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Canine Scent and Microbial Source Tracking in Santa Barbara, California

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CANINE SCENT AND MICROBIAL SOURCE TRACKING IN SANTA BARBARA, CALIFORNIA

by:

Jill Murray, Ph.D.
City of Santa Barbara

Scott Reynolds
Environmental Canine Services, LLC

Patricia Holden, Ph.D.
University of California, Santa Barbara

Laurie Van De Werfhorst
University of California, Santa Barbara

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For more information, contact:
Water Environment Research Foundation
635 Slaters Lane, Suite G-110
Alexandria, VA 22314-1177
Tel: (571) 384-2100
Fax: (703) 299-0742
www.werf.org
werf@werf.org

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Research Team

Principal Investigator:
Jill Murray, Ph.D.
City of Santa Barbara Creeks Division

Project Team:
Scott Reynolds
Karen Reynolds
Canine helpers Sable and Logan
Environmental Canine Services, LLC

Patricia Holden, Ph.D., P.E.
Laurie Van De Werfhorst
University of California, Santa Barbara

WERF Project Subcommittee

Keith Linn
Retired from Northeast Ohio Regional Sewer District

Dustin Bambic, M.S.
AMEC Earth & Environmental

Sharon Avila
K-9 Training Centers

Water Environment Research Foundation Staff

Director of Research: Daniel M. Woltering, Ph.D.
Program Director: Rhonda Kranz, M.S.

ABSTRACT AND BENEFITS

Abstract:

Advances in microbial source tracking have enabled communities to gain more information about the specific hosts that may be responsible for elevated indicator bacteria levels in recreational waters. However, even when human-specific contamination can be traced to general areas, finding exact origins remains challenging due to sample costs and processing times. This study sought to test the use of a new qualitative tool for source tracking, canine scent tracking (sewage-sniffing dogs), to provide real-time results and low sample cost for illicit discharge detection.

Canine responses were compared against traditional wastewater indicators, illicit discharge detection tracers, and emerging human-specific waste markers in storm drain locations in Santa Barbara, California. Canine scent tracking was also tested for effectiveness in locating contaminated inputs to storm drains, addressing a specific hypothesis of contamination arising from illicit dumping from recreational vehicles, and conducting systematic watershed reconnaissance. Based on the statistical and qualitative results presented in this pilot-scale study, canine scent tracking is a tool that should be expanded for use by researchers and stormwater managers.

Benefits:

- Demonstrates that canine scent tracking is an efficient and effective method that can be added to the toolbox of water quality researchers and stormwater managers.
- Demonstrates that canine responses can be used effectively with traditional and newer, DNA-based methods for assessing contamination with human waste.
- Demonstrates that major advantages of canine scent tracking are the real time results, high number of sites that can be tested per day, and low cost per sample.
- Demonstrates that canine scent tracking can be used to locate sources of contamination to storm drains, as well as bracket areas for further study.
- Demonstrates that canine scent tracking can be used to test specific hypotheses, e.g. that illicit RV dumping may contaminate storm drains, as well as be used for systematic watershed reconnaissance.

Keywords: Microbial source tracking, stormwater contamination, illicit discharge, canine scent tracking.

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LIST OF ACRONYMS

DO	Dissolved Oxygen
ECS	Environmental Canine Services
ELISA	Enzyme-Linked Immunosorbent Assay
HBM	Human-Specific *Bacteroidales* Marker
IDDE	Illicit Discharge, Detection, and Elimination
FIB	Fecal Indicator Bacteria
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
UCSB	University of California, Santa Barbara
USDA	United States Department of Agriculture
U.S. EPA	United States Environmental Protection Agency
WERF	Water Environment Research Foundation

EXECUTIVE SUMMARY

The City of Santa Barbara, located in coastal Southern California, has implemented concerted efforts to address high levels of fecal indicator bacteria (FIB) in recreational waters. While multiple FIB sources including wild and domesticated animals likely exist, in past work with the University of California, Santa Barbara (UCSB), microbial source tracking has shown that human-specific waste markers are present in some storm drains (municipal separate storm sewers, or MS4s) that discharge to creeks and coastal areas. The DNA-based methods used to discover sewage contamination in storm drains are time consuming and expensive, making such approaches impractical for surveying large areas of the City infrastructure. Also, efforts to locate specific inputs to storm drains have been hindered by the lack of a relatively inexpensive, real-time method to assess contamination at large numbers of sites. Canine scent tracking for use in illicit discharge, detection, and elimination (IDDE) work was developed recently, beginning in 2006. One canine, Sable, was trained by Mr. Scott Reynolds, at the time with Tetra Tech and now with Environmental Canine Services, LLC (ECS), to alert to raw sewage and detergents. The dog's sensitivity, based on FIB measurements, was demonstrated previously in field trials.

The City of Santa Barbara, UCSB, and ECS, teamed up to further investigate the use of sewage sniffing dogs to aid in source tracking work with financial support from the Water Environment Research Federation (WERF). The objectives of the project were to: 1) compare canine responses to chemical and microbial source tracking indicators; 2) use canine scent tracking for working upstream of known problem areas to locate or bracket inputs; 3) investigate canine scent tracking for use in testing a hypothesis about recreational vehicle dumping to storm drains; 4) determine the feasibility of canine scent tracking for use in systematic watershed reconnaissance; and 5) conduct training to introduce the approach to stormwater professionals.

ECS provided two trained dogs, Sable and Logan, and two highly experienced human handlers for the field work. Sable is a rescued German shepherd mix which has been trained to alert "Yes" to the scent of contamination by barking (Figure ES-1); Logan is a rescued collie mix which has been trained to alert "Yes" by sitting (Figure ES-2).

Figure ES-1. Sable Alerts to the Scent of Contamination by Barking.

Figure ES-2. Logan Alerts by Sitting.

Over 130 sites were visited, with 26 water samples collected. Methods for water testing included field parameters (temperature, conductivity, and dissolved oxygen), traditional waste water indicators (FIB), IDDE tracers chemicals (potassium, fluoride, ammonia, and surfactants), contemporary human-specific waste markers (human-specific *Bacteroidales*, *Methanobrevibacter smithii* nifH gene) and chemical markers for sewage (caffeine and cotinine). FIB (total coliform, *E. coli* and enterococci) were quantified using defined substrate methods from IDEXX Laboratories (Westbrook, ME). Enterococci were also enumerated using quantitative polymerase chain reaction (qPCR), as was human-specific Bacteroidales (HBM-qPCR). The *Methanobrevibacter smithii* nifH gene was assessed for presence or absence using polymerase chain reaction (Mnif-PCR). Caffeine and cotinine were quantified using commercial enzyme-linked immunosorbent assay (ELISA) test kits. Statistical analyses included nonparametric tests (Mann-Whitney and Chi-square) to determine if the samples with negative and positive canine responses came from different populations, based on chemical and microbial indicators. Statistical tests of canine responses were conducted separately for each dog.

Results from comparing canine and wastewater indicators showed that the dogs' responses were more often positive for samples with higher levels of most microbial and human-specific waste markers. Statistically significant results ($p<0.05$) were obtained for the comparison of both dogs to HBM-qPCR and total coliform and for one of the dogs to *E. coli*, enterococci (by cultivation and qPCR methods), Mnif-PCR, and caffeine. For samples with detectable levels of any of the four human-specific waste markers (11 samples), the two dogs alerted positively 70% and 100% of the time, with associated Chi-square probabilities of 0.13 and 0.0035, respectively. For samples in which both dogs responded negatively (seven samples), no human-waste specific markers were detected.

Efforts to use canine scent tracking to work upstream from known sites of storm drain contamination were successful. In several locations, canine responses led to the bracketing of smaller areas for future work with camera, smoke, and/or dye testing. At one storm drain site known to harbor human waste contamination, on-the-ground field work with the dogs allowed the research team to trace the input to an exact location where leaking sanitary sewer and storm drain pipes were causing untreated sewage to enter the storm drain. The real time results and high number of sites tested per hour by the canines provided a substantive advantage in this type of investigation.

An investigation of hypothesized illicit recreational vehicle dumping of black water tanks to catch basins and drop inlets to storm drains was aided greatly by the inclusion of canine scent tracking. The research team covered over ten city blocks and two parking lots frequented by long-term recreational vehicle dwellers for overnight parking. By investigating every catch basin, drop inlet, and wet spots in the gutter and parking lane, two RVs with leaking black water tanks were identified. No signs of deliberate dumping were observed. Previous efforts by the City to address this hypothesis were stymied by the lack of ability to discriminate between catch basins or gutters that were wet from dumping versus irrigation runoff.

Previous efforts have employed canine scent tracking for illicit discharge detection in rural areas. Here we show that the approach also works in urban settings, where manholes were opened systematically to investigate large areas of Mission Creek watershed. Work in overgrown creek channels was less successful due to the difficulty of observing flowing outfalls.

The primary recommendation from this project is that canine scent tracking can be used effectively in urban source tracking work, primarily to qualitatively survey large areas as a first

tier of investigation that could precede and prioritize quantitative assessment using microbial and chemical human-specific waste markers. As the pool of available canines and handlers expands, attention should be paid to training some dogs to alert to broad suites of markers, while others should be limited to a narrow indicator, in an effort to discriminate among wash water, sewage, and grease trap overflows. Last, an unexpected benefit of canine scent tracking during this project was the increased interest and cooperation from residents, recreational vehicle dwellers, and business owners in illicit discharge detection and stormwater pollution when the dogs were present.

CHAPTER 1.0

BACKGROUND AND OBJECTIVES

1.1 Project Background

This report describes a collaborative research project designed to test the approach of canine scent tracking as a potential tool to be used in conjunction with microbial source tracking by stormwater managers and water quality researchers. The City of Santa Barbara (City) collaborated with Environmental Canine Services, LLC (ECS), along with their trained dogs Sable and Logan, and the University of California, Santa Barbara (UCSB). This project brought together academic research on microbial source tracking, consulting on illicit discharge detection and elimination (IDDE), and a municipality that is conducting research and voluntary efforts to locate and mitigate contamination of recreational waters.

The mission of the City of Santa Barbara's Creeks Division is to improve water quality and restore creeks in Santa Barbara. One of the main goals is to improve recreational water quality at Santa Barbara beaches, which are often posted with warnings due to high levels of fecal indicator bacteria (FIB). The City has collaborated with Dr. Patricia Holden of UCSB to test and employ microbial source tracking methods to delineate the sources of waste in storm drains and creeks that may lead to the high bacteria levels observed at beaches. Results have shown the consistent presence of DNA-based human waste markers in several areas (e.g. Sercu et al., 2009). The City has constructed several capital projects to treat or divert runoff coming from contaminated storm drains before discharging to receiving waters.

However, many areas in Santa Barbara are not suitable for treatment because they are natural creek systems that provide habitat for aquatic organisms, including federally protected species (See Figure 1-1 for study location). In addition, diversion of water that could reach stream channels conflicts with the mission of restoration, and particularly counteracts efforts to restore Southern steelhead populations. Therefore, the City has continued working with Dr. Holden's research group to trace contaminated drainage further upstream in storm drain networks, with the goal of identifying the physical sources of human waste (e.g., illicit connections). Using DNA-based, chemically-based, and conventional fecal indicator-based approaches, contamination has been traced for some areas in the City. However, due to the temporal and spatial variability of the contamination, along with expense and turnaround time for the methods (<12 samples per day, and >10 days for results), extensive sampling and analysis in defined areas has been required to obtain actionable results. A first tier of field data that defines potential regions of contamination would be useful for focusing more expensive and specific quantitative approaches. Human-waste specific approaches will be useful over the long term for confirming and quantifying human waste in water samples, but an accurate and inexpensive method of surveying for sewage contamination is sorely needed to guide efforts.

1.2 Canine Scent Tracking

An emerging source tracking tool is the use of canine scent tracking, or sewage-sniffing dogs, much as canines have been used in other fields. Since 1984, the United Stated Department of Agriculture (USDA) has been using Beagles to detect fruits, vegetables, and meats brought

into the country by international travelers (USDA, 2007). Current medical research is testing the accuracy of canines to detect skin, lung and bladder cancer in human subjects (Horvath et al., 2008 and references in Helton, 2009). Canine scent tracking is also used in ecological contexts, such as mapping populations of fox species based on discrimination of scat by detector dogs (Smith et al., 2005) and aiding researchers in locating floating whale feces (Rolland et al., 2006) and woodland carnivore feces (Long et al., 2007).

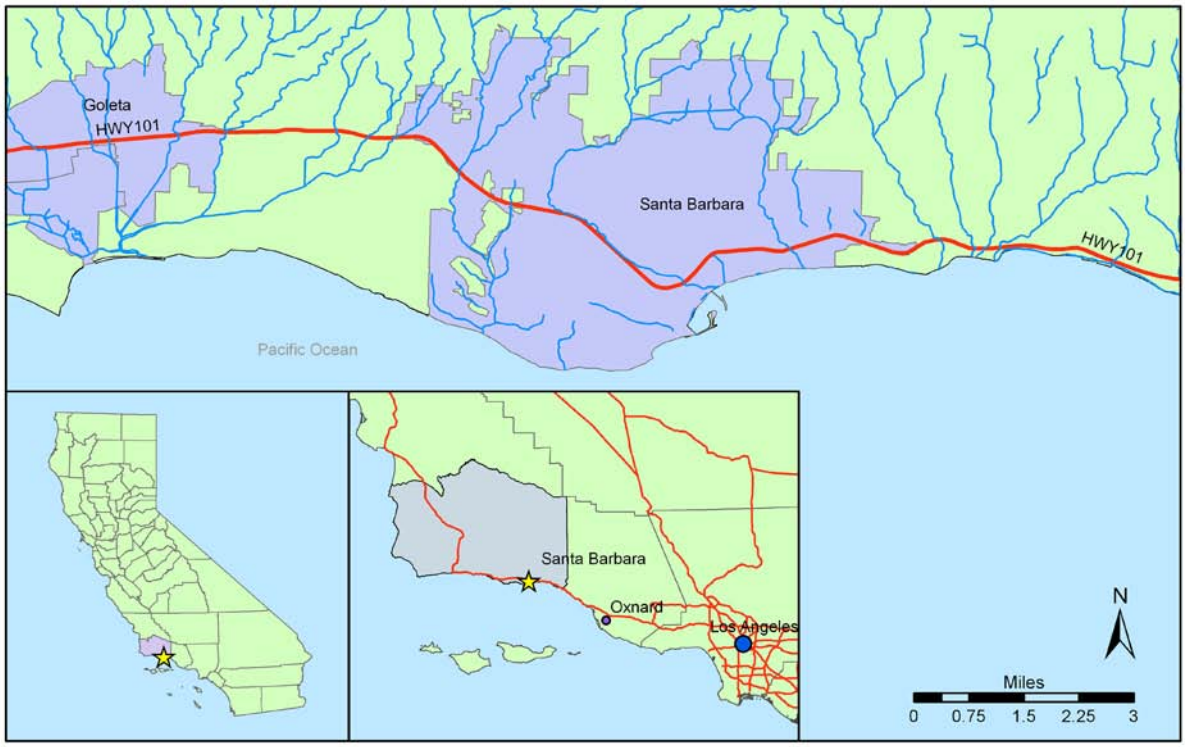

Figure 1-1. Study Location.

In December 2006 Mr. Scott Reynolds of ECS, formerly and Environmental Scientist with Tetra Tech, and Mr. Dan Christian of Tetra Tech, began developing the idea to train a dog to locate illicit connections to municipal storm sewer systems. Based on the most common illicit discharges, raw human sewage and detergents were used for the first dog's training. In 2007, a German shepherd mix shelter dog named Sable was selected and trained by Mr. Reynolds for three months with the target scents in various terrain and environments. Sable was taught to alert by barking when a target scent was located, and double blind trials were conducted. Mr. Reynolds and Sable then conducted field work with IDDE field crews who were conducting investigations on storm sewer systems. Results from the first two years of training and field work were promising and suggested the success of a tool for source tracking that can effectively guide field personnel to locations for sampling and quantitative analyses of water contaminants. By summer 2010, when the research described in this report was conducted, an additional dog handler (Mrs. Karen Reynolds) and dog (Logan, a rescued collie mix) had also been trained.

Canine scent tracking has the potential for low cost per sample, throughput of up to 50 sites per day, and real-time results. Sable was able to locate illicit connections and broken sewer lines that leaked into storm drains in Michigan (Reynolds et al., 2009). One limitation in the method gaining widespread acceptance is that canine responses had not been tested against

definitive sewage markers such as the DNA-based methods used in academic research and now employed by commercial labs.

1.3 Human Waste-Specific Markers

Several microbial and chemical methods are used in this project to ascertain the presence of human waste in storm drain samples.

- The HBM-qPCR assay measures the quantity of human-specific HF183 *Bacteroidales* 16S rRNA markers present in a sample. The presence of this marker indicates human fecal contamination (human feces, septage, sewage), and the number of marker copies detected indicates the relative degree of contamination. This assay has high specificity and a wide geographic range (Seurinck et al., 2005; Shanks et al., 2010).
- The Mnif-PCR assay determines presence or absence of the *nifH* gene of *Methanobrevibacter smithii*, which is a methanogen found in human feces and sewage (Ufnar et al., 2006).
- The caffeine measurement is a direct competitive enzyme-linked immunosorbent assay (ELISA) that measures the quantity of caffeine in a sample. Caffeine is a widely consumed drug (e.g. coffee, tea, soft drinks, chocolate, pharmaceuticals), and is excreted by humans into the wastewater stream.
- The cotinine measurement is an ELISA method that measures the quantity of cotinine in a sample. Cotinine is a metabolite of nicotine, and is excreted by humans into the wastewater stream.

Additional microbial and chemical methods, e.g. FIB and surfactants, employed in this research can be used as wastewater indicators, but they are not human specific and can be found in sources other than wastewater.

1.4 Objectives

The existing collaboration between the City and UCSB, along with the results and knowledge generated thus far, provided an ideal testing ground for canine scent tracking. With assistance from UCSB, the City recently completed a study in the Laguna Watershed (Geosyntec 2009) which is entirely developed and contains mostly sub-surface storm drains. Consistent signals for human waste were detected in approximately half of the 17 sites tested, providing a defined area in the City for studying the relationships between scent tracking and molecular laboratory results. In addition, the City had specific hypotheses about sewage sources in this area, e.g. illicit dumping from recreational vehicles, that could be tested with canine scent tracking. The City also sought to test the use of canine scent tracking in systematic watershed reconnaissance in other parts of the City.

The overall aim of the proposed study was to test the use of canine scent tracking for several different applications of illicit discharge detection. The study included the following objectives:

1) Correlation Study: Determine if sampling locations historically contaminated with sewage as determined by quantitative DNA-based microbial source tracking, traditional indicator bacteria tests, and chemical fingerprinting are detected as contaminated by canine scent tracking.

2) Detection of Physical Locations of Human Waste Signals: Determine if canine scent tracking can be used to further locate or bracket sources of human waste in a storm drain network that

have been indicated by microbial source tracking.

3) Illicit Recreational Vehicle (RV) Dumping: Determine if canine scent tracking can be used to test the hypothesis that storm drain contamination arises from illicit dumping of black water tanks by RV owners.

4) Systematic Watershed Reconnaissance: Determine the efficacy of canine scent tracking to aid a systematic approach to illicit discharge detection and outfall reconnaissance.

5) Training: Determine the feasibility of bringing canine scent tracking to stormwater groups.

CHAPTER 2.0

PROJECT APPROACH

2.1 Correlation Study

The study design for this objective was to compare canine responses with traditional wastewater indicators (fecal indicator bacteria), tracers used in IDDE efforts (ammonia, fluoride, potassium and surfactants; Pitt 2004), and contemporary human-specific waste markers (human-specific *Bacteroidales*, *Methanobrevibacter smithii* nifH gene). While not originally proposed, it was decided after this project was initiated to also benchmark canine responses to two other potential chemical indicators of sewage (caffeine and cotinine). Based on previous studies in the Laguna Watershed, it was expected that approximately half of the sites would exhibit contamination with human-specific waste markers. The approach was a single-blind study because the dogs and handlers did not have direct access to previous results and thus did not know which sites within the study area had previously been shown as contaminated with human waste or sewage.

2.2 Physical Locations of Human Waste Signals

Based on previous results and canine response during the correlation study, several contaminated sites were chosen for further investigation. Canine scent tracking was used to test many additional manholes and catch basins located around the sites that had been tested in the correlation study and in other areas of Santa Barbara. A subset of the sites were sampled and tested as in the correlation study. The canine responses were used to more narrowly bracket problem reaches. The study design was double-blind, in that ECS, UCSB, and City staff did not know results of testing until the study was complete.

2.3 Detection of Illicit Recreational Vehicle Dumping

Canine scent tracking was used to canvas a neighborhood in which RV dwellers are known to park for long periods of time (one day to several weeks), testing the hypothesis that illicit dumping of RV black water tanks in storm drains could be a source of waste to Santa Barbara creeks. All catch basins, drop inlets, and wet spots on gutters or in the parking lane were tested for canine responses. If positive response were detected, a sample would be collected if sufficient liquid could be recovered. The design of this study was test-of-concept, i.e. to explore whether the canine approach could be used for covering large geographic areas to test a specific hypothesis.

2.4 Systematic Watershed Reconnaissance

The City and ECS sought to determine how many square blocks of urban area and how many miles of creek can be surveyed in a set time period using canine scent tracking and sample collection upon a positive alert. From this effort, the City determined the feasibility of using canine scent tracking for a systematic approach to detecting illicit connections. The design of this study was proof of concept.

2.5 Training

ECS conducted a training session with stormwater professionals. The goal was to demonstrate the technique and conduct a dialogue in which the feasibility of the approach was discussed, rather than to train dogs and their human partners in this short period of time.

CHAPTER 3.0

METHODS

3.1 Canine Responses

ECS provided two dog-trainer pairs to conduct canine scent tracking. Sable is a rescued German shepherd mix that had completed training, field trials, and field work with Mr. Scott Reynolds prior to arriving in Santa Barbara, CA. Sable is trained to alert by barking. Logan is a rescued rough coat collie who began scent training with ECS in late 2009 and began field trials during this project. Logan is trained to alert by sitting.

At each storm drain test site, City personnel opened the manhole lid while the handler and canine waited. Without looking into the structure the handler gave the canine its individual search command and walked to the open structure. If the canine gave the trained alert, he was rewarded and led away. If no alert was given the canine was given verbal praise only and led away. The second team repeated this procedure with no contact with the previous handler. Each canine's results were recorded as a yes or no for alerts at each designated site (see Chapter 4.0, Results for exceptions).

To eliminate the potential for influencing the reactions of the canines during investigation, the canine handlers had no prior knowledge of previous testing results of the storm drainage systems. It was also decided that Logan and handler would check designated areas first. Due to Logan's "silent" alert, Sable and handler would not be able to hear the results and would position themselves so the investigation site was not visible. This method further reduced the chance of influencing the handlers interactions with their canine based on visual or auditory cues.

A portion of the sewage obtained for positive control use in the microbial assays was also used to test the dogs' responses on two separate occasions. For the first, approximately 50 mL of sewage was placed into a 500 mL plastic beaker and covered with a piece of plastic window screening material to prevent the dogs from direct contact with the liquid. Each dog's response to this single sample was recorded twice. For the second positive control testing a few days later, approximately 50 mL of sewage from the same initial sample (refrigerated during storage and warmed to room temperature before testing) was placed into three 100 mL FIB bottles (IDEXX Laboratories, Westbrook, ME). One bottle at a time was placed on the ground and covered with small metal cage to prevent the dogs from direct contact with the liquid. Each dog's response to each of the three samples was recorded.

3.2 Sampling and Canine Response Locations

Maps of all sampling locations are presented in Chapter 3.0.

3.2.1 Correlation Study

For the correlation study phase, water samples were taken regardless of the dogs' responses. From June 7-9, 2010, 15 water samples were grab sampled from 13 locations (Table

3-1). All samples except for S-09 were from storm drain, i.e., MS4 or storm sewer, manholes in the street, unless noted (e.g. in sidewalk). Sample S-09 was taken from an open creek channel. Two locations were sampled twice to capture temporal changes (S-04 & S-08; S-13 & S-18).

Due to the temporal variability observed in past studies, the correlation study was interrupted at times to pursue the second effort, detection of known inputs to the storm drain network. After taking the dogs to multiple manholes and catch basins, thereby bracketing an area for future analysis, we returned to the sites for the correlation study. In addition, some types of field work were best conducted in the morning hours, i.e. due to potentially higher sewage flow rates, so efforts towards each objective were not completed sequentially.

Table 3-1. Sampling Locations for Correlation Study.

ID	Date	Latitude	Longitude	Street Intersection or Site
S-01	6/7/2010	N34 25.386'	W119 41.329'	Salsipuedes & Haley
S-02	6/7/2010	N34 25.460'	W119 41.394'	Cota @ Salsipuedes (in sidewalk)
S-03	6/7/2010	N34 25.684'	W119 41.423'	De La Guerra & Nopal (in sidewalk)
S-04	6/7/2010	N34 25.696'	W119 41.276'	Milpas & Ortega
S-05	6/7/2010	N34 25.564'	W119 41.257'	Nopal & Cota
S-08	6/8/2010	N34 25.696'	W119 41.276'	Milpas & Ortega
S-09	6/8/2010	N34 25.133'	W119 41.279'	Laguna channel near Highway 101 overpass
S-10	6/8/2010	N34 25.210'	W119 41.241'	Montecito & Olive
S-11	6/8/2010	N34 25.210'	W119 41.386'	Laguna & Gutierrez
S-13	6/8/2010	N34 25.398'	W119 41.635'	Laguna between Ortega & De La Guerra
S-15	6/9/2010	N34 25.068'	W119 40.731'	Quarantina & Cacique
S-16	6/9/2010	N34 25.345'	W119 40.962'	Yanonoli @ Nopal
S-17	6/9/2010	N34 25.405'	W119 41.177'	Quarantina & Gutierrez
S-18	6/9/2010	N34 25.398'	W119 41.635'	Laguna between Ortega & De La Guerra
S-19	6/9/2010	N34 25.434'	W119 41.694'	Laguna & De La Guerra

3.2.2 Detection of Physical Locations of Known Human Waste Signals

For this study phase, samples were taken either to bracket known "hot spot" locations or when one or both dogs displayed positive responses. Several areas were chosen for further investigation. From June 7-9 and 15, 2010, canine tests were conducted at nine locations (see Results for locations), and water samples were collected from eight of these sites (Table 3-2). All samples except for S-14 were from storm sewer manholes in the street, unless noted (e.g. in sidewalk). Sample S-14 was taken from an open creek channel, approximately 30 minutes after the dogs assessed the site.

Table 3-2. Sampling Locations for Detection of Physical Locations of Known Human Waste Signals.

ID	Date	Latitude	Longitude	Street Intersection or Site
S-06	6/7/2010	N34 25.740'	W119 41.498'	Nopal & Canon Perdido
S-07	6/7/2010	N34 25.761'	W119 41.479'	Canon Perdido between Philinda & Nopal (in sidewalk)
S-12	6/8/2010	N34 25.326'	W119 41.535'	Laguna & Cota
S-14	6/8/2010	N34 25.398'	W119 41.636'	Old Mission Creek upstream of Westside drain
S-20	6/9/2010	N34 26.290'	W119 44.856'	Hope drain diversion
S-21	6/9/2010	N34 26.429'	W119 44.937'	State & Plaza (in sidewalk)
S-24	6/15/2010	N34 24.944'	W119 41.799'	Haley & Chapala
S-25	6/15/2010	N34 24.995'	W119 43.108'	Micheltorena & Gillespie

3.2.3 Illicit RV Dumping

No water samples were taken during this sampling phase. The canine testing area for illicit RV dumping included two parking lots identified by the City as available for overnight recreational vehicle parking and 10 city blocks with frequent overnight RV parking. Each canine was taken to the catch basins and curb inlets in the parking areas and given a search command. Dogs also investigated RVs parked along city streets and the beach front. Each canine was given a search command and walked around each parked RV, paying particular attention to wet areas under or around the RV and adjacent curbs. They were also directed to inspect any damp areas along curbs or low spots. Each canine's results were recorded. Although the dogs identified two RVs with liquid leaking from their septic tank areas, there was insufficient liquid for microbial source tracking analysis.

3.2.4 Systematic Watershed Reconnaissance

For watershed reconnaissance, samples were taken only when one or both dogs displayed a positive response. Two areas were chosen for systematic watershed reconnaissance. For creek outfall tracking, the lower portion of Sycamore Creek was walked on June 11, 2010. No samples were collected due to the lack of positive alerts from the dogs. For storm drain surveying, a portion of Mission Creek watershed was surveyed during June 14-15, 2010. The effort was aided by a very detailed Storm Drain Atlas and City crews to direct traffic when necessary. Positive and negative responses were recorded at each location. Three water samples were taken from three different locations (Table 3-3) from June 14-15, 2010. Sample S-22 was taken from a daylight section of a storm drain. Samples S-23 and S-26 were from storm sewer manholes.

Table 3-3. Sampling Locations for Watershed Reconnaissance Study.

ID	Date	Latitude	Longitude	Street Intersection or Site
S-22	6/14/2010	N34 24.251'	W119 41.860'	Honda drain @ Santa Barbara City College
S-23	6/14/2010	N34 25.227'	W119 42.182'	Chapala & Carrillo
S-26	6/15/2010	N34 25.015'	W119 40.358'	Milpas @ Cabrillo

3.3 Water Sampling and Field Measurements

Water samples (approximately 2 L) were grabbed from each sampling location. Depending on access and depth, samples were taken using either a sterile plastic beaker or Isco

6712 Full-size Portable Sampler (Teledyne Isco Inc., Lincoln, NE). When used, the Isco sampler lines were flushed with Nanopure DI water in between samples, and rinsed with sample water prior to collection. All water samples were passed through 25 μm pore size Miracloth (Calbiochem, San Diego, CA) to remove large debris, and stored on ice until processing (within 6 hours). Dissolved oxygen (DO), temperature, and conductivity were measured in the field with a Hach HQ40d meter with laser DO and conductivity probes (Hach Company, Loveland, CO).

A sewage sample was obtained from El Estero Wastewater Treatment Plant (Santa Barbara, CA) on June 11, 2010, to serve as a positive control. The sample (approximately 1 L) was processed and analyzed in the same way as the water samples, except it was not passed through Miracloth.

3.4 Microbial Analyses

All microbial analyses were carried out in the laboratory of Dr. Patricia Holden at the University of California, Santa Barbara, similarly to prior studies (Sercu et al., 2009; UCSB Laguna Channel study report, 2009).

3.4.1 Fecal Indicator Bacteria (FIB)

FIB (total coliform, *E. coli* and enterococci) most probable numbers (MPNs) were quantified using the Quanti-Tray®/2000 method, according to the manufacturer's instructions (IDEXX Laboratories, Westbrook, ME). Aliquots from each water sample were diluted in sterile Nanopure water prior to analysis. Dilution amount was based on previous FIB results for locations that were sampled in earlier studies. Samples from new locations were diluted at least tenfold. Sample duplicates were performed for at least every 10 samples processed, and results were averaged before reporting. If one of the sample duplicates had a value out of range (< or >) for one of the analytes, the duplicate with values in range for that analyte was reported.

3.4.2 DNA Extraction

The PowerWater® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) was used to filter and extract DNA from the water samples. Water samples were vacuum filtered through 0.22 μm filters (included in the kit) until either the collected volume was filtered or to the point of refusal. Actual volume filtered was recorded, and the filters were removed using sterile forceps and stored at -20°C in the bead beating tubes provided with the extraction kit. DNA was extracted following the manufacturer's protocol, followed by ethanol precipitation. Total DNA was quantified using the Quant-iT™ dsDNA Broad-Range Assay Kit (Invitrogen, Carlsbad, CA) on a BioTek Synergy 2 plate reader. Standard curve and sample concentrations were calculated using BioTek's Gen5™ Reader Control and Data Analysis Software.

3.4.3 Quantitative Polymerase Chain Reaction (qPCR)

The qPCR assay for salmon testes DNA was performed prior to the *Enterococcus* spp. and human-specific *Bacteroidales* qPCR assay and the *Methanobrevibacter smithii* nifH gene polymerase chain reaction (PCR) assay in order to determine the lowest template dilution without inhibition.

3.4.4 Salmon Testes DNA qPCR

The salmon testes DNA qPCR assay was performed using a dual-labeled (BHQ-FAM) probe (Eurofins MWG Operon, Huntsville, AL) and TaqMan chemistry (Haugland et al., 2005; Morrison et al., 2008) in a Bio-Rad CFX 96 thermocycler. The qPCR MasterMix for Probe

Assay (no ROX) (Anaspec, Fremont, CA) was used in final reaction volumes of 25 µl, including 2.5 µl of diluted DNA template.

The thermocycling program was: 2 min at 50°C, 10 min at 95°C, 45 cycles of 15 sec at 95°C and 60 sec at 60°C. The qPCR master mix was spiked with salmon testes DNA, to a final concentration of 0.25 ng/reaction. Four no-sample DNA reactions (= no inhibition control) were run on each plate, in which only salmon testes DNA, PCR reagents and PCR-grade water were added. In addition, a 3 log salmon testes DNA standard curve was run to determine amplification efficiency. Samples were run in duplicate.

Baseline threshold was set to 200 for data analysis. Using the no-inhibition controls, the average + 3 × standard deviation cycle threshold value (Ct_{ni}) was calculated. This value was used as the upper Ct value for no inhibition. All reactions with sample DNA that produced an average $Ct > Ct_{ni}$ were considered to be inhibited. The assay was first run using 1/5 diluted DNA template to determine the occurrence of reaction inhibition. If inhibition occurred, twofold dilutions were analyzed until no inhibition occurred. The lowest template dilution without inhibition was used for other qPCR and PCR assays utilized in this project.

3.4.5 *Enterococcus* spp. qPCR

The *Enterococcus* spp. qPCR assay was performed according to published methods (Haugland et al. 2005) using the same chemistry and qPCR parameters as detailed for the salmon testes DNA qPCR assay. The primer and probe concentrations were 900 nM (forward primer), 300 nM (reverse primer) and 100 nM (probe). Final reaction volume was 25 µl, including 2.5 µl of diluted DNA template (dilution determined by salmon testes DNA qPCR). Standard concentrations ranged from 1E+06 to 2.5E+01 markers/reaction. All samples and standards were analyzed in triplicate.

Each plate was standardized by adjusting the baseline threshold position until the coefficient of variation was less than 3% for each of the standard dilutions from run to run. The resulting sample Ct values were then used to calculate the number of *Enterococcus* spp. markers per liter of sample filtered, and the triplicate values for each sample were averaged. Only samples with two or more analytical replicates that amplified within the range of the standards were quantified. The cell equivalents (c. eq.) per 100 ml were also calculated, by assuming an rrn operon (ribosomal RNA operon) copy number of 6 for *Enterococcus*.

3.4.6 Human-Specific *Bacteroidales* qPCR (HBM-qPCR)

The assay for human-specific *Bacteroidales* qPCR was performed using SYBR® Green I chemistry (Sercu et al., 2009) in a Bio-Rad iQ5 thermocycler. The qPCR Core Kit for SYBR® Green I (Anaspec, Fremont, CA) was used in final reaction volumes of 25 µl, including 2.5 µl of diluted DNA template (dilution determined by salmon testes DNA qPCR). Primer concentrations were 250 nM.

The thermocycling program was: 2 min at 50°C, 10 min at 95°C, 40 cycles of 30 sec at 95°C, 60 sec at 53°C and 60 sec at 60°C. Melt curve analysis was performed from 60 to 94.8°C, in increments of 0.4°C per 10 sec. A human-specific *Bacteroidales* marker (HBM) standard was previously created by purifying PCR products from amplified DNA extracted from multiple sewage samples. Standard concentrations ranged from 5E-03 to 5E-09 ng/reaction. All samples and standards were run in triplicate.

Each plate was standardized by adjusting the baseline threshold position until the

coefficient of variation was less than 3% for each of the standard dilutions from run to run. The resulting sample Ct values were then used to calculate the number of HBM per liter of sample filtered, and the triplicate values for each sample were averaged. Only samples with two or more analytical replicates that amplified within the range of the standards were quantified. Melt curves were validated for all sample replicates amplifying within the quantification range.

3.4.7 *Methanobrevibacter smithii* PCR for the nifH Gene (Mnif-PCR)

The assay for detection of nifH genes of *Methanobrevibacter smithii* was modified from an existing method (Ufnar et al., 2006). A two step PCR method was used, where the PCR product of round 1 was diluted and amplified again with the same protocol in a 2nd round of PCR. All PCR reactions were performed in 25 µl reactions using the Taq PCR Core Kit (Qiagen, Valencia, CA). Each reaction contained 1x PCR buffer, 1x Q-solution, 0.2 mg/ml bovine serum albumin, 0.2 mM of each dNTP, 500 nM of each primer, and 1.25 U of Taq Polymerase. In round 1, 1 µl of diluted DNA template (dilution determined by salmon testes DNA qPCR) was added, while in round 2, 1 µl of 1/10 diluted PCR product from round 1 was added. For each set of samples, a non-template control and positive control (sewage) were included and analyzed.

Thermocycling conditions were 3 min at 92°C for initial denaturation, 30 cycles of 1 min at 92°C, 30 sec at 55.1°C and 1 min at 72°C, and final extension for 6 min at 72°C. PCR products were visualized by running 3 µl PCR product on a 2% agarose gel stained with 0.5 mg/ml ethidium bromide. Electrophoresis was run at 60 V for approximately 20 minutes. Samples with a band at 222 bp as bright as the positive control were scored as '++' (strongly positive), and samples with the correct band but fainter than the positive control were scored as '+' (weakly positive). Samples without a visible band at 222 bp were considered '-' (negative). All positive samples were analyzed in full for a second time to validate results.

3.5 Chemical Analyses

3.5 ELISA (Caffeine and Cotinine)

Water samples were also archived for ELISA analysis by UCSB. While not specified in the original proposal, these assays were performed to complement the DNA-based assays for sewage markers. Using a sterile 10 ml syringe, approximately 5-10 ml of each sample was grabbed from the sampling beaker (prior to its filtration through Miracloth). The ELISA samples were then filtered through 0.2 µm PTFE syringe filters into amber glass vials and stored on ice until transport to the lab. Samples were archived at -20°C until analysis for caffeine and cotinine (a metabolite of nicotine).

3.5.2 Surfactants

As an indicator of detergents, anionic surfactants were measured as Methylene Blue Active Substances (SM 5540C). Samples were kept on ice and delivered by overnight courier to Test America, Inc. (Irvine, CA) where the analysis was completed within recommended hold times.

3.5.3 Potassium, Fluoride, and Ammonia

Samples for potassium, fluoride, ammonia were delivered on ice to the City of Santa Barbara's Wastewater Treatment Laboratory and tested within acceptable hold times. Potassium was determined using the Direct Air-Acetylene Flame Method with an Atomic Absorption Spectrophotometer (SM 3111B). Fluoride was analyzed by an inorganic anion determination using ion chromatography (U.S. EPA 300.0), and ammonia was analyzed using a selective electrode method (SM 4500 NH3).

3.6 Statistical Analyses

Statistical analyses were performed with Systat 12 (Systat Software, Inc.). Due to small samples sizes, non-normal distributions, and high frequencies of non-detectable results, i.e. censored data (Helsel, 2004), we chose nonparametric statistical methods (Conover, 1998). Samples from all phases were combined in the analysis due to the small number of contaminated samples identified in the correlation study samples. As described in the results, site S-21 was considered an outlier and was not included in the analyses for Sable. The research team reports central tendencies as medians, and describe distributions using the maximums and minimums. Statistical analyses included Chi-square and Mann-Whitney, with results considered significant with $p<0.05$.

The Mann-Whitney test is similar to a Student's t test, but it is conducted on the ranks of the data rather than the magnitude. Therefore, procedures such a log transformations do not change the test outcomes. In addition, non-detects are treated as equal ties, ranked below the lowest detected value (Helsel, 2004). Here, non-detects were assigned the value 0 for calculations. Right-censored data, i.e. values greater than n upper threshold were treated as equal to the highest threshold for the variable, e.g. all total coliform >24,196 MPN/100 mL were coded as 241,960 MPN/100 mL for the analysis, because there was one result >241,960 MPN/100 mL. Again, because the tests are carried out on ranks, the values chosen for censored data do not influence the outcome.

Each dog's responses to the microbial, chemical and field variables were compared. For each variable, results were separated into two samples, those with "Yes" canine responses and those with "No" canine response (the analysis was conducted separately for each dog). We used the Mann-Whitney test to test whether the "Yes" samples and "No" samples were likely to have come from the same population. A positive correlation was reported when the average rank for the "Yes" sample was higher than the average rank for the "No" sample. For FIBs, human-specific markers, potassium and MBAS, one-tailed probabilities are reported unless the correlation was negative. For these exceptions, and for fluoride and field parameters, two-tailed probabilities are reported. For Mnif-PCR, which is reported only by presence/absence, Chi-square tests were used, with probabilities reported for Fisher's exact test (one-tailed). In addition, the combined human-specific markers were compared with the canine responses. A categorical variable was created that was marked as positive if any human specific markers were detected, and compared this variable to each of the dogs' responses using the Chi square test (one tail).

CHAPTER 4.0

RESULTS

4.1 Canine Field Work

Over the course of seven field days, both dogs were taken to:

- A total of 133 sites.
- 13 sites that had been tested during the previous Laguna Watershed Study.
- 13 additional sites that were sampled for water testing.
- 107 sites that were tested for canine responses but not sampled.

The dogs also:

- Walked one mile of creek.
- Walked and investigated recreational vehicles parked along 10 blocks plus two parking lots.

Both dogs (Sable and Logan) performed without any hesitation at the first site, with Logan providing a positive alert ("Yes") by sitting, and Sable providing a positive alert by barking (Figures 4-1 and 4-2). Canine responses at each site are shown in Figures 4-3, 4-4, and 4-5.

Figure 4-1. Logan Alerting ("Yes") by Sitting After Sniffing a Storm Drain Manhole.

Figure 4-2. Sable Alerting ("Yes") By Barking After Sniffing a Storm Drain Manhole.

Both dogs were able to work full days, and both appeared able and willing to work longer hours than their human companions, even during hot weather. The dogs interacted very well with members of the public, who were quite interested in the field work. The dogs most frequently gave very clear positive alerts or were clearly not responding, but in some cases their responses were ambiguous, e.g. a subdued bark, or sitting prior to sniffing the test site. The dog handlers used their experience and expertise to record "Yes" or "No" answers for the sites where water samples were collected. Results of "Unclear" were recorded for sites where no samples were collected. At a limited number of sites we only have data from one dog because the other was taking a short break.

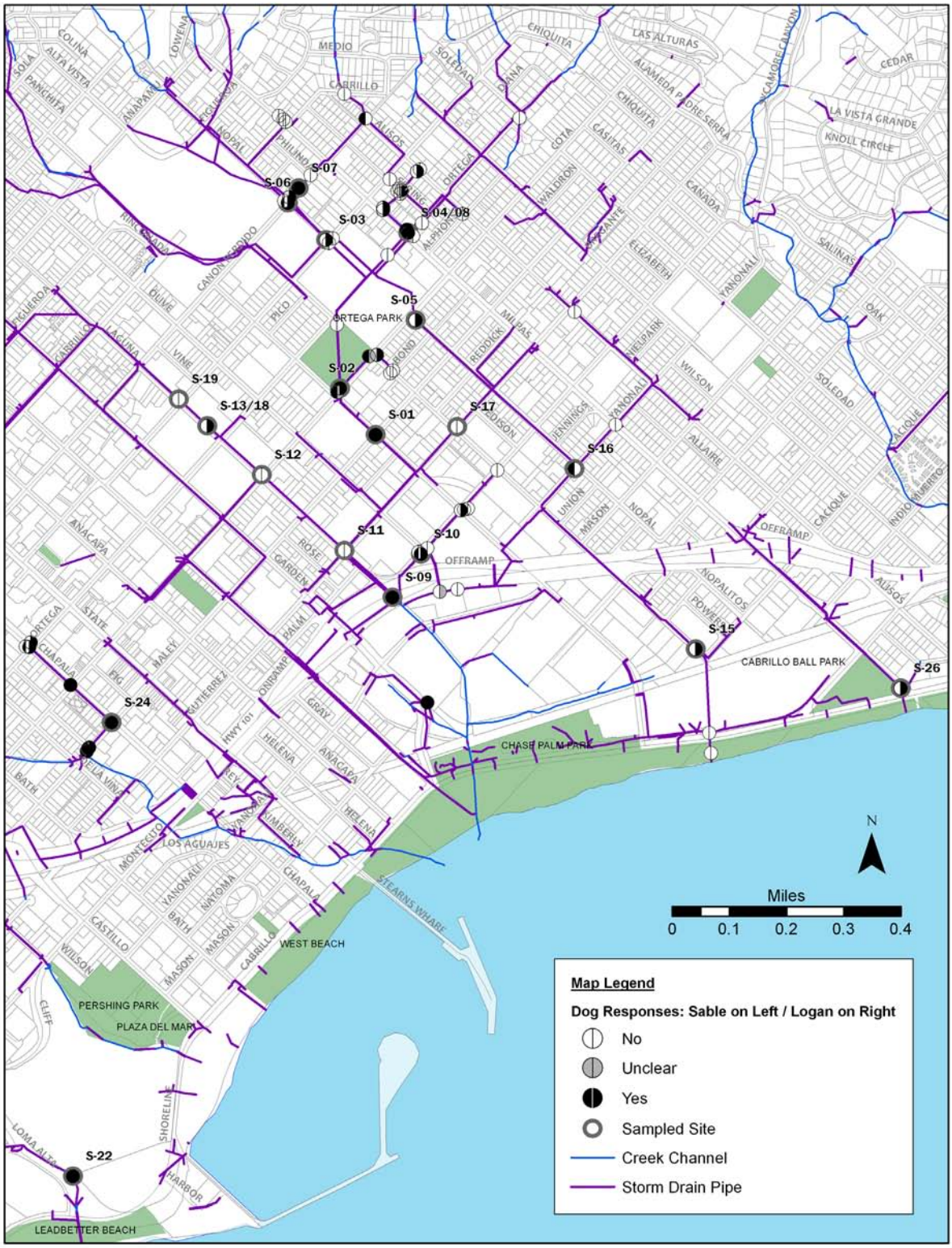

Figure 4-3. Canine Responses in the Laguna Watershed and Haley Diversion Area. Site Numbers Indicate That Water Samples Were Also Collected, with Results Shown in Tables 4-1 to 4-3.

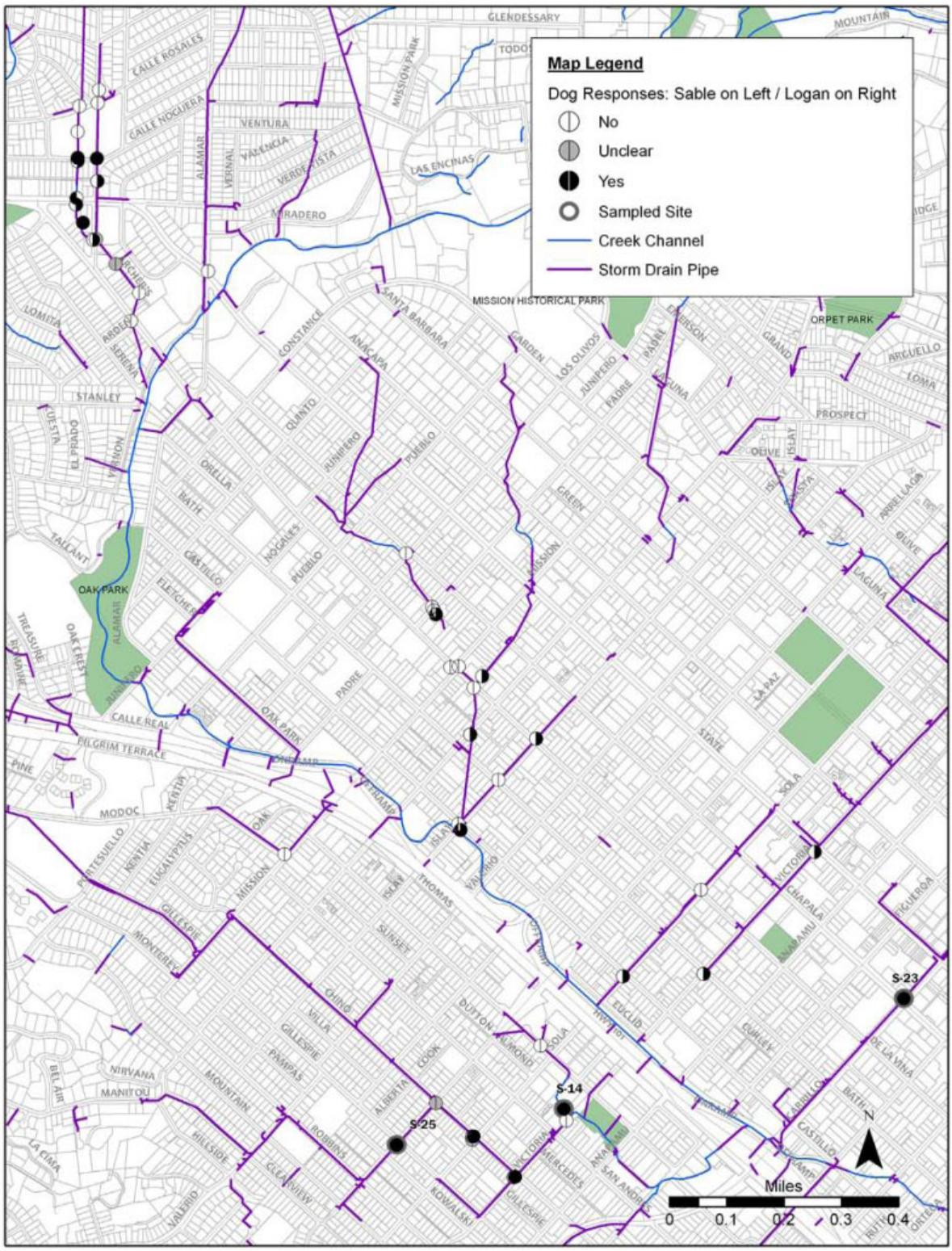

Figure 4-4. Canine Responses in the Mission Creek Area. Site Numbers Indicate That Water Samples Were Also Collected, with Results Shown in Results Shown in Tables 4-1 to 4-3.

Figure 4-5. Canine Responses in the Hope Diversion Area. Site Numbers Indicate That Water Samples Were Also Collected, with Results Shown in Tables 4-1 to 4-3.

4.2 Correlation Study

Over the course of seven field days with ECS and UCSB, 26 water samples were collected from storm drains and open channels (Figures 4-3, 4-4, and 4-5). Samples were collected for the Correlation Study, the Detection of Physical Locations of Human Waste, and Watershed Reconnaissance. Due to the small number of samples collected, the results from all samples are grouped together for presentation and statistical analyses. Samples ranged from relatively uncontaminated groundwater pumped to a storm drain, to highly contaminated runoff. As discussed below, samples S-20 and S-21 represent storm drain samples that were contaminated with a direct input of untreated sanitary sewage due to an underground leak. A positive control for sewage was also conducted, and results that were obtained for this sample are also presented in the appropriate tables below.

4.2.1 Field Measurements

Field measurements showed that temperature, dissolved oxygen, and conductivity were relatively consistent among samples (Table 4-1).

Table 4-1. Field Measurements.

Sample ID	Temperature, °C	Dissolved Oxygen, % Saturation[1]	Dissolved Oxygen, mg/L[1]	Conductivity, mS/cm
S-01	20.2	76.7	6.98	1.98
S-02	19.6	45.8	4.22	5.52.
S-03	19.7	47.6	4.38	2.82
S-04	19.5	60.1	5.52	1.60
S-05	20.6	45.1	4.06	2.21
S-06	21.1	70.5	6.29	2.45
S-07	19.1	101.1	9.34	0.01
S-08	19.3	68.4	6.34	1.59
S-09	19.2	71.1	6.58	1.26
S-10	21.9	93.5	8.22	2.36
S-11	21.0	69.9	6.27	1.23
S-12	21.0	76.9	6.85	1.18
S-13	21.7	74.3	6.57	1.65
S-14	19.0	63.3	5.86	1.15
S-15	21.0	76.4	6.79	4.37
S-16	19.9	71.9	6.55	3.17
S-17	21.1	85.4	7.61	1.87
S-18	22.1	84.1	7.33	1.66
S-19	21.5	N/A	N/A	1.77
S-20	22.3	62.4	5.37	1.95
S-21	25.3	N/A	N/A	2.18
S-22	22.1	158	13.8	4.05
S-23	24.0	2.1	0.18	2.42
S-24	22.9	N/A	N/A	1.45
S-25	22.4	103.8	8.96	1.37
S-26	22.8	68.3	5.88	1.61

[1]N/A indicates the LDO probe was unable to provide a dissolved oxygen reading for the sample.

4.2.2 Fecal Indicator Bacteria

As shown in Table 4-2, FIB results ranged from non-detectable (<10 MPN/100ml) to above quantification thresholds (>241960 MPN/100 mL at the dilution levels used here). *Enterococcus* concentrations measured by Enterococcus-qPCR were highest in samples S-23, S-20, and S-21, corresponding to approximately 72%, 0.9%, and 1.9% of the sewage sample, respectively. Two samples (S-13, S-18) did not have concentrations within quantification range. These two samples were taken from the same location on two different dates (6/8/10 and 6/9/10).

4.2.3 Human Specific Markers

Human-specific markers were detected in 11 samples (Table 4-2). Human specific *Bacteroidales* markers (HBM-qPCR) were detected in seven samples, and human waste-specific *Methanobrevibacter smithii nifH* gene targets (Mnif-PCR) were detected in five of these samples. Caffeine and human waste-specific cotinine were detected in two of the HMB-qPCR positive samples, and four and one additional sample, respectively. Concentrations ranged from 1 to>100% of those found in the sewage sample.

Table 4-2. Fecal Indicator Bacteria, Human-Specific Waste Markers, and Canine Results.

	Fecal Indicator Bacteria				Human-Specific Waste Markers				Canines	
ID	Total Coliform, IDEXX, MPN/100 ml	*E. coli*, IDEXX, MPN/100 ml	*Enterococcus* IDEXX, MPN/100 ml	*Enterococcus* qPCR, Average C.eq./100 mL	HBM-qPCR, Average Copies/L	Mnif-PCR, ++/+/-[1]	Caffeine, Average ppb	Cotinine, Average ppb	Sable, Yes/No	Logan, Yes/No
S-01	> 24196	1314	1935	301	4.7×10^3	+	ND	ND	Yes	Yes
S-02	24196	10	317	1122	4.9×10^4	+	ND	ND	Yes	Yes
S-03	3448	160	169	1099	1.5×10^4	+	ND	ND	No	Yes
S-04	> 24196	17329	1968	353	ND	ND	ND	ND	No	No
S-05	1607	ND	20	34	ND	ND	ND	ND	No	Yes
S-06	> 24196	4352	455	210	5.5×10^3	ND	ND	ND	Yes	Yes
S-07	201	ND	ND	45	ND	ND	ND	ND	Yes	No
S-08	> 24196	218	238	219	ND	ND	ND	ND	Yes	Yes
S-09	> 24196	292	554	92	ND	ND	ND	ND	Yes	Yes
S-10	> 24196	2098	6488	637	ND	ND	ND	ND	Yes	Yes
S-11	5794	41	173	85	ND	ND	ND	ND	No	No
S-12	1236	10	31	43	ND	ND	ND	ND	No	No
S-13	627	ND	20	ND	ND	ND	ND	ND	No	No
S-14	19863	309	262	1747	ND	ND	ND	ND	Yes	Yes
S-15	9208	10	536	81	ND	ND	ND	ND	No	Yes
S-16	6488	41	63	25	ND	ND	ND	ND	Yes	No
S-17	> 24196	41	185	1023	ND	ND	ND	ND	No	No
S-18	771	10	ND	ND	ND	ND	ND	ND	No	Yes
S-19	12515	10	80	107	ND	ND	ND	ND	No	No
S-20	> 24196	> 24196	> 24196	39852	1.5×10^7	++	124	2.40	Yes	Yes
S-21	> 24196	> 24196	> 24196	87444	6.3×10^6	++	101	1.95	No	Yes
S-22	> 24196	4611	2046	552	ND	ND	1.20	ND	Yes	Yes
S-23	> 241960	1100	54750	3350000	ND	ND	49.8	8.66	No	Yes
S-24	> 24196	> 24196	2489	874	ND	ND	2.80	ND	Yes	Yes
S-25	6488	ND	135	154	4.1×10^2	ND	ND	ND	Yes	Yes
S-26	> 24196	1600	14136	369	ND	ND	0.517	ND	No	Yes
sewage	> 24196000	14136100	6131400	4644444	8.8×10^7	++	91.9	5.89	n/a	n/a
ND[2]	<10	<10	<10	~20	$\sim 10^2$	N/A	0.175	0.05	n/a	n/a

[1]For Mnif-PCR: '++' strongly positive, '+' weakly positive, 'ND' negative.
[2]Lower Detection or Quantification Limit. N/A indicates that there is not a quantitative detection limit for the assay. For human-specific waste markers, ND indicates that no targets were detected within the quantification range of the assay.

Sample S-25 was initially run at 1/5 dilution (as determined by salmon testes DNA-qPCR), and was just outside the range of quantification for the HBM-qPCR assay (data not shown). The sample was run again at a lesser dilution (1/2), and the HBM concentrations were then just within the range of quantification. Salmon testes DNA qPCR was also run on this sample at the 1/2 dilution, and was not inhibited, indicating that it was appropriate to analyze this sample at this dilution. No other HBM-qPCR samples had results that indicated a lesser dilution (1/2) would bring the analysis into the quantification range.

4.2.4 Chemical Tracers

Illicit discharge tracers results showed that ammonia and MBAS was detected in 9 samples each (6 of the samples were the same; Table 4-3). Ammonia, MBAS, was highest in the two samples associated with sewage (S-20 and S-21). Fluoride and potassium results were variable and did not show obvious patterns with other indicators.

Table 4-3. Illicit Discharge Tracer Results.

ID	Ammonia, mg/L	Fluoride, mg/L	Potassium, mg/L	Surfactants, mg/L
S-01	1.52	0.54	7.63	ND
S-02	ND	0.82	35.5	0.11
S-03	ND	0.68	19.4	0.12
S-04	ND	1.5	2.71	0.26
S-05	ND	0.87	6.32	ND
S-06	ND	1.1	4.34	ND
S-07	ND	0.41	3.69	ND
S-08	ND	1.3	1.41	ND
S-09	ND	0.51	3.99	ND
S-10	ND	1.1	17.0	ND
S-11	ND	0.52	3.13	ND
S-12	ND	0.43	2.34	ND
S-13	6.87	1.1	6.68	0.14
S-14	1.1	0.56	1.54	ND
S-15	ND	0.68	23.9	ND
S-16	ND	0.85	12.0	ND
S-17	ND	1.9	0.67	ND
S-18	6.61	1.1	6.48	0.17
S-19	1.16	0.58	1.7	ND
S-20	32.4	0.45	23	5.9
S-21	39.4	0.24	27.1	13
S-22	1.08	0.42	12.3	0.14
S-23	3.76	0.51	37.4	4.7
S-24	ND	0.26	3.57	ND
S-25	ND	0.54	1.04	ND
S-26	20.2	0.55	57.5	ND
Detection Limits (ND)[1]	<1	N/A[2]	N/A	<0.1

[1]ND indicates that the analyte was not detected within quantification range of the assay.
[2]N/A indicates that no samples were below detection limits for this assay.

4.2.5 Canine Responses

As shown in Table 4-2, canine responses varied from both dogs alerting positive, to one dog or the other alerting positive, to neither dog alerting positive. Sable's "No" response at site S-21 was likely due to the phenomenon seen with scent volume detection (see next paragraph). This response was considered an outlier and was removed from statistical tests.

Two positive control tests for sewage were also conducted with the dogs. For the initial positive control test, both dogs responded correctly to two replicates of 100% fresh sewage influent samples. For the second positive control test, Sable did not respond positively to any of the triplicate 100% sewage samples, indicating the potential phenomenon of high scent volume noted in other canine scent detection fields. According to Mr. Fred Johnson (personal communication) of Canada West Canine Centre, many chemicals or substances with strong scent can sometimes temporarily deaden or wash out a canine's ability to scent. Logan responded positively for all three replicates. A positive control sample for RV discharge was not located.

4.2.6 Statistical Analyses

In the comparisons of results in which the dogs had responded "Yes" to the samples in which the dogs responded "No," all microbial and most human-specific waste markers showed a positive relationship with the dogs "Yes" responses, as based on higher average rank values (Tables 4-4). Because Sable responded "No" at site S-23, which had the highest levels of Enterococcus-qPCR and cotinine (but did not show detectable HBM-qPCR or Mnif-PCR), the maximum values for the "No" responses are greater than for the "Yes" responses.

The Mann-Whitney tests revealed several significant differences between the individual dog's responses and some of the microbial and chemical variables (Table 4-4). When the results for each variables were separated into two groups based on Logan's "Yes" responses and "No" responses, significant differences ($p<0.05$) were found with total coliform, *E. coli*, *Enterococcus* (as measured by IDEXX and qPCR) and HBM-qPCR. When analyzing Sable's responses, significant differences were found among total coliform and HBM-qPCR. A Chi-square test for association showed that positive Mnif results were associated with "Yes" responses in both dogs (Table 4-5). A chi-square test also showed that when at least one human-waste specific marker was detected, positive canine responses were more likely, especially for Logan ($p=0.0035$; Table 4-6).

For both dogs, stronger discrimination (lower p values) was seen with the microbial indicators (indicator bacteria and human-specific markers HBM-qPCR and Mnif-PCR) than with the chemical indicators (Table 4-4). Small sample sizes and high numbers of non-detectable results likely influenced the ability of the study to detect significant differences with both dogs.

Table 4-4. Comparison of Canine Responses and Water Sample Results.

	Sable								Logan							
	Mann-Whitney		"No" Responses (n=12)[1]			"Yes" Responses (n=13)[1]			Mann-Whitney		"No" Responses (n=8)[2]			"Yes" Responses (n=18)[2]		
Variable	Corr.	p	Min.	Median	Max.	Min.	Median	Max.	Corr	p	Min.	Median	Max.	Min.	Median	Max.
Total coliform, MPN/100 ml	+	0.030	627	7501	>24196	201	> 24196	> 24196	+	0.016	201	6141	> 24196	771	> 24196	> 241960
E. coli, MPN/100 ml	+	0.054	ND	26	17329	ND	309	> 24196	+	0.027	ND	26	17329	ND	704.5	> 241960
Enterococcus, MPN/100 ml	+	0.11	ND	171	54700	ND	455	> 24196	+	0.006	ND	72	1968	ND	545.1	> 241960
Entero. qPCR , Average C.eq./100 mL	+	0.12	ND	96	3350000	25	301	39852	+	0.03	ND	ND	1023	ND	460	3350000
HBM-qPCR, Copies/L	+	0.048	ND	ND	15000	ND	ND	1.5×10^7	+	0.023	ND	ND	ND	ND	ND	1.5E+07
Caffeine, ppb	+	0.32	ND	ND	49.8	ND	ND	124	+	0.036	ND	ND	ND	ND	ND	124.114
Cotinine, ppb	-	0.91	ND	ND	8.66	ND	ND	2.40	+	0.12	ND	ND	ND	ND	ND	8.656
Ammonia, mg/L	-	0.43	ND	ND	20.2	ND	ND	32.4	+	0.17	ND	ND	6.87	ND	ND	39.4
Fluoride, mg/L	-	0.24	0.43	0.68	1.93	0.26	0.54	1.32	-	0.51	0.41	0.72	1.93	0.24	0.56	1.32
Potassium, mg/L	-	0.83	0.67	6.40	57.5	1.04	4.34	35.5	+	0.015	0.67	2.92	12.00	1.04	9.97	57.50
MBAS, mg/L	-	0.31	ND	ND	4.70	ND	ND	5.90	+	0.24	ND	ND	0.26	ND	ND	13
Temperature	-	0.36	19.5	21.1	24.0	19	20.2	22.9	+	0.26	19.1	21	21.7	19.0	21.5	25.3
DO, % Saturation	+	0.24	2.1	69.9	85.4	45.8	71.5	158	-	0.32	60.1	74.3	101.1	2.1	69.5	157.9
DO, mg/L	+	0.20	0.18	6.27	7.61	4.22	6.565	13.8	-	0.39	5.52	6.57	9.34	0.18	6.32	13.82
Conductivity, mS/cm	+	0.96	1.18	1.71	4.37	0.01	1.95	5.52	+	0.10	0.01	1.63	3.17	1.15	2.08	5.52

[1] For DO, n = 11 "No" and n = 12 for "yes"
[2] For DO, n = 7 for "No" and n=16 for "Yes"

Table 4-5. Comparison of Canine Responses and Mnif-PCR Results.

Sable			Logan		
	Yes	No		Yes	No
Mnif+	3	2	Mnif+	5	0
Mnif-	10	11	Mnif-	13	8
Phi	0.22		Phi	0.34	
p	0.30		p	0.14	

Table 4-6. Comparison of Canine Responses and Combined Human Marker Responses.

Sable			Logan		
	Yes	No		Yes	No
At least one marker +	7	3	At least one marker +	11	0
All markers -	6	9	All markers-	7	8
Phi	0.29		Phi	0.57	
p	0.13		p (one tail)	0.0035	

Evaluation of the entire dataset using both dogs' responses together revealed that there were no false negatives according to the human-waste specific assays measured in this project (HBM-qPCR, Mnif-PCR, caffeine, cotinine). For samples S-04, S-11, S-13, S-17, S19, and S-12, both dogs responded negatively. In these samples, no human-waste specific markers were detected.

Seven samples were positive for the HBM-qPCR assay. Sable identified five of these, while Logan identified all seven. As discussed above, Sable's 'No' response at site S-21 was likely due to the scent volume phenomenon.

Six samples had quantifiable levels of caffeine detected. Sable indicated three of them were positive, while Logan indicated all six were positive. Three of the caffeine-positive samples (S-20, S-21, S-23) also had quantifiable level of cotinine detected. Sable only identified one (S-20), while Logan identified all three.

4.3 Detection of Physical Locations of Known Human Waste Signals

Nine areas of known human waste contamination in storm drains, based on previous research, were targeted for canine exploration in this study (Figures 4-6 and 4-7, Table 4-7). In many cases, while UCSB researchers collected water samples for the correlation study, Sable and Logan were taken to surrounding catch basin inlets and manholes and their responses were recorded. As shown in Table 4-7, one area targeted was the drainage upstream of a low-flow diversion that had been installed several years ago to divert contaminated runoff to the sanitary sewer. Previous efforts to locate inputs, using fecal indicator bacteria tests and molecular methods, were not successful. Real time results greatly aided the investigation, and immediate positive canine responses led urgency to the situation, compelling the research team and additional City of Santa Barbara staff to uncover a leaking sanitary sewer line. The sanitary sewer crossed above a cracked storm drain line, allowing raw sewage to enter the pipe.

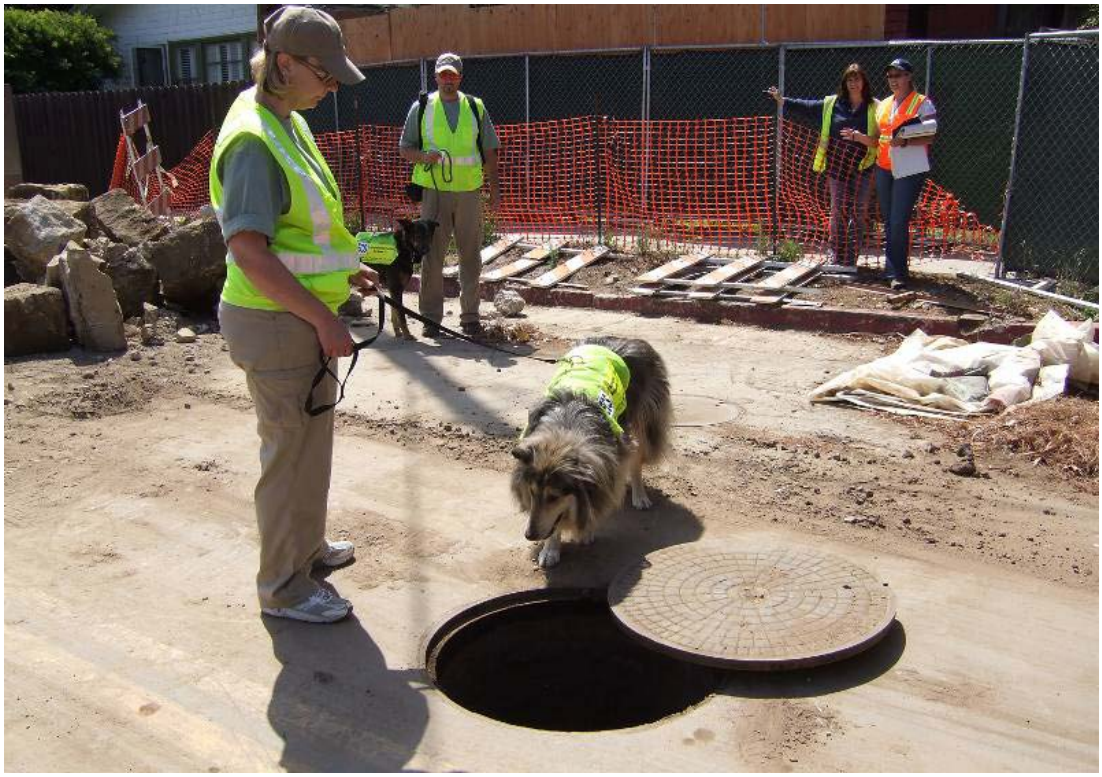

Figure 4-6. Logan Testing a Site Known from Previous Studies to be Contaminated.

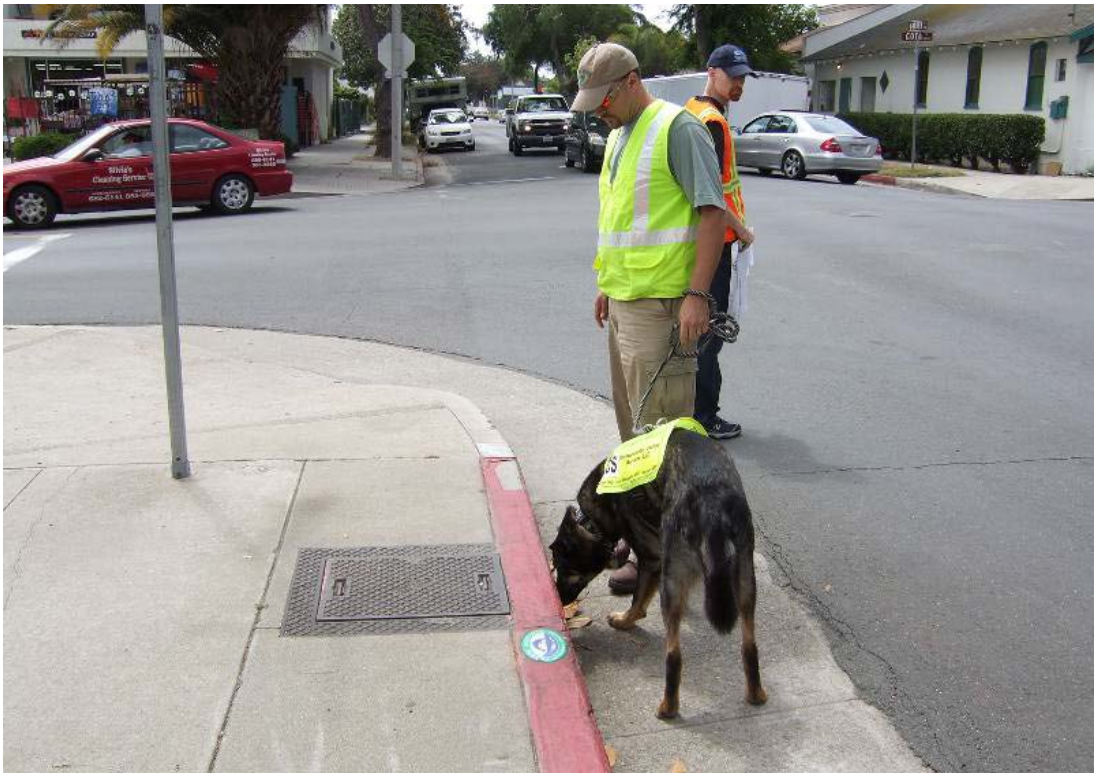

Figure 4-7. Sable Testing a Catch Basin Inlet.

Table 4-7. Summary of Detection of Physical Locations of Known Human Waste Signals.

Area	Relevant Water Samples	No. of Sites Positive By At Least One Dog/Total Sites Tested by Dogs	Summary of Outcome and/or Future Investigation Planned
Haley Drain Diversion	S-24	8/8	The Haley Street storm drain is the site of a low-flow diversion to sanitary sewer. Past results from storm drain samples were variable for human-specific waste markers. Canine responses also showed that contamination may originate upstream. One unmarked pipe (according to the City's highly detailed storm drain atlas) discharging to the storm drain is suspect. Sample S-24 had a positive response for caffeine.
Hope Drain Diversion	S-20, S-21	11/13	Sanitary sewer leak to storm drain detected and repaired. Additional testing is planned to determine if sewage markers are still entering the storm drain, perhaps from the NW area, with positive canine responses. Insufficient flow existed in the NW manholes for sample collection.
Laguna St. Housing Complex	S-12, S-13, S-18	1/3	Previous results have shown very high levels of ammonia at this location. Canine responses suggested that there may be contamination; water testing in this study found no human-waste specific markers, relatively high ammonia, and the presence of surfactants. Prior to obtaining the human-waste specific marker results, the storm drains in the housing complex were smoke tested and no illicit connections were detected. It is likely that wash water and possibly urine is leading to the observed results.
Montecito Street	S-10	3/7	Due to positive canine responses in the morning, and detection of unmarked flow to the storm drain, camera work will be conducted in this area.
Nopal and Canon Perdido	S-05, S-03, S-04 S-06, S07, S-08	13/31	Canine responses and water sample results (current and previous) suggest that contamination exists in this area. Ongoing dye studies are being used to investigate the sources from the area that was narrowed down by this study.
Ortega Park area	S-02	5/7	Canine responses suggest that contamination may derive from a school facility built in the early 20th century. Dye tests of every toilet in the facility were carried out after the canine field work, but did not identify any illicit connections.
San Pascual Drain	S-14	1/3	A very small creek is subjected to frequent human defecation nearby. Sable responded positively but sample results did not confirm that waste entered the trickle of creek flow. Both dogs responded negatively to an adjacent creek that was known to be disinfected (see Westside Drain, below).
Serena Drain		8/17	Historical observations at this location suggested potential contamination. Canine responses and subsequent observations suggested that mop water, wash water, and/or grease trap overflows were entering the storm drain. Flow was insufficient for sample collection.
Westside Drain	S-25	5/6	The Westside Drain is the site of a low-flow ultraviolet disinfection facility. Both dogs responded negatively downstream of the disinfection facility, but had positive or unclear responses upstream. Both dogs responded positively at site S-25, where results came back positive for HBM-qPCR. Additional survey work identified two unmarked flows entering the storm drain, but tests on both showed no contamination. Future work will involve smoke testing.

4.4 Illicit RV Dumping

During the illicit RV dumping investigation, ten city blocks and two parking lots were surveyed in approximately 3.5 hours; during this time, approximately 100 RVs were investigated. In addition, all catch basins and drop inlets in the vicinity were tested by the dogs. During the investigation, no signs of deliberate dumping were observed. However, two RVs with leaking black water tanks were identified (Figures 4-8, 4-9, 4-10). The wet areas below the RVs did not contain sufficient depth for water to be collected, nor did flow from these leaks enter the storm drain according to visual inspection. The results supported the conclusion that RV dwellers do not conduct widespread dumping into storm drains. However, runoff during storm events could wash contamination from leaks into storm drains.

Figure 4-8. Canines and Handlers Walking Along Recreational Vehicles.

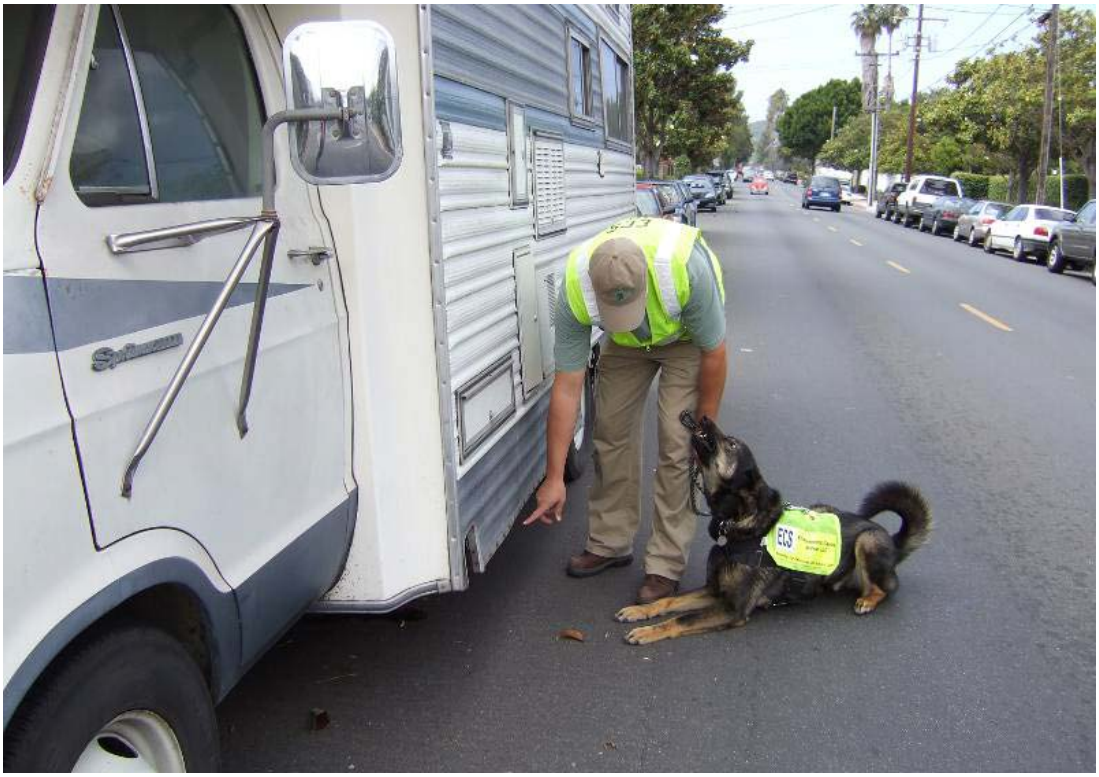

Figure 4-9. Sable Checking a RV Suspected of Leaking Waste.

Figure 4-10. Recreational Vehicle Leak that Elicited Positive Alerts from Both Canines.

4.5 Watershed Reconnaissance

Systematic outfall reconnaissance in Sycamore Creek showed that the dogs could easily walk through wet, overgrown creek beds with low flow conditions (Figure 4-11). Due to greater than average rainfall over the previous winter, the creek banks were highly overgrown with brush, which made the detection of flowing outfalls nearly impossible. This study showed that the dogs could cover approximately one mile of creek in 2.5 hours, but no positive responses were observed nor were samples collected.

Figure 4-11. Testing Sycamore Creek for Contaminated Outfalls Discharging to Creek.

Systematic storm drain reconnaissance in urban areas covered 19 sites in Mission Creek watershed in four hours. Areas included single family housing, school properties, and commercial zones. The efficiency of using the canine scent tracking technique depends on density of storm drain inlets and manholes, and their accessibility.

4.6 Training

In addition to a wall-attended community forum for interested Santa Barbara residents, the City hosted a workshop for professionals to learn about and discuss canine scent tracking work for stormwater investigations. Although substantive efforts were made to draw participation from law enforcement staff involved in canine handling, all participants were stormwater practitioners or researchers. Participants learned about the project and what had been gleaned in the field and the training techniques used by ECS. The canine and microbial tracking were met with general enthusiasm from all attendees.

CHAPTER 5.0

DISCUSSION

The purpose of this study was to test the use of a promising new method, canine scent tracking (sewage sniffing dogs), for source tracking in municipal stormwater investigations. The objectives of the study included: 1) compare canine responses to other indicators of contamination; 2) determine the feasibility of using canine scent tracking to locate inputs of contamination to a storm drain system; 3) determine the feasibility of testing specific hypotheses about contamination sources; 4) investigate the time required to conduct systematic watershed reconnaissance; and 5) conduct training for stormwater professionals and canine handlers. As discussed below, the project was successful in meeting each of these objectives. The results illustrate the benefits of adding canine scent tracking to the source tracking toolbox used to locate and ameliorate contamination problems in storm drains, surface waters, and recreational areas.

5.1 Canine Performance and Human-Specific Waste Markers

The positive and significant correlations observed in this study, even with small sample sizes, are a strong indicator of the success of using canine scent tracking to identify and locate storm drain contamination. Despite the potential complications in comparing vastly different techniques and the variability between dogs, the canine responses corresponded to locations diagnosed as contaminated using a battery of contemporary, quantitative methods. At 26 of 133 locations investigated by the canines, water samples were also collected and tested for FIB, chemical tracers used in illicit discharge detection (potassium, fluoride, ammonia, and surfactants), and contemporary human-specific waste markers (human-specific *Bacteroidales*, *Methanobrevibacter smithii* nifH gene, caffeine and cotinine).

Counting sites that were contaminated with caffeine or cotinine, eleven of the samples were positive for at least one human-specific waste marker and the canine responses were significantly associated with several of the human-specific waste markers. Also, when at least one DNA-based or chemical (caffeine or cotinine) human waste marker was detected, Sable responded positively 70% of the time, and Logan responded positively 100% of the time.

One of the strongest results of the correlation study was the small number of false negatives. For all sample locations where both dogs responded negatively, there were no human waste specific markers detected.

For samples in which the canine responses and wastewater indicators did not align, several factors may be at play, including:

- The dogs may respond to components of sewage that were not analyzed in our study. For example, the dogs were trained to detect detergents, which were assessed in this study with MBAS, an imperfect test for anionic surfactants that are found in some detergents. In some cases, the dogs were likely alerting to discharges of wash water or grease trap overflows, rather than human-specific waste.
- The dogs may have responded to scents emanating from water flowing somewhat distant

(hundred of feet) from the immediate location where the water samples were collected, or they may have responded to lingering scent from temporally varying contamination. For most samples, the canines sniffed from the street level, but manholes were typically 6-20' deep. Prior experience in the study area has shown pronounced temporal variability at several locations.

Both of these factors are likely related to the response of the dogs to volatile compounds, in contrast to the chemical and microbiological tests, which are performed on aqueous solutions. A third factor that may explain some of the discrepancy between canine responses and test results is:

- The dogs may respond to lower concentrations than detection limits of the human-specific markers. The majority of the samples were below the detection limit for one or more human-specific waste markers.

By several measures, Logan performed more in line with the markers for human waste than did Sable. Interestingly, the dog handlers predicted the more sensitive response of Logan prior to the start of field work. Sable was observed to have sensitivity to high scent volume, in that there was a negative response to 100% sewage (in highly contaminated storm drain and in a positive control sample). This phenomenon has been observed in other fields of canine scent work, as previously described by scenting expert Mrs. Sharon Avila (Avila, personal communication).

5.2 Benefits of Canine Field Work

Several hotspots were chosen for further investigation based on historical data and canine responses during this study. A dramatic advantage of using canine scent tracking was the real-time responses, which were used to guide tracking efforts (e.g. which direction to take when a storm drain forked), and to guide additional sample collection. In two cases, the dogs' responses led us to find unmarked discharges to the storm drain, with temporally variable flow. Camera testing will be used in these two locations. Additional techniques that will be used are dye and smoke testing. Having the canine responses help narrow the zone of interest from many blocks to a single block makes pursuing dye, camera, and smoke testing economically feasible. A distinct benefit of having the dogs present for illicit discharge detection work was the interest that people had in the dogs and what they were investigating. Interest and confidence in the dogs' abilities generated more conversation and cooperation than we often find when investigating and fixing illicit discharges.

A highlight of this project was work conducted at the Hope Diversion, where the study led to the detection and repair of a site where raw sewage was entering the storm drain network (Table 4-7). The Hope Drain had previously been diagnosed as contaminated with human waste based on DNA markers (Sercu et al., 2009). Both dogs signaled positively for site S-20 (Hope drain diversion), which is located in a shopping center parking lot. The dogs were then taken around to all drains upstream around the shopping center to further pinpoint the source. Site S-21 (State & Plaza) was indicated as a source and sampled accordingly. Based on the dogs' responses, the historical data available, the strong smell of sewage emanating from the catch basin, and a periodic trickle of water that could be heard entering the storm sewer, the City of Santa Barbara Wastewater Division was called in to televise the storm sewer. Defects were discovered in both the sanitary sewer and storm sewer lines that were allowing sewage to periodically enter the storm sewer at this location when the lift station upstream turned on and created a full pipe scenario. Repairs were conducted within 48 hours (Figure 5-1). Fortunately, a

low-flow diversion to the sanitary sewer prevented this contamination from reaching the creek and Arroyo Burro Beach downstream. The diversion was installed based on high FIB levels, and corroborated by DNA-based results during project design and construction.

Figure 5-1. Repair of Leaking Sanitary Sewer and Storm Drain Detected with Canine Tracking.

One hypothesized source of human fecal contamination in the storm drain system was that of illicit dumping of RV black water storage tanks into catch basin and drop inlets. In previous years, the City has attempted to survey neighborhoods with high numbers of RVs parked along streets, and did not find any obvious signs of dumping. However, the investigation was incomplete because City staff did not have a way to determine if a suspected catch basin, for example, was wet from irrigation runoff or from an RV discharge. In addition, it is not possible to see into every catch basin. Canine scent tracking was a very effective tool for investigating this hypothesis. In the areas we investigated, the dogs tested every catch basin and every wet area in the gutter and parking lane (walking along the street side of the RVs), resulting in two positive responses (by both dogs). Interestingly, both sources were slowly leaking black water tanks rather than deliberate discharges. In one case, a note was left on the RV and the vehicle owner, an RV-dwelling Santa Barbara resident, contacted the City and rectified the problem. In the second case, the visiting RV left the area before fixing the problem. As noted above, the dogs were able to facilitate informative discussions with populations that are not often eager to talk with municipal staff members.

Canine scent tracking was also effective for systematic watershed reconnaissance, although more so for testing enclosed storm drains than for testing overgrown creek channels. While there is still much to learn about what exactly the dogs respond to during an investigation,

the great number of sites that can be tested in a single day (50-100 depending on their spacing and accessibility) is logistically advantageous. The ability to narrow problem areas down to a single block or facility makes follow up investigations with water testing, dye, smoke, and/or camera testing a viable approach. We are unaware of any other method that can provide rapid results in the field, and that correlates well with waste water indicators.

5.3 Recommendations

The use of canine scent tracking for source tracking and illicit discharge, detection, and elimination work has become more attractive as a result of this study. As pressures increase on communities facing National Pollutant Discharge Elimination System (NPDES) regulations to reduce or eliminate illicit discharges of microbial contamination, the potential for expanding this field is immense. As an illustration of how rapid this technology can advance, the USDA first began its use of beagles to detect fruit transported by international travelers in 1984. In 1987 the agency opened three regional training centers, and in 1997 the National Dog Detector Training Center was opened. The agency now has thousands of trained Beagles at all major airports, and uses other breeds at border crossings, ports, and mail facilities to search for imported fruit, agricultural pests, and invasive species.

This project has resulted in several recommendations for future canine scent tracking work:

- Additional dogs and handlers should be trained in source tracking methods. Proper evaluation of training methods and performance will need a larger sample of individual canines to examine.
- Second, some canines should be trained to respond to a broad suite of indicators for contamination, whereas others should be trained for narrower foci, such as just wash water indicators, just human-waste specific markers, and/or just restaurant grease trap indicators (e.g. fats, oils, and greases).
- Canines should be trained to alert to a variety of scent volumes for each marker.
- Trained dogs and handlers should be located throughout the country, so that complications with airplane travel and or long drives can be avoided.

Last, two methods are suggested for municipalities interested in pursuing the use of canine and microbial scent tracking. Communities can bring in canine scent tracking to cover broad areas of storm drain networks, and then conduct a spatial analysis to create a sampling strategy for future investigation with wastewater indicators. Conversely, communities with suspected areas of wastewater contamination in storm drains can pursue indicator testing at key nodes, and then bring in canine scent tracking to work upstream of positive results.

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