due to the extended battery life of the unit, flow measurements were not retrieved until October 1. Unfortunately, there was an apparent error in the installation of the level sensor and no level data were collected. Velocity data did reveal some small increases in velocity in the mornings, and therefore the initial plan was set up automated sampling to collect samples before, during and after these expected flow increases.

On October 5, 2010, the Sigma 920 flow equipment was removed and the Isco 6712 Portable Sampler with a 750 Area Velocity Flow Module with low-profile AV sensor was installed. Due to the prediction of an early first storm event, the sampling plan was altered so that samples would be collected every 3 hours, over 3 days to collect 24 samples. To conserve battery life, velocity and level measurements were set for 5 minute intervals. The sampler was retrieved only once per day, to download data, exchange battery packs, collect samples, and replenish the sampler with fresh bottles and ice. Prior to deployment for sampling, new sample, pump, and distribution tubing was installed in the unit. Bottles and caps were sterilized as specified for location L15. As before, the sampler was programmed to rinse the sampling line 3 times before collecting each sample, and to collect samples in 2x 1L bottles per sampling event.

However, on the second sampler retrieval date (October 7), multiple sampler errors were observed, and no samples had been collected. The most likely cause of the errors was incomplete submersion of the sample strainer. This sampling location is very deep, near the limit of the maximum pump height for vinyl tubing, and the baseline flow was very low. In total, 7 samples were collected, in 3 hour intervals, from October 5 – 6, 2010. The sampler was then re-programmed to collect samples in 2 hour intervals from October 7 – 8, 2010, resulting in 11 additional samples. Of the 18 total samples collected from this location, the first 7 samples captured the pre-storm flow, and the rising and falling of two peak flow events. Due to the delay in sampling the remaining 11 samples, they captured the system 24 hours after the first-flush storm event.

8.3 Results

8.3.1 Location L15

Preliminary flow measurements (9/23-9/27) at L15 indicated fairly constant level (0.068 ± 0.002 m, average ± standard deviation) and velocities (0.064 ± 0.008 m/s) in the storm drain (Fig. 8.1A). The measured values were within the operating ranges of the sensor of the low-profile AV sensor (Table 4.3). The measurement accuracies are ± 0.0006 m at 0.07 m depth (± 0.8%) for level, and ± 0.03 m/s at 0.06 m/s (± 50%) for velocity. Therefore, the low velocity accuracy will greatly affect flow accuracy. Since velocity accuracy is absolute, relative error will be lower at increasing velocities. Calculated flow was relatively constant at 1.6 ± 0.2 l/s (Fig. 8.1B). Based on level and velocity accuracies, flow accuracy was approximately ± 0.8 l/s at the average of 1.6 l/s. A negative flow was observed for one data point (9/26), due to a negative velocity. This was likely caused by turbulence, and sporadic negative flow values were deleted in following experiments, as recommended by Isco.

Flow during sampling was 1.5 ± 0.1 l/s, and of similar magnitude variability and accuracy as during preliminary flow measurements (Fig. 8.2). One small flow peak was observed on 9/29, with a maximum flow of 2.2 l/s, lasting approximately 40 minutes. However, no water sample was taken during the peak.

The concentrations of nutrients were 0.17 ± 0.06 mg/L NH_3-N, 0.55 ± 0.23 mg/L PO_4, 1.85 ± 0.26 mg/L NO_3-N, and 240 ± 15 mg/L SO_4 (Fig. 8.2). Anionic surfactant concentrations were 0.26 ± 0.41 mg/L (Fig. 8.2). Coefficient of variation (CV) values were lowest for SO_4 (6%) and NO_3-N (14%), medium for NH_3-N (35%) and PO_4 (41%), and highest for anionic surfactants (155%). Concentrations peaks were observed for all nutrients, except SO_4, and for anionic surfactants. However, concentrations of NH_4-N, PO_4 and (NO_2+NO_3)-N were less variable when analyzed using FIA, with CVs of 11%, 23% and 6%, respectively (Fig. 8.3). The concentrations of NH_4-N, PO_4 and NO_3-N determined by both methods were not significantly correlated ($p > 0.05$). Therefore, colorimetry is not accurate enough for measuring rather subtle concentrations changes for nutrients, even at the same location, and other methods such as FIA are recommended.

Caffeine and cotinine were only detected twice, and once, respectively, at concentrations near the limit of detection (0.175 ppb and 0.05 ppb, respectively) (Fig. 8.4).

Concentrations of EC and ENT varied greatly in time, from approximately 100 MPN/100 ml to over 25,000 MPN/100 ml, with a CV exceeding 170% (Fig. 8.4). Concentrations of EC and ENT increased sharply, at least 2 orders of magnitude, on 9/30 between 3 and 9 PM, suggesting a new source of FIB. A diurnal pattern could be observed for EC, with increasing concentrations during nighttime and decreasing concentrations during daytime (Fig. 8.5). A cyclic concentration pattern was observed as well for ENT, but not with a consistent day- and nighttime pattern (Fig. 8.5). FIB concentrations appeared negatively correlated ($p < 0.01$) with air temperature (obtained from station NOAA 9411340, Santa Barbara harbor) (Fig. 8.6). However, correlations were greatly affected by high FIB concentrations (>25,000 MPN/100 ml) for the last 4-5 observations. When excluding the latter data points from the correlation analysis, significant correlations were no longer observed, and therefore a real effect of temperature is questionable.

HBM were consistently detected at L15 (Fig. 8.4), and concentrations varied between approximately 4,000 and 68,000 copies/L (CV = 72%). MNIF were not detected. HBM concentrations did not increase, but even decreased slightly, for the last 5 observations with high FIB concentrations. Therefore, it was unlikely that the source of these FIB was related to sewage.

The cyclic pattern for EC and ENT concentrations has important implications for monitoring storm drains, or calculating daily FIB loads. EC concentrations were at least an order of magnitude higher in the morning compared to the evening, and ENT concentrations varied similarly although they were less predictable. Therefore, 2-3 samples per day during at least 3 days are required for reliable quantification of average FIB concentrations. However, more sampling might be required if concentrations spikes, such as the one observed for the last 5 observations, occur, or if diurnal variations need to be quantified. Since HBM concentrations were more stable at this location, 3-5 samples taken on different days appear sufficient for quantifying average concentrations.

Correlation analyses were performed to determine the relations between flow and sewage-specific and alternative indicators (Table 8.1). Measurements by colorimetry were not included, as they are considered too inaccurate and imprecise for identifying small concentration variations. The Pearson correlation coefficient was used to screen for significant correlations, and scatter plots for significantly correlated variables are provided (Fig. 8.7).

Flow was significantly correlated only with NH_4-N concentrations (determined by FIA). This correlation was not observed using colorimetry for NH_3-N (data no t shown). Log-transformed HBM concentrations were not correlated to log-transformed FIB or any of the alternative indicator concentrations, suggesting that either other sources of FIB and alternative indicators are present, or fate and transport in the storm drains differ. When excluding the last 5 observations from the correlations, because of a suspected non-sewage related source of FIB, better correlations appear between FIB and HBM, although correlation coefficients are still low and not-significant (Fig. 8.8).

From all alternative indicators tested, only log-transformed EC and ENT concentrations were significantly correlated (Table 8.1, Fig. 8.7).

8.3.2 Location A5

A Sigma 920 flow meter with low-profile velocity sensor and ultrasonic in-pipe sensor was deployed at this location because of the challenging site conditions (low velocity and flow depth). However preliminary deployment of the Sigma 920 configuration did not provide level data, probably because of bad installation. The ultrasonic in-pipe level sensor was installed using the provided level sensor, in order to direct the beam vertically to the wet pipe bottom. However, Sigma technical support informed us that this sensor can be slightly inaccurate, and given the low flow depth and corresponding small flow width, the ultrasonic beam may have missed the flow at the bottom of the storm drain pipe. To avoid such problems in the future, installation of the level sensor needs to be checked by placing an obstacle in the ultrasonic beam path near the water surface, and real-time data collection, especially with very low flows and large pipe diameters (54“ in this case).

For most data points, velocity was below the limit of detection (0.01 m/s), which could be caused by low level (< 0.01 m, limit for velocity measurements), low velocities, or both (Fig. 8.9A). However, during installation water level was sufficient (0.01 – 0.02 m) for velocity measurements. Also, some single data point outliers were observed, both as positive and negative velocities. Those were considered as erroneous measurements, as positive and negative flow spikes for less than a minute are unlikely to occur. Still, several episodes occurred with measurable velocities (Fig. 8.9B). Velocity peaks between 0.6-0.9 m/s occurred between 4 and 8 AM on 3 days, in addition to smaller peaks that were consistently detected around 8-9 PM and 2 AM. Based on these velocity data, it can be concluded that intermittent flow occurred at A5, at fairly regular intervals.

Due to the anticipation of rain during sampling between 10/4-10/9, it was decided not to focus sampling around the flow peaks observed at this location, but to sample at 3-hour intervals in order to capture storm flow. Therefore, diurnal variations in dry weather flow could not be determined at this location. Flow was measured using the Isco 750 module, as the Sigma 920 module could not be connected to the Isco 6712 autosampler.

Before the rain event, water level in the storm drain was too low for velocity measurements (< 0.02 m). Consequently, all velocity measurements were zero. During the rain event, level and velocity increased to maxima of 0.26 m and 1.67 m/s, respectively, resulting in flows up to 294 l/s (Fig. 8.10-8.12). After peak flow, water level decreased again and was mostly below the cutoff of 0.025 m for velocity measurements. Besides the flow peak related to rainfall, a few additional small flow peaks were observed (Fig. 8.11). On 10/7 and 10/8, these peaks occurred between 4 and 7 AM, confirming the dry weather flow patterns observed during preliminary flow measurements. Only one sample was obtained during a dry weather flow peak (small peak on 10/8 at 4:32 AM). Therefore the influence of flow variations on contaminant concentrations during dry weather flow could not be reliably determined.

Significant variations in all nutrient and anionic surfactants concentrations were observed (Fig. 8.12). Concentrations before, during and after the high flow event did not appear different, except for SO_4 concentrations, which decreased during higher flow. Concentration peaks were observed for NH_3-N, PO_4, anionic surfactants and NO_3-N by colorimetry, although not necessarily at the same time. Colorimetry and FIA analyses agreed very well, except for NH_4-N where dry weather peaks were not observed using FIA (Fig. 8.13).

Caffeine and cotinine concentrations varied greatly as well, and appeared to be influenced by flow as well as other sources during dry weather (Fig. 8.14). EC and ENT concentrations also varied greatly, apparently unrelated to flow (Fig. 8.14). HBM and MNIF were not detected during this sampling event at A5.

When only considering dry weather flow samples (excluding 4 samples taken on 10/6), CVs were lowest for SO_4 (16%), medium for PO_4 (36%) and highest for NO_3-N (80%), NH_3-N (103%), anionic surfactants (101%), caffeine (145%), cotinine (87%), and FIB (> 143%). CVs of nutrient concentrations by FIA were very similar for NH_4-N (105%), PO_4 (42%) and (NO_2+NO_3)-N (84%). Diurnal variations of FIB concentrations could not be observed here, but limited dry weather sampling was available for a complete assessment of this phenomenon.

The sample taken on 10/8 at 4:32 AM (third last sample, taken during small dry weather flow peak) had nutrient, anionic surfactants, caffeine and cotinine concentrations within range of the other dry weather flow samples (Fig. 8.12, Fig. 8.14). However, EC and ENT concentrations for that sample were higher than other dry weather flow samples (Fig. 8.14). Therefore, the dry weather flow peaks could be FIB sources, but more samples need to be analyzed to reliably identify these patterns.

Correlation analysis confirmed that none of the measured variables, except SO_4, correlated significantly with flow (Table 8.2, Fig. 8.15). The correlation with SO_4 suggests that SO_4-rich baseflow was diluted during rainfall. Nutrient concentrations by colorimetry and FIA correlated significantly at A5, with correlation coefficients of 0.86 (NH_4-N), 0.96(PO_4) and 0.97 (NO_3-N). Therefore, nutrients by FIA were not correlated with flow either (data not shown).

Since HBM and MNIF were not detected, the correlations between sewage-specific indicators and alternative indicators could not be performed. Some, but not all, of the alternative indicators indicative of fresh sewage identified in Chapter 7 (NH_3-N, PO_4, anionic surfactants, caffeine, cotinine) were significantly and positively correlated (Table 8.2, Table 8.3, Fig. 8.16). However, strong and consistent correlations were not expected as sewage contamination was not detected by HBM. Correlations between alternative indicators not indicative of fresh sewage (NO_3-N and SO_4) and all other alternative indicators and FIB were negative, although they were not always significant (Table 8.2, Table 8.3, Fig. 8.16). When analyzing nutrients by FIA, correlations with other variables were usually similar but a little stronger (Table 8.3).

Table 8.1. Pearson correlation coefficients between flow and concentrations of FIB, human-specific and alternative indicators at L15. FIB and HBM concentrations are log-transformed. Significant correlations are indicated by one ($p < 0.05$) or two ($p < 0.01$) asterisks. Caffeine and cotinine were not detected. Nutrient concentrations were determined by FIA, colorimetric assays were not included in correlation analysis.

	Flow	NH_4-N	PO_4	(NO_2+NO_3)-N	logEC	logENT
NH_4-N	0.48*	1.00				
PO_4	-0.18	-0.22	1.00			
(NO_2+NO_3)-N	0.09	0.20	0.19	1.00		
logEC	0.1	-0.35	0.02	0.05	1.00	
logENT	0	-0.22	0.14	0.15	0.62**	1.00
logHBM	-0.14	-0.22	-0.08	-0.14	-0.04	-0.03

Table 8.2. Pearson correlation coefficients between flow and concentrations of FIB and alternative indicators at A5. FIB concentrations are log-transformed. Significant correlations are indicated by one ($p < 0.05$) or two ($p < 0.01$) asterisks.

	Flow	NH_3-N	PO_4	Surf.	NO_3-N	SO_4	Caf.	Cot.	logEC	logENT
NH_3-N	0.08	1.00								
PO_4	-0.12	0.78**	1.00							
Surf.	-0.15	0.44	0.45	1.00						
NO_3-N	-0.24	-0.41	0.00	-0.23	1.00					
SO_4	-0.60**	-0.38	-0.31	0.10	0.20	1.00				
Caf.	0.15	0.52*	0.41	0.03	-0.46	-0.42	1.00			
Cot.	-0.15	0.42	0.15	0.52*	-0.55*	0.45	0.19	1.00		
logEC	0.02	0.61**	0.20	0.19	-0.47	-0.45	0.56*	0.23	1.00	
logENT	0.00	0.79**	0.53*	0.52*	-0.43	-0.49*	0.49*	0.26	0.83**	1.00

Table 8.3. Pearson correlation coefficients between nutrients by FIA and FIB and alternative indicators at A5. FIB concentrations are log-transformed. Significant correlations are indicated by one ($p < 0.05$) or two ($p < 0.01$) asterisks. Concentrations determined by FIA and colorimetry are indicated by superscript "F" and "C", respectively.

	NH_4-N^F	PO_4^F	(NO_2+NO_3)-N^F	Surf.^{C}	SO_4^C	Caf	Cot	logEC	logENT
NH_4-N^F	1.00	0.87**	-0.01	0.20	-0.62**	0.59*	-0.04	0.25	0.92**
PO_4^F	0.87**	1.00	-0.03	0.42	-0.52*	0.49*	0.12	-0.01	0.69**

(NO_2+NO_3)-N^F	-0.01	-0.03	1.00	-0.30	0.14	-0.40	-0.59*	-0.38	-0.08

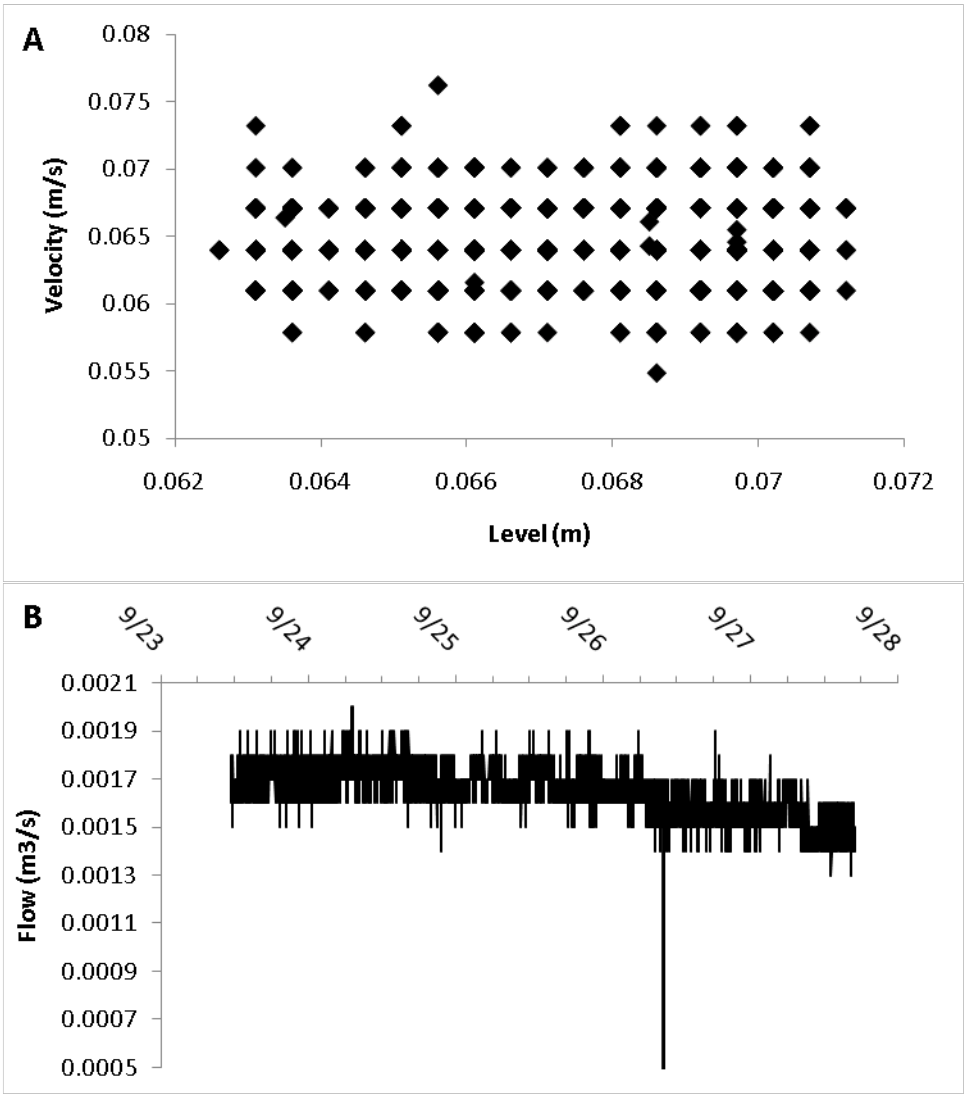


Figure 8.1. Preliminary flow measurements at L15 during the period 9/23-9/28, using Isco 750 flow module: level-velocity measurements, excluding negative velocity data point (A) and flow (B).

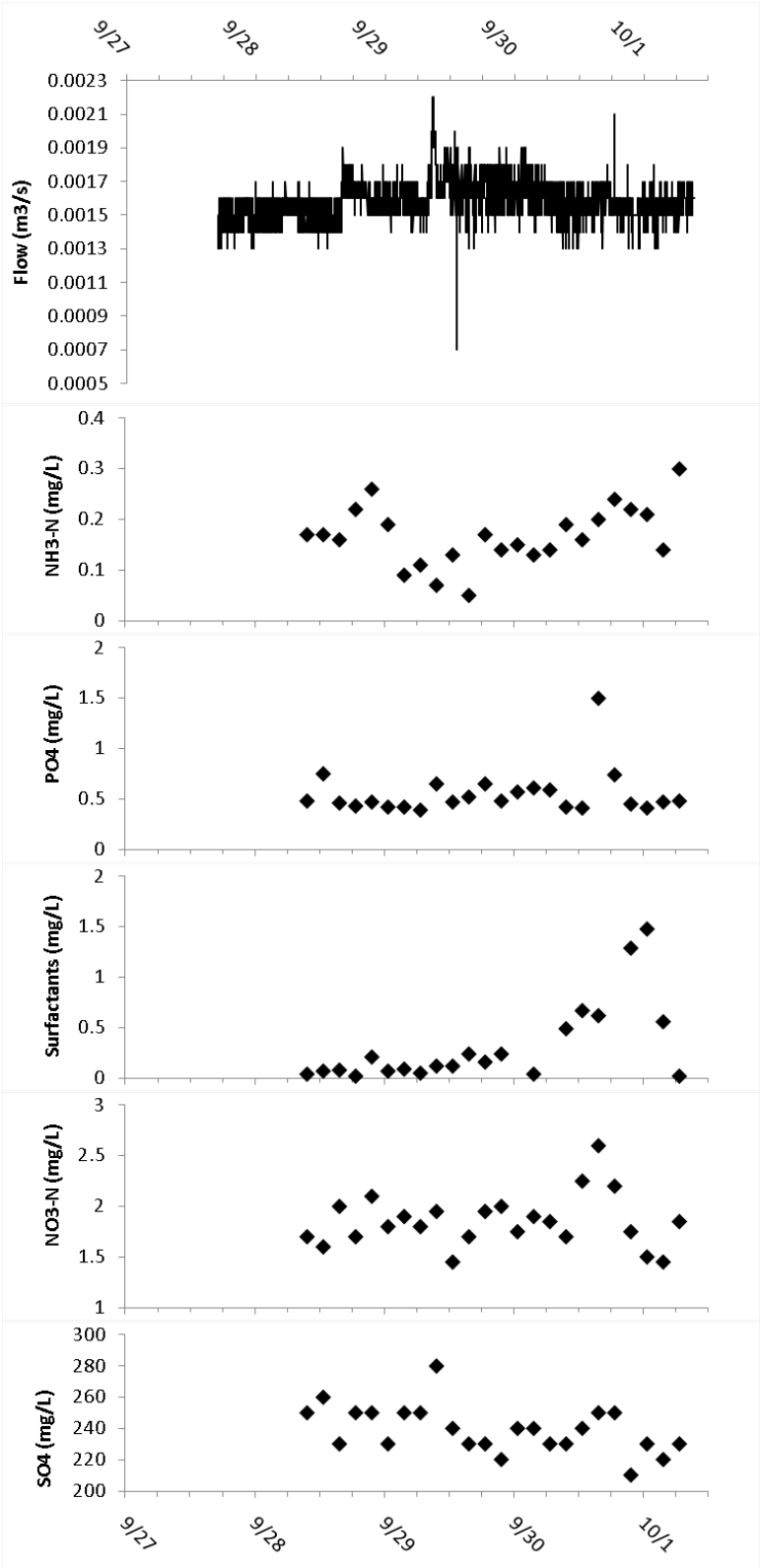


Figure 8.2. Flow and concentrations of NH_3-N, PO_4, anionic surfactants, NO_3-N and SO_4 at L15 during the period 9/27-10/1.

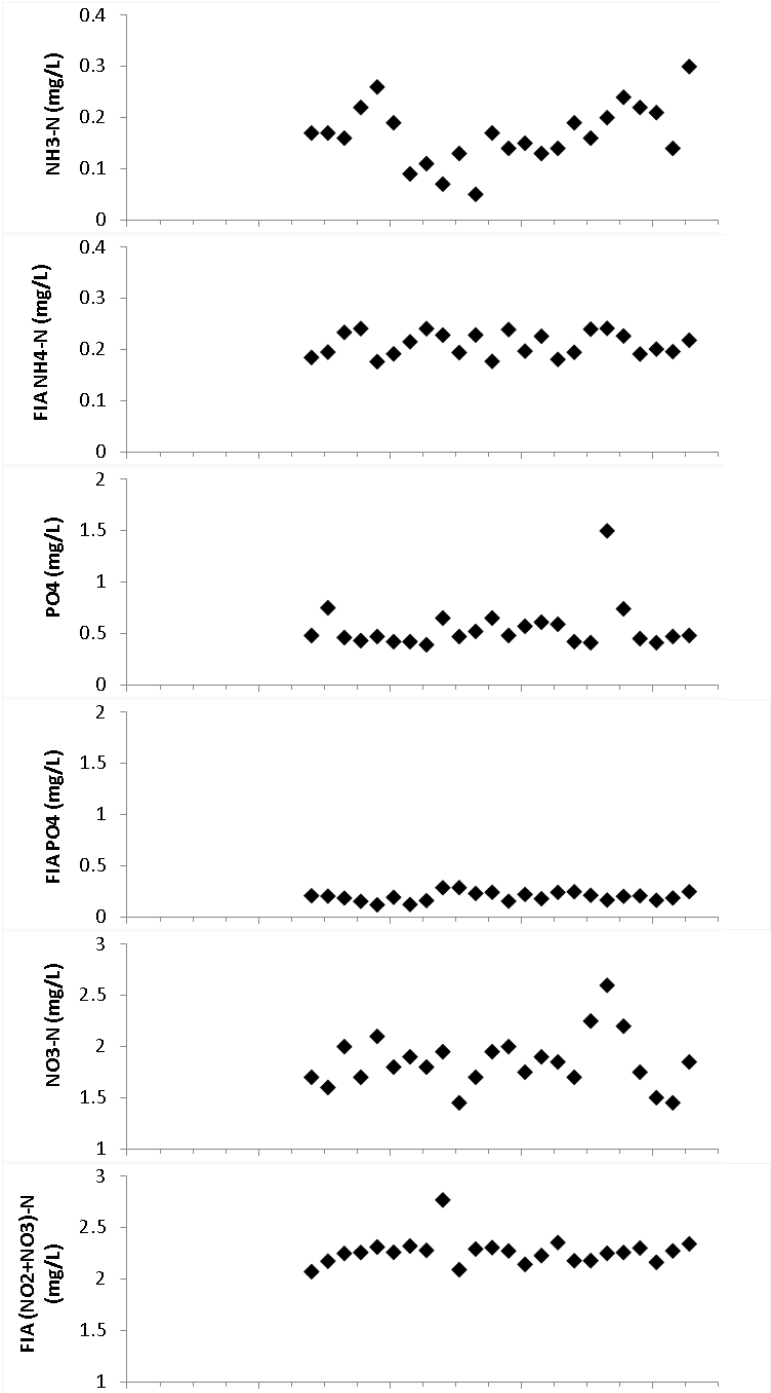


Figure 8.3. Comparison of temporal concentration patterns of NH_4-N, PO_4, and NO_3-N by colorimetry and FIA at L15 during the period 9/27-10/1. Y-axis scales are the same for each analyte.

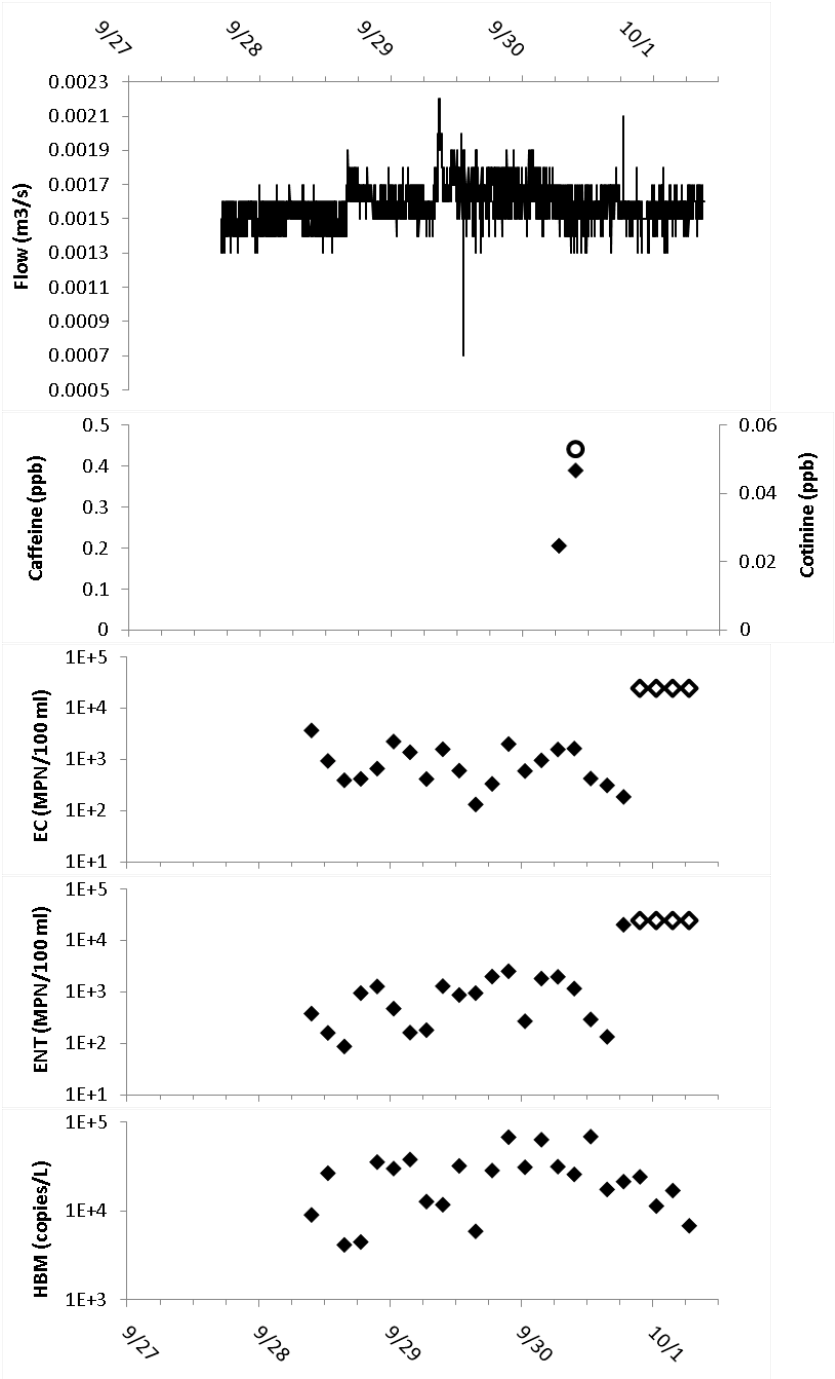


Figure 8.4. Flow and concentrations of caffeine (closed symbols), cotinine (open symbol), *E. coli* (EC), *Enterococcus* (ENT) and human-specific *Bacteroidales* markers (HBM) at L15 during the period 9/27-10/1. EC and ENT concentrations that are over-range (>25,000 MPN/100 ml) are indicated by open symbols.

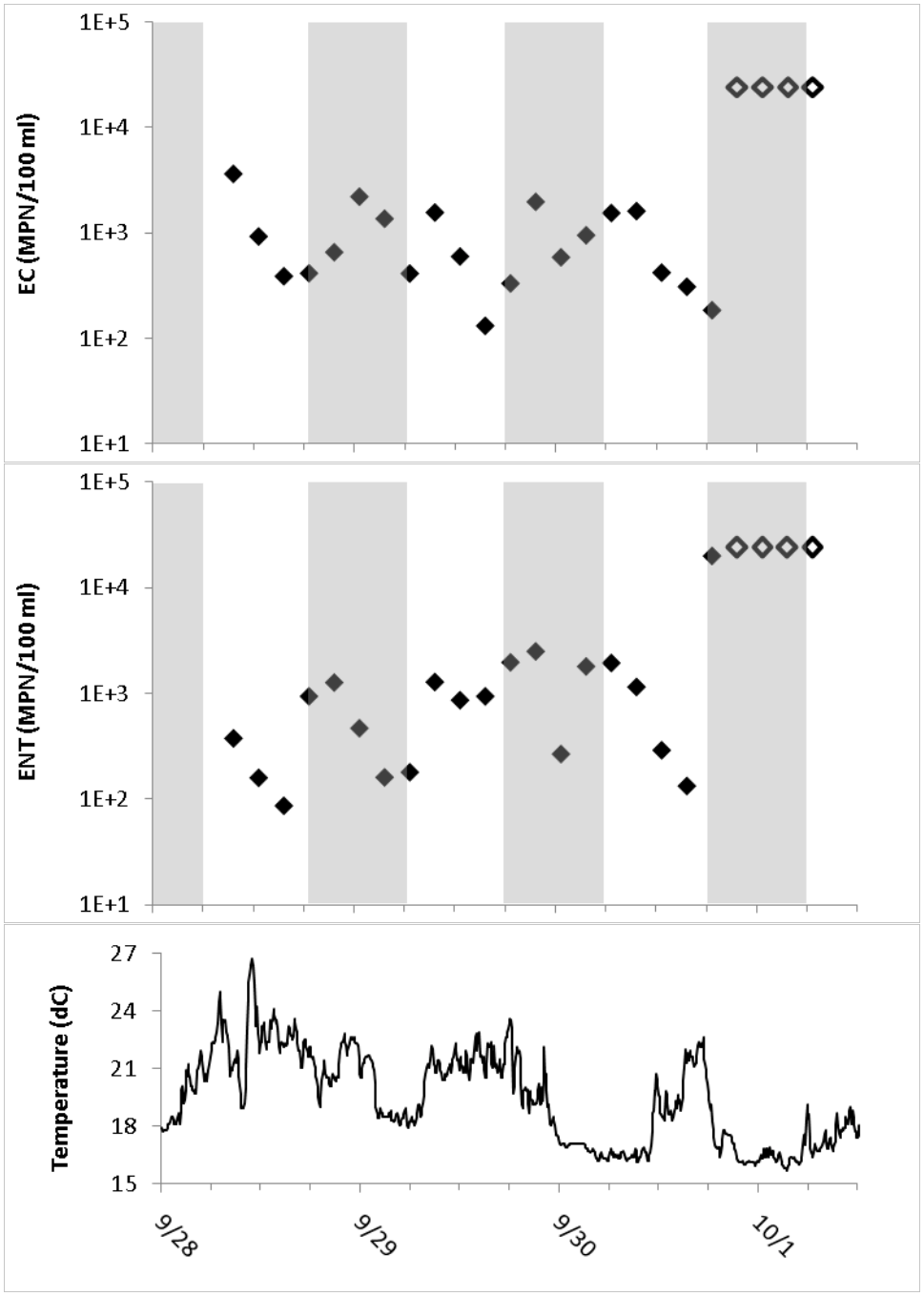


Figure 8.5. Concentrations of *E. coli* (EC) and *Enterococcus* (ENT) at L15 and temperature trends for Santa Barbara. Periods between 6 pm and 6 am are indicated in grey. EC and ENT concentrations that are over-range (>25,000 MPN/100 ml) are indicated by open symbols.

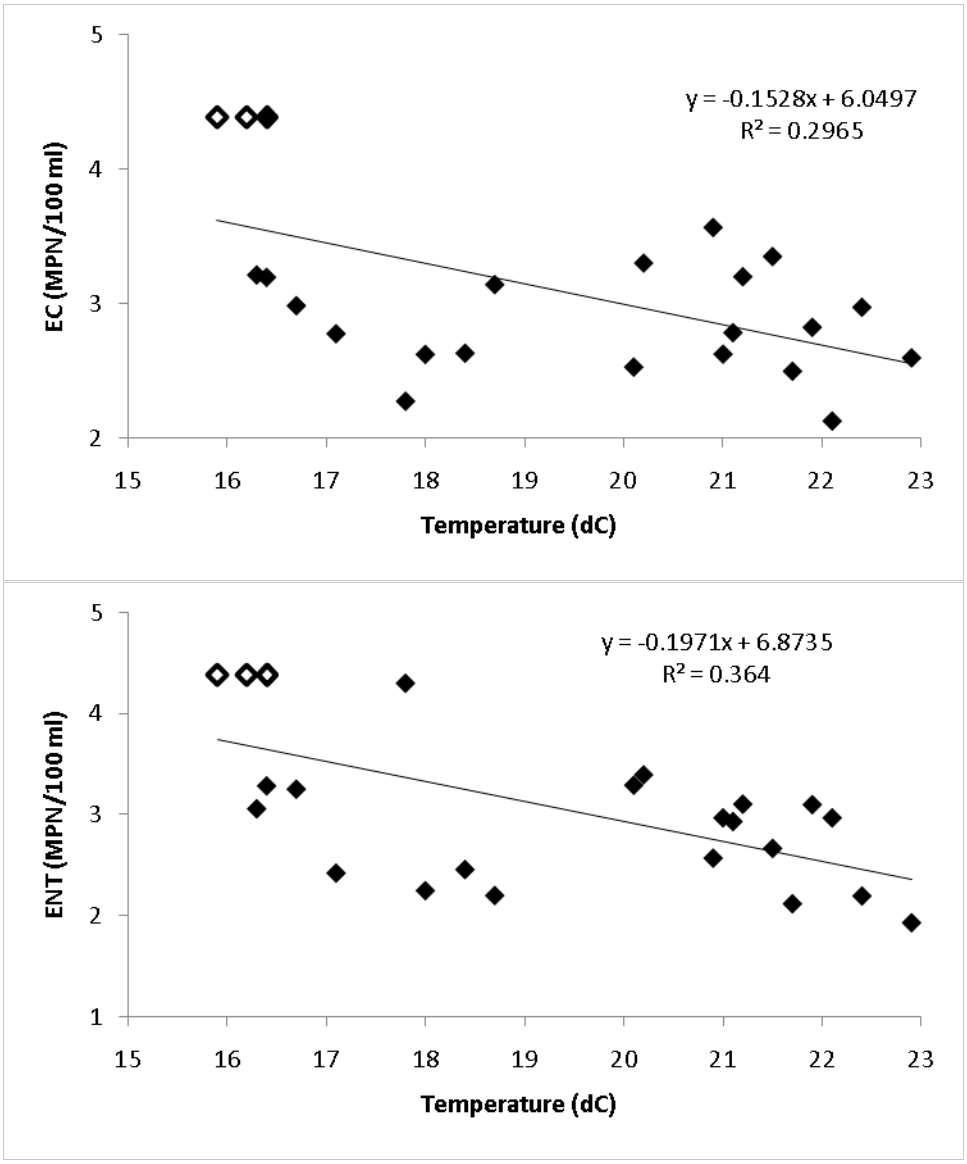


Figure 8.6. Correlations between temperature and log-transformed EC and ENT concentrations at L15. Linear regression plots are shown in each graph with regression equation and R^2. EC and ENT concentrations that are over-range (>25,000 MPN/100 ml) are indicated by open symbols.

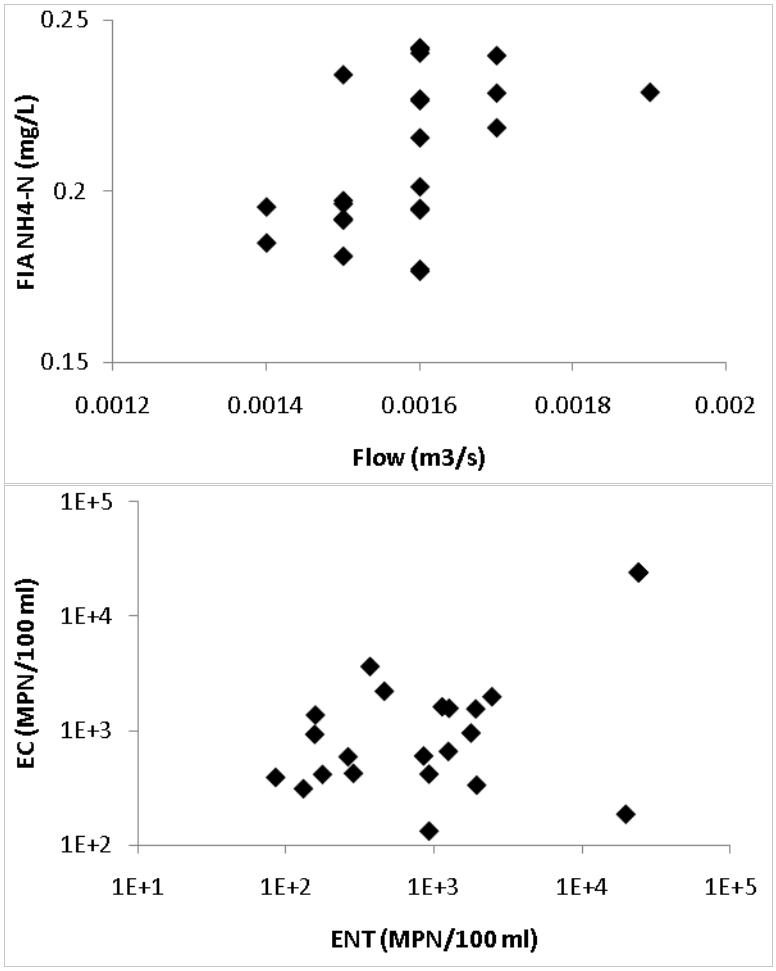


Figure 8.7. Scatter plots of FIB and alternative indicators that are significantly correlated at L15 ($p < 0.05$).

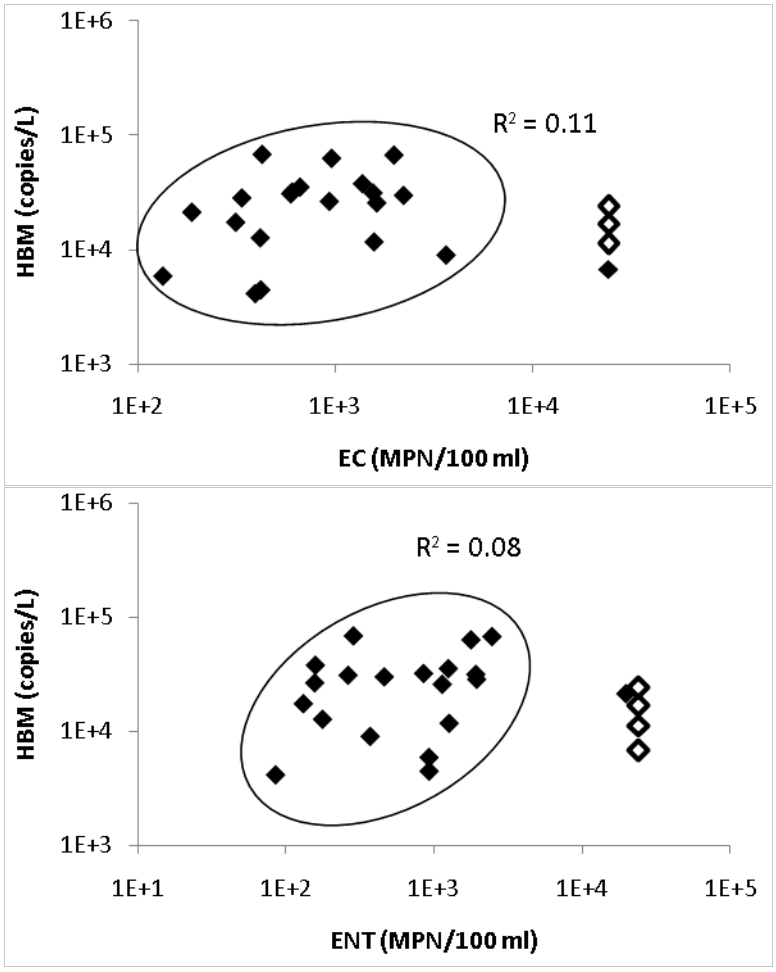


Figure 8.8. Scatter plots showing correlations between HBM and FIB concentrations. EC and ENT concentrations that are over-range (>25,000 MPN/100 ml) are indicated by open symbols. Correlation coefficients are indicated for subset of data in circles.

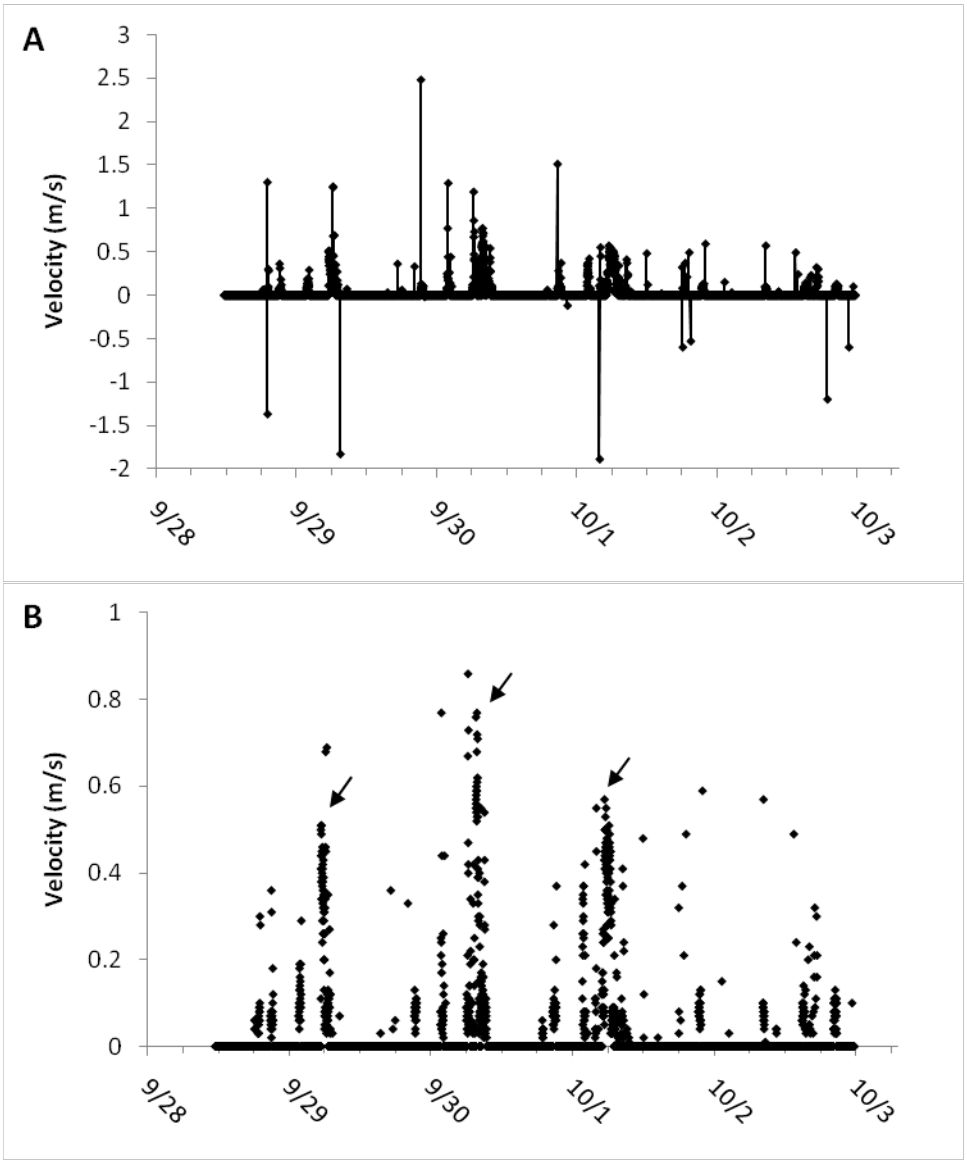


Figure 8.9. Preliminary velocity measurements at A5 during the period 9/28-10/1: all velocity measurements (A), positive velocity measurements less than 1 m/s, peaks between 4 and 8 AM are indicated by arrows (B).

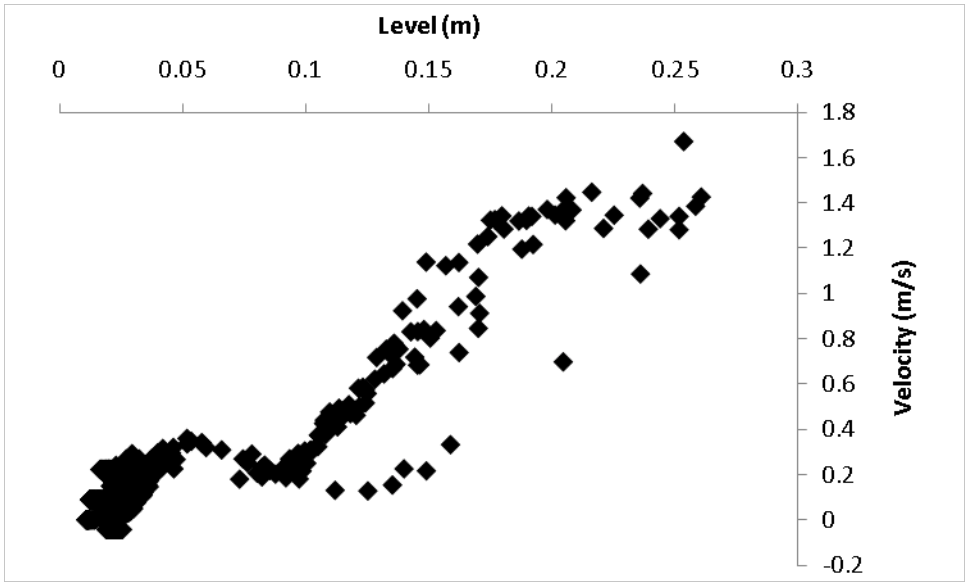


Figure 8.10. Level and velocity at A5 during the period 10/5-10/8.

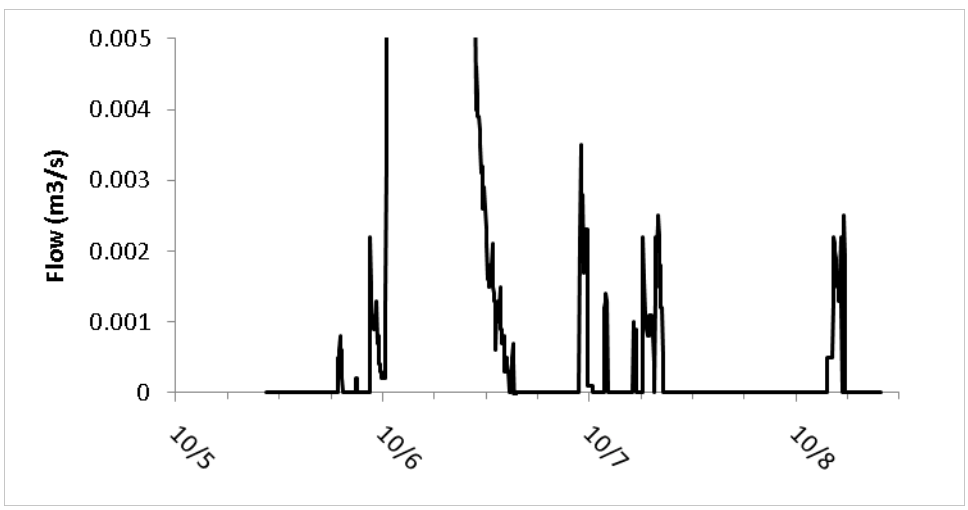


Figure 8.11. Flow at A5 during the period 10/5-10/8. Data points with level measurement below 0.025 m were set to zero m^3/s.

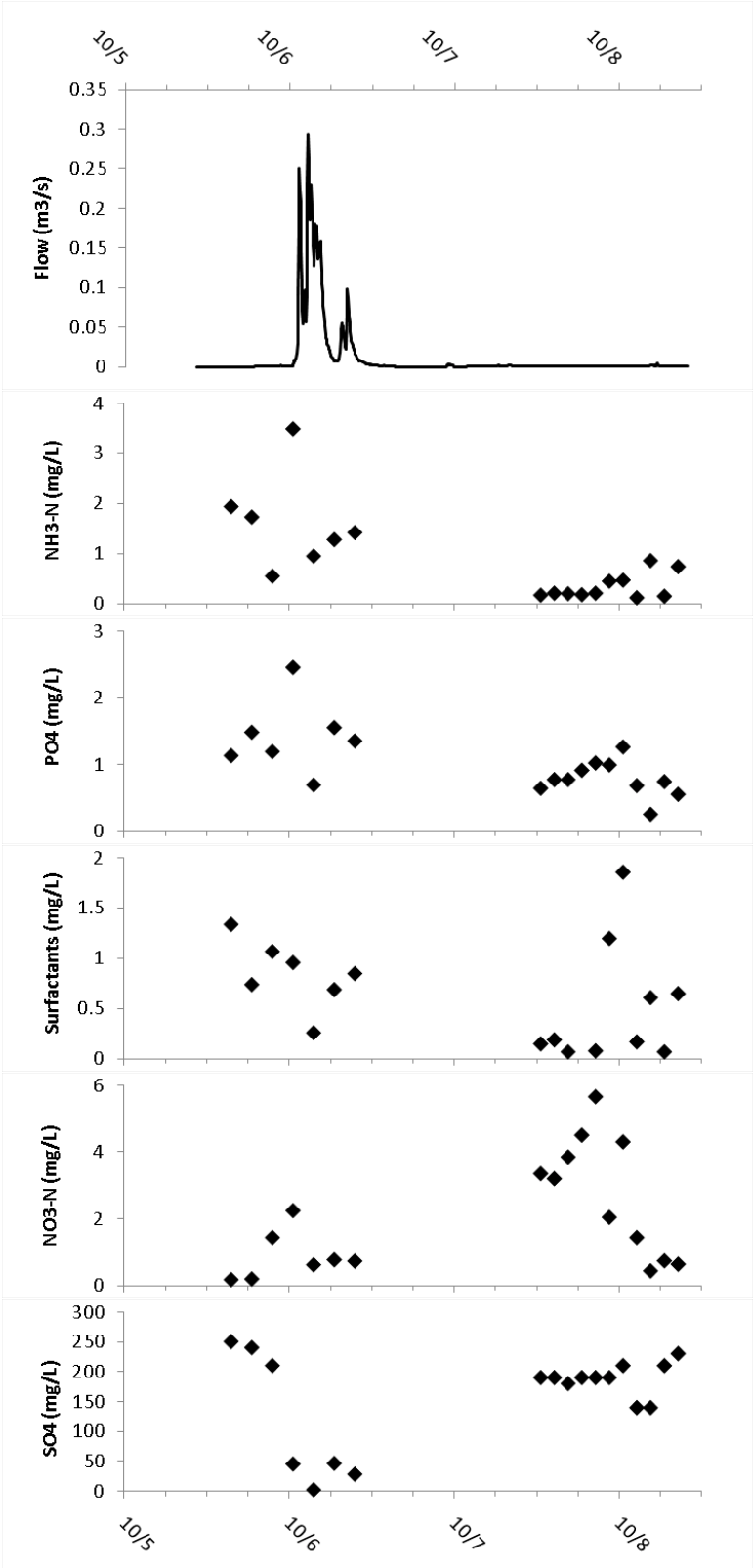


Figure 8.12. Flow and concentrations of NH_3-N, PO_4, anionic surfactants, NO_3-N and SO_4 by colorimetry at A5 during the period 10/5-10/8.

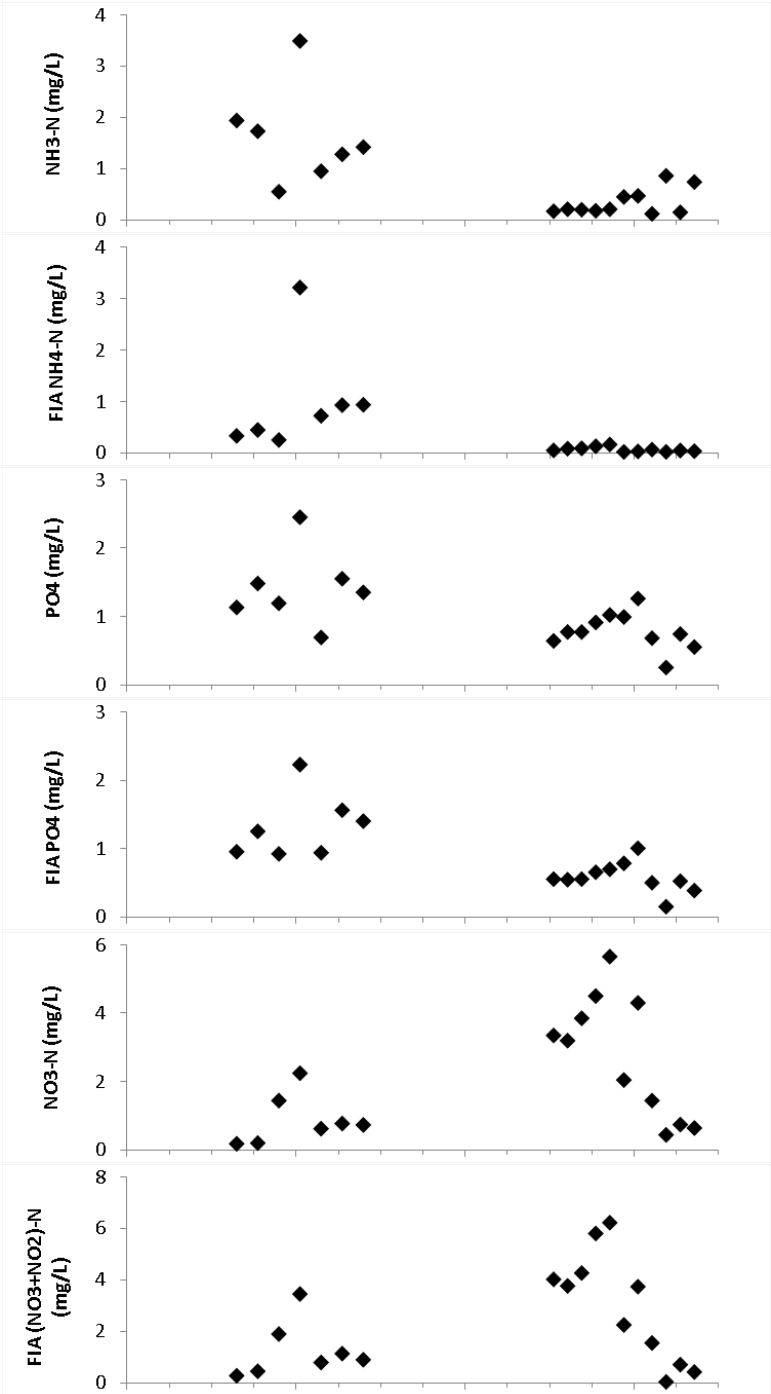


Figure 8.13. Comparison of temporal concentration patterns of NH_4-N, PO_4, and NO_3-N by colorimetry and FIA at A5 during the period 10/5-10/8. Y-axis scales are the same for each analyte.

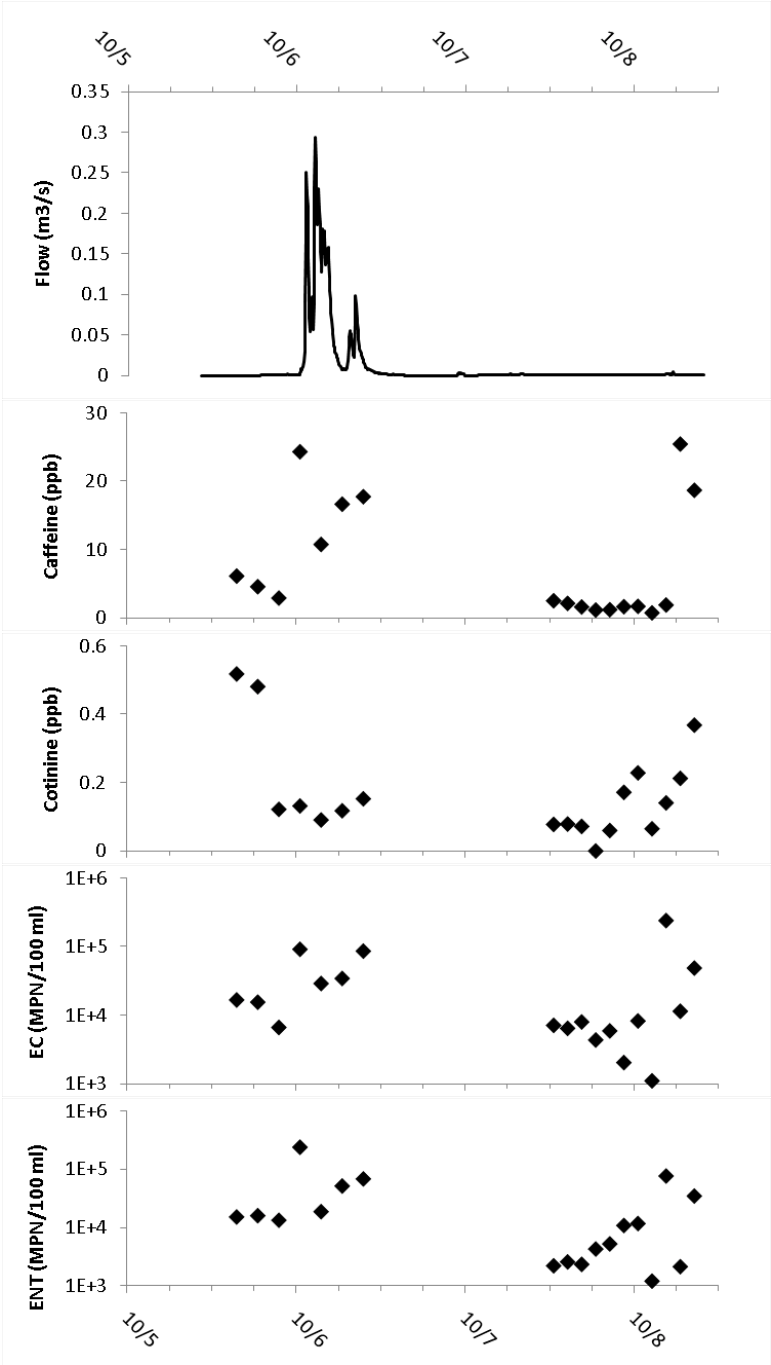


Figure 8.14. Flow and concentrations of caffeine, cotinine, *E. coli* (EC) and *Enterococcus* (ENT) at A5 during the period 10/5-10/8.

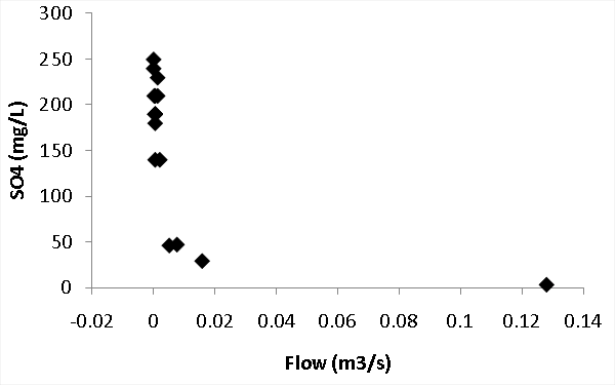


Figure 8.15. Scatter plot of flow versus SO_4 concentrations at A5.

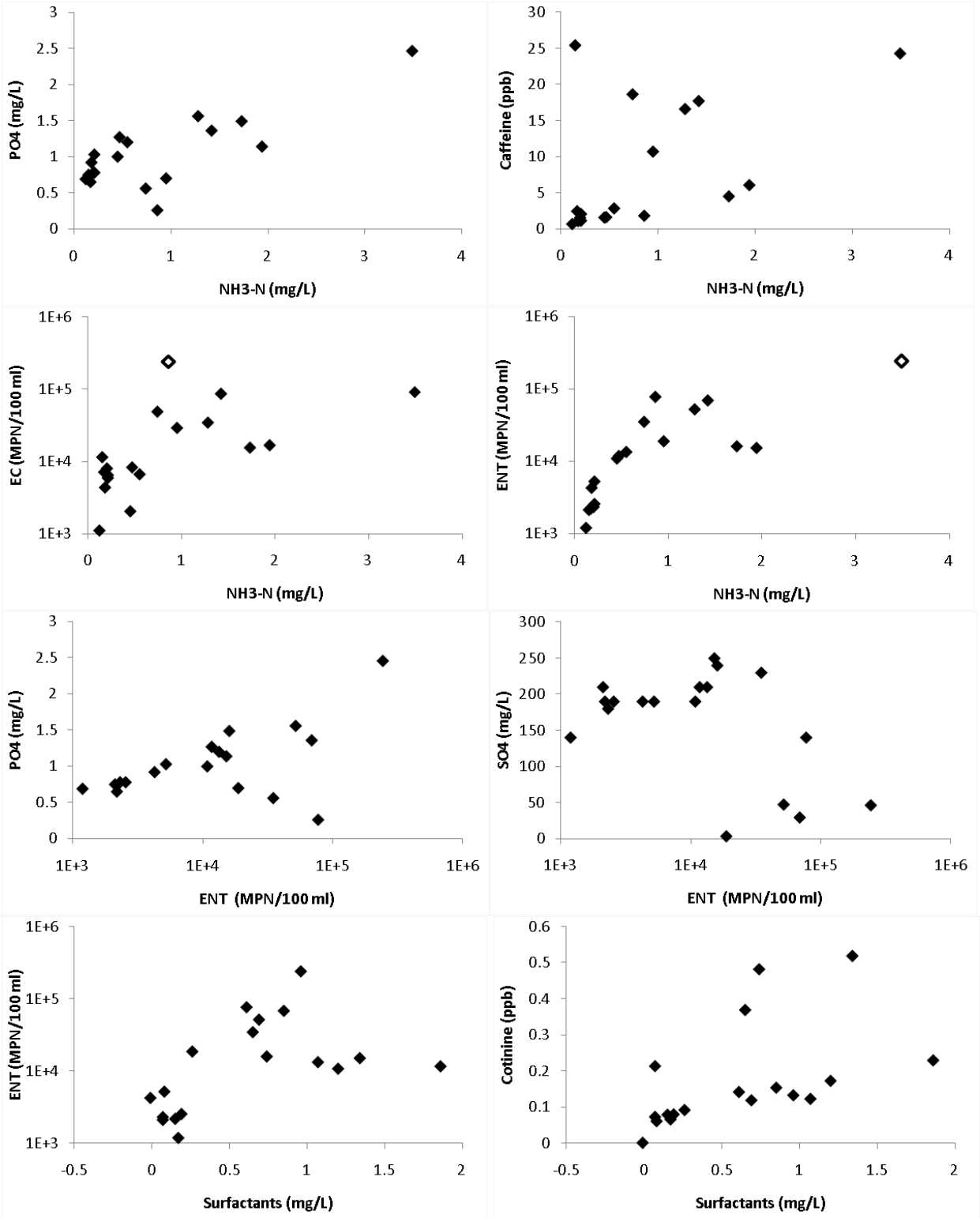


Figure 8.16. Scatter plots of alternative indicators and FIB that are significantly correlated at A5 ($p < 0.05$). Nutrients were determined by colorimetry.

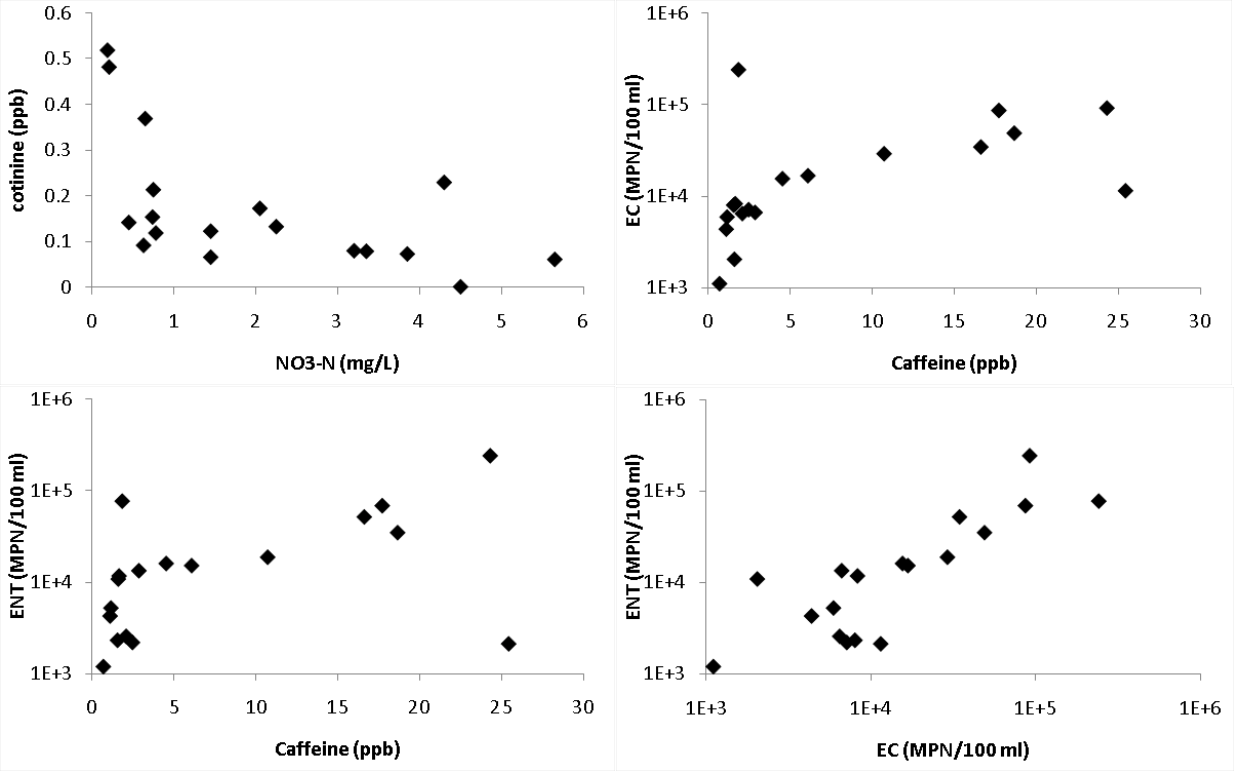


Figure 8.16. Continued

IX. RHODAMINE WT DYE STUDIES

Note: This chapter is formatted as a manuscript, as it will be submitted for publication after further editing and approval by all authors. Dye studies at two locations (Chino/Micheltorena and Nopal/Cota) are described separately in Sections 9.5 and 9.6, as they were not included in the manuscript.

9.1 Abstract

Separating storm drains and sanitary sewers is expected to control diffuse sewage pollution, for example from combined sewer overflows, and to regulate seasonal wastewater treatment plant flow exceedances in urban areas. However, research in Santa Barbara, CA and other urban areas have indicated sewage contamination in municipal separate storm drain systems during dry weather flow. To determine if transmission of sewage is occurring from sanitary sewers to separated storm drains, field experiments were performed in three watersheds in Santa Barbara, CA. Areas with high and low risk for sewage exfiltration into storm drains were identified, and rhodamine WT (RWT) dye pulses were added to the sanitary sewers in both areas. RWT was monitored in nearby storm drain manholes using optical probes set up for unattended continuous monitoring. Above-background RWT peaks were detected in storm drains in high risk areas, and multiple locations of sewage contamination were found. Sewage contamination in the storm drains during the RWT field studies was confirmed using the human-specific *Bacteroidales* HF183 and *Methanobrevibacter smithii nifH* DNA markers. This study is the first to provide direct proof that leaking sanitary sewers can contaminate nearby storm drains during dry weather, and suggests chronic sanitary sewer leakage as a possible source of downstream fecal contamination of coastal beaches.

9.2 Introduction

Modern cities use a network of sanitary sewers to transport municipal and industrial wastewater to centralized treatment plants. However, sewer leakage is a known problem in many cities worldwide, and can be due to structural defects caused by aging, excessive demand, insufficient rehabilitation and poor construction and materials (*1*). Sewage exfiltration, i.e. losses of wastewater from the sewer system, can occur when the groundwater table is below the sanitary sewers, and has been extensively described in literature (*1-4*). Exfiltration rate estimates in literature vary greatly, but the overall magnitude of exfiltration is unclear due to differences in measurement methods and experimental designs (*5, 6*). A literature review suggested likely sewer exfiltration rates in the range 0.01-0.1 l/s per km for the UK and countries with similar sewer networks (*1*). In the U.S., an exfiltration rate of 19,000 m^3 per day was estimated for the city of Albuquerque, NM, corresponding to an exfiltration rate of about 2 l/s per km (*7*). Sewage exfiltration can cause contamination of groundwater (*8-10*), drinking water wells (*8*), and even drinking water distribution systems during pressure loss events (*11*). The detection of sewage contamination in the latter environments relied on monitoring a variety of sewage tracer chemicals (*8, 9, 12*), fecal indicator bacteria (*10*) or viruses (*8*). The risks for human health

upon exposure to sewage are mainly associated with pathogenic viruses and bacteria, although toxic chemicals can pose a risk as well (*7*).

Besides sanitary sewers and drinking water infrastructure, most cities in the West and Northeast of the U.S. also built municipal separate storm drains to transport stormwater runoff from impervious surfaces to oceans or lakes, and to avoid combined sewer overflows and overloading of treatment plants. However, storm drains can be contaminated by sewage through illicit connections and discharges (*13-15*), and possibly by exfiltration from leaky sanitary sewers (*16-18*), although the latter has not been directly proven.

The goal of this study was to provide proof of principle of hydrological connections between sanitary sewers and storm drains. More specifically, we hypothesize that sewage exfiltrating from sanitary sewers can travel through unsaturated soil during dry weather, and infiltrate into nearby storm drains. In order to be able to provide direct evidence for the hypothesized hydrological connections, rhodamine WT fluorescent dye was dosed into sanitary sewer sewers at multiple locations, and monitored in nearby storm drains downstream of the dosing locations. The urban watersheds in Santa Barbara, CA, were selected based on recent data (unpublished data) and a previous study, which showed sewage contamination in storm drains during dry weather from unidentified sources (*16*).

9.3 Materials and Methods

9.3.1 Study sites and infrastructure details

Field experiments were performed in three watersheds in Santa Barbara, CA (Fig. 9.1A). Field study 1 (Fig. 9.1B) was performed at a location where field observations suggested severe sewage pollution in the storm drain. Surcharge flow occurred in the sanitary sewer every 15-30 minutes, due to pump operation at an upstream lift station. At the time of surcharge flow, liquid was heard trickling into the storm sewer upstream of P1-D. In addition, sewage odor was observed at the storm sewer manhole P1-D. The storm sewer section was located upstream from a storm drain where significant but intermittent sewage contamination was observed before (*16*).

Field study 2 (Fig. 9.1C) was performed upstream of a location (N1-D) where sewage contamination was recently observed, based on detection of the human-specific *Bacteroidales* marker (Holden et al., submitted). However, the exact origin of the contamination was not identified. Sanitary sewers were mostly vitrified clay pipes from the 1950s or older, although some sections were rehabilitated in PVC since the 1990s. The storm drains in this area were all below the sanitary sewers, and large part of the storm drains run parallel to the sewers at a distance between 2-3 m. In addition, sanitary sewers cross above the storm drains at several locations. A high potential for sewage exfiltration in the storm drains was assumed in this area.

Field study 3 (Fig. 9.1D) was also performed at a location where human-specific *Bacteroidales* marker concentrations suggested sewage pollution, with unknown origin (*16*). In contrast with field study 2, the storm drains in this area were all above the sanitary sewers. Sanitary sewers were all vitrified clay pipes from the 1950s or older, and run parallel to storm drains at a distance between 2-5 m. A low potential for sewage exfiltration in the storm drains was assumed in this area.

9.3.2 RWT dosing

In field study 1, there was one sanitary sewer crossing the storm drain upstream of P1-D, and the first sanitary sewer manhole upstream of the suspected contamination area was selected for dosing RWT (Fig.9.1B). For field studies 2 and 3, the locations for dosing RWT were selected based on the spatial information in the storm drain atlas and GIS database of the City of Santa Barbara. Locations for which a high risk for sewage exfiltration into the storm drains was assumed had the following characteristics: sanitary sewers made of vitrified clay, sanitary sewers positioned above storm drains, sanitary sewers crossing storm drains or running parallel within 5 m (Fig. 9.1). In field study 2, RWT was dosed into all sanitary sewer manholes with at least one location downstream with high risk for sewage exfiltration into storm drains (Fig. 9.1C). RWT dosed to those sanitary sewer manholes will flow through the high risk locations and potentially make its way into the storm drains. In field study 3, locations with high risk for sewage exfiltration into storm drains were not identified. However, RWT was dosed into sanitary sewers to determine if sewage exfiltrates into storm drains in areas with assumed low risk. RWT was dosed into all sanitary sewer manholes with at least one location downstream where sanitary sewers run parallel or cross the storm drains (Fig. 9.1D). The details of RWT dosing are shown in Table 9.1. In field study 1, RWT was added twice with a 0.5 h interval. Field studies 2 and 3 were more complex, and RWT pulses were added once or more to multiple sanitary sewer manholes. RWT was dosed to the sanitary sewers in all studies except study 2*c*, in which RWT was dosed to the storm sewers.

9.3.3 RWT monitoring

RWT was monitored using a 600 OMS V2 sonde equipped with temperature and conductivity sensors and a rhodamine WT optical probe (YSI Incorporated, Yellow Springs, OH). The sonde was programmed for unattended monitoring, at 1 or 2 minute intervals. The sonde was calibrated using a 2-point calibration curve (0 and 100 ppb), has a detection limit of 1 ppb and a linear range of 1-200 ppb. RWT was purchased as Keyacid rhodamine WT liquid, and consisted of 20 % true dye concentration (Keystone Aniline Corporation, Chicago, IL). Background signal was collected during 20 days in field study 2, and 8 days in field study 3.

9.3.4 Flow rate calculations

Flow rates in sanitary sewers were calculated based on the Manning's Equation, assuming a roughness coefficient n of 0.015. Slopes were calculated from manhole invert depths and spatial information contained in a GIS database from the City of Santa Barbara.

9.3.5 Water sampling and microbiological analyses

One or more water samples were taken in the storm drains during each of the three field studies. Storm drain samples were taken at P1-D and P2-D (field study 1), N1-D and N2-D (field study 2), and H1-D and H2-D (field study 3). One sewage sample was collected from the nearby El Estero wastewater treatment plant influent, on 6/11/2010. Water samples (2

l) were collected using a sterile plastic beaker and filtered through Miracloth (20-25 μm pore size) into a sterile plastic bottle in the field. Samples were stored on ice in the dark until filtering in the lab through 0.22 μm filters (within 6 hours).

DNA was extracted from the archived filters (- 20 ºC) using the PowerWater DNA Isolation kit (MoBio Laboratories, Carlsbad, CA) according to manufacturer's instructions, followed by ethanol precipitation in a final volume of 50 μl. PCR inhibition was tested by using salmon testes DNA from *Oncorhynchus keta* as an internal control, based on the protocol of Morrison et al. (*19*). The PCR conditions were as previously described (*19*), except for an annealing temperature of 62 ºC, and the addition of 0.2 mg/ml bovine serum albumin and dNTP concentrations of 0.2 mM each. A separate Sybr Green qPCR reaction was run after adding 0.25 ng salmon testes DNA to each sample reaction (in duplicate) and a no sample control (in quadruplate). At first, 2.5 μl of 1:5 diluted DNA template was run. Samples were considered inhibited if the average threshold cycle value (Ct) of samples exceeded the average + 3 times standard deviation of the blank control with salmon testes DNA. Samples were diluted twofold until inhibition was removed. Concentrations of human-specific HF183 *Bacteroidales* markers were determined using SybrGreen qPCR, based on a previously described protocol (*16, 20*). A volume of 2.5 μl DNA template was used per 25 μl qPCR reaction, with the template dilution based on the inhibition assay (i.e. 1:5 or higher). All qPCR reactions were run in an iQ5 thermocycler (Bio-Rad, Hercules, CA), using the qPCR Core Kit for Sybr Green I (Eurogentec, San Diego, CA). The presence or absence of the *Methanobrevibacter smithii nifH* gene was determined by two rounds of PCR, using the protocol of Ufnar et al. (*21*). The DNA template in round 1 consisted of 1 μl of diluted template DNA (per salmon testes DNA inhibition assay), in round 2 of 1 μl of 1:10 diluted PCR product from round 1. PCR reactions were performed in 25 μl reactions, using a Hybaid PCR Sprint thermocycler and the Taq PCR Core Kit including Q-mix (Qiagen, Valencia, CA).

9.4 Results and Discussion

9.4.1 Field study 1: leaking sanitary sewer due to surcharge conditions

In field experiment 1 (Fig. 9.1B), multiple lines of indirect and qualitative evidence indicated that the storm sewer was receiving leakage from a sanitary sewer that was under surcharge conditions at regular intervals. Direct evidence was obtained by detection of two RWT peaks in the storm sewer at P1-D, after dosing two separate pulses of RWT in the sanitary sewer at P1, during surcharge conditions (Table 9.1, Fig. 9.2). The time of travel of RWT between dosing and detection was 20-30 minutes. Based on an estimated flow of 0.1 m^3/s in the sanitary sewer (Manning's equation), a RWT dosing rate of 0.17×10^{-3} m^3/s and assuming no longitudinal dispersion, a 1-minute pulse of 1.4 ppm RWT occurred in the sanitary sewer after mixing of RWT with the sewage flow. A maximum of 0.25 ppm RWT was detected in the storm sewer (Fig. 9.2), suggesting ~20 % sewage in the storm drain shortly after surcharge conditions in the sanitary sewer. Concentrations of HBM indicated approximately 7 % sewage at P1-D (Table 9.2), agreeing well with the estimates based on RWT concentration. The detection of the MNIF marker also confirmed the sewage pollution.

Sewage contamination was observed in 2005 at P2-D, based on elevated HBM concentrations (*16*). The current study indicates that most the sewage contamination originates from the branch at P1-D, receiving the sewage exfiltrate, as HBM concentrations were similar at both locations (Table 9.2). After the sanitary sewer pipe was repaired, shortly after the leakage was detected, follow-up monitoring did not reveal any HBM and MNIF at P2-D (data not shown).

Field study 1 indicated that lift station operation and surcharge conditions in sanitary sewers can lead to exfiltration and severe contamination of nearby storm drains. Continuous monitoring of RWT in the storm drains appeared a promising approach for obtaining direct evidence of such contamination.

9.4.2 Field study 2: storm drains below sanitary sewers

Background RWT concentrations were consistently between -1 and 2 ppb, and no peaks could be distinguished (Fig. 9.3A). After RWT addition to sanitary sewer in phase 2a, two RWT peaks were observed in the storm sewer at N1-D, different from the background signal (Fig. 9.3B). The first peak was detected after dosing RWT at N1-N4, the second after dosing at all manholes. Therefore, at least one RWT peak could be attributed to RWT dosing at N1-N4, and phase 2b focused on the latter manholes. Multiple RWT peaks were detected during phase 2b, but not in 1-hour intervals, the time interval between pulses at each manhole (Fig. 9.2C). Therefore, the approach of dosing multiple pulses into each manhole was effective in confirming the occurrence of sewage to storm drain contamination in this area, but did not allow better localization of the contamination. In addition, the detection of multiple RWT peaks suggested multiple locations of sewage exfiltration into the storm drains. Detection of HBM and MNIF markers at two storm drain locations during phase 2a, confirmed the occurrence of sewage contamination during RWT testing. HBM concentrations were 2-3 orders of magnitude lower than during field study 1, and corresponded to sewage concentrations between 0.004 and 0.15 % (Table 9.2). Based on RWT dosing concentrations in sewage of 200-4000 ppm and an observed RWT peak concentration of 5 ppb, the sewage dilution at N1-D was 10^4 – 10^6. This corresponds to approx. 10^2-10^4 HBM copies/L, which is on the low end of the HBM concentration range observed (10^4-10^5 copies/L). Because of RWT adsorption in soil (*22, 23*) and longitudinal dispersion in the sanitary sewer, estimates of sewage contamination in storm drains based on RWT may be lower than estimates based on HBM concentrations.

During phase 2c, RWT was injected at 3 storm sewer locations (Fig. 9.1C) to determine travel times for the different storm drain branches with confluent at N1-D. After dosing 100 and 1000 ppm RWT at N2-D, where continuous low flow was present, RWT was observed at N1-D within 2 hours (Fig. 9.4A). After dosing the same concentrations at N3-D, one small RWT peak was observed 74 hours after the second dosing, and after flushing the storm drain with clean water. A trickle flow was observed during the first dosing, but no flow during the second dosing. After dosing of 1000 ppm RWT at N4-D, where flow was absent, RWT pooled in the manhole. Still, a small RWT peak was observed at N1-D after 18 hours, indicating the intermittent flow occurred. After flushing the storm sewer, a second RWT peak was observed at N1-D after 4 hours. Overall, the results from phase 2c indicate that the travel time of water in the storm sewers in this area is on a scale of hours when flow is present, but can increase to day or weeks due to periods without flow. Time of

travel of RWT in storm drains is unpredictable, and is unlikely to provide useful information regarding the location of exfiltration and storm drain pollution in this case. However, intermittent flow patterns can explain why the time between RWT dosing and detection in phases 2a and 2b were not reproducible. Since HBM concentrations at N2-D and N1-D were similar, the area near N2-D should contribute significantly to the sewage pollution downstream.

Detection of RWT in storm drains after dosing in sanitary sewers during field study 2 indicated that the experimental approach is useful for obtaining direct evidence of sewage exfiltration into storm drains with relatively low concentrations of sewage contamination (< 0.15 %). Exact localization of the source of contamination using RWT tracing is challenging because of variable travel times in storm drains. However, HBM concentrations helped localizing at least one of the contamination locations.

9.4.3 Field study 3: storm drains above sanitary sewers

Background RWT concentrations in this area were mostly between -1 and 1 ppb (Fig. 9.5A). However, multiple RWT spikes up to 5 ppb, but consisting of only one data point, were observed (Fig. 9.5A, inset A1). Storm drain flow at this location was usually very low, with water levels below 3 cm, although episodes of increased flow occurred at regular intervals. Sand bags were used to dam the flow and provide enough water depth for submerging the probe during low flow episodes. Because sufficient water level could not always be sustained, the probe was not submerged at times, as evident from decreased conductivity at regular intervals (Fig. 9.5A, inset A1). Only one small RWT peak consisting of multiple datapoints was observed (Fig. 9.5A, inset A2), and should be considered part of the background signal.

One small RWT peak could be observed at H1-D, 13-14 days after dosing RWT into the sanitary sewers at 6 locations (Fig. 9.5B, inset). Due to its small size, this peak could not be reliably distinguished from the background signal. Multiple one-datapoint peaks were also observed, and were considered background, perhaps due to incomplete submersion of the probe. HBM or MNIF markers were not detected at H1-B and H2-B. Therefore, both RWT and microbiological data suggest no or low sewage contamination due to exfiltration from sanitary sewers in this area.

9.4.4 Importance of exfiltration to storm drain water quality

Studies about the impact of exfiltration on the environment or human health, have mostly focused on potential contamination of groundwater (*8-10*). However, this study provided multiple lines of evidence that storm drains, and therefore surface waters and oceans, can be contaminated by sewer exfiltration as well. Poor condition of the sanitary sewer infrastructure and sufficient depth to groundwater increase the susceptibility for sewer exfiltration in the U.S. (*7*). It has been suggested that especially arid urban areas are at risk, e.g. in Arizona and New Mexico (*7*). However, this study demonstrates exfiltration and contamination of surface waters, in a small city along the California coast. Given that large parts of California are highly urbanized and have a similar climate and sanitary/storm sewer infrastructure as Santa Barbara, it can be reasonably assumed that similar contamination issues occur there as well.

In the case of the sewage exfiltration under surcharge conditions (phase 1), transport from sanitary sewer to storm drain was fast, and sewage concentrations were in the range of 10 %. The surcharge conditions caused a flow of about 1 l/min in the storm drains (visual observations), approximately 3 times an hour lasting 2 minutes, corresponding to a load on the order of 14 liters of fresh sewage per day. In the case of the diffuse sewage exfiltration (phase 2), a maximum loading estimate of 120 liters sewage per day is obtained, based on an estimated storm drain flow of 55 l/min (Manning's equation) and 0.15 % sewage concentration. Based on the current study, over 100 l of sewage enters the storm drains per day, but this quantity will likely increase if more storm drains are investigated. For assessing the risk to human health, it is important to consider that sewage properties may be altered by transport through soil, e.g. through reductions in virus and bacterial concentrations (*24, 25*). For instance, after exfiltration near N2-D, filtration of sewage through soil is expected to take several days.

9.4.5 RWT dye studies for localizing sewage exfiltration pollution

This study shows that RWT dosing experiments combined with unattended monitoring are a promising method for assessing the occurrence of sewage exfiltrate contamination in storm drains. The methods are within reach of communities and municipalities, as screening watersheds requires no other equipment than the optical probe set up (~ $7,000) and a PC or laptop. While tracers such as bacteriophage PRD-1 can be detected to very low concentrations, and may be more relevant for pathogen transport in the environment (*22, 26*), the inability to use unattended monitoring makes their use very impractical. Commonly used non-reactive tracers such as bromide did not provide sufficiently low detection limits for this study.

Our data suggest that spatial information can be used to estimate the risk for sewage exfiltration into storm drains, as most (if not all) contamination occurred in an area with all risk factors present: old vitrified clay sanitary sewer pipes, sanitary sewers above storm drains and multiple locations where sanitary sewers and storm drains cross or run parallel within 3 m. In the area where one of these factors was missing, i.e. storm drains were above the sanitary sewers, evidence for sewage exfiltration into storm drains was lacking. Therefore, identifying all areas that match the above criteria should be a first step for preliminary assessment of the contamination potential, or when designing field studies. The latter will require a spatial database with age, construction material, and depth of sanitary sewers and storm drain infrastructure. Based on the detection of sewage exfiltration pollution in multiple storm drain locations in this study, more research is recommended in other urban areas to assess the magnitude of the problem.

9.5 RWT testing at Chino/Micheltorena

9.5.1 Introduction

Infrastructure details of sewer and storm drains are shown in Fig. 9.6. Both "sewage-sniffing" dogs signaled at Micheltorena/Gillespie storm drain manhole (MH1). Inspection of

the manhole revealed flow input from the CB north of MH (CB1) and from pipe south of MH (CB2). However, CB1 and CB2 were dry.
Inspection of CB1 revealed seepage entering pipe at 2 joints downstream of CB1, at bottom of pipe (groundwater?). In addition, inflow was trickling in from the top, near the end of the pipe, probably close to the overlying sanitary sewer (~ 5ft deep). Invert depths were approximately 6ft (CB1) and 12ft (MH1). Therefore, infiltration of sewage was suspected. However, the sanitary sewer section downstream of SEW MH was made of PVC (1999). The influent from CB2 had etched and colored (black) the storm drain pipe. The source of this influent is unknown.

9.5.2 Experiments and results

First, the time of travel between MH1 and MH2 was determined by releasing 10L of ~1000 ppm RWT in MH1 and detecting with probe at MH2. Second, 10L of ~4000 ppm rhodamine WT was released at SEW (during ~ 1min) twice in 15 min, and detected by the probe at MH2. Samples for FIB and NH_3 analysis were taken before adding rhodamine and analyzed by the City of Santa Barbara.

Time of travel between MH1 and MH2 was 45-60 mins (Fig. 9.7). The RWT peak was detected during at least 1hr, indicating significant longitudinal dispersion. No RWT was detected at MH2 after dosing rhodamine twice at SEW (Fig. 9.8). The probe was deployed for 23 hrs.

In conclusion, we found no evidence for sewage infiltration in storm drain near Gillespie/Micheltorena. Sewage exfiltration was unlikely because of the PVC-lined sewer.

9.6 RWT testing at Nopal/Cota

9.6.1 Introduction

Illicit connections of sewers to storm drain were suspected at Santa Barbara Jr High School, based on high HBM concentrations in the Nopal/Cota storm drain and the old sewer infrastructure in school.

9.6.2 Experiments and results

RWT was added to all Jr. High restroom facilities starting around 10 am on 6/24/10. The RWT probe was deployed in the Nopal/Cota storm drain, downstream of the school, for continuous RWT detection, and one person was stationed at the Salsipuedes/Cota storm drain manhole for visual detection of dye. A fire hydrant was opened approx 0.5 miles upstream of Nopal/Cota to provide additional flow to storm drain. The flow from the fire hydrant was temporarily increased after all dye was added. The probe was retrieved on 6/28/10.

No RWT was detected in the Nopal/Cota storm drain (Fig. 9.9-9.10). Data collection stopped at approx. 19:00 on 6/26/10, possibly due to low battery. However, an increase in

temperature and decrease in conductivity occurred around 13:00 on 06/24/10, likely due to increased flow from the fire hydrant. Therefore, any RWT should have been detected before the temperature peak. Time of increased fire hydrant flow needs to be verified by City.

In conclusion, there is no evidence for illicit connections at Jr. High causing human fecal contamination at the Nopal/Cota storm drain. Time interval during which data were collected was long enough for detection of dye.

9.7 References

1. Chisala, B. N.; Lerner, D. N. Distribution of sewer exfiltration to urban groundwater. *Proceedings of the Institution of Civil Engineers-Water Management* **2008,** *161*, 333-341.
2. Blackwood, D. J.; Ellis, J. B.; Revitt, D. M.; Gilmour, D. J. Factors influencing exfiltration processes in sewers. *Water Science And Technology* **2005,** *51*, 147-154.
3. DeSilva, D.; Burn, S.; Tjandraatmadja, G.; Moglia, M.; Davis, P.; Wolf, L.; Held, I.; Vollertsen, J.; Williams, W.; Hafskjold, L. Sustainable management of leakage from wastewater pipelines. *Water Science And Technology* **2005,** *52*, 189-198.
4. Ellis, J. B.; Revitt, D. M.; Lister, P.; Willgress, C.; Buckley, A. Experimental studies of sewer exfiltration. *Water Science And Technology* **2003,** *47*, 61-67.
5. Rutsch, M.; Franz, T.; Krebs, P. Transferability of exfiltration rates from sewer systems. *Journal of Soils and Sediments* **2007,** *7*, 69-74.
6. Rutsch, M.; Rieckermann, J.; Cullmann, J.; Ellis, J. B.; Vollertsen, J.; Krebs, P. Towards a better understanding of sewer exfiltration. *Water Research* **2008,** *42*, 2385-2394.
7. Amick, R. S.; Burgess, E. H. *Exfiltration in sewer systems*; U. S. Environmental Protection Agency, Report EPA/600/R-01/034.: 2000.
8. Hunt, R. J.; Borchardt, M. A.; Richards, K. D.; Spencer, S. K. Assessment of Sewer Source Contamination of Drinking Water Wells Using Tracers and Human Enteric Viruses. *Environmental Science & Technology 44*, 7956-7963.
9. Fenz, R.; Blaschke, A. P.; Clara, M.; Kroiss, H.; Mascher, D.; Zessner, M. Monitoring of carbamazepine concentrations in wastewater and groundwater to quantify sewer leakage. *Water Science And Technology* **2005,** *52*, 205-213.
10. Paul, M.; Wolf, L.; Fund, K.; Held, I.; Winter, J.; Elswirth, M.; Gallert, C.; Hotzl, H. Microbiological condition of urban groundwater in the vicinity of leaky sewer systems. *Acta Hydrochimica et Hydrobiologica* **2004,** *32*, 351-360.
11. Teunis, P. F. M.; Xu, M.; Fleming, K. K.; Yang, J.; Moe, C. L.; LeChevallier, M. W. Enteric Virus Infection Risk from Intrusion of Sewage into a Drinking Water Distribution Network. *Environmental Science & Technology*, null-null.
12. Wolf, L.; Eiswirth, M.; Hotzl, H. Assessing sewer-groundwater interaction at the city scale based on individual sewer defects and marker species distributions. *Environmental Geology* **2006,** *49*, 849-857.
13. Hoes, O. A. C.; Schilperoort, R. P. S.; Luxemburg, W. M. J.; Clemens, F.; de Giesen, N. C. V. Locating illicit connections in storm water sewers using fiber-optic distributed temperature sensing. *Water Research* **2009,** *43*, 5187-5197.

14. Field, R.; Pitt, R.; Lalor, M.; Brown, M.; Vilkelis, W.; Phackston, E. INVESTIGATION OF DRY-WEATHER POLLUTANT ENTRIES INTO STORM-DRAINAGE SYSTEMS. *Journal of Environmental Engineering-ASCE* **1994,** *120*, 1044-1066.
15. Li, T.; Zhou, Y. C.; Li, H. Quantifying Nonstorm-Water Discharges to Storm-Water Systems with Model Analysis. *Journal of Environmental Engineering-ASCE* **2008,** *134*, 928-932.
16. Sercu, B.; Van De Werfhorst, L. C.; Murray, J.; Holden, P. A. Storm drains are sources of human fecal pollution during dry weather in three urban Southern California watersheds. *Environmental Science & Technology* **2009,** *43*, 293-298.
17. Ana, E. V.; Bauwens, W. Modeling the structural deterioration of urban drainage pipes: the state-of-the-art in statistical methods. *Urban Water J.* **2010,** *7*, 47-59.
18. Fenner, R. A. Approaches to sewer maintenance: a review. *Urban Water* **2000,** *2*, 343-356.
19. Morrison, C. R.; Bachoon, D. S.; Gates, K. W. Quantification of enterococci and bifidobacteria in Georgia estuaries using conventional and molecular methods. *Water Research* **2008,** *42*, 4001-4009.
20. Seurinck, S.; Defoirdt, T.; Verstraete, W.; Siciliano, S. D. Detection and quantification of the human-specific HF183 Bacteroides 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater. *Environmental Microbiology* **2005,** *7*, 249-259.
21. Ufnar, J. A.; Wang, S. Y.; Christiansen, J. M.; Yampara-Iquise, H.; Carson, C. A.; Ellender, R. D. Detection of the nifH gene of Methanobrevibacter smithii: a potential tool to identify sewage pollution in recreational waters. *Journal Of Applied Microbiology* **2006,** *101*, 44-52.
22. Harden, H. S.; Chanton, J. P.; Rose, J. B.; John, D. E.; Hooks, M. E. Comparison of sulfur hexafluoride, fluorescein and rhodamine dyes and the bacteriophage PRD-1 in tracing subsurface flow. *Journal Of Hydrology* **2003,** *277*, 100-115.
23. Smart, P. L.; Laidlaw, I. M. S. EVALUATION OF SOME FLUORESCENT DYES FOR WATER TRACING. *Water Resources Research* **1977,** *13*, 15-33.
24. Hua, J. M.; An, P. L.; Winter, J.; Gallert, C. Elimination of COD, microorganisms and pharmaceuticals from sewage by trickling through sandy soil below leaking sewers. *Water Research* **2003,** *37*, 4395-4404.
25. Horswell, J.; Hewitt, J.; Prosser, J.; Van Schaik, A.; Croucher, D.; Macdonald, C.; Burford, P.; Susarla, P.; Bickers, P.; Speir, T. Mobility and survival of Salmonella Typhimurium and human adenovirus from spiked sewage sludge applied to soil columns. *Journal Of Applied Microbiology 108*, 104-114.
26. Blanford, W. J.; Brusseau, M. L.; Yeh, T. C. J.; Gerba, C. P.; Harvey, R. Influence of water chemistry and travel distance on bacteriophage PRD-1 transport in a sandy aquifer. *Water Research* **2005,** *39*, 2345-2357.

Table 9.1. Rhodamine WT dye injection details for all field studies. RWT was added into the sanitary sewers in all experiments, except in Field 2c, when RWT was added into the storm sewers.

Experiment	Date	Sewer	# RWT pulses (interval)	RWT conc. (ppm)	RWT vol (l)
Field 1	6/11/10	P1	2 (0.5 h)	800	10
Field 2a	7/1/10	N1, N2	1	800	10
	7/6/10	N3, N4	1	400	10
	7/9/10	N5-N8	1	800	10
Field 2b	7/22/10	N1	2 (1 h)	800	10
		N2	3 (1 h)	800	10
		N3	4 (1 h)	800	10
		N4	5 (1 h)	800	10
Field 2c	9/16/10	N2-D	1	100	0.05
	9/17/10	N2-D	1	1000	0.05
	9/21/10	N3-D	1	100	0.05
	9/22/10	N3-D	1	1000	0.05
	9/17/10	N4-D	1	1000	0.05
Field 3	9/7/10	H1-H3	1	2,000	10
	9/8/10	H4-H6	1	2,000	10

Table 9.2. Microbial source tracking results: human-specific *Bacteroidales* (HBM) concentrations, presence (+) or absence (-) of *Methanobrevibacter smithii nifH* gene (MNIF). Standard errors for analytical replicates (n = 3) are shown in parentheses.

Experiment	Sample	Date	HBM[1] (copies/L)	HBM (% sewage)	MNIF
Sewage	WWTP	6/11	8.8E7 (4.2E6)	100	+
Field 1	P1-D	06/09	6.3E6 (6.0E5)	7	+
	P2-D	06/09	1.5E7 (1.8E5)	17	+
Field 2	N2-D	07/01	n.d.	n.d.	+
	N2-D	07/06	3.9E3 (5.9E2)	0.004	-
	N2-D	07/09	1.7E4 (1.1E3)	0.02	+
	N1-D	07/01	1.3E5 (6.8E3)	0.15	+
	N1-D	07/06	n.d.	n.d.	-
	N1-D	07/09	5.3E4 (3.4E3)	0.06	+
Field 3	H2-D	09/08	n.d.	n.d.	-
	H2-D	09/13	n.d.	n.d.	-
	H2-D	09/20	n.d.	n.d.	-
	H1-D	09/08	n.d.	n.d.	-
	H1-D	09/13	n.d.	n.d.	-
	H1-D	09/20	n.d.	n.d.	-

[1]n.d.: not detected

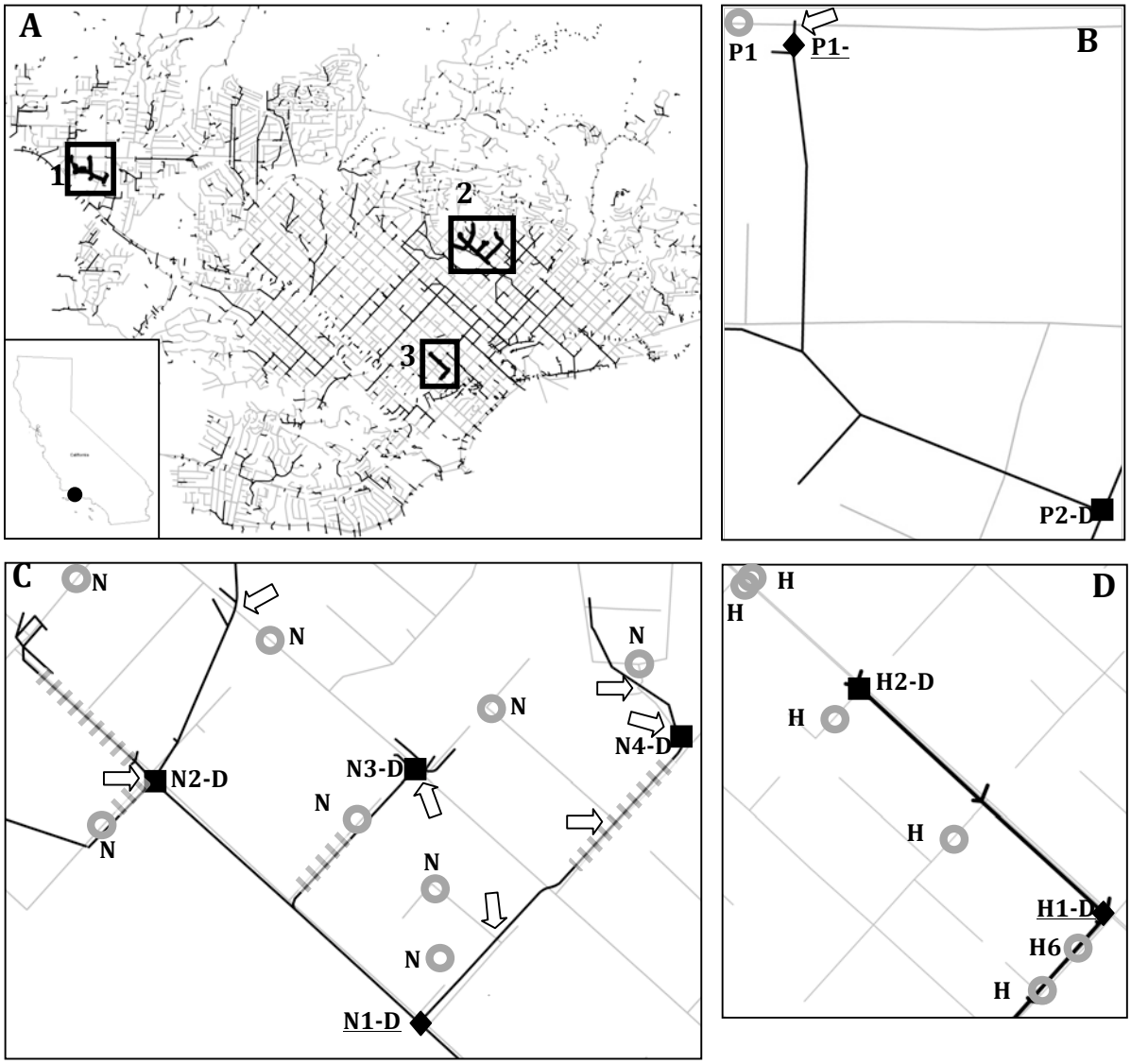


Figure 9.1. Field sites and locations of rhodamine WT (RWT) dye addition and detection, with subsurface storm sewers as black lines, and sanitary sewers as grey lines. Numbered symbols identify the RWT dye injection locations in sanitary sewers (grey circles), RWT dye injection locations or sampling in storm sewers (black squares), and RWT dye detection locations in storm sewers (black diamonds, underlined). Areas with potential for sewage exfiltration into storm drains are indicated by grey diagonal lines (pipes run parallel) or arrows (pipes cross). A Location of Santa Barbara, CA (black circle) and map of the downtown area with field study locations (numbered squares). B Infrastructure details for field study 1. C Infrastructure details for field study 2. D Infrastructure details for field study 3.

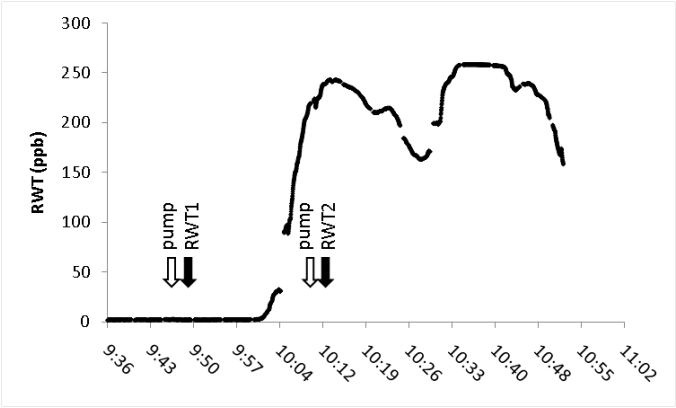


Figure 9.2. Detection of RWT at P1-D after dosing into sanitary sewers P1. Arrows indicate start of surcharge flow (open arrows) and RWT dosing (black arrows) in the sanitary sewer.

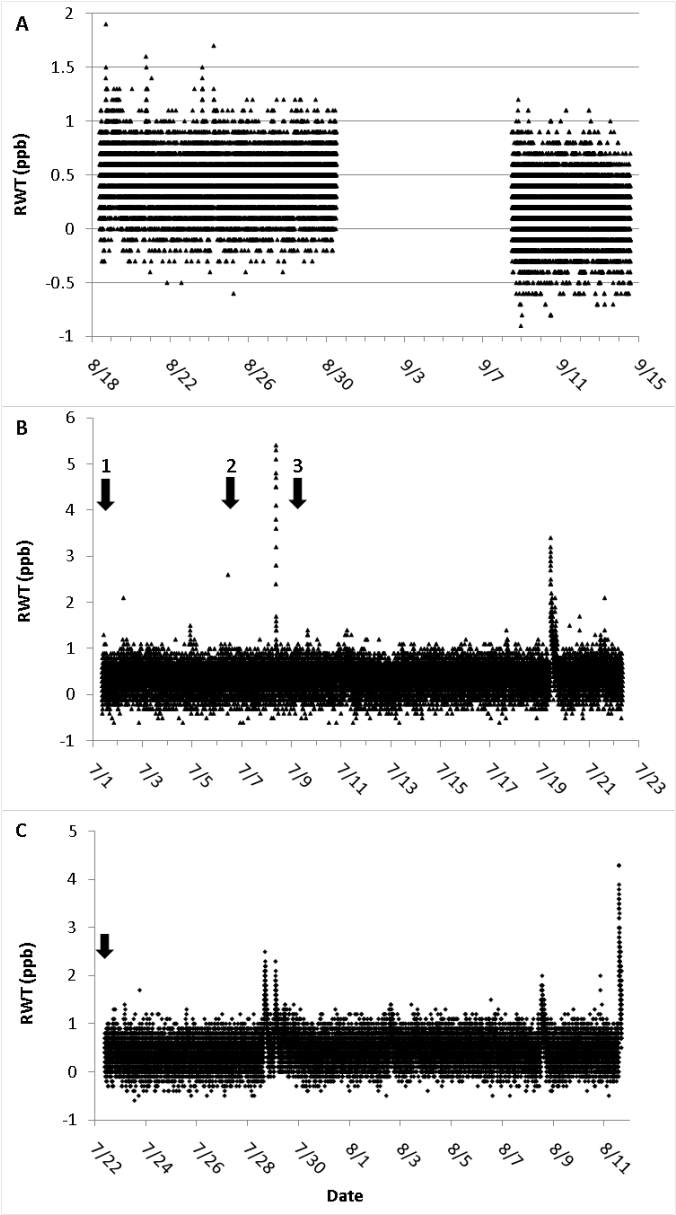


Figure 9.3. RWT concentrations at N1-D. A. Background. B. Phase 2a: one RWT pulse in each storm sewer manhole (indicated by arrows). Pulse 1: N1 and 2, pulse 2: N3 and 4, pulse 3: N5-8. C. Phase 2b: multiple RWT pulses in each storm sewer manhole.

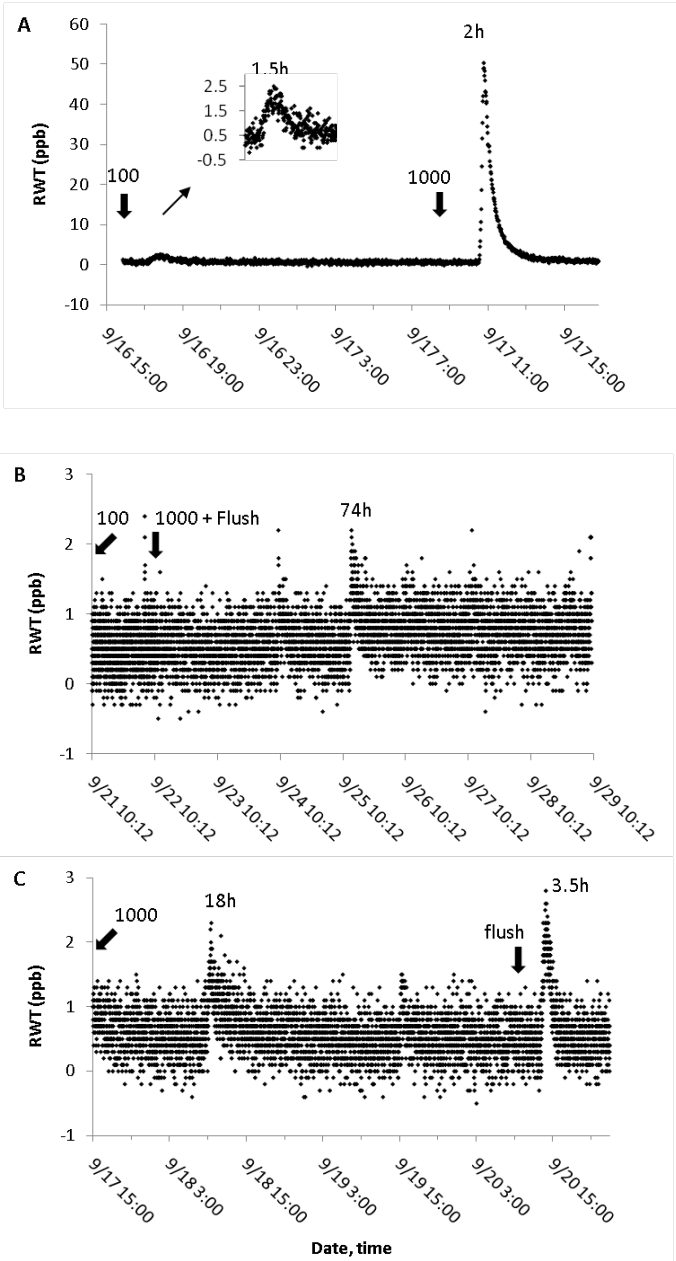


Figure 9.4. Detection of RWT at N1-D after dosing into storm sewers in N2-D (A), N3-D (B) and N4-D (C). RWT pulse volumes (100 or 1000 mL) are indicated with each injection, as well as flushes with water (without RWT). Time of travel between injection and detection location is indicated for each peak.

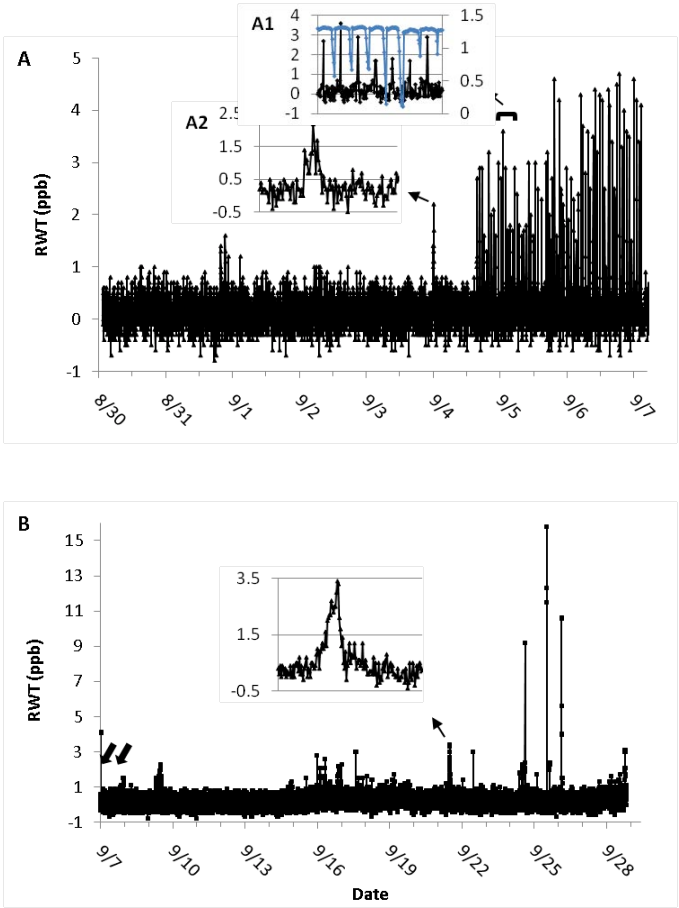


Figure 9.5. RWT concentrations at H1-D. A. Background. Inset A1 shows detail with conductivity in blue lines on right y-axis. Inset A2 shows detail of RWT peak. B. RWT concentrations after dosing one RWT pulse in each sanitary sewer manhole (dosing times indicated by arrows).

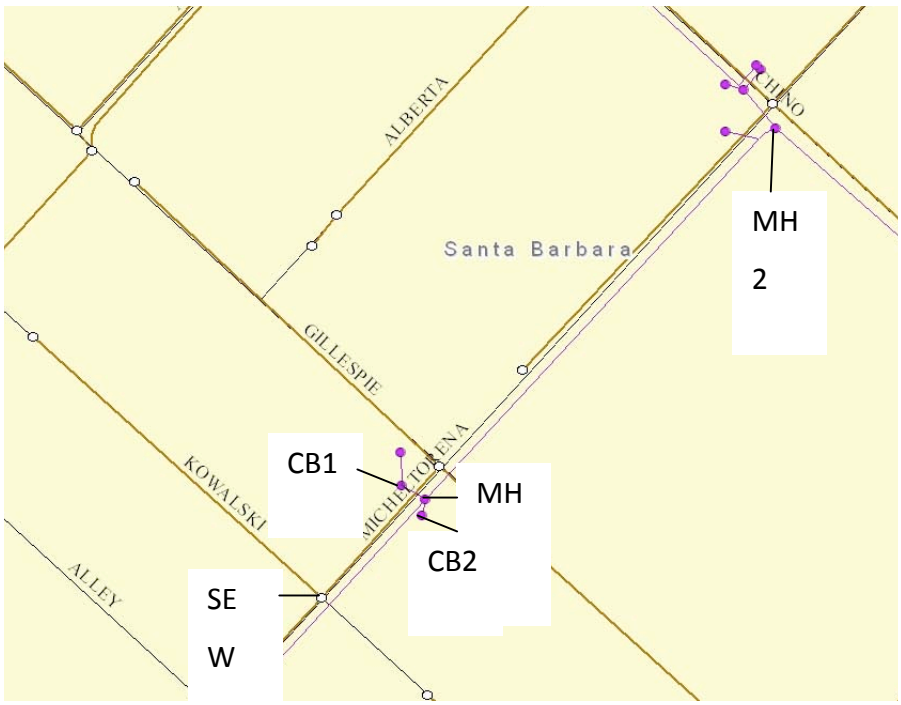


Figure 9.6. Location of sanitary sewer (brown) and storm drains (purple) for the RWT study at Micheltorena/Chino.

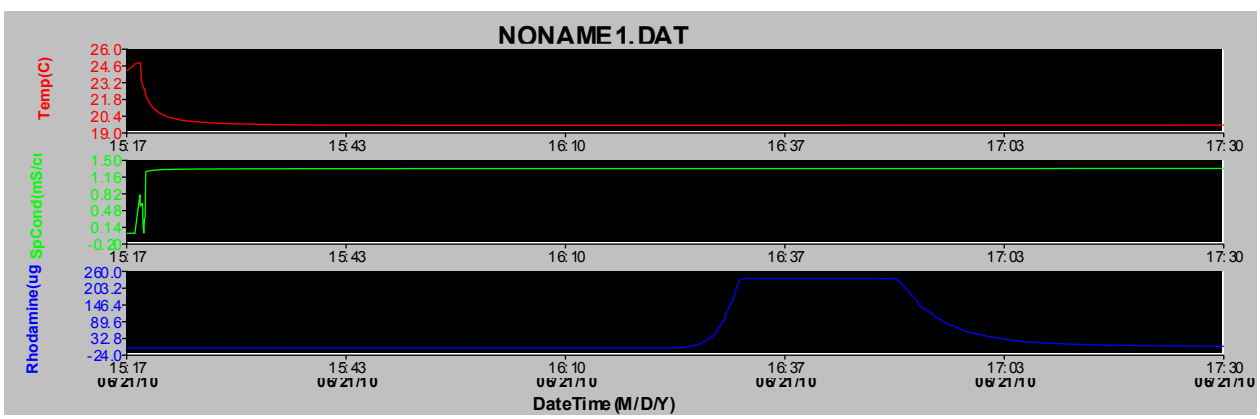


Figure 9.7. Temperature (red), Conductivity(green) and RWT (blue) in MH2 after RWT dosing at MH1.

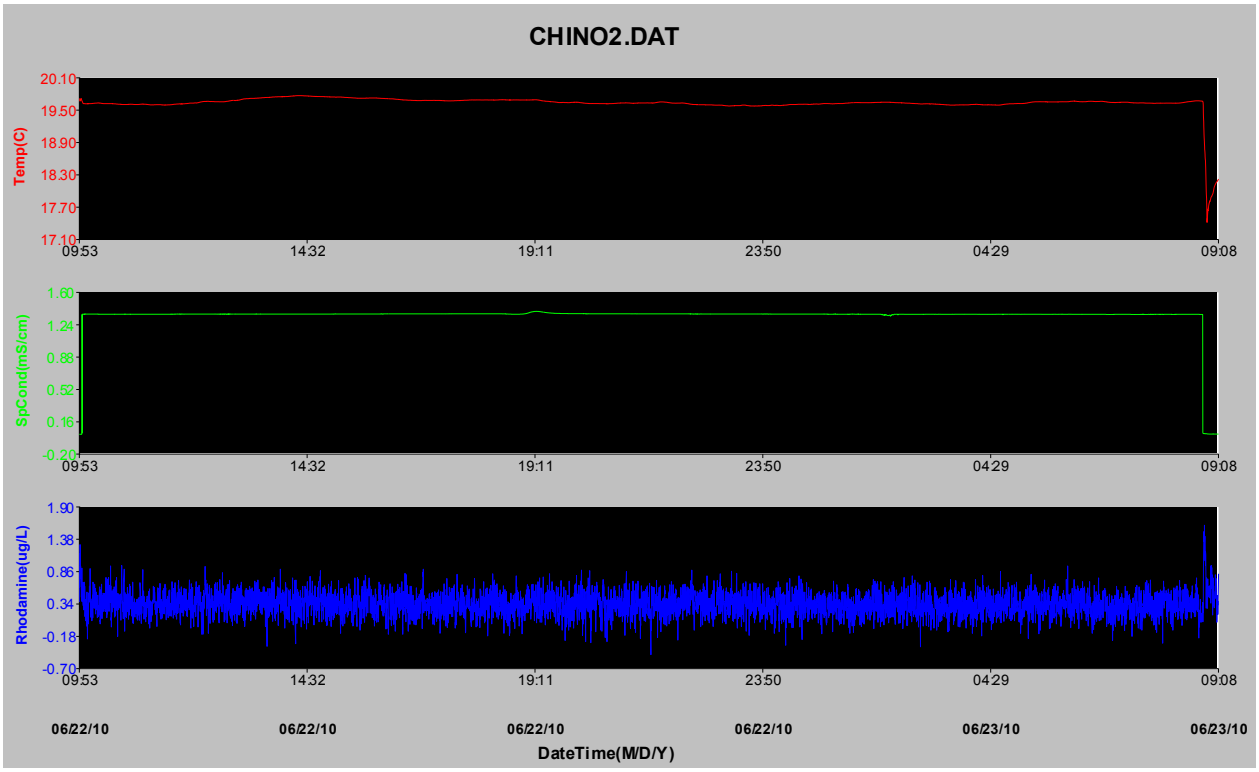


Figure 9.8. Temperature (red), Conductivity (green) and RWT (blue) in MH2 after RWT dosing at SEW.

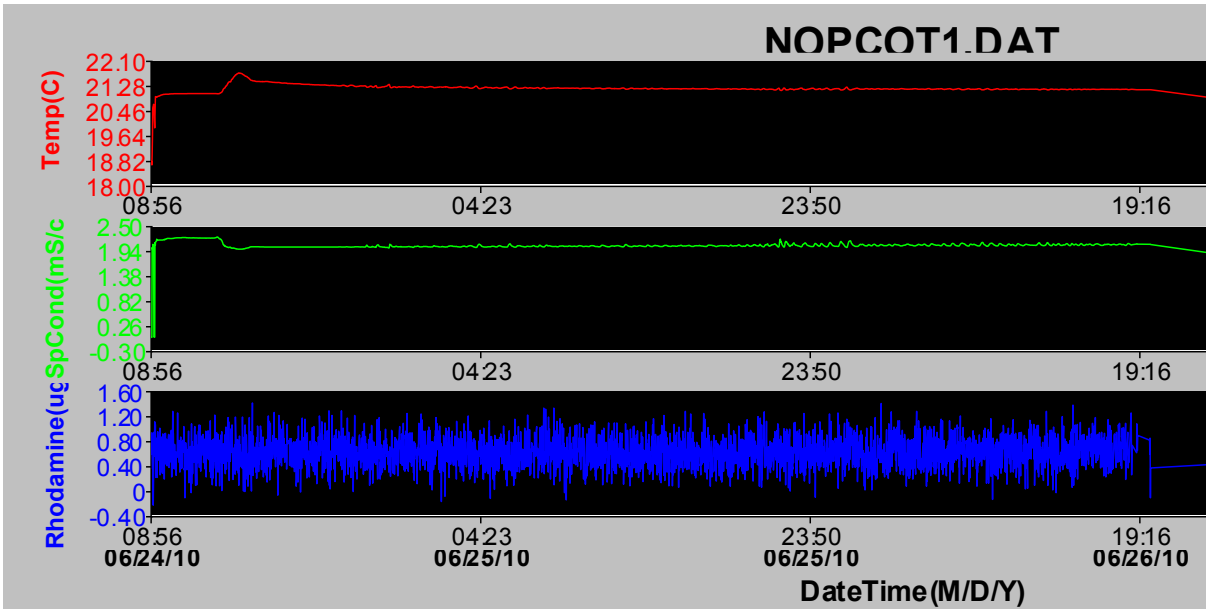


Figure 9.9. Temperature (red), Conductivity (green) and RWT (blue) in the Nopal/Cota storm drain after RWT dosing in SB Jr High.

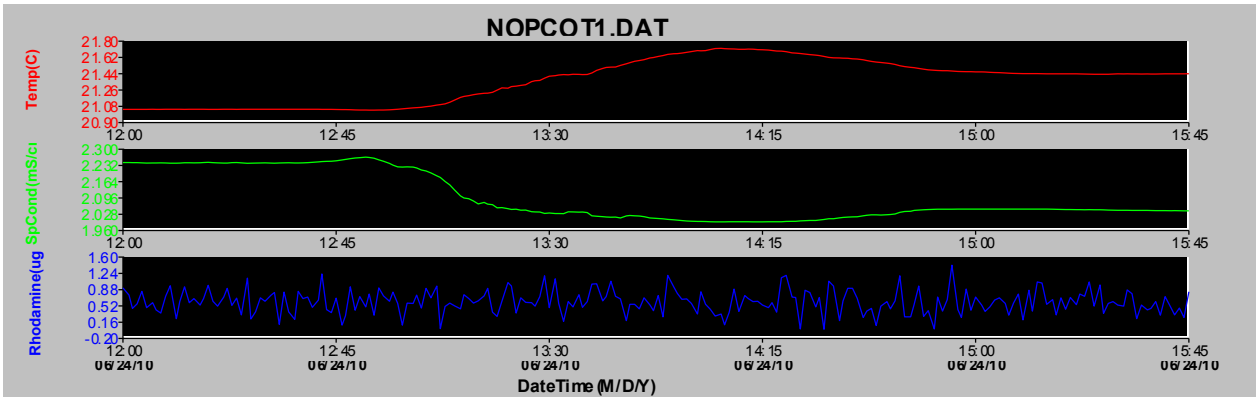


Figure 9.10. Temperature (red), Conductivity (green) and RWT (blue) in the Nopal/Cota storm drain after RWT dosing in SB Jr High, zoomed.

X. GIS Analyses

10.1 Background & Objectives

To compile and analyze the storm drain water contamination data collected over several years and multiple sampling sites, a geographic information system (GIS) was created. The objectives defined for the GIS analysis were to:

1. Compile the project data in a consistent format in a single Excel database;
2. Create a GIS database using ArcGIS software that incorporates spatial and temporal project data with the existing City of Santa Barbara basemap data in order to display tested parameters in a variety of ways;
3. Create an index to combine results from multiple contamination parameters for visual comparison.
4. Use modeling within ArcGIS to identify areas with a potentially high risk for human-specific fecal contamination due to proximity of storm drain pipe to:
    a. Adjacent or overlapping sewer infrastructure situated at a shallower depth
    b. Adjacent or overlapping sewer pipes composed of vitrified clay material;
5. Use modeling to identify patterns between the high-risk areas identified in objective 3 and the sampling locations with high and/or present human fecal indicator data

10.2 Data Collection & Compilation

To achieve objective 1 above, data was collected and compiled in Microsoft Excel from each location sampled over the span of the project for some or all of the following parameters:

1. *Sampling location information*: latitude; longitude; date sampled; time sampled.
2. *Field Measurements*: temperature (°C); salinity (ppt); conductivity (mS/cm); dissolved oxygen (mg/L); pH.
3. *Microbiology (fecal indicator bacteria)*: Total coliform (MPN/100mL); E. coli (MPN/100mL); Enterococcus (IDEXX – MPN/100mL; qPCR – c.e./100mL).
4. *Chemistry*: Human *Bacteroidales* markers (copies/L); *Methanobrevibacter smithii nifH* PCR; Caffeine (ppb); Cotinine (ppb); NH_4 (ppm); NO_3 (ppm); PO_4 (ppm); SO_4 (ppm).

Compiled data was spot-checked by project researchers on several occasions to ensure accuracy and consistency.

10.3 Method & Results

To achieve objective 2 above, a feature layer was created in ArcCatalog to identify each sampling location using its coordinates in latitudinal and longitudinal degrees to six (6) decimal places. The compiled field, microbiological and chemical data was then linked to its spatial location in ArcMap using the Join function. With this completed, the data was visualized in a number of ways. Each parameter was mapped and symbolized separately by

date or location using graduated symbols and/or colors to best exemplify temporal data for locations that were sampled multiple times, or indexed in a manner that displayed multiple related parameters adjacent to one another for a single location. For instance, creating an index using maximum, median or mean values for E. coli, Enterococcus, HBM, and Mnif data visually displayed the degree of consistency and correlation between locations with high FIB data and locations with high and/or positive human-specific fecal indicator data. Figure 10.1 shows the standard parameter symbology using Mission Project Enterococcus results as an example.

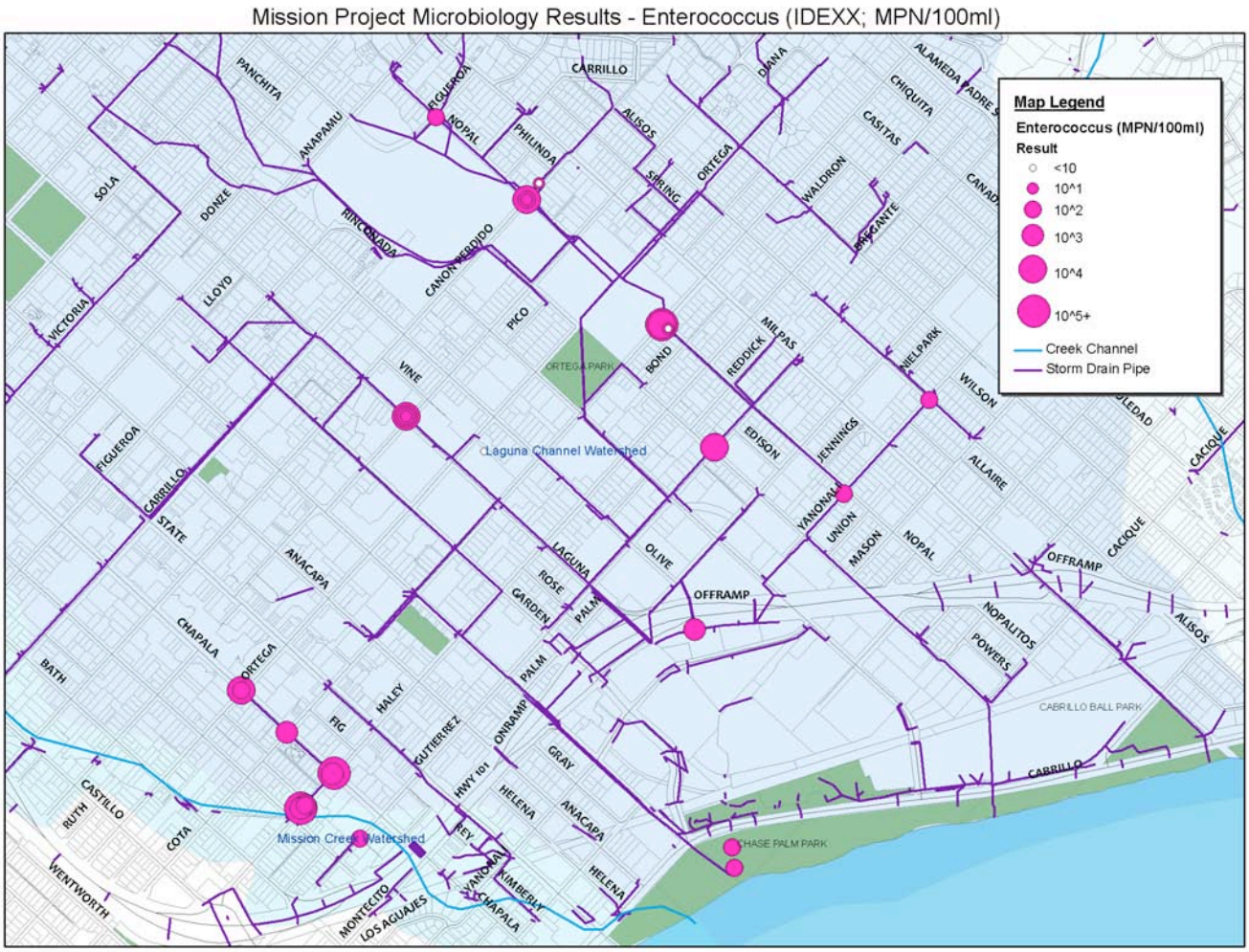


Figure 10.5.

The City of Santa Barbara's existing GIS database contained the following relevant base-map features and related attributes: aerial imagery, City parcel data, watersheds, storm drain system (nodes, storm drain pipe, creek channel), and sewer system (structures, mains, laterals, lift stations). In order to achieve objective 3 above, data gaps relating to the depths of sewer mains had to be filled using modeling within ArcMap. To attain the approximate depths of the City's sewer mains, the Spatial Join tool was used to relate the depth information provided for individual sewer structures with the sewer main pipes to which they are connected.

Once sewer main depth information was extrapolated, all points where storm drain pipe and sewer mains overlap were identified using the Intersect tool in ArcMap. As a first step to find the locations where sewer mains are positioned at a shallower depth than the storm drains they overlap, the overlap points where the sewer main depth is six feet or less were isolated. This depth boundary was based on information provided by City of Santa Barbara Creeks Department staff who indicated that storm drain pipe is generally located an average of six feet or greater below ground level. Running this model yielded the result that 513 points exist in the City of Santa Barbara where there is overlap between a storm drain pipe and a sewer main that is less than six feet in depth.

In order to identify the storm drains with highest risk for human fecal contamination from sewage exfiltration and to test the assumption that sewer mains less than six feet in depth were likely shallower than the storm drains at these points, the storm drain pipe depths at these intersections were verified using the City's storm drain atlas. The paper storm drain atlas was referenced in place of storm drain data from the City's GIS database attribute tables because existing GIS layer data proved to be inconsistent and inaccurate as a result of several spot checks. This storm drain atlas inquiry confirmed that there are 127 overlap points where sewer mains are shallower than storm drain pipe, 116 points where sewer mains are likely shallower but not verifiable due to incomplete data in the storm drain atlas, and at 270 points the storm drain pipe was confirmed shallower. While there are overlap points distributed throughout the City of Santa Barbara, they exist in higher concentrations in the Eastside, Laguna, Westside and Mesa neighborhoods. Of the 127 confirmed sewer overlap points, 91 were also locations where the sewer main material consisted of vitrified clay pipe (VCP) averaging over 70 years old, with the oldest being 122 years old. Figure 10.2 maps the location of the 127 locations where sewer mains were confirmed to be shallower than storm drain pipes they overlap throughout the City of Santa Barbara.

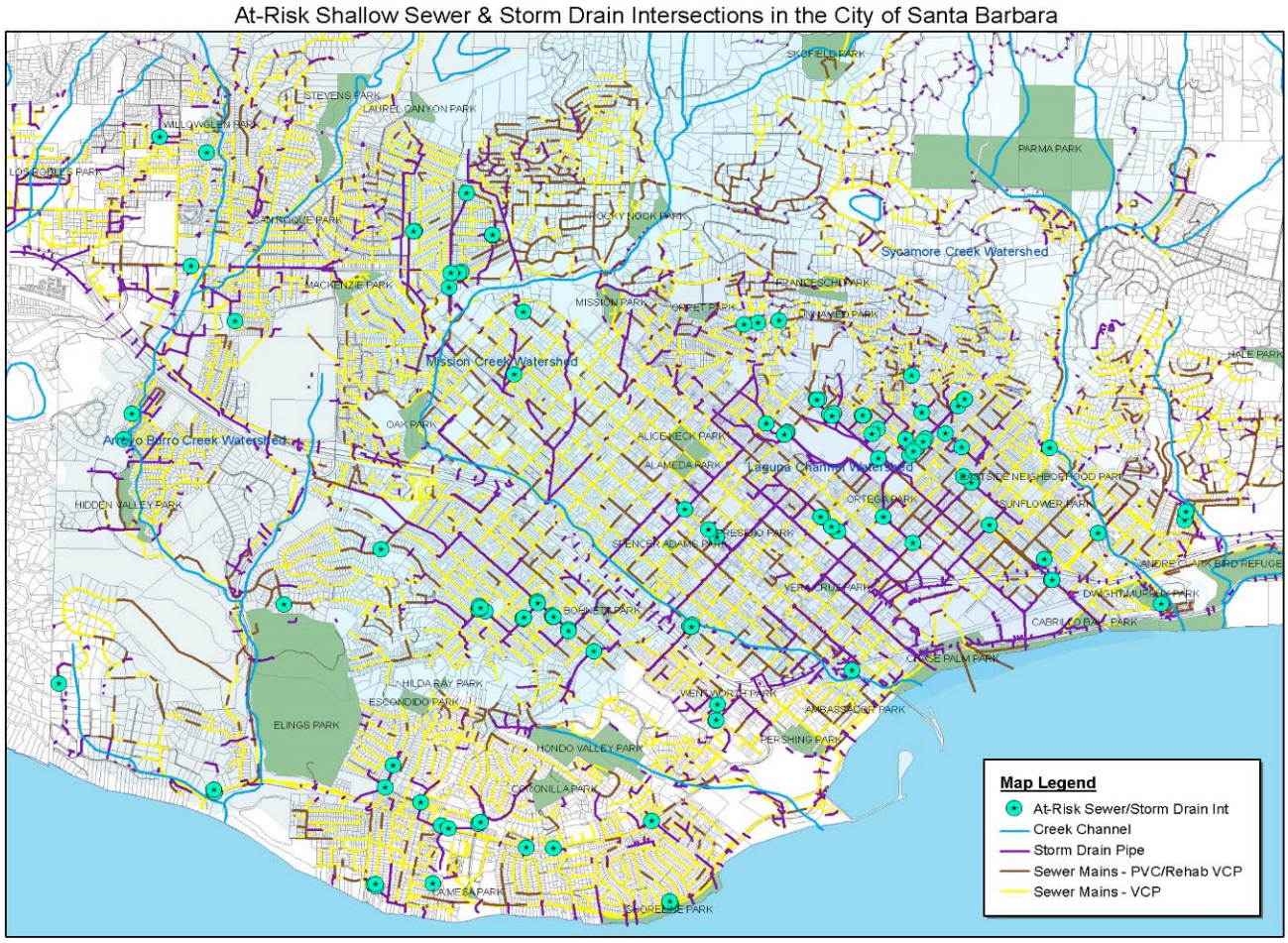


Figure 10.2.

After identifying these 127 locations, they were mapped with the selected project sampling data in order to visually assess the proximity of these at-risk sewer/storm drain intersections to locations with tested and confirmed contamination data. Figure 10.3 provides a project area subsection of this map illustrating HBM results. This assessment does not indicate a definitive, consistent correlation between areas with confirmed positive HBM results and their proximity to at-risk sewer / storm drain overlaps due to the broad spatial and temporal distribution of sampling locations, however there are several sampling locations with positive contamination results in relatively close proximity to at-risk intersections.

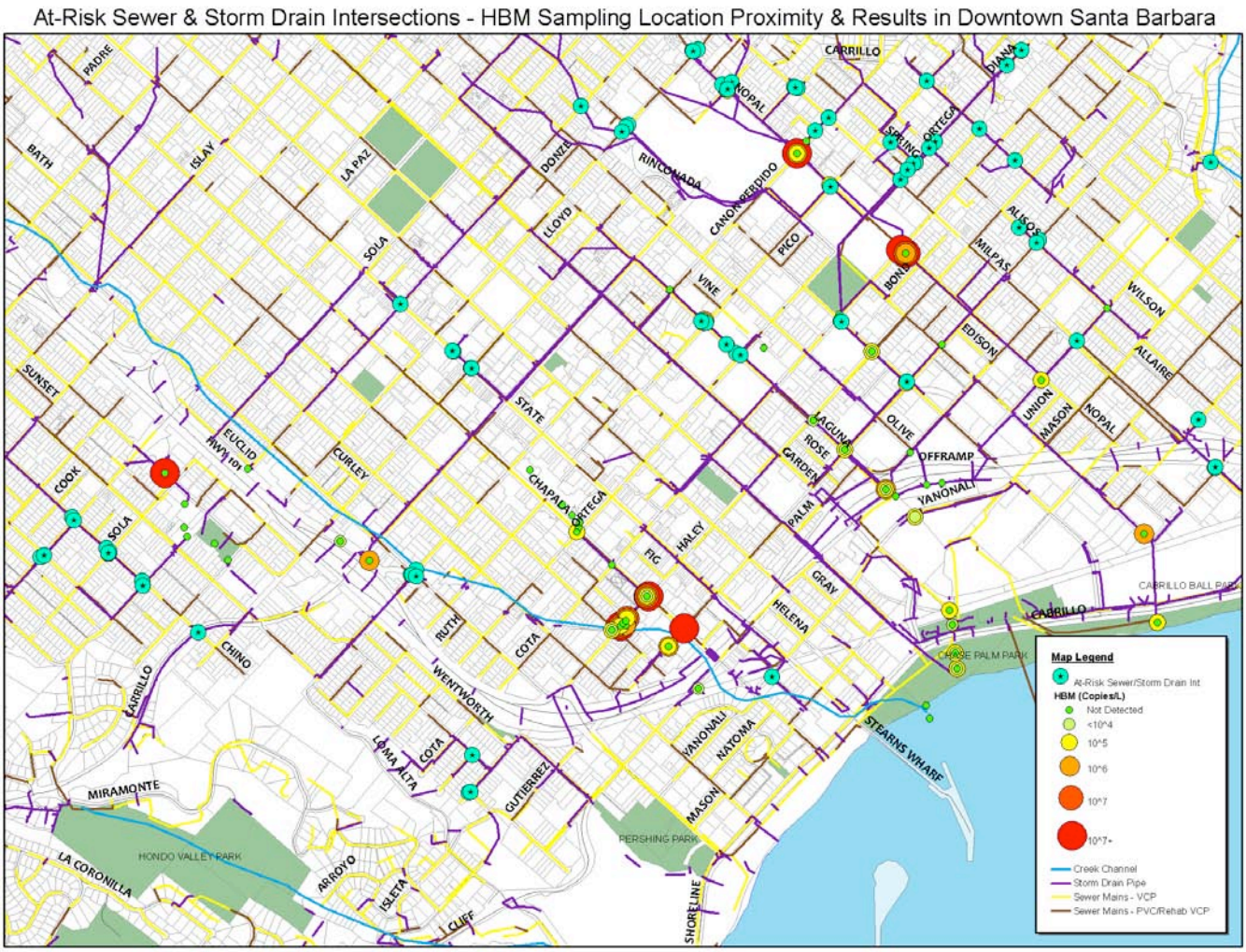
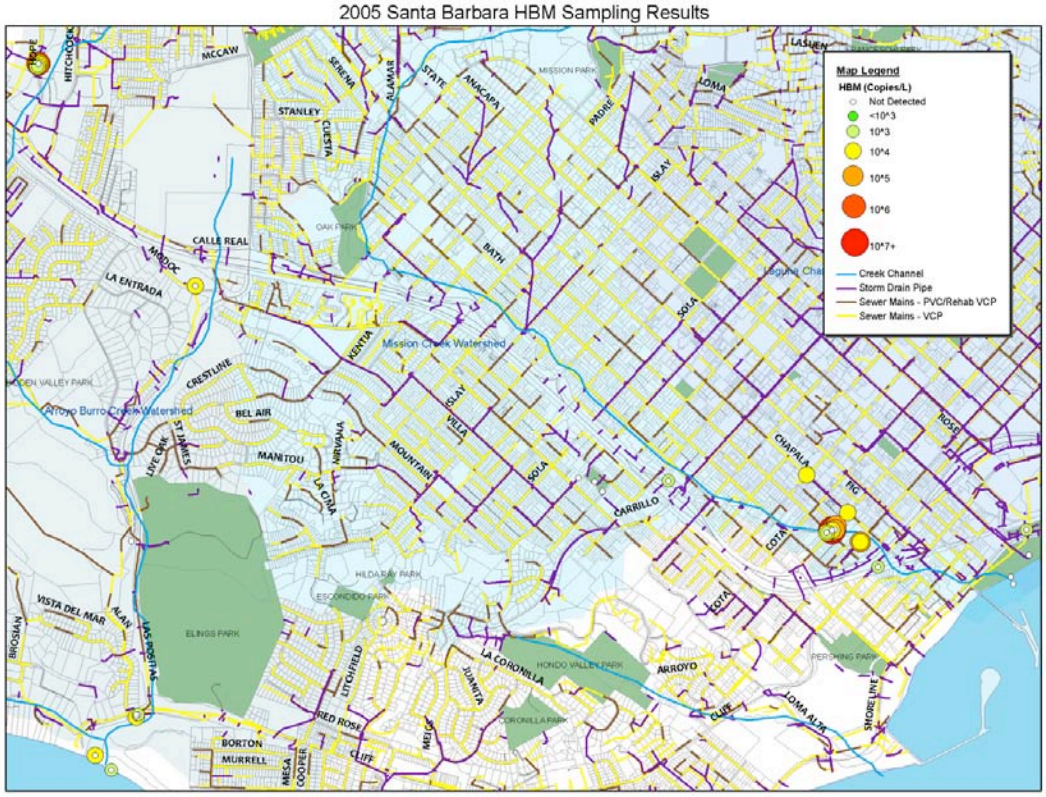


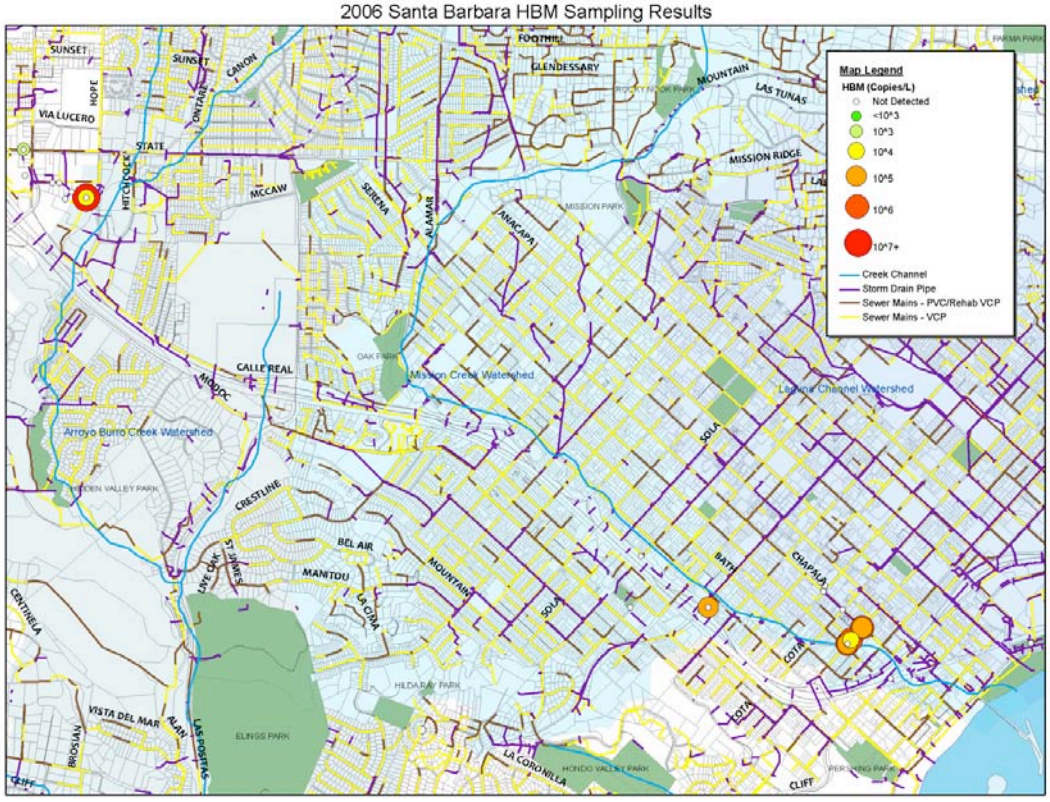
Figure 10.3.

In addition to this analysis, both a broad and a focused temporal contamination pattern analysis were performed using the data collected. For visual purposes, aggregated annual data was mapped to show how sample locations and results varied from year to year. Figure 10.4 below provides an example of this mapping using HBM data for each of the project's sampling stations. Analysis of the data attribute tables also offered a targeted temporal approach to contamination data. For example, there are several sampling locations that yielded consistently high HBM data over the period of several days or weeks indicating a persistent source of human fecal contamination entering the storm drains upstream of the sampling location. Many stations, however, yielded results ranging from no HBM detected to significantly high levels over a timespan of days, months or years. This appears to indicate the existence of more isolated, inconsistent contamination sources and events.

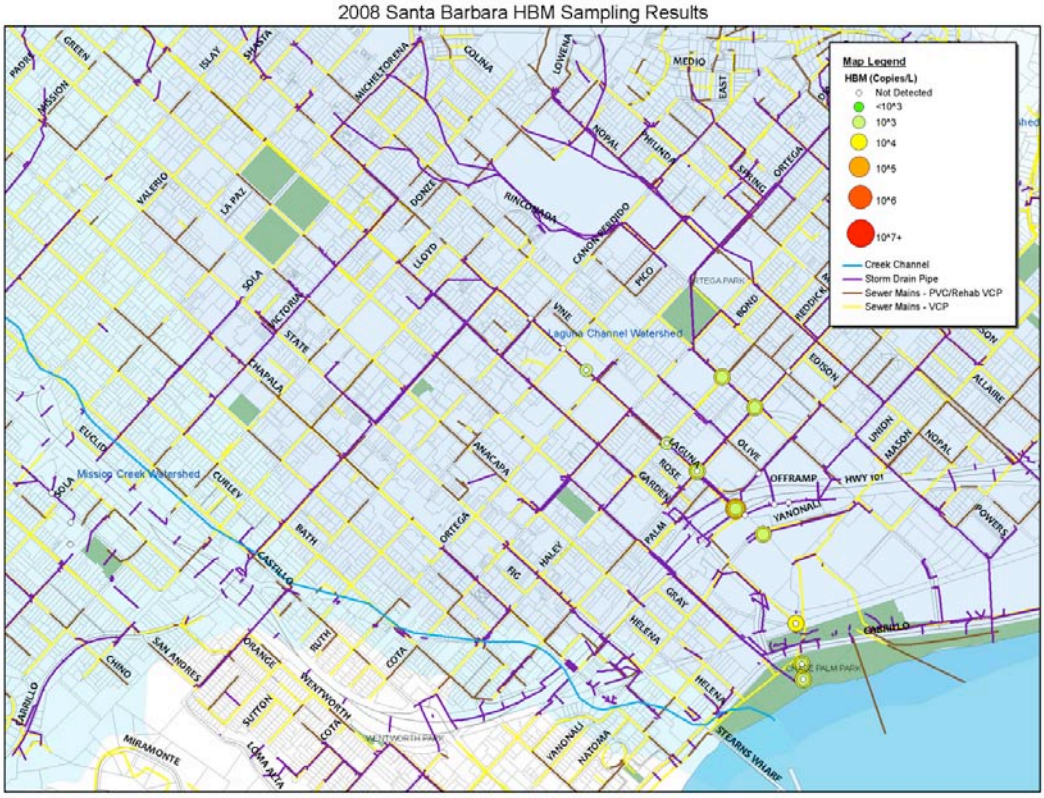
2005 Santa Barbara HBM Sampling Results



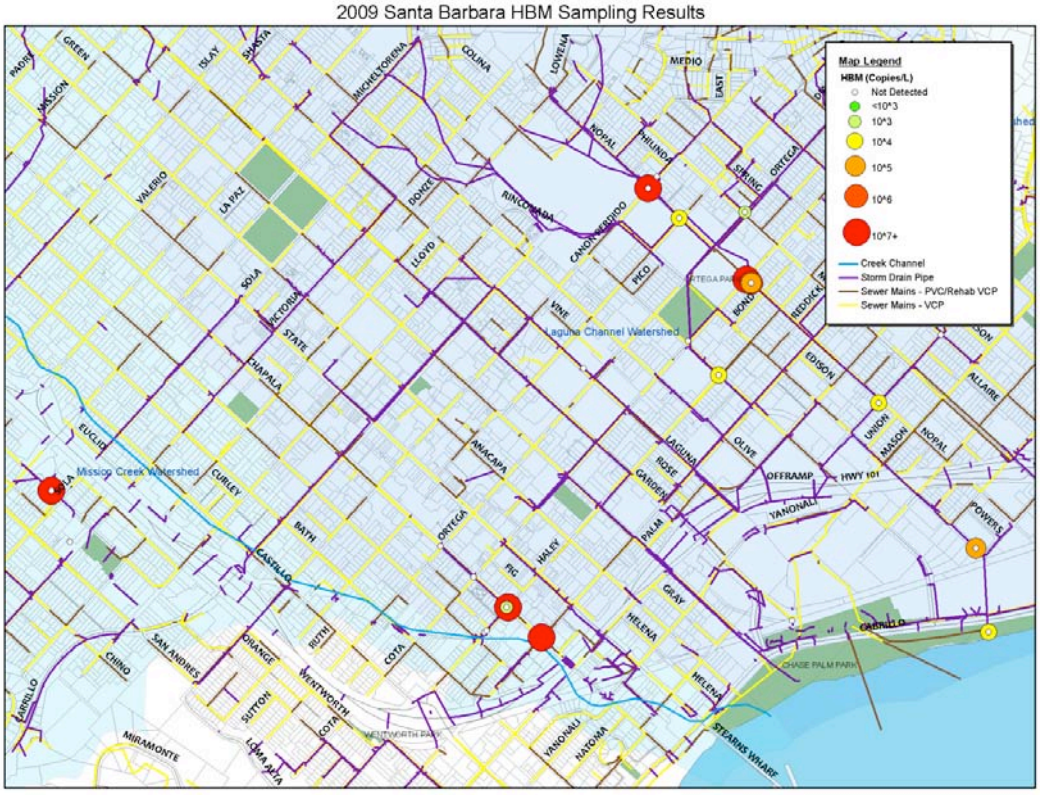
2006 Santa Barbara HBM Sampling Results



2008 Santa Barbara HBM Sampling Results



2009 Santa Barbara HBM Sampling Results



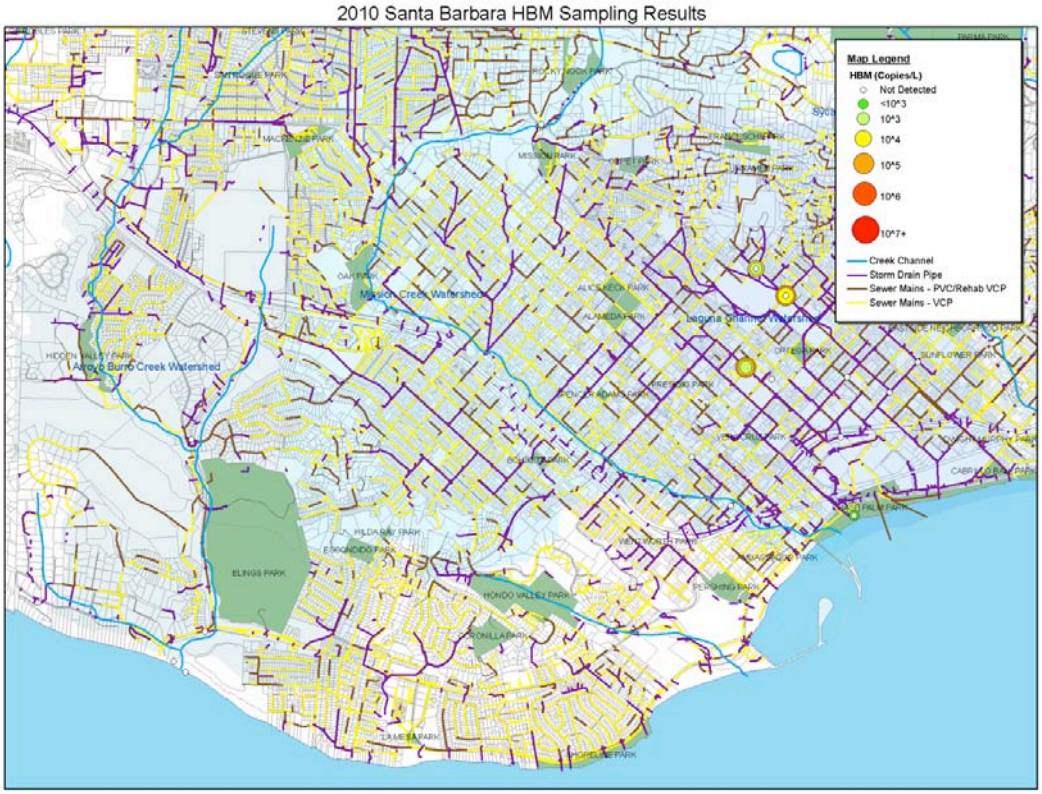


Figure 10.4.

Using ArcGIS, project researchers were able to spatially and temporally visualize and analyze water quality data for fifteen different microbiological, chemical and field parameters in sampling locations throughout the City of Santa Barbara. Objectives of data compilation, visualizing and analysis were achieved, and researchers should continue to utilize the powerful tool of GIS in future water quality studies. These initial assessments and visualizations seem to indicate that contamination pattern analysis using GIS will be most useful by supplementing existing data with new data from more targeted and consistently-sampled locations in the City. Moving forward, it is recommended that researchers expand their sampling locations to include storm drains in areas not yet sufficiently sampled such as the Eastside and Mesa neighborhood in addition to storm drains in close downstream proximity to areas where shallow sewer mains were identified in this study, particularly vitrified clay sewer mains.

XI. Microbial Source Tracking Protocol

11.1 Background to this Section and Relation to Other Sections

This section recommends an approach for source tracking human waste in storm drains in urban coastal communities, and draws significantly on recommendations and conclusions made in sections IV through X of this report. This section also considers recommendations made in other related guidance documents (section III). Therefore, the detailed justifications supporting the recommendations here are within prior report sections, and this section provides a synthetic view on what should be an overall protocol.

11.2 Overall approach to "drain tracking"

As discussed previously, storm drains have been shown to carry DNA-based human waste markers which indicate contamination from sewage. Such evidence for sewage contamination in storm drains occurs during dry weather in coastal Santa Barbara, CA [1, 2] and in other coastal communities in California [3]. The widespread occurrence of human waste markers in waters along the central California coast [4] may signal that urban sanitary infrastructure is contributing broadly to coastal contamination. However, there are variations in the specificity of different DNA-based approaches for diagnosing human waste contamination [5, 6], and thus---as described in section V of this report—careful verification of DNA-based methods is recommended, especially for new methods as they emerge. Still, DNA-based approaches are just one aspect of a community's toolkit in assessing the presence and origins of human waste in coastal waters, creeks, and drainage. Also, DNA-based approaches should not be used in isolation of a broader context related to the watershed and possible fecal sources. Rather, a systematic protocol is needed for determining waste origins. Ideally, the protocol is one that any coastal urban community in CA could adopt. While communities may not operate laboratories for DNA-based analyses, there are commercial labs that can be hired and university groups available for collaborative research (e.g. this study). The protocol in this report is oriented towards empowering communities to take advantage of the myriad technologies available for tracking FIB sources, particularly sources of sewage contamination. A stepwise approach is recommended. Within each major step, actions are suggested to advance to the next step. Those actions are likely to vary, depending on the setting and its characteristics, for each community and situation. This Protocol is intended to provide detailed guidance that can be readily implemented and that can be expected to produce results upon which water quality managers can act. The Protocol is also a suitable starting place for writing applications for funding from water quality improvement grant programs.

This project was motivated by addressing FIB levels as beaches which frequently exceeded State of California beach water quality criteria ("AB411 criteria"). However, it is clear from this work that raw sewage, and the associated health risk, could enter beaches that do not receive regular beach warnings. Any beach with storm drain discharges or creek outlets, that is suspected of being impacted by aging, decaying sanitary sewer infrastructure or illicit connections, is an appropriate candidate for the Protocol proposed here.

11.3 Recommended Steps in Source Tracking Human Waste in Urban Coastal Communities

The following steps are recommended, in order. Detailed advice, within particular steps where choices are available, is also provided. Costs associated with implementing these steps are provided (in prior report sections) for some of the recommended approaches. Other costs are inherent to general water quality management and would be estimated within an agency by their budgetary standards.

NOTE: Steps need not be fully complete prior to advancing through the protocol. For example, water quality samples can be collected while the GIS is being completed. Furthermore, if limited funds are available, the community can prioritize subsets of this protocol based on the greatest concerns (e.g., FIB levels vs. human health risk), and the strongest suspicions of contamination (e.g. sewage exfiltration vs. illicit connections).

1. **Identify, inventory, collect and organize information, including**:
    a. Maps of the natural physical setting, identifying the beach locations, monitoring locations, watershed and subwatershed boundaries, and creeks. A GIS representation is best, as this will allow for building a more comprehensive database for use in planning research and management actions. Interns recruited from environmental studies or geography programs can be very helpful for this stage.
    b. All FIB monitoring data at all locations hydrologically connected to the beach that is failing water quality criteria. Old and recent data are equally relevant. Ideally, these data are entered into a computerized spreadsheet which makes data analysis approachable.
    c. Other water quality data that is available for the same watershed areas, as it may pertain to tracing the origins of fecal pollution.
    d. Maps of civil infrastructure and infrastructure metadata (pipe diameters, materials of construction, pipe depths, ages of the infrastructure) within the boundaries of the watershed discharging into the beach of concern. Infrastructure of interest includes wastewater treatment plants, roads, sanitary sewer systems including manholes and pump stations, and storm drain systems including manholes and trash separators. Ideally, this information would be within a GIS database, likely within separate layers for each infrastructure category.
    e. An inventory of other potential FIB sources and where they are likely to enter the watershed including seabirds at the beach, pets near creeks, wildlife leaving near creeks or in storm drains, and localized livestock (expected to be minimal in urban areas targeted by this protocol).
2. **Evaluate, correct, complete and analyze the collected information:**
    a. FIB and other water quality data should be organized in a spreadsheet according to sampling location, type of sample, date, and time. The methods of sample analysis are important because analysis results can vary by method (e.g. membrane filtration for FIB versus IDEXX-based reagents in an MPN format [7]), and thus results should be grouped according to analysis

method. Data should be graphed: like data over time for single locations to assess temporal variability at a location, and like data at different locations at the same time points to assess spatial variability. For many types of source tracking data, nonparametric statistics are appropriate due to small sample sizes, non-normal distributions, and especially high frequencies of data above and below thresholds. Using parametric statistics with such data sets can lead to misinterpretation of patterns and correlations [8]. Box plots are ideal for exploration of nonparametric data, such as comparing multiple data points across sites. For statistical tests, medians are used in place of means, and correlations and tests of significance are computed on ranked data. Most statistical packages provide tests such as Mann-Whitney, Wilcox, or the Sign Test. Nonparametric correlations are also available [9]. When parametric statistics are warranted, the means for individual sites, calculated as an average plus or minus the standard error of the mean, should be calculated, and appropriate (e.g. one way-ANOVA, Tukey's honestly significant difference, or Student's *t*-test) means comparisons statistical tests should be employed to determine if there are differences between sites over time. Means (plus and minus standard error of the mean) should be plotted, or tabulated, for FIB and other pertinent water quality data by site. Where high concentrations of FIB exist, these should be flagged as "hot spots". *NOTE: the choice of a statistical method is important, and it may be necessary to seek professional advice.*

b. GIS data should be checked for completeness and accuracy. For example, metadata for piping systems may be entered using differing units. Incomplete data should be filled in by making field measurements (e.g. surveying elevations and measuring depths in manholes, for example) and questionable data should be checked for accuracy.

c. Merge water quality (FIB and other pertinent) and spatial infrastructure and watershed data into one GIS database that can then be used to display water quality data in a spatial context. As additional water quality data are acquired, they are entered into this "living" GIS database for the watershed. Produce at least two different types of displays: a) one showing water quality "hot spots" in the spatial context of watershed features and civil infrastructure, and b) one showing "infrastructure hot spots" where civil infrastructure may be causing surface water contamination—in particular where old sanitary sewers cross above and in close vertical plus lateral proximity to storm drains.

3. **Plan, schedule and conduct water sampling at and around "water quality hot spots" to determine human waste concentration patterns**:

a. Delineate the "FIB hotspots" from the data analysis above and select the locations for follow-up sampling and more specific analysis of human waste or potentially other sources of waste. Initially, prioritize the source tracking of human waste as it is most likely to be unhealthful for people in the community. Plan to sample along a transect for each hotspot:

i. Upstream and downstream of "hot" drain discharges in creeks, as well as

ii. upstream in drain systems towards putative sources

b. Delineate the "infrastructure hotspots" from the GIS analysis above and select the locations for follow-up sampling and more specific analysis of human waste or potentially other sources of waste. Initially, prioritize the source tracking of human waste as it is most likely to be unhealthful for people in the community. Plan to sample:
   i. In the storm drain network, downstream of clusters of infrastructure risk areas identified.
   ii. Upstream of inputs of uncontaminated water, e.g. groundwater pumps, that can dilute signals of human waste.

c. Choose sampling times that best represent conditions of concern, e.g. dry weather periods when there is sufficient drain discharge to sample. Choose multiple times to capture temporal variations, which can arise from industrial or domestic water use and waste generation, including:
   i. day-to-day by sampling several days in a row
   ii. diurnal by sampling over a 24-hour time frame. As described in section VIII, automated flow measurement and sampling may be needed to better understand temporal variations. It is recommended that such equipment be employed after the need is established, due to its expense and the expertise required to use it.

d. Identify and acquire reference fecal source material which is most likely sewage for the urban settings using this protocol. Sewage from the influent to the wastewater treatment plant (upstream of screening and primary sedimentation) is suitable; also fine is sewage from manholes in the vicinities of "hot spots" in the drain discharges. Fecal source material should be sampled with the "hot spot" sampling event, but can also be pre-sampled and analyzed so that reference concentrations of sewage tracers (e.g. chemicals and DNA-based markers) are known in advance.

e. Sample, and handle samples according to the appropriate procedures so that the analyses can be performed without introducing contamination and without losing analytes due to degradation. Sampling and handling procedures must be pre-planned such that adequate:
   i. sampling equipment and supplies are ready and available
   ii. temporary sample storage (e.g. in ice chests in the field) is ready
   iii. sample pre-treatment supplies (e.g. filters for caffeine or cotinine analysis, or prefilters for performing DNA-based analyses) are field-ready
   iv. equipment and reagents in the case of field analyses (e.g. for nutrients, or dissolved oxygen) are ready and available

f. Prioritize sample analyses to first emphasize inexpensive and rapid methods, then to progress to more specific and complex methods as such prioritization will be invaluable for discerning between acute sewage contamination that may arise from illicit connections versus chronic sewage contamination that may arise from exfiltration. Based on the studies in this report, the following hierarchy of sample analysis is recommended, with samples preserved for each more-complex (and thus expensive analysis).

Wastewater or public health laboratories may be able to complete the following techniques with relatively small investments in equipment and training:

i. analyze in the field for ammonia and phosphate using colorimetry (section VII, this report) plus standard probe based measurements (temperature, dissolved oxygen, and conductivity), but also
ii. collect samples for caffeine and/or cotinine analysis in the field (syringe filtration through 0.2 mm and store at -20° C in glass vials, section IV) and
iii. return split samples to the lab for FIB analysis and for
iv. surfactant analysis (as per section VII and IV) and for
v. filtration (0.2 mm, section IV) for collecting cells in the event that DNA-based analysis is required, while
vi. reserving filtrate (-20° C) for other analyses (section IV and VII), depending on the concentrations of ammonia and phosphate

4. **<u>Analyze samples and data</u>**, first focusing on field measurements and also FIB measurements as these are always recommended. Make decisions about additional analyses, e.g. of archived (frozen) water for cotinine or caffeine analyses or of frozen filters for DNA-based measurements, based on the following criteria:
    a. where nutrient (ammonia and phosphate) and surfactant concentrations appear to equal sewage concentrations of 10% or higher, consider not performing additional analyses as acute sewage contamination is indicated.
    b. where nutrient and surfactant concentrations appear to equal less than 10% sewage, analyze for sewage concentrations using more specific methods:
        i. caffeine and/or cotinine
        ii. DNA-based markers of human waste (as per IV and V, this report).
5. **<u>Evaluate the need for assessing other fecal sources and then act</u>** if human fecal sources are not implicated using the above methods at FIB and infrastructure "hot spots" in urban drains. In these cases, other host-specific DNA-based markers or community DNA analysis for water samples in comparison to fecal sources (section VI) may be required. *Consultation and assistance from professionals who develop and employ DNA-based MST technologies will be required.* See section IV for recommendations including companies that currently market such services.
6. **<u>Assess temporal variability more extensively</u>** if the multiple samples acquired and analyzed do not provide consistent impressions of source presence or concentration. *Flow and sampling equipment for automated assessment can be procured and used as per section VIII, or water resource professionals in the private sector can provide this service.*
7. **<u>Confirm evaluations based on acquired data</u>** including if, as above, acute sewage discharge into storm drains appears likely or if exfiltration may be indicated, as per low nutrient (and surfactant) concentrations but the repeated presence of DNA-based human waste markers. Specifically, perform the following physical investigations under the following conditions:
    a. if acute sewage contamination is implicated, use standard IDDE methods [10] such as smoke testing storm drains to determine if sewer laterals are illicitly connected

b. if chronic human contamination is suspected to be derived from leaking (exfiltrating) sanitary sewers into storm drains, plan a rhodamine dye tracer study (as per section IX) in a drain network vicinity using methods described in this research (again, section IX). *NOTE: as this approach is relatively inexpensive, i.e. compared to sampling in the field and analysis by DNA-based approaches in the laboratory, it may be desirable to use this approach before more expensive approaches.*

These measures will indicate if, and approximately where, sewage is entering storm drains. Actions by infrastructure managers can then be prioritized around correcting and reducing sewage contamination into storm drains that enters creeks and, eventually, coastal beaches.

11.4 References

1. Sercu, B., et al., *Storm drains are sources of human fecal pollution during dry weather in three urban southern California watersheds.* Environmental Science & Technology, 2009. **43**(2): p. 293-298.
2. Geosyntec Consultants, *Laguna Watershed Study and Water Quality Improvement Feasibility Analysis, Laguna Channel*, 2009, City of Santa Barbara, Creeks Division: Santa Barbara, California. p. 264.
3. Noble, R.T., et al., *Multitiered approach using quantitative PCR to track sources of fecal pollution affecting Santa Monica Bay, California.* Applied and Environmental Microbiology, 2006. **72**(2): p. 1604-1612.
4. Schriewer, A., et al., *Presence of Bacteroidales as a Predictor of Pathogens in Surface Waters of the Central California Coast.* Applied and Environmental Microbiology, 2010. **76**(17): p. 5802-5814.
5. Ahmed, W., et al., *Comparison of molecular markers to detect fresh sewage in environmental waters.* Water Research, 2009. **43**(19): p. 4908-4917.
6. Ahmed, W., et al., *Evaluation of Bacteroides markers for the detection of human faecal pollution.* Letters in Applied Microbiology, 2008. **46**(2): p. 237-242.
7. Noble, R.T., et al., *Comparison of beach bacterial water quality indicator measurement methods.* Environmental Monitoring and Assessment, 2003. **81**(1-3): p. 301-312.
8. Helsel, B., *Nondetects and Data Analysis: Statistics for censored environmental data*2004: Wiley.
9. Conover, W.J., *Practical Nonparametric Statistics*. 3rd ed1998, New Jersey: John Wiley and Sons.
10. Brown, E., D. Caraco, and R. Pitt, *Illicit Discharge Detection and Elimination: A Guidance Manual for Program Development and Technical Assessments*, 2004, Department of Civil and Environmental Engineering, University of Alabama, Tuscaloosa, AL. p. 378.

APPENDIX I: Standard Operating Procedures (SOPs)

STANDARD OPERATING PROCEDURE	SOP# H02
Holden Lab Donald Bren School of Environmental Science and Management University of California, Santa Barbara	
TITLE: **Microbiological Water Sampling**	
EFFECTIVE DATE: 01/10	REVISION: 2
AUTHORS: Bram Sercu & Laurie C. Van De Werfhorst	FINAL REVIEWER: Patricia Holden

1.0 Purpose and Scope

This Standard Operating Procedure (SOP) is for sampling of freshwater and ocean water for microbiological analysis. It also includes sample processing details for fecal indicator bacteria (FIB) via IDEXX, filtration and cell capture for DNA, and archiving for possible nutrients analysis. Sample capture and processing details for additional methods (e.g. caffeine and cotinine via ELISA, optical brighteners) will be covered in separate SOPs.

2.0 Applicability

This SOP should be followed for all water sampling activities performed by the Holden lab (UCSB), when sampling creeks, lagoons, ocean and storm drain water from banks, bridges, shoreline, etc. Samples are further processed for fecal indicator bacteria (FIB) concentrations and DNA-based analysis

3.0 Personnel Qualifications/Responsibilities

All field and laboratory staff must be familiar with this document. The Field Lead directing sample collection must be knowledgeable of all aspects of project's Quality Assurance Project Plan (QAPP) to ensure that credible and useable data are collected. All field staff should be briefed by the Field Lead or designate on the sampling goals and objectives prior to arriving to the site.

4.0 Health and Safety Warnings

Water samples are potentially contaminated with fecal waste, and can contain chemical and/or biological agents that can cause illness, even when they appear clean. Thus, the sampler should treat all water as though it is polluted. The sampler should avoid water contact, wear protective gloves, and the sample containers should be handled with care. The sampler should test surfaces for slipperiness and

pay particular attention to conditions when working on the creek banks. Also, the sampler should be aware of traffic when sampling in or near roads.

Storm drain (or other sampling) that occurs on a street surface needs to be coordinated with City staff so that traffic flow can be managed, and safety rules enforced. Field personnel need to stay within the demarked safety cone area and wear bright, reflective safety vests when sampling.

Safety glasses must be worn at all times by all personnel in the laboratory during vacuum filtration of samples.

5.0 Equipment, Reagents, and Supplies

5.1 Sampling Equipment and Supplies

- Nitrile or latex exam gloves
- Sample coolers with ice
- Sterile 2 or 4 L Nalgene bottles with labels (1 per site)
- Sterile 500 mL beakers
- Sterile Miracloth squares
- Extendable sampling pole
- Field log notebook and pens
- Digital camera
- Kimwipes
- Waterproof boots or chest waders
- Squirt bottle with DI water
- Hand-held GPS unit (GARMIN GPSMAP 76, with extra AA batteries)
- HACH HQ40d portable meter with Luminescent Dissolved Oxygen (LDO) and Conductivity probes
- ISCO 6712 portable sampler (optional)

5.2 Sample Processing Equipment and Supplies

- Nitrile or latex exam gloves
- Safety glasses
- Lab notebook, pen and Sharpie pen
- Sterile serological pipets & electronic pipet-aid
- Forceps
- Squirt bottle with 70% ethanol
- Sterile (autoclaved) Nanopure water
- 100 mL bottles (IDEXX Laboratories, cat# WV120-200)
- Quanti-Tray®/2000 trays (IDEXX Laboratories, cat# WQT-2K)
- Colilert® reagent snap packs (IDEXX Laboratories, cat# WP020 or WP200)

- Enterolert® reagent snap packs(IDEXX Laboratories, cat# WENT020 or WENT200)
- IDEXX Quanti-Tray® Sealer
- **Colilert Quanti-Tray®/2000 Comparator (IDEXX Laboratories, cat#** WQT2KC**)**
- UV light (6-watt, 365 nm in a dark enclosure)
- Incubators (35°C and 41°C)
- Digital thermometers with Max/Min memory
- 0.22 µm filters with housing (included in PowerWater® DNA Isolation Kit, MoBio Laboratories, cat# 14900-50-22 or 14900-100-22)
- 2 L vacuum filter flasks
- 5 mL sterile tubes (with beads, included in PowerWater® DNA Kit, MoBio Laboratories, cat# 14900-50-22 or 14900-100-22)
- 1 L graduated cylinder
- 60 mL HDPE wide-mouth square bottles (Naglene, cat# 2114-0002)

6.0 Summary of Sampling Procedure

6.1 Preparation

All sampling and sample processing equipment required to be sterile will be purchased sterile or autoclaved using aluminum foil, if necessary, and autoclave tape attached to ensure effectiveness of the procedure. Care will be taken that sterile equipment is not contaminated during storage, transportation and use in the field. Bottles will be labeled beforehand whenever possible, and will be attached so that labels and writing are waterproof and will not come off of the bottle. All sample bottles will be labeled on both the caps and the bottles themselves.

6.2 Sampling

Wearing Nitrile or latex gloves, a sterile beaker is dipped into the water, just under the water surface to collect approx. 500-600mL of water. The water is then poured through sterile Miracloth into a sterile 2 or 4 L Nalgene bottle. The Miracloth can either be hand held by field personnel or attached with a rubber band around the neck to hold it in place. When handling sterile Miracloth, gloves should be worn and only the edges should be touched. The Miracloth ensures removal of larger particles (>22-25 µm) from the water. Nalgene bottles are immediately placed in a cooler containing ice.

Note: If necessary, the sterile beaker can be attached to the sampling pole to collect the water sample.

Note: If the sample is not able to be safely reached using the sampling pole but is still within 21' of sampler access, an ISCO 6712 portable sampler may be used instead.

Prior to sample collection, the pump lines will be rinsed 3x with a volume of Nanopure water equal to the sample volume.

Note: If necessary, the water can be collected directly in Nalgene bottles, with or without using the sampling pole, and filtered through Miracloth back in the lab into a new sterile Nalgene bottle.

Note: Within 6 hours, samples should be transported back to the lab, completely processed for IDEXX based FIB measurements, and filtration initiated, to ensure representative sampling of the microbial community.

6.3 Sample measurements

Whenever possible, water samples will also have temperature, dissolved oxygen, and conductivity/salinity recorded. The HACH HQ40d portable meter with LDO and Conductivity probes will be used as specified in the instrument manuals. The probes can either be directly lowered into the sample in situ, or they may be placed in the sampling beaker to record the measurements. Both probes will be rinsed with DI water after reading each sample.

Note: In order to prevent contamination to other samples, the probes should not be used on raw sewage or septage samples.

Note: For best accuracy, probes will be gently stirred while taking sample measurements.

Note: Even though the HQ40d will electronically log the results, all probe measurements will also be recorded in a field notebook.

6.4 Sampling details

At each sampling location, GPS coordinates will be recorded (if not done previously), as well as any other pertinent information (flow, sample color or odor, weather, etc.). Whenever possible, a digital camera will be used to document each site sampled, at each sampling event.

7.0 Sample Processing

7.1 Preparation

If possible, label and add the desired amount of sterile Nanopure water to all of the 100 mL IDEXX bottles (2 per sample) beforehand, using sterile serological pipets and working inside a certified biological safety cabinet (biohood).

Note: Ocean samples must be diluted at least ten-fold for use with the Enterolert reagent. To simplify setup, all marine samples will also be diluted at least ten-fold for use with the Colilert assay. This corresponds to adding 10 mL of sample to 90 mL of sterile Nanopure water in the 100 mL IDEXX bottles. The appropriate dilution for

freshwater samples is based on the expected FIB concentrations of the water, and is usually 1:10 or 1:100.

Note: It is recommended to prepare the vacuum filtering setup before sampling. Label vacuum filtration flasks and connect to vacuum lines. Attach and label 0.22 µm filters to the filtration flasks and secure set-up with a ring stand.

7.2 Fecal Indicator Bacteria (FIB)

Invert the 2 or 4 L Nalgene sample bottle three times prior to removing an aliquot for FIB testing via the IDEXX Quanti-Tray®/2000 method. To prepare the Colilert® assay, remove the appropriate water volume from the sample bottle using a sterile serological pipet and dilute it in the prepared 100 mL IDEXX bottle, containing the desired amount of sterile Nanopure water. Repeat this step with the desired dilution for the Enterolert® assay. Add the appropriate reagent packs (Colilert® and Enterolert®) to the bottles and shake to mix.

After all the reagents have dissolved, the IDEXX sample bottles are poured into IDEXX Quanti-Tray®/2000 trays and sealed with the Quanti-Tray® Sealer. Enterolert® trays are then incubated for 24 hours at 41°C, and Colilert® trays for 24 hours at 35°C.

After the 24 hour incubation, the trays are read. Colilert sample trays are compared to the **Colilert® Quanti-Tray®/2000 Comparator**, and any wells with yellow color intensity equal or greater to the Comparator are counted positive for total coliforms and marked with a Sharpie pen. The Colilert® trays are then examined in the UV light enclosure (trays 5" or less from the light source), and any positive total coliform wells with fluorescence intensity equal or greater to the Comparator are counted positive for *E. coli*. Enterolert® trays are also examined in the UV light enclosure, and any wells with blue fluorescence are counted positive for enterococci.

The number of large and small wells counted for each tray and assay are then entered into the IDEXX MPN Generator software (IDEXX Laboratories), along with the undiluted sample volume (e.g. 1:10 dilution = 10 mL of undiluted sample), and the MPN (Most Probable Number) and 95% Confidence Limits are generated and recorded into a Microsoft Excel spreadsheet.

7.3 DNA

Note: Safety glasses must be worn at all times by all personnel in the laboratory during vacuum filtration of samples.

Invert the 2 or 4 L Nalgene sample bottle three times prior to pouring through the 0.22 µm filter. Vacuum filtration is carried out until the entire sample has filtered or

the filter reaches the point of refusal, not to exceed 4 hours. Keep the sample bottles in the cooler during filtration. Record actual volume filtered.

Archive a portion of the filtrate for possible nutrients analysis. Rinse a new 60 mL Naglene bottle once with sample, then fill approximately halfway and cap. Store the bottles at -20°C until analysis.

Working inside a certified biological safety cabinet (biohood), filters are then removed from the housings using sterile forceps. Forceps are sterilized by immersing the tips in 70% ethanol and shaking off the excess drops. Filters are transferred to sterile 5 mL tubes (containing beads, included in PowerWater® **kit**) and stored at -20°C until extraction.

Note: Sterile 15mL tubes may be substituted if filters are being archived, or if another DNA extraction kit/method will be used.

8.0 Records Management

All field and sampling notes will be recorded in a field notebook (site#, sample IDs, probe sample measurements, sample notes, GPS coordinates). All sample processing details (sample IDs, volume filtered, IDEXX incubation times, IDEXX incubation results, any problems or deviations from protocols) will be recorded in a laboratory notebook.

Summary tables of field and sampling notes and results will be prepared in Microsoft Excel.

Electronic data files and digital photographs will be stored on a computer connected to the Bren/ICESS network that is backed up daily.

9.0 Quality Control and Quality Assurance Section

9.1 At least one lab duplicate for FIB measurements via IDEXX will be run for each sampling date, and another duplicate added for every 10 samples. Duplicate testing involves performing two separate analyses of a particular parameter on the same sample. The sample will be inverted three times prior to dividing into duplicates. The precision of the duplicates will be evaluated according to section 9020B of Standard Methods for the Examination of Water and Wastewater (Clesceri et al., 1998).

9.2 Each incubator used for IDEXX assays will be set up with a digital thermometer with Max/Min memory capability. The memory will be reset the morning of each sampling event. The incubator temperature will be checked prior to starting IDEXX incubation, and again at the completion of incubation the following day. The maximum and minimum temperatures for each incubation will be recorded in a log book. If the temperature varies by more than 2°C from its setpoint during an

incubation, notations will be made for the affected samples, maintenance/repairs will be initiated, and that incubator will not be used for IDEXX assays again until fixed.

9.3 All reagents will be used prior to expiration dates.

9.4 The HACH LDO and Conductivity probes were calibrated at the factory. As suggested by the manufacturer, regular calibration will also be performed, according to the protocols as listed in each probe's manual. During active sampling season, each probe will calibrated 1x per week.

9.5 Biological safety cabinets (biohoods) are inspected and certified annually.

10.0 References

Clesceri LS, Greenberg AE, Eaton AD (1998). Standard Methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, D.C.

STANDARD OPERATING PROCEDURE	SOP# H03
Holden Lab Donald Bren School of Environmental Science and Management University of California, Santa Barbara	
TITLE: **DNA extraction from water samples**	
EFFECTIVE DATE: 01/10	REVISION: 2
AUTHORS: Bram Sercu & Laurie C. Van De Werfhorst	FINAL REVIEWER: Patricia Holden

1.0 Purpose and Scope

This Standard Operating Procedure (SOP) describes how to perform DNA extraction to extract total DNA from bacteria collected from water samples.

2.0 Applicability

This SOP should be followed for DNA extractions from water samples, in all water quality research projects under the supervision of Dr. Patricia Holden.

3.0 Personnel Qualifications/Responsibilities

All laboratory staff performing the analysis must be familiar with this document, and have completed the UCSB EH&S Lab Safety Class.

4.0 Health and Safety Warnings

The person performing the analysis should always wear safety gloves and lab coat. No especially toxic or hazardous materials are used in the described protocols, but general lab safety rules should be followed.

5.0 Equipment, Reagents, and Supplies

5.1 Equipment

- Vortex
- Vortex adapter (MoBio Laboratories; cat# 13000-V1-15)
- Sorvall RC5B Plus Centrifuge, with applicable rotors
- Benchtop microcentrifuge
- 1000, 200 & 10 µL pipettes and tips
- Water bath set at 55°C
- -80°C freezer

5.2 Reagents and Supplies

- PowerWater® DNA Isolation Kit (MoBio Laboratories; cat# 14900-50-22 or 14900-100-22)
- 100% ethanol (ice cold)
- Sterile 5M NaCl
- Polyacryl carrier (Molecular Research Center; Inc. cat# PC152)
- DNA grade water (e.g. Fisher; cat# BP2470-1)
- Nitrile or latex exam gloves

6.0 Procedure

6.1 Samples are collected according to SOP# H02.

6.2 DNA is extracted from the filters according to the procedure recommended by the manufacturer of the PowerWater® DNA Isolation Kit. A protocol is provided with each kit and utilized with no modifications.

Note: The required pre-warming step for solution PW1 & recommended pre-warming for solution PW3 will be performed with every batch of samples extracted.

Note: The recommended centrifuge step after bead-beating will be performed with every batch of samples extracted.

6.3 No further concentration is needed for sewage or septage samples.

6.4 For environmental samples, an ethanol precipitation will be performed in order to concentrate the DNA and ensure enough for DNA-based analyses:

To each sample tube, 1 µL of polyacryl carrier is added to facilitate pellet visualization. Next, 5 µL of 5M NaCl and 200 µl of cold ethanol are added to each tube, and the samples are vortexed briefly on low setting. Samples are incubated for 20 minutes at -80°C, and then centrifuged at 10,000 x g for 20 minutes at 4°C. Tubes are then allowed to air dry for 1-2 hours. Pellets are re-suspended in 50 µL of diluted PW6 solution (0.1x dilution of PW6 provided in PowerWater® kit with DNA grade water).

6.5 All DNA extracts will be stored at -20°C until further processing. SOP# H04 contains the specifications for DNA quantification.

7.0 Records Management

DNA extraction details (sample IDs, personnel performing extraction, any problems or deviations from protocol) will be recorded in a laboratory notebook.

8.0 Quality Control and Quality Assurance Section

No specific QA/QC measures are needed as long as protocol is followed completely. Any problems that arise with sample recovery/yield will be promptly investigated.

Pipettes and biological safety cabinets (biohoods) are inspected and calibrated/certified annually.

STANDARD OPERATING PROCEDURE	SOP# H04
Holden Lab Donald Bren School of Environmental Science and Management University of California, Santa Barbara	
TITLE: **DNA quantification**	
EFFECTIVE DATE: 01/10	REVISION: 2
AUTHORS: Bram Sercu & Laurie C. Van De Werfhorst	FINAL REVIEWER: Patricia Holden

1.0 Purpose and Scope

This Standard Operating Procedure (SOP) describes how to quantify DNA from DNA extracts and purified PCR products.

2.0 Applicability

This SOP should be followed for quantification of DNA from DNA extracts and purified PCR products in all water quality research projects under the supervision of Dr. Patricia Holden.

3.0 Personnel Qualifications/Responsibilities

All laboratory staff performing the analysis must be familiar with this document, and have completed the UCSB EH&S Lab Safety Class.

4.0 Health and Safety Warnings

The person performing the analysis should always wear safety glasses, nitrile gloves and lab coat. The DNA quantification kit may contain toxic compounds. Plates with reagents and spent reagents should be treated as hazardous waste and disposed according to UCSB regulations.

5.0 Equipment, Reagents, and Supplies

5.1 Equipment

- Synergy 2 microplate reader (BioTek Instruments)
- 1000, 200 & 10 µL pipettes and tips

5.2 Reagents and Supplies

- 96-well flat bottom black polystyrene microplates (Corning; Fisher cat# 07-200-590)
- Quant-iT™ dsDNA Assay Kit, Broad Range (Molecular Probes; cat# Q33130)

- Nitrile exam gloves
- Safety glasses

6.0 Procedure

DNA samples are quantified according to the procedure described by the kit manufacturer. A protocol is included in every kit and utilized with no modifications. Standards will be analyzed in duplicate. Samples will be analyzed with no replications. Volume of standards used is 10 uL per well. Volume of samples will start at 4 uL for DNA extracts and 2 uL for purified PCR products.

Blank (zero standard) fluorescence will subtracted from each sample and standard. Sample concentration will be extrapolated from the standard curve fluorescence either automatically using protocols developed with the BioTek Gen5 software (DNAQuant-iT(top-50)_DNAsamples.prt or DNAQuant-iT(top-50)_PCRsamples.prt), or manually in Microsoft Excel.

Note: Sample volumes might need to be adjusted in order for sample fluorescence to fall within range of standards.

Note: All kit reagents will be allowed to equilibrate to room temperature before use.

Note: The Quant-iT™ dsDNA Assay Kit, High Sensitivity (Molecular Probes; cat# Q33120) may be used instead of the specified Broad Range kit. However, volume of samples will need to be adjusted to ensure their fluorescence falls within the range of the standards. Using the High Sensitivity kit, an aliquot of purified PCR products will need to be diluted with DNA grade water in order to fall within standard range.

7.0 Records Management

The DNA quantification details (sample IDs, personnel performing quantification, any problems or deviations from protocol) will be recorded in a laboratory notebook, along with the standard curve R^2 value.

Data will not be stored on the computer connected to the plate reader. All data files will be transferred and stored on a computer connected to the Bren/ICESS network that is backed up daily.

8.0 Quality Control and Quality Assurance Section

Standard curves will be run with every quantification run, and will be run in duplicate (at minimum). If the standard curve R^2 is < 0.99, the run will be aborted and a new plate with new standards will be run. Similarly, if any of the standard duplicates appear out of range, or if samples are outside of the range of standards, a new set of standard will be run.

All kit reagents will be used until the expiration date or standard curve performance becomes unacceptable.

Pipettes are inspected and calibrated/certified annually.

STANDARD OPERATING PROCEDURE	SOP# H05
Holden Lab Donald Bren School of Environmental Science and Management University of California, Santa Barbara	
TITLE: **quantitative PCR analysis of human-specific *Bacteroidales* via SYBR® Green I**	
EFFECTIVE DATE: 01/10	REVISION: 3
AUTHORS: Bram Sercu & Laurie C. Van De Werfhorst	FINAL REVIEWER: Patricia Holden

1.0 Purpose and Scope

This Standard Operating Procedure (SOP) describes how to perform quantitative PCR using SYBR® Green I as the detected fluorophore and the human-specific *Bacteroidales* marker (HBM) as target, on previously extracted DNA. The ultimate purpose of the method is to quantify the extent of human fecal pollution in water or other environmental samples. Sample DNA template dilution should be determined via SOP# H08 (Salmon testes DNA quantitative PCR).

2.0 Applicability

This SOP should be followed for quantifying HBM in DNA extracts from water or other samples, in all water quality research projects under the supervision of Dr. Patricia Holden, unless utilizing SOP# H09 (quantitative PCR analysis of human-specific *Bacteroidales* via TaqMan).

3.0 Personnel Qualifications/Responsibilities

All laboratory staff performing the analysis must be familiar with this document, and have completed the UCSB EH&S Lab Safety Class.

4.0 Health and Safety Warnings

The person performing the analysis should always wear safety glasses, nitrile gloves and lab coat when handling any solution containing SYBR® Green I. Reagents used in the assays may be toxic. Plates with reagents and spent reagents should be treated as hazardous waste and disposed according to UCSB regulations.

5.0 Equipment, Reagents, and Supplies

5.1 Equipment

- Bio-Rad iCycler iQ5 thermocycler
- Plate centrifuge (e.g. “salad spinner” with 96 well plate attachments)

- USB flash drive
- 1000, 200, 20 & 10 µL filter tips and pipettes
- Vortexer
- Biological safety cabinet (biohood)

5.2 Reagents and Supplies

- qPCR core kit for SYBR Green I (Eurogentec; cat# RT-SN10-05 or RT-SN10-05NR)
- Fluorescein (Eurogentec; cat# RT-FLUO-ADD)
- iQ 96-well real-time PCR plates (Bio-Rad; cat# 223-9441)
- Optical-quality sealing tape (Bio-Rad; cat# 223-9444)
- Plate sealer
- Primers HF183 and qBacR (Seurinck et al., 2005) (e.g. Operon Biotechnologies, Alameda, CA)
- Sterile TE buffer pH 7 or 7.4
- 500 µl microcentrifuge tubes
- Molecular biology grade water (e.g. Fisher; cat# BP2470-1)
- HBM standard (2 ng/µL, prepared by Laurie C. Van De Werfhorst)
- Nitrile gloves
- Safety glasses

6.0 Procedure

6.1 Thaw all reagents and DNA samples and store on ice, except for the SYBR Green/DMSO vial which should be kept at room temperature after thawing as it will re-freeze on ice.

Note: Keep the DNA polymerase in the freezer until just before use and immediately return to freezer after use. The enzyme will degrade gradually when stored on ice.

Note: New primers should be dissolved in sterile TE buffer pH 7 or 7.4. After all material has been re-suspended and mixed well, multiple aliquots should be created for each primer to minimize number of freeze-thaw cycles to no more than 3x per tube.

6.2 Label microcentrifuge tubes for samples and standards (1 each).

6.3 Prepare sample dilutions using DNA grade water. Sample DNA template dilution should be determined via SOP# H08.

6.4 Make sure to vortex all samples before pipetting. Kit reagents should not be vortexed, but instead should be inverted 3-5 times to mix before pipetting.

6.5 Prepare tenfold dilutions of HBM standards from $2x10^{-3}$ to $2x10^{-9}$ ng/µlL

6.6 Prepare Master Mix, at 82 µL per sample. This is sufficient for 1 sample in triplicate (3 x 25 µL), with extra volume to account for pipetting errors.

	Volume added (µL)	Final concentration
Water	54.9	-
HF183 (F primer)	0.205	0.25 µM
qBacR (R primer)	0.205	0.25 µM
dNTP	3.28	200 µM each dNTP
$MgCl_2$	3.28	2 mM
PCR 10X buffer	8.2	1X
Hot GoldStar DNA polymerase	0.41	2.5 U/100 µL
SybrGreen I (1:10)	2.46	1:333
Fluorescein (1:1000)	0.82	1:100000

6.7 Add 75 µL of Master Mix to all sample tubes.

6.8 Add 8.2 µL of DNA template (diluted to 4ng/µl) or standard to the sample tubes and vortex briefly. DNA template should be quantified according to SOP #H04.

6.9 Aliquot 3 x 25 µL of each sample or standard in the 96-well PCR plates.

6.10 Seal the PCR plates using the sealing tape and plate sealer, and spin briefly using a plate centrifuge or modified salad spinner to remove air bubbles.

Note: Make sure the plate is sealed well, as a weak seal could lead to evaporation during the reaction.

6.11 Shield PCR plate from light (e.g. wrap in foil, place in opaque box) and transport to the iQ5 thermocycler.

6.12 Start thermocycler and computer and wait until lamp is warmed up (~10 minutes).

6.13 Input plate lay-out and standard concentrations in the software.

6.14 Use the method saved in C:\Program Files\Bio-Rad\iQ5\User1\Laurie\HF183Bacteroides.tmo. The cycling parameters should be the following:

	50°C	2min
Polymerase activation		95°C 10min
40 cycles of:		
Denaturation	95°C	30sec
Annealing	53°C	60sec
Extension	60°C	60sec

6.15 Collect well factors on experimental plate, and analyze melting curve.

7.0 Records Management

The run details (sample IDs, personnel performing qPCR, any problems or deviations from protocol) will be recorded in a laboratory notebook, along with the plate layout.

Data will not be stored on the computer connected to the thermocycler. All data files will be transferred and stored on a computer connected to the Bren/ICESS network that is backed up daily.

8.0 Quality Control and Quality Assurance Section

The qPCR detector will be calibrated for use with SybrGreen fluorescent dyes at the time the dyes are first used. Negative controls and positive controls are always included on every plate, and all samples including controls are run in triplicate. If target concentrations in the negative control exceed those of the lowest standard, the run will be repeated. Internal well factors will be recorded every run, to standardize the fluorescence signal in each well and correct for pipetting errors.

Run efficiency shall be between 90-110%, or else the run will be repeated. Standard and sample melt curves will be compared and verified for each sample to ensure the specificity of this assay. The baseline for each run will be adjusted so that the coefficient of variation for the standard curve Ct values are 3% or less between all plates.

Pipettes and biological safety cabinets (biohoods) are inspected and calibrated/certified annually.

9.0 References

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

STANDARD OPERATING PROCEDURE	SOP# H07
Holden Lab Donald Bren School of Environmental Science and Management University of California, Santa Barbara	
TITLE: **DNA extraction from fecal, soil and sediment samples**	
EFFECTIVE DATE: 01/10	REVISION: 1
AUTHORS: Bram Sercu & Laurie C. Van De Werfhorst	FINAL REVIEWER: Patricia Holden

1.0 Purpose and Scope

This Standard Operating Procedure (SOP) describes how to perform DNA extraction to extract total DNA from bacteria from fecal, soil and sediment samples.

2.0 Applicability

This SOP should be followed for DNA extractions from fecal, soil and sediment samples, in all water quality research projects under the supervision of Dr. Patricia Holden.

3.0 Personnel Qualifications/Responsibilities

All laboratory staff performing the analysis must be familiar with this document, and have completed the UCSB EH&S Lab Safety Class.

4.0 Health and Safety Warnings

Soil, sediment and fecal samples may contain chemical and/or biological agents that can cause illness. Thus, the sampler should treat all samples carefully. The sampler should avoid contact, wear protective gloves, and the sample containers should be handled with care to minimize exposure.

The person performing the analysis should always wear safety gloves and lab coat. No especially toxic or hazardous materials are used in the described protocols, but general lab safety rules should be followed.

Solution C5 contains ethanol and therefore is flammable and should be treated with caution in use and storage.

5.0 Equipment, Reagents, and Supplies

5.1 Equipment

- Balance

- Reference weights (Denver Instrument Company cat# 854254.4)
- Bead beater
- Vortex
- Benchtop microcentrifuge
- Sorvall RC5B Plus Centrifuge, with applicable rotors (optional)
- 1000 & 200 µL pipettes and tips

5.2 Reagents and Supplies

- PowerSoil® DNA Isolation Kit (MoBio Laboratories; cat# 12888-50 or 12888-100)
- 100% ethanol (ice cold) (optional)
- Sterile 5M NaCl (optional)
- Polyacryl carrier (Molecular Research Center, Inc. cat# PC152) (optional)
- DNA grade water (e.g. Fisher cat# BP2470-1) (optional)
- Nitrile or latex exam gloves

6.0 Procedure

6.1 Weigh ~ 0.25 g (wet) of sample in to the bead beating tubes provided in the PowerSoil® DNA Isolation Kit. Record actual weight added.

6.2 DNA is extracted from the tubes according to the procedure recommended by the manufacturer of the PowerSoil® DNA Isolation Kit. A protocol is provided with each kit and followed with a few modifications:

DNA is extracted using a bead beater instead of the vortex adapter. Bead beater setting is marked on the bead beater controller, and duration shall be 60 seconds.

Final elution will be in 50 uL instead of 100 uL. After the addition of the final solution (C6) to the spin columns, the solution will be allowed to soak into the columns for 60 seconds before centrifugation.

Note: It is best to use the specified 0.25 g of sample per tube. If concerned about DNA yield, multiple tubes may be used (see note below).

6.3 All DNA extracts will be stored at -20°C until further processing. SOP# H04 contains the specifications for DNA quantification.

Note: If extracted DNA concentration is too low for further analyses (as quantified in SOP# H04), multiple tubes may be extracted for each sample. The tubes are then combined and concentrated via an ethanol precipitation:

For each 100 µL of combined sample:
1 µL of polyacryl carrier is added to facilitate pellet visualization, followed by

5 µL of 5M NaCl & 200 µL of cold ethanol, and the samples are vortexed briefly on low setting.

Samples are incubated for 20 minutes at -80°C, and then centrifuged at 10,000 x g for 20 minutes at 4°C. Tubes are then allowed to air dry for 1-2 hours. Pellets are re-suspended in 50 µL of diluted C6 solution (0.1x dilution of C6 provided in PowerSoil® kit in DNA grade water) and then quantified according to SOP# H04.

7.0 Records Management

The DNA extractions details (sample IDs, personnel performing extraction, any problems or deviations from protocol) will be recorded in a laboratory notebook.

8.0 Quality Control and Quality Assurance Section

No specific QA/QC measures are needed as long as protocol is followed completely. Any problems that arise with sample recovery/yield will be promptly investigated.

Balance(s) used for weighing samples shall be checked for accuracy prior to weighing out samples or 1x per month as needed. Balance accuracy will be checked by weighing the reference balance weights (100 g through 1 mg) and recording the weights in a log book.

Pipettes are inspected and certified/calibrated annually.

STANDARD OPERATING PROCEDURE	SOP# H08
Holden Lab Donald Bren School of Environmental Science and Management University of California, Santa Barbara	
TITLE: **Template dilution determination via quantitative PCR analysis of Salmon testes DNA using TaqMan®**	
EFFECTIVE DATE: 01/10	REVISION: 2
AUTHORS: Bram Sercu & Laurie C. Van De Werfhorst	FINAL REVIEWER: Patricia Holden

1.0 Purpose and Scope

This Standard Operating Procedure (SOP) describes how to perform quantitative PCR of salmon testes DNA via TaqMan® to determine the how much sample template to use in the other TaqMan® quantitative PCR assays in our lab (SOP# H09 human-specific *Bacteroidales*, and H10 *Enterococcus* spp.). The ultimate purpose of the method is to determine the lowest sample template dilution without inhibition.

2.0 Applicability

This SOP should be followed for determining the lowest sample dilution without inhibition to use in other TaqMan® quantitative PCR assays in all water quality research projects under the supervision of Dr. Patricia Holden.

3.0 Personnel Qualifications/Responsibilities

All laboratory staff performing the analysis must be familiar with this document, and have completed the UCSB EH&S Lab Safety Class.

4.0 Health and Safety Warnings

The person performing the analysis should always wear safety glasses, nitrile gloves and lab coat. Reagents used in this assay may be toxic. Plates with reagents and spent reagents should be treated as hazardous waste and disposed according to UCSB regulations

5.0 Equipment, Reagents, and Supplies

5.1 Equipment

- Bio-Rad CFX96 thermocycler
- Plate centrifuge (e.g. “salad spinner” with 96 well plate attachments)
- USB flash drive
- 1000, 200, 20 & 10 µL filter tips and pipettes

- Vortexer
- Biological safety cabinet (biohood)

5.3 Reagents and Supplies

- qPCR MasterMix Plus No Rox kit (Eurogentec; cat# RT-QP2X-03NR)
- Hard-shell thin-wall 96-well PCR plates (Bio-Rad; cat# HSP-9601)
- Optical-quality sealing tape (Bio-Rad; cat# MSB1001)
- Plate sealer
- Primers (SketaF2 and SketaR3, Haugland et al, 2005) (Operon Biotechnologies, Alameda, CA)
- Probe (SketaP2, Haugland et al, 2005) (Operon Biotechnologies, Alameda, CA)
- TE buffer pH 7 or 7.4 and pH 8
- Salmon testes DNA standard (1E+03 ng/µL , prepared by Bram Sercu)
- 500 µL microcentrifuge tubes
- DNA grade water (e.g. Fisher; cat# BP2470-1)

6.0 Procedure

6.1 Thaw all reagents and DNA samples and store on ice.

Note: Keep the MasterMix buffer in the freezer until a few minutes before use and return to freezer after use. The enzyme will degrade gradually when stored on ice.

Note: New primers should be dissolved in sterile TE buffer pH 7 or 7.4. After all material has been re-suspended and mixed well, multiple aliquots should be created for each primer to minimize number of freeze-thaw cycles to no more than 3x per tube.

Note: Probes are ordered in single-use aliquots. Dissolve in sterile TE buffer pH 8 and mix well. Keep protected from light until added to master mix.

6.2 Label microcentrifuge tubes for samples and standards (1 each).

6.3 Make sure to vortex all samples before pipetting. Kit reagents should not be vortexed, but instead should be inverted 3-5 times to mix before pipetting.

6.4 Prepare Salmon testes DNA dilutions:

	ng/uL DNA	**ng/well**	**std/water uL**
T1	1.00E+03	stock	

T2	1.00E+02		3/27
T3	1.00E+01	2.53E+01	3/27
T4	9.09E-01	2.52E+00	3/30.3

6.5 Prepare sample dilutions using DNA grade water. Start with 1:5 dilution for samples from the PowerWater® kit.

6.6 Prepare Reagent Mix, at 55 µL per sample/standard. This is sufficient for 1 sample in duplicate (2 x 25 µL) (and ST4 4 x 25 µL).

	Volume added (µl)
Water	1063.9
SketaF2	8.6
SketaR3	8.6
MasterMix buffer	1430.0
BSA	57.2
SketaP2	2.9
T3	2.9

6.7 Add 49.5 µL of Master Mix to all sample/standard tubes (99 µL for ST4).

6.8 Add 5.5 µL of DNA template to the sample tubes and vortex briefly. DNA template should be quantified according to SOP #H04.

6.9 Use table below to add the appropriate volume of salmon testes DNA to the standards tubes:

std	**µL**
ST2	5.5 of T3
ST3	5.5 of T4
ST4	11 of water

6.10 Aliquot 2 x 25 µL of each sample or standard in the 96-well PCR plates (4 x 25 µL for ST4).

6.11 Seal the PCR plates using the sealing tape and plate sealer, and spin briefly using a plate centrifuge or modified salad spinner to remove air bubbles.

Note: Make sure the plate is sealed well, as a weak seal could lead to evaporation during the reaction.

6.12 Turn on thermocycler and computer.

6.13 Input plate lay-out and standard concentrations in the software.

6.14 Start run.
The cycling parameters should be the following:

50°C 2min
95°C 10min

45 cycles of:
95°C 15sec
60°C 1min
Plate Read

7.0 Records Management

The run details (sample IDs, personnel performing qPCR, any problems or deviations from protocol) will be recorded in a laboratory notebook, along with the plate layout.

Data will not be stored on the computer connected to the CFX 96. All data files will be transferred and stored on a computer connected to the Bren/ICESS network that is backed up daily.

8.0 Quality Control and Quality Assurance Section

The qPCR detector was calibrated for use with all fluorescent dyes by the manufacturer. Negative controls and positive controls are always included on every plate, and all samples including controls are run in duplicate. If target concentrations in the negative control exceed those of the lowest standard, the run will be repeated.

Run efficiency shall be between 90-120%, or else the run will be repeated.
The baseline for each run will be adjusted to 200 RFU for data analysis.

Biological safety cabinets (biohoods) and pipettes are inspected and certified/calibrated annually.

9.0 References

Haugland, R.A., Siefring, S.C., Wymer, L.J., Brenner, K.P., Dufour, A.P. (2005). Comparison of Enterococcus measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Research 39(4): 559-568.

STANDARD OPERATING PROCEDURE	SOP# H09
Holden Lab Donald Bren School of Environmental Science and Management University of California, Santa Barbara	
TITLE: **quantitative PCR analysis of human-specific *Bacteroidales* via TaqMan®**	
EFFECTIVE DATE: 01/10	REVISION: 1
AUTHORS: Bram Sercu & Laurie C. Van De Werfhorst	FINAL REVIEWER: Patricia Holden

1.0 Purpose and Scope

This Standard Operating Procedure (SOP) describes how to perform quantitative PCR using TaqMan® and the human-specific *Bacteroidales* marker (HBM) as target, on previously extracted DNA. The ultimate purpose of the method is to quantify the extent of human fecal pollution in water or other environmental samples. Sample DNA template dilution should be determined via SOP# H08 (Salmon testes DNA quantitative PCR).

2.0 Applicability

This SOP should be followed for quantifying HBM in DNA extracts from water or other samples, in all water quality research projects under the supervision of Dr. Patricia Holden, unless utilizing SOP# H05 (quantitative PCR analysis of human-specific *Bacteroides* via SYBR® Green I).

3.0 Personnel Qualifications/Responsibilities

All laboratory staff performing the analysis must be familiar with this document, and have completed the UCSB EH&S Lab Safety Class.

4.0 Health and Safety Warnings

The person performing the analysis should always wear safety glasses, nitrile gloves and lab coat. Reagents used in this assay may be toxic. Plates with reagents and spent reagents should be treated as hazardous waste and disposed according to UCSB regulations

5.0 Equipment, Reagents, and Supplies

5.1 Equipment

- Bio-Rad CFX96 thermocycler
- Plate centrifuge (e.g. “salad spinner” with 96 well plate attachments)

- USB flash drive
- 1000, 200, 20 & 10 µL filter tips and pipettes
- Vortexer
- Biological safety cabinet (biohood)

5.2 Reagents and Supplies

- qPCR MasterMix Plus No Rox kit (Eurogentec; cat# RT-QP2X-03NR)
- Hard-shell thin-wall 96-well PCR plates (Bio-Rad; cat# HSP-9601)
- Optical-quality sealing tape (Bio-Rad; cat# MSB1001)
- Plate sealer
- Primers (BacHum-160f and BacHum-241r, Kildare et al, 2007) (Operon Biotechnologies, Alameda, CA)
- Probe (BacHum-193p, Kildare et al, 2007) (Operon Biotechnologies, Alameda, CA)
- TE buffer pH 7 or 7.4 and pH 8
- HBM standard (4E+10 copies/µL, prepared by Bram Sercu)
- 500 µL microcentrifuge tubes
- Molecular biology grade water (e.g. Fisher; cat# BP2470-1)

6.0 Procedure

6.1 Thaw all reagents and DNA samples and store on ice.

Note: Keep the MasterMix buffer in the freezer until a few minutes before use and return to freezer after use. The enzyme will degrade gradually when stored on ice.

Note: New primers should be dissolved in sterile TE buffer pH 7 or 7.4. After all material has been re-suspended and mixed well, multiple aliquots should be created for each primer to minimize number of freeze-thaw cycles to no more than 3x per tube.

Note: Probes are ordered in single-use aliquots. Dissolve in sterile TE buffer pH 8 and mix well. Keep protected from light until added to master mix.

6.2 Label microcentrifuge tubes for samples and standards (1 each).

6.3 Make sure to vortex all samples before pipetting. Kit reagents should not be vortexed, but instead should be inverted 3-5 times to mix before pipetting.

6.4 Prepare HBM standard dilutions:

std	copies/uL	copy/well	std/water

			uL
x1	4.00E+10	stock	
x2	4.00E+09		3/27
x3	4.00E+08		3/27
x4	4.00E+07		3/27
x5	4.00E+06		3/27
x6	4.00E+05	1.00E+06	3/27
x7	4.00E+04	1.00E+05	3/27
x8	4.00E+03	1.00E+04	3/27
x9	4.00E+02	1.00E+03	3/27
x10	4.00E+01	1.00E+02	3/27
x11	1.00E+01	2.50E+01	5/15
x12	4.00E+00	1.00E+01	4/6

Note: Only standard dilutions **x6** through **x12** will be run on the plate.

6.5 Prepare Reagent Mix, at 80 µL per sample/standard. This is sufficient for 1 sample in triplicate (3 x 25 µL).

	Volume added (µL)
Water	1009.7
BacHum-160f	10.9
BacHum-241r	10.9
MasterMix buffer	1360.0
BSA	54.4
BacHum-193p	2.2

6.6 Add 72 µL of Master Mix to all sample/standard tubes.

6.7 Add 8 µL of DNA template to the sample tubes and vortex briefly. DNA template should be quantified according to SOP #H04.

6.8 Aliquot 3 x 25 µL of each sample or standard in the 96-well PCR plates.

6.9 Seal the PCR plates using the sealing tape and plate sealer, and spin briefly using a plate centrifuge or modified salad spinner to remove air bubbles.

Note: Make sure the plate is sealed well, as a weak seal could lead to evaporation during the reaction.

6.10 Turn on thermocycler and computer.

6.11 Input plate lay-out and standard concentrations in the software.

6.12 Start run.
The cycling parameters should be the following:

50°C 2min
95°C 10min

45 cycles of:
95°C 15sec
60°C 1min
Plate Read

7.0 Records Management

The run details (sample IDs, personnel performing qPCR, any problems or deviations from protocol) will be recorded in a laboratory notebook, along with the plate layout.

Data will not be stored on the computer connected to the CFX 96. All data files will be transferred and stored on a computer connected to the Bren/ICESS network that is backed up daily.

8.0 Quality Control and Quality Assurance Section

The qPCR detector was calibrated for use with all fluorescent dyes by the manufacturer. Negative controls and positive controls are always included on every plate, and all samples including controls are run in triplicate. If target concentrations in the negative control exceed those of the lowest standard, the run will be repeated.

Run efficiency shall be between 90-110%, or else the run will be repeated. The baseline for each run will be adjusted so that the coefficient of variation for the standard curve Ct values are 3% or less between all plates.

Biological safety cabinets (biohoods) and pipettes are inspected and certified/calibrated annually.

9.0 References

Kildare, B. J., C. M. Leutenegger, et al. (2007). 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: A Bayesian approach. Water Research 41(16): 3701-3715.

STANDARD OPERATING PROCEDURE	SOP# H10
Holden Lab Donald Bren School of Environmental Science and Management University of California, Santa Barbara	
TITLE: **quantitative PCR analysis of *Enterococcus* spp. via TaqMan®**	
EFFECTIVE DATE: 01/10	REVISION: 2
AUTHORS: Bram Sercu & Laurie C. Van De Werfhorst	FINAL REVIEWER: Patricia Holden

1.0 Purpose and Scope

This Standard Operating Procedure (SOP) describes how to perform quantitative PCR using TaqMan® for *Enterococcus* spp. markers (ENT), on previously extracted DNA. The ultimate purpose of the method is to quantify the extent of fecal pollution in water or other environmental samples. Sample DNA template dilution should be determined via SOP# H08 (Salmon testes DNA quantitative PCR).

2.0 Applicability

This SOP should be followed for quantifying ENT in DNA extracts from water or other samples, in all water quality research projects under the supervision of Dr. Patricia Holden.

3.0 Personnel Qualifications/Responsibilities

All laboratory staff performing the analysis must be familiar with this document, and have completed the UCSB EH&S Lab Safety Class.

4.0 Health and Safety Warnings

The person performing the analysis should always wear safety glasses, nitrile gloves and lab coat. Reagents used in this assay may be toxic. Plates with reagents and spent reagents should be treated as hazardous waste and disposed according to UCSB regulations

5.0 Equipment, Reagents, and Supplies

5.1 Equipment

- Bio-Rad CFX96 thermocycler
- Plate centrifuge (e.g. “salad spinner” with 96 well plate attachments)
- USB flash drive
- 1000, 200, 20 & 10 µL filter tips and pipettes
- Vortexer

- Biological safety cabinet (biohood)

5.2 Reagents and Supplies

- qPCR MasterMix Plus No Rox kit (Eurogentec; cat# RT-QP2X-03NR)
- Hard-shell thin-wall 96-well PCR plates (Bio-Rad; cat# HSP-9601)
- Optical-quality sealing tape (Bio-Rad; cat# MSB1001)
- Plate sealer
- Primers (ECST748F and ENC854R, Haugland et al, 2005) (Operon Biotechnologies, Alameda, CA)
- Probe (GPL813TQ, Haugland et al, 2005) (Operon Biotechnologies, Alameda, CA)
- TE buffer pH 7 or 7.4 and pH 8
- ENT standard (2E+11 copies/µL, prepared by Bram Sercu)
- 500 µL microcentrifuge tubes
- DNA grade water (e.g. Fisher; cat# BP2470-1)

6.0 Procedure

6.1 Thaw all reagents and DNA samples and store on ice.

Note: Keep the MasterMix buffer in the freezer until a few minutes before use and return to freezer after use. The enzyme will degrade gradually when stored on ice.

Note: New primers should be dissolved in sterile TE buffer pH 7 or 7.4. After all material has been re-suspended and mixed well, multiple aliquots should be created for each primer to minimize number of freeze-thaw cycles to no more than 3x per tube.

Note: Probes are ordered in single-use aliquots. Dissolve in sterile TE buffer pH 8 and mix well. Keep protected from light until added to master mix.

6.2 Label microcentrifuge tubes for samples and standards (1 each).

6.3 Make sure to vortex all samples before pipetting. Kit reagents should not be vortexed, but instead should be inverted 3-5 times to mix before pipetting.

6.4 Prepare ENT standard dilutions:

std	copies/uL	copies/well	std/water uL
x1	2.00E+11	stock	
x2	4.00E+10	1.00E+11	4/16

x3	4.00E+09	1.00E+10	3/27
x4	4.00E+08	1.00E+09	3/27
x5	4.00E+07	1.00E+08	3/27
x6	4.00E+06	1.00E+07	3/27
x7	4.00E+05	1.00E+06	3/27
x8	4.00E+04	1.00E+05	3/27
x9	4.00E+03	1.00E+04	3/27
x10	4.00E+02	1.00E+03	3/27
x11	4.00E+01	1.00E+02	3/27
x12	1.00E+01	2.50E+01	5/15

Note: Only standard dilutions **x7** through **x12** will be run on the plate.

6.5 Prepare Reagent Mix, at 82 µL per sample/standard. This is sufficient for 1 sample in triplicate (3 x 25 µl).

	Volume per well (µL)	**Volume for full plate (µL)**
Water	9.175	1053.3
ECST748F	0.225	25.8
ENC854R	0.075	8.6
MasterMix buffer	12.5	1435.0
BSA	0.5	57.4
GPL813TQ	0.025	2.9

6.6 Add 73.8 µL of Master Mix to all sample/standard tubes.

6.7 Add 8.2 µL of DNA template to the sample tubes and vortex briefly. DNA template should be quantified according to SOP #H04.

6.8 Aliquot 3 x 25 µL of each sample or standard in the 96-well PCR plates.

6.9 Seal the PCR plates using the sealing tape and plate sealer, and spin briefly using a plate centrifuge or modified salad spinner to remove air bubbles.

Note: Make sure the plate is sealed well, as a weak seal could lead to evaporation during the reaction.

6.10 Turn on thermocycler and computer.

6.11 Input plate lay-out and standard concentrations in the software.

6.12 Start run.
The cycling parameters should be the following:

50°C 2min
95°C 10min

45 cycles of:
95°C 15sec
60°C 1min
Plate Read

7.0 Records Management

The run details (sample IDs, personnel performing qPCR, any problems or deviations from protocol) will be recorded in a laboratory notebook, along with the plate layout.

Data will not be stored on the computer connected to the CFX 96. All data files will be transferred and stored on a computer connected to the Bren/ICESS network that is backed up daily.

8.0 Quality Control and Quality Assurance Section

The qPCR detector was calibrated for use with all fluorescent dyes by the manufacturer. Negative controls and positive controls are always included on every plate, and all samples including controls are run in triplicate. If target concentrations in the negative control exceed those of the lowest standard, the run will be repeated.

Run efficiency shall be between 90-110%, or else the run will be repeated. The baseline for each run will be adjusted so that the coefficient of variation for the standard curve Ct values are 3% or less between all plates. Sample replicates amplifying past the lowest standard are considered not detectable within quantification range of the assay (ND). Only samples with 2 or more replicates within quantification range of the assay are assigned concentration values.

Biological safety cabinets (biohoods) and pipettes are inspected and certified/calibrated annually.

9.0 References

Haugland, R.A., Siefring, S.C., Wymer, L.J., Brenner, K.P., Dufour, A.P. (2005). Comparison of Enterococcus measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Research 39(4): 559-568.

STANDARD OPERATING PROCEDURE	SOP# H11
Holden Lab Donald Bren School of Environmental Science and Management University of California, Santa Barbara	
TITLE: **PCR – Mnif**	
EFFECTIVE DATE: 01/10	REVISION: 2
AUTHORS: Bram Sercu & Laurie C. Van De Werfhorst	FINAL REVIEWER: Patricia Holden

1.0 Purpose and Scope

This Standard Operating Procedure (SOP) describes how to perform polymerase chain reaction (PCR) for *Methanobrevibacter smithii nifH* gene target (Mnif) on previously extracted DNA. The ultimate purpose of the method is detect/confirm human fecal pollution in water or other environmental samples.

2.0 Applicability

This SOP should be followed for Mnif PCR analysis of DNA extracts from water or other samples, in all water quality research projects under the supervision of Dr. Patricia Holden.

3.0 Personnel Qualifications/Responsibilities

All laboratory staff performing the analysis must be familiar with this document, and have completed the UCSB EH&S Lab Safety Class.

4.0 Health and Safety Warnings

The person performing the analysis should always wear safety gloves and lab coat. No especially toxic or hazardous materials are needed for PCR, but general lab safety rules should be followed.

Ethidium bromide, a highly toxic substance, is used as a visualizing agent in gel electrophoresis. All solid waste associated with preparing and running a gel (e.g. used gel, gloves, pipet tips) should be treated as hazardous waste and disposed according to UCSB regulations. Spent TE buffer may either be disposed as hazardous waste or treated with a commercial kit (e.g. Whatman Extractor EtBr System; cat# 10448030 or 10448031). Keep all ethidium bromide contaminated objects (gel box, designated pipet, etc.) in the designated and labeled ethidium bromide area.

Note: Nitrile gloves must be used when working with ethidium bromide. Latex gloves do not offer adequate protection. Safety glasses must be worn when working with or near ethidium bromide.

Note: A UV shield must be used when the UV transilluminator is turned on. Either the UV shield on the transilluminator must be kept lowered, or a UV face shield must be worn.

5.0 Equipment, Reagents, and Supplies

5.1 Equipment

- Thermocycler
- Benchtop centrifuge
- 1000, 200, 20 & 10 µL filter tips and pipettes
- Vortexer
- Bio Rad power PAC 300 power supply
- Bio Rad mini-sub cell GT gel box with gel trays & combs
- 125 mL Erlenmeyer flask
- Microwave
- UV transilluminator
- Digital or Polaroid camera
- Biological safety cabinet (biohood)

5.2 Reagents and Supplies

- Taq PCR Core kit (Qiagen; cat# 201223 or cat# 201225)
- Primers (Mnif-342f and Mnif-363r, Ufnar et al, 2006) (e.g. Operon Biotechnologies)
- BSA (10 mg/mL) (New England BioLabs, cat# B9001S)
- DNA grade water (e.g. Fisher; cat# BP2470-1)
- Positive control (sewage DNA or any other sample that has shown positive on a previous gel)
- Thin walled 0.5 mL PCR tubes (Fisher; cat# 07-200-252)
- DNA grade agarose powder (e.g. Fisher; cat# BP164-25)
- 0.5X TBE buffer
- 1kb DNA ladder (Promega, cat# 17355204)
- Blue/orange 6x loading dye (Promega; cat# G190A), diluted to 2x with DNA grade water
- Ethidium bromide staining solution, 0.5 mg/mL
- Disposable specimen cups or 50 mL tubes
- Nitrile or latex exam gloves
- Safety glasses

6.0 Procedure

6.1 Thaw all reagents and DNA samples and store on ice.

Note: Keep the DNA polymerase in the freezer until just before use and immediately return to freezer after use. The enzyme will degrade gradually when stored on ice.

Note: New primers should be dissolved in sterile TE buffer pH 7 or 7.4. After all material has been re-suspended and mixed well, multiple aliquots should be created for each primer to minimize number of freeze-thaw cycles to no more than 3x per tube.

6.2 Label all tubes for samples.

6.3 Make sure to vortex all samples before pipetting. Kit reagents should not be vortexed, but instead should be inverted 3-5 times to mix before pipetting.

6.4 Prepare sample dilutions using DNA grade water. Use the same dilution as used in SOP# H09 & H10, determined by H08.

6.5 Prepare Master Mix. Make 24 µL per sample and add 10% extra volume to account for pipetting errors.

	Volume added (µL)	Final concentration
Water	15.1	-
10X-QIA	2.5	1X
Q mix	5	1X
BSA	0.5	0.2 mg/mL
dNTP	0.5	0.2 mM ea
Mnif-342F	0.125	0.5 uM
Mnif-363R	0.125	0.5 uM
TOTAL	**24**	

6.6 Add 24 µL of Master Mix to all sample tubes.

6.7 Add 1 µL of diluted sample and vortex briefly at a low speed.

Note: Make sure the tubes are closed well, as a weak seal could lead to evaporation during the reaction.

6.8 Put the tubes in the thermocycler and run program B: 30 MNIF. Use settings: "hot lid = manual" and "lid on after final hold step = no". .

6.9 The complete thermocycler program is the following:

Initial denaturation 92°C 2min

30 cycles of

Denaturation	92°C	1min
Annealing	55.1°C	30sec
Extension	72°C	1min
Final extension	72°C	6min
Hold	4°C	

6.13 Collect the samples after the run terminates and store at -20°C if not immediately running the 2nd required PCR.

6.14 Dilute the PCR products from the first round of PCR 1:10 using DNA grade water. This includes the positive and negative controls.

6.15 Prepare 2nd round of PCR, exactly as before (starting at step 6.5).

6.16 Collect the samples after the run terminates, and store at -20°C if not immediately running a gel.

6.17 Prepare and run gel as described in SOP #H06, except that a 2% gel is used here (for small gel box = 0.75 g agarose powder and 35 mL of 0.5x TBE buffer). Gel results will be captured/archived via a Polaroid camera or through a digital imaging setup.

6.18 All positive samples will be confirmed by running through both PCR rounds again.

7.0 Records Management

Gel image and PCR run details (sample IDs, personnel performing extraction, any problems or deviations from protocol, notes) will be recorded in a laboratory notebook.

All data files will be stored on a computer connected to the Bren/ICESS network that is backed up daily.

8.0 Quality Control and Quality Assurance Section

If the negative control amplifies, as checked on a 2% agarose gel, both PCR runs will have to be performed again. With persistent amplification of the negative control, all reagents will be checked for contamination.

Biological safety cabinets (biohoods) and pipettes are inspected and certified/calibrated annually.

9.0 References

Ufnar, J. A., S. Y. Wang, J. M. Christiansen, H. Yampara-Iquise, C. A. Carson and R. D. Ellender. 2006. Detection of the *nifH* gene of *Methanobrevibacter smithii*: a potential

tool to identify sewage pollution in recreational waters. J. Appl. Microbiol. 101:44-52.

STANDARD OPERATING PROCEDURE	SOP# H12
Holden Lab Donald Bren School of Environmental Science and Management University of California, Santa Barbara	
TITLE: **ELISA - Caffeine**	
EFFECTIVE DATE: 01/10	REVISION: 2
AUTHORS: Bram Sercu & Laurie C. Van De Werfhorst	FINAL REVIEWER: Patricia Holden

1.0 Purpose and Scope

This Standard Operating Procedure (SOP) describes how to collect, process and analyze environmental freshwater samples for caffeine via an ELISA method. The ultimate purpose of the method is to quantify the amount of caffeine in aqueous samples and infer/confirm human fecal contamination.

2.0 Applicability

This SOP should be followed for caffeine ELISA analysis of freshwater samples, in all water quality research projects under the supervision of Dr. Patricia Holden.

3.0 Personnel Qualifications/Responsibilities

All laboratory staff performing the analysis must be familiar with this document, and have completed the UCSB EH&S Lab Safety Class.

4.0 Health and Safety Warnings

Water samples are potentially contaminated with fecal waste, and can contain chemical and/or biological agents that can cause illness, even when they appear clean. Thus, the sampler should treat all water as though it is polluted. The sampler should avoid water contact, wear protective gloves, and the sample containers should be handled with care. The sampler should test surfaces for slipperiness and pay particular attention to conditions when working on the creek banks. Also, the sampler should be aware of traffic when sampling in or near roads.

Storm drain (or other sampling) that occurs on a street surface needs to be coordinated with City staff so that traffic flow can be managed, and safety rules enforced. Field personnel need to stay within the demarked safety cone area and wear bright, reflective safety vests when sampling.

Safety glasses must be worn by all nearby personnel during syringe filtration of samples.

The person performing the ELISA analysis should always wear safety gloves, lab coat and safety glasses. No especially toxic or hazardous materials are used in the analysis, but general lab safety rules should be followed. Contact with the substrate solution (contains tetramethylbenzidine) and the stop solution (contains diluted sulfuric acid) should be avoided.

5.0 Equipment, Reagents, and Supplies for Sample Collection

5.1 Sampling Equipment and Supplies

- Nitrile or latex exam gloves
- Sample cooler with ice
- Sterile 500 mL beakers
- Extendable sampling pole
- ISCO 6712 portable sampler (optional)
- Sterile 10 mL syringes with Luer-Lok™ Tips (e.g. BD, cat# 309604)
- Sterile 25 mm, 0.2 µm pore size Anotop or PTFE syringe filters (e.g. Whatman Anotop25, cat# 6809-2122 or Whatman GD/X, cat# 6874-2502)
- 20 mL glass amber vials with Teflon lined caps
- Styrofoam or plastic rack to hold 20 mL vial(s)
- Field log notebook and pens
- Safety glasses

5.2 Sample Analysis Equipment

- Synergy 2 microplate reader (BioTek Instruments)
- Electronic multi-channel pipet (12 channel preferred)
- 200 µl tips
- Timer/stopwatch

5.3 Sample Analysis Reagents and Supplies

- Caffeine ELISA (Microtiter Plate) kit (Abraxis cat# 515575)
- 5 reagent reservoirs/basins for multi-channel pipet
- Parafilm
- 500 mL or 1 L glass bottle
- Nanopure water
- Paper towels
- Nitrile or latex exam gloves
- Safety glasses

6.0 Sample Collection Procedure

Note: Only freshwater sample may be analyzed using this protocol. Saltwater samples can only be analyzed if they are diluted at least 50%.

6.1 As detailed in SOP #H02, water samples are obtained by dipping a sterile beaker into the water source, while wearing Nitrile or latex gloves. Depending on the depth of the water source, the beaker may be dipped by hand, attached to a sampling pole, or an ISCO 6712 portable sampler may be used to pump the sample up to the surface.

6.2 Once the sample is in the sterile beaker, a sterile 10 mL syringe is used to pull out ~ 10 mL of sample (volume does not have to be exact). A sterile 25 mm, 0.2 µm pore size Anotop or PTFE syringe filter is then attached to the tip of the syringe. The tip of the syringe filter is then inserted into a 20 mL vial, and the syringe plunger is depressed to filter the sample into the vial. Samples are stored on ice until transport to the laboratory, where they are archived at -20°C until analysis.

Note: The same sample vial may also be used for the cotinine ELISA assay (SOP #H13).

Note: Safety glasses should be worn by all nearby personnel during syringe filtration.

Note: It is easier to filter the sample if the glass vial is in a styrofoam or plastic rack, to prevent tip over.

Note: If the sample contains particles (especially sewage samples), a full 10 mL will be too difficult to push through the syringe filter. As only 150 µL are needed to run one ELISA assay in triplicate, obtaining only a few milliliters is acceptable. It is preferred to obtain at least a couple of milliliters so that a portion may be archived for future ELISA analyses.

Note: Multiple syringe filters may be used on a single sample if the filter gets clogged. If there is little or no sample flow despite pressure on syringe plunger, do not continue pressing on the syringe plunger as the pressure could cause the filter to separate from the syringe and the sample could be sprayed upon personnel.

7.0 Sample Assay Procedure

The caffeine ELISA test preparation and assay procedure is performed according to the protocol described by the kit's manufacturer. A protocol is included in every kit and utilized with no modifications. All samples and standards are analyzed in triplicate.

Note: Use only the reagents, standards and plate from the same package, as they have been adjusted in combination.

Note: All kit reagents, standards and samples will be allowed to reach room temperature before use.

Note: The 5X wash solution will be diluted with 400 mL of Nanopure water and mixed well prior to use.

Note: Reagent sequence and incubation times for each step must be followed exactly according to the protocol. It is important that the reagents are added to the plates in the same row order, with the same amount of time in between the addition to each row. Variations in incubation time between wells will produce inconsistent results between replicates and increase error.

Note: Wash step is best performed by adding 300 µL diluted wash solution to the wells with the multi-channel pipet. If the multi-channel pipet can only dispense smaller volumes, add multiple aliquots until the volume in each well is 300 µL. Shake the wash buffer out of the wells into a sink, and tap plate on a stack of paper towels to dry the wells in between each wash step. After the final wash step, continue tapping to try to remove as much of the residual buffer in the wells as possible. If air bubbles appear after buffer removal, gently pop using a clean pipette tip before adding the substrate/color solution.

Note: Protect the plate from sunlight, especially during the substrate/color and stop solution steps. Keep plate protected from light until ready to read the plate. Plate must be read within 15 minutes after stopping the assay.

8.0 Sample Analysis

Evaluation of ELISA results is performed using the ELISA_Caffeine.prt protocol, saved on the BioTek plate reader. This protocol automatically corrects for blank absorbance, creates a 4 Parameter nonlinear regression of the standards, and calculates caffeine concentration (ppb) in the samples.

8.1 Adjust sample and standard layout and concentration, if different from the protocol template.

8.2 Place plate in the BioTek plate reader and initiate run.

8.3 Check results and export standard curve and quantification values to Microsoft Excel.

8.4 If sample was diluted, multiply results by dilution factor used.

8.5 The lowest replicate of the lowest standard will be used as the lower limit of detection for samples on that plate. Sample replicate wells with values lower than this value will be treated as zeros.

8.6 The highest replicate of the highest standard will be used as the upper limit of detection for samples on that plate. Sample replicate wells with values higher than this value will be reported as '>5 ppb'. If time and resources allow, these samples may be diluted and run on a second plate.

9.0 Records Management

All field and sampling notes will be recorded in a field notebook (site#, sample IDs, sample notes).

Sample analysis details (sample IDs, personnel performing analysis, any problems or deviations from protocol) will be recorded in a laboratory notebook, along with the standard curve R^2 value.

Data will not be stored on the hard drive of the computer connected to the plate reader. All data files will be transferred and stored in a labeled folder on the Bren/ICESS network that is backed up daily.

10.0 Quality Control and Quality Assurance Section

Standard curves will be run on every plate, and will be run in triplicate. If the standard curve R^2 is < 0.99 the run will be aborted and a new plate with new standards will be run.

All kit reagents will be before the expiration date.

Pipettes are inspected and certified/calibrated annually.

STANDARD OPERATING PROCEDURE	SOP# H13
Holden Lab Donald Bren School of Environmental Science and Management University of California, Santa Barbara	
TITLE: **ELISA - Cotinine**	
EFFECTIVE DATE: 01/10	REVISION: 2
AUTHORS: Bram Sercu & Laurie C. Van De Werfhorst	FINAL REVIEWER: Patricia Holden

1.0 Purpose and Scope

This Standard Operating Procedure (SOP) describes how to collect, process and analyze environmental freshwater samples for cotinine via an ELISA method. The ultimate purpose of the method is to quantify the amount of cotinine in aqueous samples and infer/confirm human fecal contamination.

2.0 Applicability

This SOP should be followed for cotinine ELISA analysis of freshwater samples, in all water quality research projects under the supervision of Dr. Patricia Holden.

3.0 Personnel Qualifications/Responsibilities

All laboratory staff performing the analysis must be familiar with this document, and have completed the UCSB EH&S Lab Safety Class.

4.0 Health and Safety Warnings

Water samples are potentially contaminated with fecal waste, and can contain chemical and/or biological agents that can cause illness, even when they appear clean. Thus, the sampler should treat all water as though it is polluted. The sampler should avoid water contact, wear protective gloves, and the sample containers should be handled with care. The sampler should test surfaces for slipperiness and pay particular attention to conditions when working on the creek banks. Also, the sampler should be aware of traffic when sampling in or near roads.

Storm drain (or other sampling) that occurs on a street surface needs to be coordinated with City staff so that traffic flow can be managed, and safety rules enforced. Field personnel need to stay within the demarked safety cone area and wear bright, reflective safety vests when sampling.

Safety glasses must be worn by all nearby personnel during syringe filtration of samples.

The person performing the ELISA analysis should always wear safety gloves, lab coat and safety glasses. No especially toxic or hazardous materials are used in the analysis, but general lab safety rules should be followed. Contact with the substrate solution (contains tetramethylbenzidine) and the stop solution (contains diluted sulfuric acid) should be avoided.

5.0 Equipment, Reagents, and Supplies

5.1 Sampling Equipment and Supplies

- Nitrile or latex exam gloves
- Sample cooler with ice
- Sterile 500 mL beakers
- Extendable sampling pole
- ISCO 6712 portable sampler (optional)
- Sterile 10 mL syringes with Luer-Lok™ Tips (e.g. BD, cat# 309604)
- Sterile 25 mm, 0.2 µm pore size Anotop or PTFE syringe filters (e.g. Whatman Anotop25, cat# 6809-2122 or Whatman GD/X, cat# 6874-2502)
- 20 mL glass amber vials with Teflon lined caps
- Styrofoam or plastic rack to hold 20 mL vial(s)
- Field log notebook and pens
- Safety glasses

5.2 Sample Analysis Equipment

- Synergy 2 microplate reader (BioTek Instruments)
- Electronic multi-channel pipet (12 channel preferred)
- 200 µl tips
- Timer/stopwatch

5.3 Sample Analysis Reagents and Supplies

- Cotinine ELISA (Microtiter Plate) kit (Abraxis cat# 515565)
- 5 reagent reservoirs/basins for multi-channel pipet
- Parafilm
- 500 mL or 1 L glass bottle
- Nanopure water
- Paper towels
- Nitrile or latex exam gloves
- Safety glasses

6.0 Sample Collection Procedure

Note: Only freshwater sample may be analyzed using this protocol. Saltwater samples can only be analyzed if they are diluted at least 50%.

6.1 As detailed in SOP #H02, water samples are obtained by dipping a sterile beaker into the water source, while wearing Nitrile or latex gloves. Depending on the depth of the water source, the beaker may be dipped by hand, attached to a sampling pole, or an ISCO 6712 portable sampler may be used to pump the sample up to the surface.

6.2 Once the sample is in the sterile beaker, a sterile 10 mL syringe is used to pull out ~ 10 mL of sample (volume does not have to be exact). A sterile 25 mm, 0.2 µm pore size Anotop or PTFE syringe filter is then attached to the tip of the syringe. The tip of the syringe filter is then inserted into a 20 mL vial, and the syringe plunger is depressed to filter the sample into the vial. Samples are stored on ice until transport to the laboratory, where they are archived at -20°C until analysis.

Note: The same sample vial may also be used for the caffeine ELISA assay (SOP #H12).

Note: Safety glasses should be worn by all nearby personnel during syringe filtration.

Note: It is easier to filter the sample if the glass vial is in a styrofoam or plastic rack, to prevent tip over.

Note: If the sample contains particles (especially sewage samples), a full 10 mL will be too difficult to push through the syringe filter. As only 150 µL are needed to run one ELISA assay in triplicate, obtaining only a few milliliters is acceptable. It is preferred to obtain at least a couple of milliliters so that a portion may be archived for future ELISA analyses.

Note: Multiple syringe filters may be used on a single sample if the filter gets clogged. If there is little or no sample flow despite pressure on syringe plunger, do not continue pressing on the syringe plunger as the pressure could cause the filter to separate from the syringe and the sample could be sprayed upon personnel.

7.0 Sample Assay Procedure

The cotinine ELISA test preparation and assay procedure is performed according to the protocol described by the kit's manufacturer. A protocol is included in every kit and utilized with no modifications. All samples and standards are analyzed in triplicate.

Note: Use only the reagents, standards and plate from the same package, as they have been adjusted in combination.

Note: All kit reagents, standards and samples will be allowed to reach room temperature before use.

Note: The 5X wash solution will be diluted with 400 mL of Nanopure water and mixed well prior to use.

Note: Reagent sequence and incubation times for each step must be followed exactly according to the protocol. It is important that the reagents are added to the plates in the same row order, with the same amount of time in between the addition to each row. Variations in incubation time between wells will produce inconsistent results between replicates and increase error.

Note: Wash step is best performed by adding 300 µL diluted wash solution to the wells with the multi-channel pipet. If the multi-channel pipet can only dispense smaller volumes, add multiple aliquots until the volume in each well is 300 µL. Shake the wash buffer out of the wells into a sink, and tap plate on a stack of paper towels to dry the wells in between each wash step. After the final wash step, continue tapping to try to remove as much of the residual buffer in the wells as possible. If air bubbles appear after buffer removal, gently pop using a clean pipette tip before adding the substrate/color solution.

Note: Protect the plate from sunlight, especially during the substrate/color and stop solution steps. Keep plate protected from light until ready to read the plate. <u>Plate must be read within 15 minutes after stopping the assay</u>.

8.0 Sample Analysis

Evaluation of ELISA results is performed using the ELISA_Cotinine.prt protocol, saved on the BioTek plate reader. This protocol automatically corrects for blank absorbance, creates a 4 Parameter nonlinear regression of the standards, and calculates cotinine concentration (ppb) in the samples.

8.1 Adjust sample and standard layout and concentration, if different from the protocol template.

8.2 Place plate in the BioTek plate reader and initiate run.

8.3 Check results and export standard curve and quantification values to Microsoft Excel.

8.4 If sample was diluted, multiply results by dilution factor used.

8.5 The lowest replicate of the lowest standard will be used as the lower limit of detection for samples on that plate. Sample replicate wells with values lower than this value will be treated as zeros.

8.6 The highest replicate of the highest standard will be used as the upper limit of detection for samples on that plate. Sample replicate wells with values higher than

this value will be reported as '>5 ppb'. If time and resources allow, these samples may be diluted and run on a second plate.

9.0 Records Management

All field and sampling notes will be recorded in a field notebook (site#, sample IDs, sample notes).

Sample analysis details (sample IDs, personnel performing analysis, any problems or deviations from protocol) will be recorded in a laboratory notebook, along with the standard curve R^2 value.

Data will not be stored on the hard drive of the computer connected to the plate reader. All data files will be transferred and stored in a labeled folder on the Bren/ICESS network that is backed up daily.

10.0 Quality Control and Quality Assurance Section

Standard curves will be run on every plate, and will be run in triplicate. If the standard curve R^2 is < 0.99 the run will be aborted and a new plate with new standards will be run.

All kit reagents will be before the expiration date.

Pipettes are inspected and certified/calibrated annually.

APPENDIX II. UCSB Progress Reports to the City of Santa Barbara

UCSB Mission Project –Progress Report **October 14 – December 31, 2008**
T. Holden, UCSB

Note: During this period, Bram Sercu was employed on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Also, the City issued a "stop work" order on 12/23 due to a freeze on State funds that are supporting this project. However, research associated with a 12/4/08 sampling event was continued and the City was in email correspondence with Dr. Sercu on 12/30/08 regarding the results.

- Order field fluorometer and supplies (fluorescent dye, cuvettes,…)
- Test field fluorometer in the lab
- Prepare for flow measurements using dye dilution and fluorometer (literature search, method evaluation)
- Set up new qPCR thermocycler.
- Transfer and test *Enterococcus* spp. quantification protocol on new qPCR thermocycler
- Sample at San Pascual Drain and downstream locations (including photo-documentation)
- Perform DNA extraction/quantification for San Pascual Drain samples
- Perform qPCR human-specific *Bacteroides* for San Pascual Drain samples
- Report results for San Pascual Drain samples to City of Santa Barbara

UCSB Mission Project –Progress Report **January 1 – May 31, 2009**
Revised 6-23-09
T. Holden, UCSB

Note: During this period, Bram Sercu, Laurie Van De Werfhorst, and Aaron Sobel were each employed part time on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB. Aaron Sobel is a MESM student in the Bren School who is working as a Research Assistant (workstudy).

On February 2, 2009, the City rescinded the "stop work" order issued on 12/23/08. The "stop work" order was issued due to a freeze on State funds that are supporting this project.

- Evaluated published literature regarding tracing (single and dual tracer methods) sanitary sewage into groundwater with application to storm drain contamination in the City of Santa Barbara. Developed a staged plan for 1) dye studies, 2) implementation of chemical tracers used in Illicit Discharge studies, and 3) dye tracing as used in quantifying exfiltration; internal review of the plan and communication with the City by email (3/3/09, Holden). Work performed by Sercu.
- Compiled GIS data provided by the City and began inventory of data, including indentifying additional data needs and communicating these to the City (3/3/09 email, Holden, and also communication by Sercu in June, 2009). Work performed by Sobel, and summarized by Sercu.
- Prepared, sampled, and processed samples from San Pascual Drain & Haley Drain (@ diversion pump structure); DNA extraction, EtOH ppt. & quantification (April/May); prepared & ran salmon qPCR and SYBR green human-specific Bacteroides qPCR (April/May). Work performed by Sercu and Van De Werfhorst. This work was follow-on to the last period, during which (in December, 2008 as previously reported) one sampling and analysis event of San Pasqual Drain occurred.
- Project planning meeting, City and Santa Barbara, related to Mission but also to Laguna project. Sercu, Van De Werfhorst, Holden attended with Dr. Murray from the City.
- Developed TaqMan qPCR method for Human *Bacteroides* marker of human waste using new qPCR instrument purchased with funds from this project. This method makes use of qPCR Salmon assay results for dilution selections, and works compatibly with new qPCR *Enterococcus* assay. Work performed by Sercu.
- Researched methods for using ELISA-based assays (by Abraxis) for chemical markers cotinine, caffeine and trichlosan. Researched and ordered appropriate filters for water samples in support of these new assays. Van De Werfhorst performed this work, mostly.
- Attended and presented at EPA Beach Conference in Huntington Beach, CA. Sercu presented and Van De Werfhorst attended. Travel funds were paid out of other sources available to Holden. Work presented was based on research in Laguna watershed in Summer, 2008 and demonstrated the use of several compatible source tracking methods including qPCR HumBac plus a method for presence/ absence of a human-specific *Methanobrevibacter* by routine PCR, and lastly evaluation of optical brighteners and an associated microplate assay.
- Attended and presented at the General Meeting of the American Society for Microbiology in Philadelphia, PA. Sercu presented and attended results as above, also presented at the EPA Beach Conference in Huntington, Beach, CA. Funds were not available from this project to support conference travel and thus were used from another source.
- Edit draft of manuscripts related to research with the City of Santa Barbara, including 1) a manuscript regarding clone library analysis of Arroyo Burro dry weather samples (prior project with the City), and 2) a manuscript regarding a storm study performed in 2005/06 with samples assayed thereafter. Writing and data analysis by Sercu.

UCSB Mission Project –Progress Report June 1 – June 30, 2009
T. Holden, UCSB

During this period, no project personnel were working directly on this project because all related personnel were working in support of another project with the City of Santa Barbara.

UCSB Mission Project –Progress Report July 1 – July 31, 2009
T. Holden, UCSB

Note: During this period, Bram Sercu was employed part time on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB.

- Finalized PCR for *Methanobrevibacter smithii nifH* gene for San Pascual and Haley Drain samples.
- Sampled El Estero WWTP raw influent and confluent for ELISA tesing
- Performed analysis of salmon testes DNA, *Enterococcus* spp. and human-specific *Bacteroidales* by TaqMan qPCR for San Pasqual and Haley Drain samples acquired previously (described in prior report), and for which other analyses were previously performed (described in prior report).
- Completed data analysis in support of, and wrote plus submitted report entitled "Summary of results for San Pascual and Haley Drain (December 2008 – May 2009)" describing the results pertinent to the sampling at these locations which are in support of this project, especially with regards to site selection for dye studying sanitary to storm sewer potentially transmission.
- Completed and submitted manuscript for publication regarding clone library analysis of Arroyo Burro dry weather samples (prior project with the City). Writing and data analysis by Sercu.

UCSB Mission Project –Progress Report August 1 – August 31, 2009
T. Holden, UCSB

Note: During this period, Bram Sercu and Laurie Van De Werfhorst were each employed on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB.

- Continued developing plans for additional field sampling by building knowledge of sanitary and storm sewer locations using GIS-based information and analysis of City-provided files and maps. Acquired storm drain maps from City in support of this effort. Scheduled and met with GIS Professor in Bren School (James Frew) who is agreeing to collaborate on this project starting in October, 2009 at no charge to the project.
- Continued editing draft of manuscript related to research with the City of Santa Barbara, regarding a storm study performed in 2005/06 with samples assayed thereafter. Writing and data analysis by Sercu.
- Evaluated an improved kit for DNA extraction that is faster and provides higher purity DNA for PCR analysis. The kit is called the MoBio PowerWater kit, and is made by the same manufacturer as the prior kit, but has improved chemistry for increasing the purity and thus lowering the inhibitor concentrations in PCR. The evaluation involved co-evaluating several samples using both kits, then comparing yield and DNA purity plus qPCR results. While the new kit yields somewhat less DNA, it is more pure and more can be analyzed. Also, the variation in purity is less (i.e. better) with the new kit.
- Prepared for and conducted field water sampling in lower Mission Creek watershed (Haley Drain area, three locations in the storm drain, W Haley St diversion near Brinkerhoff Ave, W Haley St & Chapala St, Chapala St & W Ortega St), and in the Laguna watershed (4 locations in storm drains: N Nopal St & E. Cota St, E Ortega St & N Alisos St, N Nopal St & E Canon Perdido, E Canon Perdido & Philinda Ave) on August 19th. [Two additional sampling times were selected but postponed twice by the City and are now rescheduled for 9/9 and 9/10/09]. Sample analysis includes IDEXX for FIB, qPCR ENT, qPCR HBM, mnif, and ELISA for at least cotinine and caffeine.
- Continued to design dye study for planned implementation in early October: location, dye delivery method and quantity in sewer, expected dye concentration in storm drain and detection methodology.

UCSB Mission Project –Progress Report September 1 – September 30, 2009
T. Holden, UCSB

Note: During this period, Bram Sercu and Laurie Van De Werfhorst were each employed on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB.

- Continued developing plans for additional field sampling by building knowledge of sanitary and storm sewer locations using GIS-based information and analysis of City-provided files and maps. Evaluated more storm drain maps and construction drawings (of Haley Drain Diversion) acquired from City in support of this effort.

- Revised manuscript related to clone library development and analysis of IDEXX enrichments. Manuscript was submitted to Water Research in July, 2009, and was returned for revisions at the end of August, 2009. Revisions were made and the manuscript re-submitted in September, 2009.
- Prepared for and continued field water sampling at the same sites sampled on 8/19/09. Sampling locations in lower Mission Creek watershed (Haley Drain area, three to four locations in the storm drain, W Haley St diversion near Brinkerhoff Ave, W Haley St & Chapala St, Chapala St & W Cota St, Chapala St & W Ortega St (9/10/09 only due to construction)), and in the Laguna watershed (3 locations in storm drains: N Nopal St & E. Cota St, N Nopal St & E Canon Perdido, E Canon Perdido between Philinda Ave. and Nopal St.) were sampled on 9/9 and 9/10/09. Sewer system manholes were also opened and inspected at W Haley St & Chapala St (2 separate sewer lines), Chapala St & W Cota St (2 separate sewer lines), and N Nopal St & E Cota St. Sewage samples were taken from the North sewer manhole at Haley & Chapala, and at Nopal & Cota. DNA extraction, concentration and quantification was performed on all storm drain and sewage samples. Sample analysis conducted in this period included: IDEXX for FIB, qPCR of salmon testes DNA for determination of dilution needed to avoid inhibition. There were some methodological problems that precluded completing the qPCR HBM and qPCR ENT. The mnif assay and ELISA assays (caffeine and cotinine) will be performed in October, 2009. During field visits, reconnaissance of invert depths in sewers and storm drain sampling locations to correct GIS databases for continued planning of the dye studies.
- Evaluated and purchased equipment for suspending ISCO sampler in manholes.
- Evaluated and purchased dye for dye study.
- Continued to design dye study for planned implementation in early October, with continued sampling (as above), sample analysis and planning in September.

UCSB Mission Project –Progress Report October 1 – October 31, 2009
T. Holden, UCSB

Note: During this period, Bram Sercu and Laurie Van De Werfhorst were each employed on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB.

- Revised manuscript related to clone library development and analysis of IDEXX enrichments (Water Research) and submitted revised manuscript in October, 2009.
- Continued the analysis of 23 samples (water, and two sewage) acquired in (and for which DNA was extracted, purified and quantified in) September, 2009 in lower Mission Creek watershed (Haley Drain area, three locations in the storm drain, W Haley St diversion near Brinkerhoff Ave, W Haley St & Chapala St, Chapala St & W

Ortega St), and in the Laguna watershed (3 to 4 locations in storm drains: N Nopal St & E. Cota St, E Ortega St & N Alisos St, N Nopal St & E Canon Perdido, E Canon Perdido & Philinda Ave., with the last being optional) 9/9 and 9/10/09. Salmonid qPCR for determination of PCR inhibition was completed in September, 2009. Sample analysis completed in this period included qPCR HBM for all but the two sewage samples. qPCR HBM for the latter samples was attempted but failed due to probe expiration. To be efficient, the analysis of these samples was planned with others for another project that was to occur in either November or early December, depending on probe re-order and its efficacy. *Sample analyses to be performed as of this report are the two remaining qPCR HBM samples, and for all samples: chemical tracers (ELISA-based caffeine and cotinine) and Mnif.*

- In support of future dye studies, planned then conducted (during the morning of October 19th) field reconnaissance at Haley and Laguna storm drain system sites (involving Sercu, Van De Werfhorst and Holden from UCSB, and Vidal from the City) to gather storm sewer depth data and to observe (presence / absence) flows in storm drain manholes. This was necessary to verify City GIS data or to add data where GIS information was lacking.
- Incorporated field data acquired from 10/19/08 field reconnaissance into more comprehensive maps of storm and sanitary sewer locations (depth and laterally) at the Haley Drain and upper Laguna locations (as above, 2nd bullet).

UCSB Mission Project –Progress Report November 1 – November 30, 2009
T. Holden, UCSB

Note: During this period, Bram Sercu and Laurie Van De Werfhorst were each employed on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB.

- Using information gathered in the last period, continued development of accurate schematics of storm drain and sanitary sewer locations for the Haley Drain and upper Laguna (Nopal area) sites, in support of planning dye studies of storm to sanitary sewer communication.
- Identification of information gaps regarding sewer investigation history (e.g. smoke testing or televising lines) through internal meetings, in support of continued planning of dye studies.
- Organized and conducted a meeting at UCSB with Dr. Murray (Creeks Division) and UCSB staff (Sercu, Van De Werfhorst, Holden) to present updated schematic of drain and sanitary sewer configurations (Sercu presenting) and to request support from the City in the form of filling information gaps (history of smoke tests or televising lines in study areas) and in the form of further communication with an outside consultant (City contact) regarding other possible methods for assessing sewer to drain contamination.

- Continued the analysis of 23 samples (water, and two sewage) acquired in (and for which DNA was extracted, purified and quantified in) September, 2009 in lower Mission Creek watershed (Haley Drain area, three locations in the storm drain, W Haley St diversion near Brinkerhoff Ave, W Haley St & Chapala St, Chapala St & W Ortega St), and in the Laguna watershed (3 to 4 locations in storm drains: N Nopal St & E. Cota St, E Ortega St & N Alisos St, N Nopal St & E Canon Perdido, E Canon Perdido & Philinda Ave., with the last being optional) 9/9 and 9/10/09. Salmonid qPCR for determination of PCR inhibition was completed in September, 2009. Sample analysis completed in this period was ELISA-based analysis for cotinine and caffeine for all samples. *Sample analyses to be performed as of this report are the qPCR HBM for the two sewage samples (as per last report) and Mnif of all samples. Additionally, the analysis of data from chemical tracers (ELISA-based caffeine and cotinine) is yet to be performed.*

UCSB Mission Project –Progress Report December 1 – December 31, 2009

T. Holden, UCSB

Note: During this period, Laurie Van De Werfhorst was employed part time on this project. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB.

- Continued the analysis of 23 samples (water, and two sewage) acquired in (and for which DNA was extracted, purified and quantified in) September, 2009 in lower Mission Creek watershed (Haley Drain area, three locations in the storm drain, W Haley St diversion near Brinkerhoff Ave, W Haley St & Chapala St, Chapala St & W Ortega St), and in the Laguna watershed (3 to 4 locations in storm drains: N Nopal St & E. Cota St, E Ortega St & N Alisos St, N Nopal St & E Canon Perdido, E Canon Perdido & Philinda Ave., with the last being optional) 9/9 and 9/10/09. Salmonid qPCR for determination of PCR inhibition was completed in September, 2009. qPCR HBM was completed for 21 samples in October, 2009. ELISA-based analysis of cotinine and caffeine was completed in November, 2009, except for data analysis. Sample analysis completed in this period included: qPCR HBM for the remaining two samples and Mnif analysis for all samples. Additionally, the analysis of data from chemical tracers (ELISA-based caffeine and cotinine) was performed. A report based on all of the data is to be written for submission in January, 2010.

UCSB Mission Project –Progress Report February 1 – February 28, 2010

Note: During this period, Bram Sercu was employed part time, and Laurie Van De Werfhorst was employed full time on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB.

- With the assistance of Dr. Murray (Creeks Division), prepared and edited QAPP (Quality Assurance Project Plan) and MRP (Monitoring Research Plan) documents for submission to the State.

- Standard Operating Procedures (SOPs) were created and/or edited to reflect the current methods and quality control processes utilized in the Holden Lab on the Mission Project. The SOPs were included in the QAPP as required.

- Prepared and initiated animal fecal collection to assess the specificity of human-specific waste assays currently used in the Holden Lab on the Mission Project: quantitative PCR analysis of human-specific *Bacteroidales* via TaqMan®, and PCR analysis of the *Methanobrevibacter smithii nifH* gene target. Animal feces are being collected from 10 cats, 10 dogs, gulls on 2 separate occasions, multiple raccoons and multiple rats. From our previous research project in the Mission Creek watershed, 2 cats, 2 dogs and 1 gull sample will also be analyzed.

- Researched rapid field-based methods for human fecal pollution detection, including limits of detection, assay range and cost (equipment and reagents) for each.

- Bram Sercu (UCSB) met with Jill Murray (Creeks Division) to present results of rapid field-based methods research and discuss tracer studies, possible use of PhlyoChip analysis, use of GIS in sample planning and data presentation, scope of the Source Tracking Manual that will be produced from this project, and use of City funds to purchase equipment and outsource analytical or other work.

UCSB Mission Project –Progress Report March 1 – March 31, 2010

Note: During this period, Bram Sercu was employed full time, and Laurie Van De Werfhorst was employed full time on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB.

- Progress on evaluation of source specificity of human-specific waste assays:
    - Animal fecal collection initiated in February was completed. Feces were collected, processed and archived from 10 individual cats, 10 individual dogs, 2 composite gull samples from multiple individuals, 1 individual and 3 composite raccoon samples, and 1 individual and 3 composite rat samples.
    - DNA was extracted, concentrated and quantified from all 30 animal fecal samples, using standard operating procedures.
    - Salmon testes qPCR was run on all 30 animal fecal samples recently acquired, to determine the optimal dilution for human-specific *Bacteroidales* qPCR and PCR analysis of the *Methanobrevibacter smithii nifH* gene target. Salmon testes qPCR was also run on 6 animal fecal samples obtained during our previous research in the Mission Creek watershed (2 individual cats, 2 individual dogs, 1 composite gull sample, and 1 composite raccoon sample).
    - Initial testing of human-specific *Bacteroidales* qPCR via TaqMan was performed on all 36 animal fecal samples.
- Mission field work planning
    - Received historical data of sewage chemicals and nutrients in storm drains from City and updated report/recommendations for selecting methods to rapidly measure sewage chemicals
- Progress on manuscript submission
    - Submitted storm ms to ES&T
    - Edited text and initiated final review of IDEXX ms before resubmission to AEM
    - Finalized first draft Laguna ms for internal review at UCSB

UCSB Mission Project –Progress Report April 1 – April 30, 2010

Note: During this period, Bram Sercu and Laurie Van De Werfhorst were employed full time on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB.

- Progress on evaluation of source specificity of human-specific waste assays:
    - For a more complete evaluation of the human-specific waste assays, human feces, sewage and septage samples were added to the 36 animal fecal samples. Previously extracted and archived DNA from 8 individual humans, 3 septage samples, and 10 sewage samples were selected. This brings the total sample number for human-specific assay evaluation to 57.
    - Salmon testes qPCR was run on all of the human, sewage and septage samples that were not analyzed previously.
    - Human-specific *Bacteroidales* qPCR via TaqMan was performed on all of the human, sewage and septage samples that were not analyzed previously.

    - Human-specific *Bacteroidales* qPCR via SYBR Green I was performed on all 57 samples (animal, human, sewage and septage) using the Bio-Rad iQ5 located in the Hodges Lab.
- Researched ISCO sampler size specifications and requirements (full 6712 vs. compact 6712C), bottle configurations (24 x 1L for 6712, 24 x 500 mL for 6712C), sterile options for sampling (ProPak single-use bags lined with Teflon vs. PE or PP sample bottles that can't be autoclaved), and the details for obtaining custom-sized adjustable brackets to deploy the sampling unit in manholes.
- Researched specifications of ISCO 700 series, ISCO 4250 and Sigma 920 flow meters and accessories (data transfer, rings, …) in order to make recommendations to City.
- Researched field methods for quantifying sewage chemicals (colorimeters, ISEs, test kits), provided recommendations and discussed with City, ordered equipment and reagents.
- Continued to plan televising, smoke testing, tracer study and discussed plans with City.
- Finalized planning for initial tracer study, selected Rhodamine WT as tracer with continuous detection in storm drain. Researched equipment for continuous quantification of Rhodamine WT in storm drains, forwarded recommended equipment/vendor to City.
- Established weekly meetings/conference calls between Jill Murray and UCSB, starting on 4/15/10 and continuing on 4/22 and 4/29/10.
- Field visit to Haley & Chapala and Nopal & Canon Perdido study areas on 4/27/10 to interview Vidal, check manhole measurements and verify locations for proposed dye study.

UCSB Mission Project –Progress Report May 1 – May 31, 2010

Note: During this period, Bram Sercu and Laurie Van De Werfhorst were employed full time on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB.

- Progress on evaluation of source specificity of human-specific waste assays:
    - Performed PCR for *Methanobrevibacter smithii nifH* gene target on all 57 fecal source samples
- Finalized ISCO 6712 sampler specification/configuration research and submitted complete list of items to order to the City. Also provided information for sole source justification on required equipment.
- Finalized comparison of flow equipment. Submitted recommendations to City. Obtained quotes for flow equipment and accessories.
- Began ordering general field and lab supplies needed for the sampling season.
- Ordered and received equipment and supplies for quantifying sewage chemicals

- Met with Jesse Bickley from EH&S department at UCSB to discuss confined space entry requirements and training options.
- Discussed PhyloChip sampling with City and Gary Andersen (LBNL) and drafted preliminary scope of work.
- Finalized planning for initial field sampling to test protocols for quantifying sewage chemicals.
- Wrote advertisement for hiring student assistant for GIS work.
- Met with Jill Murray, Manuel Romero and Alex Alonzo (5/5/10) to discuss availability of smoke testing and televising results for sewer lines near the current study areas. Met again with Jill and Manuel (5/25/10) to go over what records are available, and to watch sample video footage to determine if quality will be sufficient for our needs.
- Obtained approval of Regional Water Board and City Water Resources dept. for dye testing study
- Continued to plan televising, smoke testing, tracer study and discussed plans with City.
- Continued weekly meetings/conference calls between Jill Murray and UCSB (5/6/10, 5/20/10 & 5/27/10).

UCSB Mission Project –Progress ReportJune 1 – June 30, 2010

Note: During this period, Bram Sercu and Laurie Van De Werfhorst were employed full time on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB. Kilty Inafuku started working full-time as a summer intern from 6/21/2010.

- Hired Bren student for performing GIS work at Creeks Division
- Meeting at UCSB on 6/17 (discuss progress and dye testing)
- Sampling for testing colorimetry methods on 6/3 and 6/28.
- Lab testing of colorimetry methods for measuring chemical indicators (NH_3, NO_3, PO_4, SO_4, K, B, anionic surfactants) and fluorescence for optical brighteners: analysis of standard curves for testing method accuracy and precision, analysis of storm drain samples for determination of concentration ranges, analysis of diluted storm drain samples for determining matrix interferences.
- Lab testing of rhodamine WT probe: connectivity to PC, programming and unattended monitoring testing.
- Field work for dye testing at State/Plaza, Chino/Micheltorena and Nopal/Canon Perdido on 6/11, 6/18, 6/21-6/24, and 6/28: dye release, rhodamine WT probe deployment and collection, water sampling.
- qPCR instrument sensitivity testing initiated on both Bio-Rad systems currently utilized (CFX96 & iQ5) to determine if the CFX96 may be used for the HBM SYBR green assay.

- Obtained and processed sewage confluent and reclaimed water samples from El Estero WWTP to confirm and/or evaluate method sensitivity for chemical indicators, caffeine & cotinine via ELISA, HBM qPCR (no reclaimed water) and Mnif PCR (no reclaimed water).

UCSB Mission Project –Progress Report July 1 – July 31, 2010

Note: During this period, Bram Sercu and Laurie Van De Werfhorst were employed full time on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB. Kilty Inafuku assisted as a summer intern.

- Kick-off meeting GIS at Creeks Division
- Phone conferences for project evaluation and planning on 7/26.
- Meeting at UCSB for discussion of dye testing experiments on 7/13.
- Assisted Jill Murray in preparing Grant Report for State: provide photo documentation, summary of field work, summary of lab work.
- Assisted Jessica Golman with data input into GIS.
- Field work:
    - Dye testing at Nopal/Canon Perdido: dye release, rhodamine WT probe deployment and collection, water sampling.
    - Collecting water samples for analysis of sewage chemical indicators, FIB, nutrients, caffeine, cotinine and DNA markers (Mnif and human-specific Bacteroidales).
    - Field days: 7/1, 7/6, 7/9, 7/14, 7/19, 7/22, 7/29
- Lab analyses:
    - FIB for all samples
    - Chemical indicators for all samples (NH_3, NO_3, PO_4, SO_4, anionic surfactants)
- Continued qPCR instrument sensitivity testing on CFX96 & iQ5 systems to determine if the CFX96 may be used for the HBM SYBR green assay.
- Performed method sensitivity analysis on diluted sewage confluent samples from El Estero WWTP for the HBM qPCR via SYBR Green® I assay.

UCSB Mission Project –Progress Report August 1 – August 31, 2010

Note: During this period, Bram Sercu and Laurie Van De Werfhorst were employed (Sercu full time, and Van De Werhorst half time) on this project. Bram Sercu is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB. Kilty Inafuku assisted as a summer intern, with financial support through the UC LEADS program.

- GIS progress meeting at UCSB (8/30)
- Assist Jessica Golman with data input into GIS.
- Phone conferences for project evaluation and planning on 8/5 and 8/31.
- Field work for:
    - Dye testing at Nopal/Canon Perdido: continue monitoring of rhodamine WT using probe, data collection, probe maintenance and battery replacement.
    - Dye testing at Haley/Chapala: lab testing new probe, probe deployment for collecting background signal.
    - Field days: 8/5, 8/12, 8/18, 8/24, 8/26, 8/30
- Lab analyses:
    - DNA extraction for all samples collected in July
    - Salmon DNA qPCR for all samples collected in July
    - Mnif PCR for all samples collected in July
    - ELISA (caffeine & cotinine) assays begun on July samples
    - HBM qPCR via SYBR Green I for samples previously run via TaqMan chemistry (August & September 2009 samples)
- Planning for Phase III dye testing at Nopal/Canon Perdido and Phase I dye testing at Haley/Chapala
- Discuss / coordinate Phylochip analyses with LBNL
- Continued qPCR instrument sensitivity testing on CFX96 & iQ5 systems to determine if the CFX96 may be used for the HBM SYBR green assay
- Contined Mnif PCR analysis of fecal study samples

UCSB Mission Project –Progress Report September 1 – September 30, 2010

Note: During this period, Bram Sercu and Laurie Van De Werfhorst were employed full time on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB.

- Field work:
    - Installation of Isco flow monitoring equipment (Salsipuedes @ Cota) on 9/23; daily battery changes and flow data downloads through 9/27.
    - Sample collection (Salsipuedes @ Cota) from 9/28 through 10/1.
    - Installation of Hach flow monitoring equipment (Hope drain diversion) on 9/28.
    - Dye testing at Haley/Chapala: rhodamine WT dosing in sewer manholes (9/7-9/8) and monitoring, sample collection in storm drains.
    - Dye testing at Nopal/Canon Perdido: troubleshoot new rhodamine probe, dosing and monitoring of KCl and rhodamine WT in storm drains.
    - Field days for dye testing: 9/7, 9/8, 9/9, 9/13, 9/14, 9/16, 9/17, 9/20, 9/21, 9/22.

- Lab analyses: ammonia and nitrate by colorimetry within 24 hours after sample collection.
- Prepared dilutions and sent fecal source samples to LBNL for PhyloChip analysis
- Continued qPCR instrument sensitivity testing on CFX96 & iQ5 systems to determine if the CFX96 may be used for the HBM SYBR green assay
- Continued Mnif PCR analysis of fecal study samples, and investigated gel interpretation standardization methods

UCSB Mission Project –Progress Report October 1 – December 31, 2010

Note: During this period, Bram Sercu and Laurie Van De Werfhorst were employed full- and part- time, respectively, on this project. Bram is an Associate Specialist I (postdoctoral level) and is a researcher in the Holden Lab at UCSB. Laurie Van De Werfhorst is a Staff Research Associate II in the Holden Lab at UCSB.

- Field work:
    - Flow monitoring at Hope drain diversion using Sigma 920 flow monitoring equipment (9/28-10/3).
    - Flow monitoring and sample collection at Hope drain diversion using Isco flow monitoring and autosampling equipment (10/5-10/8).
- Lab analyses:
    - FIB for all sample collected using automated sampling
    - Ammonia and nitrate by colorimetry within 24 hours after sample collection.
    - Sulfate and phosphate by colorimetry
    - Adaptation of Salmon testes DNA TaqMan qPCR assay for SybrGreen chemistry
    - Salmon testes DNA SybrGreen qPCR for all samples
    - Finalize HBM qPCR for all samples
    - Finalize Mnif PCR for remaining samples
    - Finalize ELISA assays for caffeine and cotinine
- Prepared dilutions and sent water sample DNA extracts to LBNL for PhyloChip analysis
- Phone conferencing with LBNL staff to discuss PhyloChip analyses and timing
- Meeting with Jill Murray at UCSB to discuss content of report from UCSB to City
- Data delivery to Jessie Golman for inclusion in GIS database
- Analysis of all project data
- Write and deliver report to City of Santa Barbara