SWAMP
Surface Water Ambient Monitoring Program

Final Technical Report	2014

FINAL REPORT
SWAMP SAFE-TO-SWIM STUDY
REGION 5
Sites Sampled: May 2012 – September 2013

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SWAMP Safe to Swim Study

May 2012-September 2013

Final Report on July 2014

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1.0 EXECUTIVE SUMMARY

Outbreaks of waterborne disease caused by microbial pathogens have been of increasing concern to public health. *Cryptosporidium* spp., *Giardia duodenalis*, *E. coli* O157:H7, and *Salmonella* are among major waterborne pathogens. Contamination of surface waters by these pathogens may impact many beneficial uses including the recreational water in watershed in the Central Valley. The objective of the study was to provide data for answering the question "Is the water safe"? The study investigated the occurrence of *Cryptosporidium* spp., *Giardia* spp., *E. coli* O157:H7, and *Salmonella* in recreational riverine and lacustrine surface waters in the Central Valley of California. The Central Valley Water Board selected and prioritized the following eleven sites for sampling activities based on data from previous monitoring projects, safety, and all-weather access across seasons:

- American River at Discovery Park (Sacramento County)
- Dry Creek at Cirby Creek confluence (Placer County)
- Folsom Lake at Beal's Point Left (Placer County)
- Kings River at Laton-Kingston Park (Fresno County)
- Kings River at Reedley Beach (Fresno County)
- Kings River at Winton Park (Fresno County)
- Lake Natoma at Nimbus Flat Left (Sacramento County)
- Linda Creek at Condor Court (Placer County)
- Miner's Ravine/Secret Ravine Confluence (Placer County)
- Squirrel Creek in Western Gateway Park (Nevada County)
- Tuolumne River at Fox Grove (Stanislaus County)

Central Valley Water Board staff collected water samples in two swimming seasons, May through September, 2012 and June through September, 2013. Surface Water Ambient Monitoring Program (SWAMP) field crew followed procedural safeguards for the State Water Board SWAMP approved Monitoring Plan (MP) that ensured actual custody and safekeeping of the water samples until delivery to the UC Davis Atwill Water & Foodborne Zoonotic Disease Laboratory (Atwill Lab). Upon delivery of the water samples with a signed Chain of Custody (COC) form, the Atwill Lab maintained a storage and analyses area that met Quality Assurance Project Plan (QAPP) requirements of the scope of work and that secured access to the water samples by a designated custodian. Central Valley Water Board Lab measured field parameters during sampling and performed analysis for detection of *E. coli* in their lab. The Atwill Lab performed analysis of *Cryptosporidium* spp., *Giardia* spp., *E. coli* O157:H7, and *Salmonella*.

Field parameters of water varied with sampling sites but values for each parameter (specific conductivity, dissolved oxygen, pH, temperature, and turbidity) were consistent in the two swimming seasons. All water samples detected positive for indicator *E. coli*. Mean concentrations of *E. coli* in positive sites ranged from 3.07 MPN (Most Probable Number)/100 ml to 219.58 MPN/100 ml in 2012 and 13.4 MPN/100 ml to 646.0 MPN/100 ml in 2013. *E. coli* concentrations in 9.09% of the samplings sites exceeded

the Basin Plan objective (400 MPN/100ml) in the two years. *E. coli* concentrations in 54.54% and 36.36% samplings sites exceeded the EPA Recreational Water Quality Criteria (RWQC) 2012 recommended geometric mean concentration of *E. coli* in freshwater (126 CFU/100 ml) in 2012 and 2013 respectively.

Cryptosporidium spp. oocysts were detected in water samples collected from all sampling sites, with an overall of 50.0% samples positive. Mean oocyst concentration in all positive samples was 0.08 oocysts/L in 2012 and 0.19 oocysts/L in 2013. Most detected oocysts were damaged. Low concentration of oocysts and damages to oocysts resulted in the failure of genotyping of *Cryptosporidium* in the present study due to limited DNA or degraded DNA. Water samples from eight of the sampling sites were detected positive of *Giardia* spp. The overall *Giardia* prevalence was 28.78% and mean cyst concentration in positive samples was 0.20 cysts/L in 2012 and 0.19 cysts/L in 2013. Most *Giardia* cysts detected were damaged. *E. coli* O157:H7 detected in 9.09% of all water samples but limited in three sites. *Salmonella* detected in water samples from all but one sampling site. Mean concentrations of *Salmonella* in positive samples were 0.94 MPN/L in 2012 and 1.85 MPN/L in 2013.

Cryptosporidium concentrations were associated with previous 30 day wind speed and rainfall, and DO concentrations in water. *Giardia* concentrations were associated with *E. coli* concentrations, turbidity and pH of surface water, and 24 hour previous air temperature. *Salmonella* concentrations were associated with previous 30-day air temperature. *E. coli* O157:H7 presences in water were associated with previous 30 day solar radiation and rainfall. No significant relationships were observed between pathogen concentrations at sampling sites and other field parameters (conductivity, water temperature, collection year, site elevation, or indicator *E. coli* concentrations). Based on findings of this study, the Atwill Lab recommends future Safe-to-Swim studies to investigate spatial and temporal pathogen occurrence on sites with higher prevalence of pathogens. We also recommend conducting epidemiology studies to identify the source of water contamination.

2.0 INTRODUCTION

Since pathogen contamination of recreational surface waters in California impacts many beneficial uses, waterborne disease outbreaks are of increasing concern to State and Federal public health officials. Given that zoonosis varies across basin and season (among other variables), long-term watershed scale pathogen reduction requires integration of monitoring protocols that detect trends in recovery and/or degradation of microbial water quality.
The Central Valley Water Board's SWAMP has completed several Safe-to-Swim studies (http://www.waterboards.ca.gov/centralvalley/water_issues/swamp/r5_activities/index.shtml). In order to better assess the impact of waterborne pathogens on beneficial uses, the Central Valley Water Board collaborated with UC Davis Atwill Lab to conduct this study to collect additional data of indicator organism concentrations and occurrence and sources of specific waterborne pathogens.

3.0 BACKGROUND

Waterborne disease outbreaks caused by microbial pathogen infection have been of increasing concern to public health. Contamination of surface waters by pathogenic organisms in California continues to impact the many beneficial uses including the recreational water in watershed in the Central Valley. *Cryptosporidium* spp., *Giardia duodenalis*, *E. coli* O157:H7, and *Salmonella* are among the major waterborne pathogens. Long-term reduction of contamination of pathogenic organisms requires an integrated approach that combines pathogen monitoring, microbial source tracking, and monitoring protocols that can detect trends of occurrences of pathogens in recreational water. The EPA RWQC 2012 recommended geometric mean concentration of *E. coli* in freshwater is (126 CFU/100 ml). The Central Valley Regional Water Quality Control Board Basin Plan identifies a water quality objective that utilizes fecal coliform (not to exceed 400 MPN/100mL in a single sample). Despite the availability of water quality guidelines using indicator organisms, indicator organism presence does not always accurately predict occurrence of pathogens. Design of this study focused on detection of specific pathogens and indicator *E. coli* in eleven recreational sites.

4.0 MONITORING OVERVIEW

This Safe-to-Swim study investigated pathogen concentrations at eleven monitoring sites in the Central Valley in order to determine occurrences and concentrations of *Cryptosporidium* spp., *Giardia* spp., *Salmonella*, and presence or absence of *E. coli* O157:H7. The budget and contract limited samples to about 60 each for detection of these pathogens. The Central Valley Water Board's Staff coordinated sampling events where field crew collected water samples between May 2012-September 2012, and June 2013-September 2013 in an attempt to evaluate water quality during the typical contact recreation season (April-August) encompassing the entire Central Valley Region.

5.0 QUALITY ASSURANCE

Weather conditions and types of land use in each site may influence the occurrence and persistence of pathogens in each site. Field parameters of water and variability due to unique unidentified site characteristics may also have influenced results. We maintained chain-of-custody forms for all sampling events and for all samples. All procedures of this study were conducted in accordance with the QAPP and MP. All data presented met Method Quality Objectives specified for this study (Table 1).

Table 1. Quality objectives overview of laboratory methods for pathogen detection

Parameter	Accuracy	Precision	Recovery/ Sensitivity	Target Reporting Limit	Complete-ness
E. coli O157:H7	Positive and negative standards test	Duplicate samples ≥80%	Distinguish from ≥1 MPN	≥1 cfu per liter	80%

Parameter	Accuracy	Precision	Recovery/ Sensitivity	Target Reporting Limit	Complete-ness
	≥90% accurate	concordant			
Salmonella	Positive and negative standards test ≥90% accurate	Duplicate samples ≥80% concordant	Distinguish from ≥1 MPN	≥1 cfu per liter	80%
Cryptosporidium	Positive and negative standards test ≥90% accurate	Lab duplicates are ≥80% concordant	1-4 gene copies per PCR reaction per vertebrate source	1-4 gene copies per PCR reaction per vertebrate source	80%
Giardia	Positive and negative standards test ≥90% accurate	Lab duplicates are ≥80% concordant	NA	NA	80%

6.0 FIELD SAMPLING and PARAMETERS

Sampling sites comprising this study ranged over a variety of watershed boundaries and land uses including timber production, grazing, recreation, fish habitat, reservoir storage, urban dwellings, and agriculture. Sampling sites were:

- American River at Discovery Park (Sacramento County)
- Dry Creek at Cirby Creek confluence (Placer County)
- Folsom Lake at Beal's Point Left (Placer County)
- Kings River at Laton-Kingston Park (Fresno County)
- Kings River at Reedley Beach (Fresno County)
- Kings River at Winton Park (Fresno County)
- Lake Natoma at Nimbus Flat Left (Sacramento County)
- Linda Creek at Condor Court (Placer County)
- Miner's Ravine/Secret Ravine Confluence (Placer County)
- Squirrel Creek in Western Gateway Park (Nevada County)
- Tuolumne River at Fox Grove (Stanislaus County)

Local land use, latitude, and longitude of these selected sites are shown in Table 2.

Table 2. Monitoring sites of the Central Valley Safe-to-Swim Study, 2012-2013

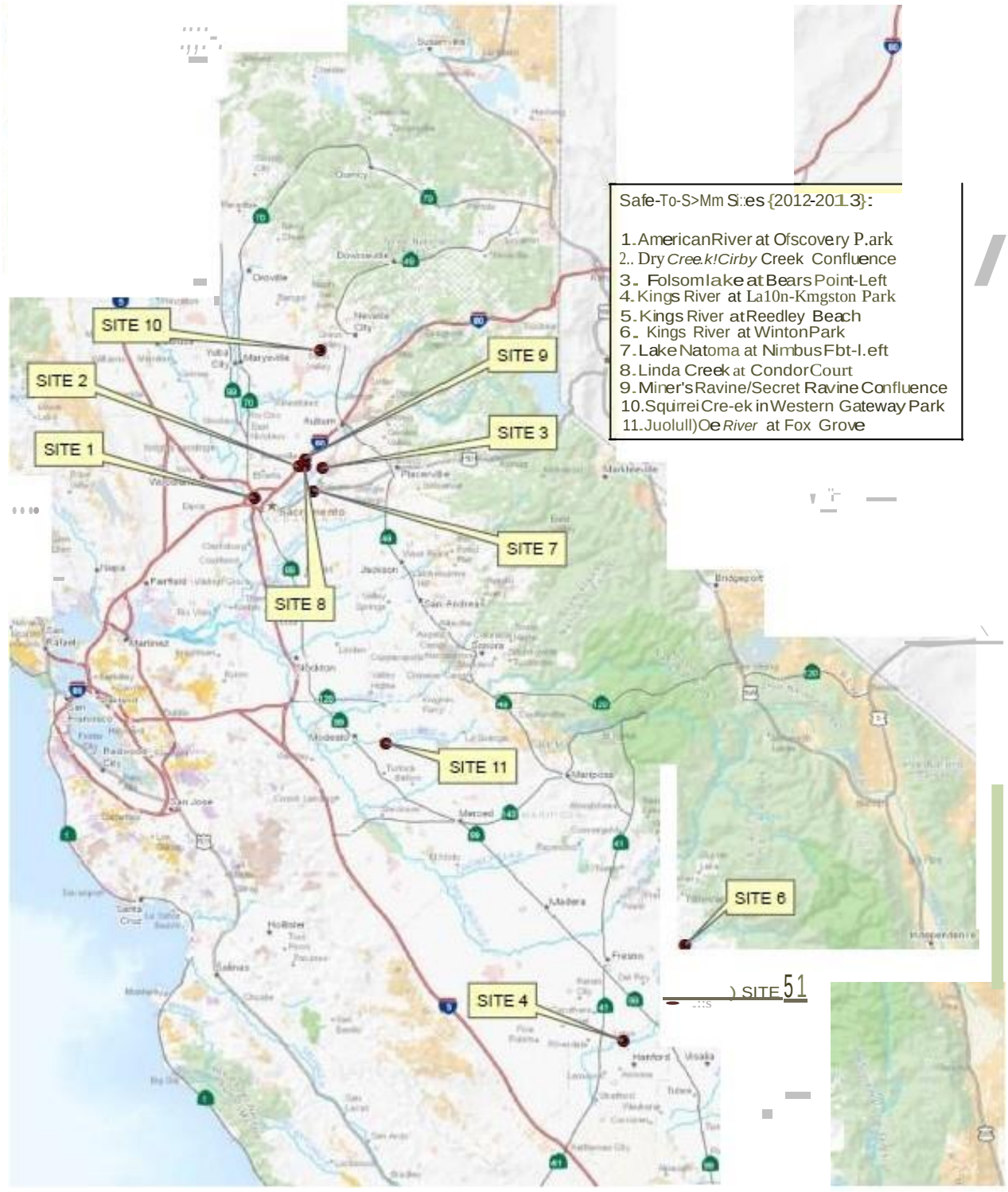
Map ID	Station Number	Site Description	Local land use (< 1 mile diameter from site)	Latitude	Longitude
1	519AMNDVY	American River at Discovery Park	D, E	38.60090	-121.50550

Map ID	Station Number	Site Description	Local land use (< 1 mile diameter from site)	Latitude	Longitude
2	531PLA900	Dry Creek/Cirby Confluence	D, E	38.73350	-121.28850
3	514PLABPL	Folsom Lake at Beal's Point Left	A, B, D, E	38.72327	-121.16933
4	551KIN060	King's River at Laton-Kingston Park	B, C, D, E	36.42760	-119.68980
5	552FRE511	King's River at Reedley Beach	B, C, D, E	36.58690	-119.45930
6	552FRE510	King's River at Winton Park	A, B, D, E	39.20410	-121.19060
7	519SACNFL	Lake Natoma at Nimbus Flat Left	A, D, E	38.63654	-121.21593
8	519PLA921	Linda Creek at Condor Court	D, E	38.73140	-121.25620
9	531PLA902	Miner's Ravine/Secret Ravine Confluence	A, D, E	38.75980	-121.25660
10	516NEV906	Squirrel Creek in Western Gateway Park	B, D, E	39.20402	-121.19040
11	535STC218	Tuolumne River at Fox Grove	A, B, C, D, E	37.61920	-120.84270

A – Integrator Site (located near discharge points of a large watershed characterized by heterogeneous land uses)
B – Irrigated Agriculture
C – Confined Animal Feeding Operation
D – Community Development (Areas of potential residential influences to water quality)
E – Recreation

Between May 2012 and September 2013, water samples were collected from 11 monitoring swimming sites selected by the Central Valley Water Board. UC Davis provided the Water Board with sterilized carboys and portable pumps for collecting water. Water Board field crew collected water samples at selected sites. Water samples were collected by directly pumping water into carboys and approximately 40 L of water were collected from each site during each sampling event. Carboys were placed in containers with ice immediately after collection and delivered to the Atwill Laboratory at UC Davis in the same day of sampling with a chain of custody. The field crew also measured Dissolved Oxygen (DO) (mg/L), Specific Conductivity (µS/m), pH, temperature (°C), and turbidity (NTU) at each site during sampling. A map of sampling sites for this study is shown in Figure 1.

Figure 1. Map of the Central Valley Safe to Swim Study sampling sites. 2012-2013



7.0 ANALYTICAL METHODS

The State Water Board Laboratory performed the analysis for detection of indicator *E. coli* using the IDEXX Colilert® QuantiTray system (USEPA, 2003). The UC Davis Atwill Laboratory performed analysis for detection of *Cryptosporidium* spp., *Giardia* spp., *E. coli* O157:H7, and *Salmonella*. Water samples were stored at 4°C immediately after arrival at the Laboratory until processing. Samples were processed for laboratory analysis within 24 h of collection. Water was filtered using hollow-fiber ultrafiltration (UF) technique (also called tangential flow) that has been reported to be effective for recovering a diverse array of microbes from water (Hill et al, 2005). F200NR filters were used for the ultrafiltration and approximately 1000 ml of concentrated water (also called retentate) were obtained for each sample.

Detection of *Cryptosporidium* spp. and *Giardia* spp.
Approximately 500 ml of retentate water was transferred into two 250 ml centrifuge tubes. Five hundred µl of 10% (vol/vol) Tween-80, combined with 10% (wt/vol) sodium dodecyl sulfate (SDS), was added to each tube. The suspensions were mixed on a wrist action shaker at setting 7 for 5 min followed by centrifuging at 1,100× g for 15 min. Supernatants were discarded by aspiration, leaving a 1:3 pellet:water ratio. Pellets were resuspended and transferred into a 15 ml tube and centrifuged at 1,100×g for 15 min. Supernatants were discarded, leaving 3 ml with pellet. The final suspension was used for isolating *Cryptosporidium* oocysts and *Giardia* cysts by Immunomagnetic Separation (IMS). IMS was performed using anti-*Cryptosporidium* and *Giardia* beads (Invitrogen, Carlsbad, CA) with a Dynal Bead Retriever (Thermo, Finland) according to manufacturer's instructions. The final solution after IMS (~ 80 µl) was mixed with 5 µl of 1 N NaOH and placed onto a well of pre-treated glass slides (Super Stick Slides, Waterborne Inc.). Slides were dried at room temperature and stained with FITC-conjunct anti-*Cryptosporidium*/*Giardia* antibodies (Waterborne Inc.). Slides were examined with fluorescent microscope (Olympus BX60) at minimum 400× magnification. *Cryptosporidium* spp. oocysts and *Giardia* cysts were counted and concentrations of (oo)cysts were calculated as no. (oo)cysts/L water.

Attempts of Genotyping of *Cryptosporidium*
All microscopic *Cryptosporidium*-positive samples were subjected to DNA extraction from slides. DNA was extracted using a DNA Stool Mini Kit (Qiagen®.) according to manufacturer's instructions. A nested Polymerase Chain Reaction (PCR) was performed using primers amplifying approximately 830 base pairs (bp) of the SSU (small subunit) rRNA gene as previously described (Xiao et al., 1999). Primers for the primary PCR were SSU-F2 5'-TTC TAG AGC TAA TAC ATG CG-3' and SSU-R2 5'- CCC TAA TCC TTC GAA ACA GGA-3'. The 100 µl PCR mixture contained 10 µl 10x PCR buffer, 6 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 mM, each primer at a concentration of 200 mM, 2.5 U of Taq polymerase, and 0.25 to 2 µl of DNA template. The mixture was subjected to 35 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min. The secondary PCR amplify from 2 µl of the primary PCR product with primers 5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3' and 5'-AAG GAG TAA GGA ACA ACC TCC A-3'. The mixture and reaction conditions were identical

to the primary PCR except that 3 mM $MgCl_2$ were used in the PCR mixture. PCR products were verified by electrophoresis in 2% agarose gel stained with ethidium bromide. If PCR was successful, secondary PCR products were purified using Qiaquick® spin columns (Qiagen®) and sequenced at the University of California DNA Sequencing Facility, where ABI 3730 Capillary Electrophoresis Genetic Analyzer was used for sequencing. A preliminary analysis of sequences would be performed using Vector NTI Advanced 11 software (Invitrogen Corporation, Carlsbad, CA) followed by a BLAST analysis to compare to existing *Cryptosporidium* spp. 18S rRNA gene sequences in the GenBank using NCBI (National Center for Biotechnology Information)'s online blasting tool (http://blast.ncbi.nlm.nih.gov/).

Qualitative Detection of *E. coli* O157:H7

A previously described enrichment and IMS method (Paton and Paton, 2003) was used for the detection of *E. coli* O157:H7. Depending on the volume of retentate, 50 to 250 µl of retentate was filtered through 0.45 µm pore size nitrocellulose membrane filters. Filters with filtrate were placed into Tryptic Soy Broth (TSB) and incubated in a Multitron programmable shaking incubator for 2 h at 25°C followed by 8 h at 42°C and held at 6°C overnight. After the incubation, 1.0 ml of the enrichment solution was used for IMS of *E. coli* O157 and 100 µl final solutions were obtained after IMS. The IMS isolation was performed using anti-*E. coli* O157 beads (Invitrogen, Carlsbad, CA) with a Dynal Bead Retriever (Thermo, Finland) according to manufacturer's instructions. After IMS, 50 µl of the final solutions was streaked onto Rainbow agar (Biolog, Hayward, CA) and the rest 50 µl on Sorbitol MacConkey Agar (BD Becton, Sparks, MD) for isolation of *E. coli* O157:H7. The plates were incubated for 24 h at 37°C. Presumptive *E. coli* O157:H7 colonies were confirmed by PCR using *E. coli* O157 specific primers 5' CGG ACA TCC ATG TGA TAT GG 3'(forward) and 5' TTG CCT ATG TAC AGC TAA TCC 3'(reverse). The 50 µl PCR reaction mix was composed of 1X PCR Buffer, 200 µM of each DNTP, 1.5 mM $MgCl_2$, 0.4 µM Forward Primer, 0.4 µM Reverse Primer and 1.25 units/reaction AmpliTaq Polymerase. PCR reaction started 95°C for 1 min to denature the DNA followed by 30 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec and extension at 72°C for 1 min. The PCR products were stained with ethidium bromide and visualized on 2% agarose gel.

Quantitative Detection of *Salmonella*

An MPN method was used for quantitative detection of *Salmonella*. For each sample, 50 ml (×3 replicates), 10 ml (×3 replicates), 5 ml (×3 replicates) and 1 ml (×3 replicates) of retentate were filtered through 0.45 µm pore size nitrocellulose membrane filters. Filters were placed into wells of a 12-well reservoir that contains 3 ml of Buffered Peptone Water (BPW) in each well followed by incubating at 37°C for 24 h. Ten microliter of the BPW enrichment solution was transferred into a well of 12-well plate reservoir contains 1.0 ml of Rappaport-Vassiliadis (RV) broth and incubated at 42°C for 24 h. Five microliters of the RV enrichment solution from each well was streaked as a lane on Xylose Lysine Deoxycholate (XLD) agar plates and incubated at 37°C for 24 h. Lanes with black colonies were presumptive positive *Salmonella* reaction which was confirmed by biochemical tests (Triple Sugar Iron Agar, Urea Agar, and Lysine Iron Agar). The numbers of confirmed positive reaction lanes of each volume tested were

used for calculating *Salmonella* concentration using computer software based MPN calculator (Mike Curiale).

Statistics

The proportions for *E. coli* O157 presence/absence, and frequencies, arithmetic means, minimum and maximum values for *E. coli* and pathogens (*Salmonella, Giardia, Cryptosporidium*) in water collected from monitoring sites were calculated. We adopted California Irrigation Management Information System (CIMIS) Weather data from weather stations near monitoring sites (Table 3) and entered data in Microsoft Excel spreadsheets and followed Quality Assurance (QA) protocol for range (min/max), and step tests for each weather variable. Weather data QA values that failed the range or step tests were not included in the analysis. When weather data and water quality data was quality assured in Excel by linking each individual cell in the spreadsheet to the original spreadsheet value and verifying data entries from Chain of Custody forms, we exported the data for analysis in STATA (STATA 2011). Initial assessments used a power sample size estimate to determine if all variables should be included in the analysis, followed by a Poisson regression to determine associations between *E. coli* concentrations with pathogens, weather, and water quality data.

Table 3: Weather stations from California Irrigation Management Information System (CIMIS) used for data analysis

Station Name	River Basin	Longitude	Latitude	Elevation (feet)	Approximate distance from monitoring site
Parlier	Kings	36.6	-119.5	337	3 miles from Kings River at Reedley Beach. 16 miles from Kings River at Laton Kingston Park.
Browns Valley	Squirrel Creek	39.27	-121.31	940	8 miles from Squirrel Creek in Western Gateway Park
Fair Oaks	American	38.65	-121.22	265	1 mile from Lake Natoma at Nimbus Flat Left. 6 miles from Folsom Lake at Beal's Point Left and Linda Creek at Condor Court. 8 miles from Miner's Ravine/Secret Ravine Confluence. 7 miles from Dry Creek/Cirby Creek Confluence.
Orange Cove	Kings	36.72	-119.39	450	7 miles from Kings River at Winton Park.
Bryte	American	38.6	-121.54	40	2 miles from American River at Discovery Park.
Denair II	Tuolumne	37.55	-120.75	150	7 miles from Tuolumne River at Fox Grove.

8.0 RESULTS

Summary of results. Water condition parameters varied with sampling sites but values or each parameter (specific conductivity, dissolved oxygen, pH, temperature, and turbidity were consistent in the two swimming seasons (Table 4). All water samples detected positive of indicator *E. coli*. Concentrations of *E. coli* in water from positive sites ranged from 3.0 MPN/100 ml to 488.4 MPN/100 ml in 2012 and 13.4 MPN/100 ml to 2419.6 MPN/100 ml in 2013 (Table 5). In 2013, mean concentration of *E. coli* in one sampling site (Lake Natoma at Nimbus Flat-Left) exceeded the Basin Plan objective (400 MPN/100 ml). In both 2012 and 2013, mean concentrations of *E. coli* in four sampling sites exceeded the EPA 2012 RWQC (Recreational Water Quality Criteria, 126 CFU/100 ml).

Cryptosporidium spp. oocysts were detected in water samples collected from all the sampling sites, with an overall of 50.0% water samples positive. Concentration of oocysts in positive samples ranged from 0.04 to 0.23 oocysts/L in 2012 and 0.04 to 1.22 oocysts/L in 2013 (Table 6). Most of the detected oocysts were damaged. Low concentration of oocysts and damages to oocysts resulted in the failure of PCR of *Cryptosporidium* in the present study due to limited DNA or degraded DNA. Water samples from eight of the sampling sites were detected positive of *Giardia* spp. Overall, 28.78% of all water samples from all sites were positive of *Giardia* spp. Concentration of cysts in positive samples ranged from 0.04 to 1.56 cysts/L in 2012 and 0.04 to 1.13 cysts/L in 2013 (Table 7). Most of the detected cysts were damaged. *E. coli* O157:H7was detected in 9.09% of all water samples but limited in three sampling sites (Table 8). *Salmonella* was detected in water samples from all but one of the sampling sites. Concentrations of *Salmonella* in positive water samples ranged from 0.08 MPN/L to 4.99MPN/L in 2012 and 0.10 MPN/L to 14.87 MPN/L in 2013 (Table 9). *Cryptosporidium* concentrations in water were associated with previous 30 day wind speed and rainfall, and DO concentrations in surface water. *Giardia* concentrations in water were associated with *E. coli* concentrations, turbidity and pH of surface water, and 24 hour previous air temperature. *Salmonella* concentrations in water were associated with previous 30-day air temperature. *E. coli* O157:H7 presences in water were associated with previous 30 day solar radiation and rainfall. No significant relationships were observed between pathogen concentrations at sampling sites and other field parameters (conductivity, water temperature, collection year, site elevation, or indicator *E. coli* surface water concentrations).

Field parameters. Field water parameters including specific conductivity, DO, pH, temperature, and turbidity are shown in Table 4. These parameters varied with sampling sites but values for each parameter were consistent in the two swimming seasons (years).

Table 4. Field parameters of the Central Valley Safe-to-Swim Study, 2012-2013

Map ID	Station Number	Site Description	Specific Conductivity (µS/cm)		DO (mg/L)		pH		Temperature (°C)		Turbidity (NTU)	
			2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
1	519AMNDVY	American River at Discovery Park	58.67	52.00	9.35	9.48	7.45	7.64	19.68	20.86	3.15	3.72
2	531PLA900	Dry Creek/Cirby Creek Confluence	140.40	96.25	8.17	8.91	7.58	7.58	21.02	20.75	6.14	3.98
3	514PLABPL	Folsom Lake at Beal's Point-Left	NA	54.33	NA	9.27	NA	7.86	NA	21.94	NA	2.80
4	551KIN060	Kings River at Laton-Kingston Park	31.43	NA	6.63	NA	7.33	NA	19.15	NA	3.00	NA
5	552FRE511	Kings River at Reedley Beach	55.88	48.47	6.93	4.78	7.25	7.28	18.48	21.23	2.00	1.21
6	552FRE510	Kings River at Winton Park	29.20	28.05	7.66	5.83	7.04	7.42	9.82	19.73	0.00	2.53
7	519SACNFL	Lake Natoma at Nimbus Flat-Left	NA	56.75	NA	8.06	NA	7.38	NA	19.49	NA	3.08
8	519PLA921	Linda Creek at Condor Court	366.20	NA	6.33	NA	7.53	NA	20.29	NA	2.80	NA
9	531PLA902	Miner's Ravine/Secret Ravine Confluence	126.80	NA	8.79	NA	7.60	NA	20.27	NA	2.83	NA
10	516NEV906	Squirrel Creek in Western Gateway Park	130.80	91.00	9.35	8.92	7.68	7.69	18.90	18.66	2.47	1.45
11	535STC218	Tuolumne River at Fox Grove	123.67	94.00	8.44	7.38	7.71	7.49	24.09	26.21	2.85	NA

NA: Not Applicable

***E. coli*.** Indicator *E. coli* was detected in all water samples (100%) collected from all the eleven sites in the two years. Concentrations of *E. coli* in water from positive sites ranged from 3.0 MPN/100 ml to 488.4 MPN/100 ml in 2012 and 13.4 MPN/100 ml to 2419.6 MPN/100 ml in 2013. In 2012, the highest concentration was 488.4 MPN/100ml in a sample collected from American River at Discovery Park. In 2013, the highest concentration was 2419.60 MPN/100 ml in a sample collected from Lake Natoma at Nimbus Flat-Left (Table 5). As shown in the table, in 2013, mean concentration of *E. coli* in one sampling site (Lake Natoma at Nimbus Flat-Left) exceeded the Basin Plan objective (400 MPN/100 ml). In both 2012 and 2013, mean concentrations of *E. coli* in four sampling sites exceeded the EPA 2012 RWQC (Recreational Water Quality Criteria, 126 CFU/100 ml).

***Cryptosporidium* spp.** *Cryptosporidium* spp. oocysts were detected in water samples collected from all the eleven sites in the two years. The overall prevalence was 50.0% in the two years and 39.47% and 64.28% for 2012 and 2013 respectively. The prevalence of *Cryptosporidium* varied among sampling sites, with highest prevalence of 80.0% in Dry Creek/Cirby Creek Confluence site in 2012 and 100% in Dry Creek/Cirby Creek Confluence and Kings River at Laton-Kingston Park sites in 2013. Overall, Dry Creek/Cirby Creek Confluence, Lake Natoma at Nimbus Flat-Left, and American River at Discovery Park were the sites of high prevalence of *Cryptosporidium* spp. (88.89%, 75.0%, and 71.4% respectively). Despite high *Cryptosporidium* spp. prevalence, concentrations of oocysts in all but one positive water sample were <1 oocyst/L. The sample that had >1 oocyst/L (1.22 oocysts/L) was collected from Lake Natoma at Nimbus Flat-Left in 2013 (Table 6). Concentration of oocysts in positive samples ranged from 0.04 to 0.23 oocysts/L in 2012 and 0.04 to 1.22 oocysts/L in 2013 (Table 6). In addition to low *Cryptosporidium* spp. oocyst concentrations in positive samples, most oocysts were damaged, such as empty shells, partially excysted oocysts, shrinked oocysts, contracted oocysts with separation of oocyst wall and contents. Low concentration of oocysts and damages to oocysts resulted in the failure of PCR of *Cryptosporidium* in the present study due to limited DNA or degraded DNA. As reported elsewhere, PCR are frequently hampered due to the low numbers of oocysts and inhibitors in samples (Kostrzynska et al., 1999; Elmore et al., 2013).

Giardia. Water samples from eight of the sampling sites detected positive of *Giardia* spp. The overall prevalence was 28.78% in the two years and 23.68% and 35.71% for 2012 and 2013 respectively. Among the eight sites detected positive of *Giardia* spp., the prevalence of *Giardia* varied with sites, with higher prevalence detected in American River at Discovery Park (71.43%), Dry Creek/Cirby Creek Confluence (66.67%), and Folsom Lake at Beal's Point-Left (66.67%). Concentrations of cysts in all but two of the positive samples were <1 cyst/L. A sample collected from Dry Creek/Cirby Creek Confluence (2012) had a concentration of 1.56 cysts/L and another sample collected from American River at Discovery Park (2013) had a concentration of 1.13 cysts/L. Concentration of cysts in positive samples ranged from 0.04 to 1.56 cysts/L in 2012 and 0.04 to 1.13 cysts/L in 2013 (Table 7). In addition to the low concentrations in *Giardia* positive samples, most cysts were damaged, such as empty shells, shrinked or folded cysts, and contracted cysts with separation of cyst wall and contents.

Table 5. *E. coli* concentrations in surface water from monitoring sites of the Central Valley Safe-to-Swim Study, 2012-2013

Map ID	Station Number	Site Description	Overall % prevalence (positive/total samples of the two years)	*E. coli* Concentrations (MPN/100ml)					
				2012 (May-September)			2013 (June-September)		
				Min	Max	Mean	Min	Max	Mean
1	519AMNDVY	American River at Discovery Park	100 (7/7)	21.60	488.40†‡	193.50†	48.10	325.50	178.70†
2	531PLA900	Dry Creek/Cirby Creek Confluence	100 (9/9)	151.50	248.10	219.58†	178.50	248.90	229.08†
3	514PLABPL	Folsom Lake at Beal's Point-Left	100 (3/3)	NA	NA	NA	43.20	116.20	80.33
4	551KIN060	Kings River at Laton-Kingston Park	100 (3/3)	35.00	93.40	71.47	NA	NA	NA
5	552FRE511	Kings River at Reedley Beach	100 (3/3)	34.50	74.90	54.40	NA	NA	NA
6	552FRE510	Kings River at Winton Park	100 (3/3)	3.00	3.10	3.07	NA	NA	NA
7	519SACNFL	Lake Natoma at Nimbus Flat-Left	100 (4/4)	NA	NA	NA	29.50	2419.60†‡	646.00†‡
8	519PLA921	Linda Creek at Condor Court	100 (5/5)	32.30	290.90	174.06†	NA	NA	NA
9	531PLA902	Miner's Ravine/Secret Ravine Confluence	100 (5/5)	41.10	160.70	96.24	NA	NA	NA
10	516NEV906	Squirrel Creek in Western Gateway Park	100 (9/9)	178.50	248.10	203.26†	108.10	156.50	138.23†‡
11	535STC218	Tuolumne River at Fox Grove	100 (5/5)	3.00	137.60	49.37	13.40	13.40	13.40
MEAN:			100 (56/56)	55.61	193.9	118.3	70.1	546.68	214.29

NA: Not applicable; †: concentration exceeded Basin Plan guideline (400 MPN/100 ml); ‡: concentration exceeded EPA 2012 RWQC (126 CFU/100 ml)

Table 6. Occurrence of *Cryptosporidium* spp. in surface water from monitoring sites of the Central Valley Safe-to-Swim Study, 2012-2013

Map ID	Station Number	Site Description	Overall % prevalence (positive/total samples of the two years)	2012 (May-September)				2013 (June-September)			
				% (positive /total samples	Concentration (no. oocyst/L)			% (positive /total samples	Concentration (no. oocyst/L)		
					Min	Max	Mean		Min	Max	Mean
1	519AMNDVY	American River at Discovery Park	71.43 (5/7)	66.67 (2/3)	0.09	0.23	0.16	75.00 (3/4)	0.09	0.32	0.18
2	531PLA900	Dry Creek/Cirby Creek Confluence	88.89 (8/9)	80.00 (4/5)	0.04	0.14	0.07	100.00 (4/4)	0.04	0.12	0.06
3	514PLABPL	Folsom Lake at Beal's Point-Left	66.67 (2/3)	NA	NA	NA	NA	66.67 (2/3)	0.08	0.12	0.10
4	551KIN060	Kings River at Laton-Kingston Park	20.00 (1/5)	0.00 (0/4)	NA	NA	NA	100.00 (1/1)	0.08	0.08	0.08
5	552FRE511	Kings River at Reedley Beach	42.86 (3/7)	25.00 (1/4)	0.07	0.07	0.07	66.67 (2/3)	0.13	0.29	0.21
6	552FRE510	Kings River at Winton Park	50.00 (3/6)	33.33 (1/3)	0.04	0.04	0.04	66.67 (2/3)	0.10	0.36	0.23
7	519SACNFL	Lake Natoma at Nimbus Flat-Left	75.00 (3/4)	NA	NA	NA	NA	75.00 (3/4)	0.18	1.22	0.53
8	519PLA921	Linda Creek at Condor Court	40.00 (2/5)	40.00 (2/5)	0.05	0.12	0.08	NA	NA	NA	NA
9	531PLA902	Miner's Ravine/Secret Ravine Confluence	40.00 (2/5)	40.00 (2/5)	0.04	0.04	0.04	NA	NA	NA	NA
10	516NEV906	Squirrel Creek in Western Gateway Park	33.33 (3/9)	60.00 (3/5)	0.04	0.14	0.07	0.00 (0/4)	NA	NA	NA
11	535STC218	Tuolumne River at Fox Grove	16.67 (1/6)	0.00 (0/4)	NA	NA	NA	50.00 (1/2)	0.13	0.13	0.13
MEAN:			50.0 (33/66)	39.47 (15/38)	0.05	0.11	0.08	64.28 (18/28)	0.10	0.33	0.19

NA: Not Applicable

Table 7. Occurrence of *Giardia spp.* in surface water from monitoring sites of the Central Valley Safe-to-Swim Study, 2012-2013

Map ID	Station Number	Site Description	Overall % prevalence (positive/total samples of the two years)	2012 (May-September)				2013 (June-September)			
				% (positive /total samples)	Concentration (no. cyst/L)			% (positive /total samples)	Concentration (no. cyst/L)		
					Min	Max	Mean		Min	Max	Mean
1	519AMNDVY	American River at Discovery Park	71.43 (5/7)	66.67 (2/3)	0.05	0.26	0.16	75.0 (3/4)	0.09	1.13	0.44
2	531PLA900	Dry Creek/Cirby Creek Confluence	66.67 (6/9)	80.00 (4/5)	0.04	1.56	0.68	50.00 (2/4)	0.19	0.31	0.25
3	514PLABPL	Folsom Lake at Beal's Point-Left	66.67 (2/3)	NA	NA	NA	NA	66.67 (2/3)	0.04	0.13	0.08
4	551KIN060	Kings River at Laton-Kingston Park	20.00 (1/5)	25.00 (1/4)	0.04	0.04	0.04	0.00 (0/1)	NA	NA	NA
5	552FRE511	Kings River at Reedley Beach	0.00 (0/7)	0.00 (0/4)	NA	NA	NA	0.00 (0/3)	NA	NA	NA
6	552FRE510	Kings River at Winton Park	16.67 (1/6)	0.00 (0/3)	NA	NA	NA	33.33 (1/3)	0.08	0.08	0.08
7	519SACNFL	Lake Natoma at Nimbus Flat-Left	50.00 (2/4)	NA	NA	NA	NA	50.00 (2/4)	0.10	0.11	0.10
8	519PLA921	Linda Creek at Condor Court	0.00 (0/5)	0.00 (0/5)	NA	NA	NA	NA	NA	NA	NA
9	531PLA902	Miner's Ravine/Secret Ravine Confluence	20.00 (1/5)	20.00 (1/5)	0.04	0.04	0.04	NA	NA	NA	NA
10	516NEV906	Squirrel Creek in Western Gateway Park	11.11 (1/9)	20.00 (1/5)	0.10	0.10	0.10	0.00 (0/4)	NA	NA	NA
11	535STC218	Tuolumne River at Fox Grove	0.00 (0/6)	0.00 (0/4)	NA	NA	NA	0.00 (0/2)	NA	NA	NA
MEAN:			28.78 (19/66)	23.68 (9/38)	0.05	0.40	0.20	35.71 (10/28)	0.10	0.35	0.19

NA: Not Applicable

***E. coli* O157:H7**. Approximately 9% (6/66) of all the water samples detected positive for *E. coli* O157:H7. *E. coli* O157:H7 was detected in three sites, American River at Discovery Park, Dry Creek/Cirby Confluence, and Squirrel Creek in Western Gateway Park. The first two sites detected positive for *E. coli* O157:H7 in 2013 only while the third site detected positive for *E. coli* O157:H7 in both 2012 and 2013 (Table 8).

Table 8. Occurrence of *E. coli* O157:H7 in surface water from monitoring sites of the Central Valley Safe-to-Swim Study, 2012-2013

Map ID	Station Number	Site Description	Overall % prevalence (positive/total samples of the two years)	*E. coli* O157:H7 positive samples	
				2012 (May-September)	2013 (June-September)
1	519AMNDVY	American River at Discovery Park	14.29 (1/7)	0 (0/3)	25.0 (1/4)
2	531PLA900	Dry Creek/Cirby Confluence	11.11 (1/9)	0 (0/5)	25.0 (1/4)
3	514PLABPL	Folsom Lake at Beal's Point Left	0.00 (0/3)	NA	0 (0/3)
4	551KIN060	King's River at Laton-Kingston Park	0.00 (0/5)	0 (0/4)	0 (0/1)
5	552FRE511	King's River at Reedley Beach	0.00 (0/7)	0 (0/4)	0 (0/3)
6	552FRE510	King's River at Winton Park	0.00 (0/6)	0 (0/3)	0 (0/3)
7	519SACNFL	Lake Natoma at Nimbus Flat Left	0.00 (0/4)	NA	0 (0/4)
8	519PLA921	Linda Creek at Condor Court	0.00 (0/5)	0 (0/5)	0 (0/0)
9	531PLA902	Miner's Ravine/Secret Ravine Confluence	0.00 (0/5)	0 (0/5)	0 (0/0)
10	516NEV906	Squirrel Creek in Western Gateway Park	44.44 (4/9)	40.0 (2/5)	50.0 (2/4)
11	535STC218	Tuolumne River at Fox Grove	0.00 (0/6)	0 (0/4)	0 (0/2)
		MEAN:	9.09 (6/66)	5.26 (2/38)	14.28 (4/28)

Salmonella. *Salmonella* was detected in water samples from all the sampling sites except for Folsom Lake at Beal's Point-Left. The overall prevalence was 68.18% in the two years and 73.68% and 60.71% for 2012 and 2013 respectively. Concentrations of *Salmonella* in positive water samples ranged from 0.08 MPN/L to 4.99MPN/L in 2012 and 0.10 MPN/L to 14.87 MPN/L in 2013. Highest concentration in 2012 was 4.99 MPN/L in a sample collected from Kings River at Laton-Kingston Park. Highest concentration in 2013 was 14.87 MPN/L in a sample collected from Squirrel Creek in Western Gateway Park (Table 9).

Table 9. Occurrence of *Salmonella* in surface water from monitoring sites of the Central Valley Safe-to-Swim Study, 2012-2013

Map ID	Station Number	Site Description	Overall % prevalence (positive/total samples of the two years)	2012 (May-September)				2013 (June-September)			
				% (positive/ total samples)	Concentration (MPN/L)			% (positive/ total samples)	Concentration (MPN/L)		
					Min	Max	Mean		Min	Max	Mean
1	519AMNDVY	American River at Discovery Park	28.57 (2/7)	33.33 (1/3)	0.14	0.14	0.14	25.0 (1/4)	0.13	0.13	0.13
2	531PLA900	Dry Creek/Cirby Creek Confluence	88.88 (8/9)	100.00 (5/5)	0.13	3.60	1.24	75.0 (3/4)	0.14	1.38	0.63
3	514PLABPL	Folsom Lake at Beal's Point-Left	0 (0/3)	NA	NA	NA	NA	0 (0/3)	NA	NA	NA
4	551KIN060	Kings River at Laton-Kingston Park	100 (5/5)	100.00 (4/4)	0.39	4.99	2.05	100.00 (1/1)	2.15	2.15	2.15
5	552FRE511	Kings River at Reedley Beach	100 (7/7)	100.00 (4/4)	0.15	1.97	0.95	100 (3/3)	0.15	6.00	2.19
6	552FRE510	Kings River at Winton Park	33.33 (2/6)	0.00 (0/3)	NA	NA	NA	66.67 (2/3)	0.15	0.55	0.35
7	519SACNFL	Lake Natoma at Nimbus Flat-Left	75.0 (3/4)	NA	NA	NA	NA	75.0 (3/4)	0.10	1.00	0.41
8	519PLA921	Linda Creek at Condor Court	60 (3/5)	60.00 (3/5)	0.18	1.42	0.82	NA	NA	NA	NA
9	531PLA902	Miner's Ravine/Secret Ravine Confluence	100 (5/5)	100.00 (5/5)	0.08	1.68	1.02	NA	NA	NA	NA
10	516NEV906	Squirrel Creek in Western Gateway Park	88.88 (8/9)	100.00 (5/5)	0.30	1.56	0.74	75.0 (3/4)	2.15	14.87	8.78
11	535STC218	Tuolumne River at Fox Grove	33.33 (2/6)	25.00 (1/4)	0.55	0.55	0.55	50.00 (1/2)	0.13	0.13	0.13
MEAN:			68.18 (45/66)	73.68 (28/38)	0.24	1.99	0.94	60.71 (17/28)	0.64	3.27	1.85

NA: Not Applicable

Statistical Results: *Cryptosporidium* concentrations in surface water were associated with previous 30 day wind speed (P=0.00), previous 30 day rainfall (P=0.046), and DO concentrations (P=0.00) in surface water. *Giardia* concentrations in surface water were associated with *E. coli* concentrations (P=0.04), turbidity (P=0.00) and pH (P=0.00) of surface water, and 24 hour previous air temperature (P=0.02). *Salmonella* concentrations in surface water were associated with previous 30-day air temperature (P=0.028). *E. coli* O157:H7 presence in water were associated with previous 30 day solar radiation (P=0.05) and previous 30 day rainfall (P=0.01). Yet, no significant relationships were observed between pathogen concentrations at sampling sites and other field parameters (conductivity, water temperature, collection year, site elevation, or indicator *E. coli* surface water concentrations).

9.0 DISCUSSION

Indicator *E. coli* concentrations in 9.09% of the sampling sites exceeded the Basin Plan objective (400 MPN/100ml) in the two years and 54.54% and 36.36% of the sampling sites exceeded EPA recommended 2012 RWQC guideline (126 CFU/100 ml) in 2012 and 2013 respectively (Tables 5 and10). Although prevalence of *Cryptosporidium*, *Salmonella*, and *E. coli* O157:H7 were not significantly associated with *E. coli* concentrations, *Giardia* concentrations were associated with *E. coli* concentrations. Sites with *E. coli* concentrations exceeded water quality objectives and guidelines especially those with high prevalence of pathogens present water quality concern and warrant a more in-depth investigation of the cause of the water quality impairment.

Table 10. Comparison of *E. coli* concentrations to water quality objectives and guidelines

Comparison to State Board Basin Plan and EPA guideline		**2012 (May-September)**	**2013 (June-September)**
Basin Plan objective (fecal coliform not to exceed 400 MPN/100 ml in >10% of samples)*	% of sites exceed objective	9.09% (1/11)	9.09% (1/11)
EPA RWQC 2012 (*E. coli* not to exceed geometric mean of 126 CFU/100 ml in any 30-day interval)*	% of sites exceed guideline	54.54% (6/11)	36.36% (4/11)

*This project sampled each site several times over the 2 years which does not match the 30-day time frame of the Basin Plan for fecal coliform monitoring or the EPA RWQC *E. coli* monitoring. Sites with one or more samples having >400 *E. coli* MPN/100 ml were classified as exceeding the State water quality objective. Sites with one or more sample having >126 *E. coli* MPN/100 ml classified as exceeding the EPA 2012 RWQC.

Parameters for indicators of Beneficial Use protection were drawn from the Central Valley Water Board Basin Plan, Calfed Guidelines, EPA RWQC 2012, State Board Objectives, and recommendations from the Food and Agriculture Organization of the United Nations and Water Quality for Agriculture. Table 11 summarizes the parameters used to determine whether beneficial uses were protected for each of the constituents measured that can be used as reference studies for water quality objectives.

Table 11. California Department of Public Health reports of risk factors of bacterial contamination related to outbreaks, 1996 - 2008

Report Title	**Year of Publication**	**Risk Factor**
Investigation of an *E. coli* O157:H7 Outbreak Linked to Fancy Cutt Farms	1996	No conclusive finding
Environmental Investigation of *Salmonella* Enteritidis, Phage Type 30 Outbreak Associated with Consumption of Raw Almonds	2001	Application of primary or secondary treated sewage effluent
E. coli O157:H7 Illnesses in Washington – July, 2002	2002	No conclusive finding
Report of Investigation of *E. coli* Outbreak at San Mateo County Retirement Facility in October 2003	2004	Flood irrigation water
Investigation of Pre-washed Mixed Bagged Salad Following an Outbreak of *E. coli* O157:H7 in San Diego and Orange County	2004	Irrigation water, drainage ditch flooding
Environmental Investigation of *Escherichia coli* O157:H7 Outbreak Associated with Taco Bell Restaurants in Northeastern States.	2007	No conclusive finding
Investigation of an *Escherichia coli* O157:H7 Outbreak Associated with Dole Pre-packaged Spinach	2007	Cattle feces, wild pig feces, soil, and river water samples
Investigation of the Taco John's *Escherichia coli* O157:H7 Outbreak Associated with Iceberg Lettuce.	2008	Lettuce growing regions in California's Central Coast and Central Valley, specifically, potential of microbial cross-contamination between growing fields of lettuce and nearby dairies.

This study allowed for a preliminary investigation of pathogen occurrence of pathogens in monitoring swimming sites in the Central Valley and provided additional data for SWAMP's Safe-to-Swim Study. Because of the limit of sample numbers and sampling frequency, as well as technology challenges, we can hardly draw conclusions regarding the source of fecal contamination and correlations between sources of contamination

and bacterial concentrations in water. Future studies with increased sampling frequencies and focus on sites of high prevalence of pathogens will provide more data regarding seasonal occurrence and sources of pathogens.

10.0 RECOMMENDATIONS

- Longitudinal studies focused on selected sites with *E. coli* concentrations exceeded water quality objectives and guidelines especially those with higher prevalence of *Cryptosporidium* spp., *Giardia* spp., *E. coli* O157:H7 and *Salmonella* in the present study. With increased sampling frequencies for detection of these pathogens in water from these sites, data of seasonal occurrence and concentrations of pathogens can be obtained.
- Epidemiology studies on pathogen positive sites would provide valuable information for identifying the water contamination source(s). Future studies should expand assessments of frequency and density of human recreational activities, seasonality, prevalence of major wildlife species surrounding sites, proximity to farm types and size, among other uninvestigated variables will help to further investigate the potential source and associations to microbial water contamination.
- The retentate volume used for detection of *Cryptosporidium* spp. and *Giardia* spp. was equivalent to ~ 20 L of water, which increased the detection possibility of the two waterborne parasites in the present study. However, genotyping failed due to the low concentrations and damages of oocysts. The Atwill Water & Foodborne Zoonotic Disease Laboratory recommends conducting future intensive (increasing sampling frequency and duration based on recreational activity and weather events) studies to focus on the sites with high prevalence of *Cryptosporidium* spp. For example, if oocysts were detected in a site, immediately resample the site to concentrate larger volumes of water to obtain more oocysts for DNA extraction. Such efforts will increase the possibility for successful genotyping of *Cryptosporidium* oocysts detected.
- Further characterize bacteria pathogens detected from recreational water.
    a. In order to better understand the virulence of *E. coli* O157:H7 and *Salmonella*, we recommend future studies that determine the presence of *E. coli* stx1, stx 2, and rfbE genes in *E. coli* O157:H7 isolates and serotyping of *Salmonella* isolates from water samples.
    b. Pulsed-field gel electrophoresis (PFGE) is among the methods for Microbial Source Tracking (MST) (Foley et al., 2009). Using well established PFGE in the laboratory, we can compare *E. coli* O157:H7 isolates and *Salmonella* isolates from water to isolates of these bacteria from humans and different animal species.
    c. DNA sequencing is another method for MST in water samples. Source of bacteria can be determined by online comparisons of sequences of DNA amplified from *E. coli* O157:H7 and *Salmonella* isolates from water to sequences of these bacteria from humans and animals published in the GenBank.

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