Beach Monitoring Phylochip Project
Clean Beaches Grant Program, Proposition 50

# Final Project Report

Agreement 07-576-550-02

Between the State Water Resources Control Board
and
County of Marin Environmental Health Services

Funding for this project was provided by the State Water Resources Control Board and came from Proposition 50: the Water Security, Clean Drinking Water, Coastal and Beach Protection Act of 2002. Additional funding provided by the Rathmann Family Foundation.

Project Cost: $942,237.10

August 2012

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## PROJECT SUMMARY

This final project report provides a brief project description, a summary of the activities completed, references to deliverables sent during the project timeline and a link to the web site developed as part of the project. Also included are several peer-reviewed scientific papers prepared by researchers associated with the project. These are attached as appendices, which are listed on page 29 of this report.

### Project Purpose, Scope and Goals

The purpose of this research project was to conduct microbial censuses, source identification and develop test procedures for water quality analysis. As described in detail in the grant agreement, the project goals can be succinctly summarized as follows:

1. Adapt the original PhyloChip to give it the capability to conduct a microbial census of recreational waters. Pathogen specific primers and probes on the microarray were to be validated. Particular attention was to be paid to the three beaches at Muir, Campbell Cove and Baker Beach, San Francisco as these sites had been identified by the Clean Beaches Task Force. (Task 1.1, 1.2).

2. Develop a suitable testing protocol and establish baselines by sampling waters in parallel with the standard AB411 beach water quality tests. Conduct baseline monitoring at these sites (Tasks 1.1 & 4).

3. Establish a website to publicize the project and disseminate information on progress. (Task 1.3).

4. Perform microbial census analyses on specific sources of water pollution such as septic system or sewage effluent, agricultural runoff etc. Specific event testing was a goal e.g. waters affected by a known sewage spill would give information about the microbial makeup of such waters. (Task 3).

5. Develop a QPCR protocol using keystone indicator species identified through the PhyloChip-based work. The test method was to be evaluated by conducting testing of retrospective samples to compare them with tests conducted using the standard methods. Diffusion chamber testing was also required to investigate the fate of bacteria in the beach environment. (Task 5).

### How the Project Addressed the Stated Goals

Previous studies and empirical field experience gained during conventional beach water quality sampling highlighted several shortcomings of the testing methods now in widespread use. Testing agencies are concerned that the present “indicator paradigm” where the presence of certain

organisms as a surrogate for human sewage may be inaccurate as only those organisms that are culturable on particular media are counted by this method and many species of concern cannot be detected. It was widely suspected that the indicator bacteria may often originate from sources other than human sewage. More recently, these bacteria have been shown to regrow in the beach environment, further calling into question the results obtained by the standard method of testing. Substantial variability between samples taken in close temporal and spatial proximity and timeliness of test results are also problematic.

A major goal of the project was to develop a testing protocol to conduct a comprehensive census of bacteria present in beach waters. Before this project, beach water quality testing was largely limited to traditional laboratory culture methods, which have significant limitations. Not all bacteria found in water bodies can be cultured and the traditional analytical methods are usually slow and time-consuming.

Water quality testing centered on recreational waters at three beaches that had been identified by the State Water Resources Control Board staff and the Clean Beaches Task Forces as "Problem Beaches." Traditional water quality sampling conducted at these sites had indicated persistent high levels of fecal bacteria. The presence of these bacteria in significant quantities is widely regarded as indicative of fecal pollution and can be cause for beach water quality advisories, with attendant possible loss of beneficial uses. For more information on the coastal monitoring program see the SWRCB web page at this link: http://tinyurl.com/7qkkbec

The three sites chosen were Campbell Cove in Sonoma County, Muir Beach in Marin County and Baker Beach in San Francisco (see Table 2 and maps on pages 10-14). Staff from the three Counties conducted routine water quality monitoring at these locations using the methods and standards contained in the monitoring program commonly known as Assembly Bill 411 (or AB 411). These routine samples were split and analyzed by both LBNL and the County laboratories that carried out the standard indicator bacteria testing methods. Samples were also frozen so that waters that were found to violate the AB411 water quality standards could be retrospectively analyzed using the PhyloChip to see which groupings of bacteria might be present and to gain an understanding of their relative abundance. For more details of the water sampling protocols and methods, see Appendices A and B of the Quality Assurance Program Plan (QAPP) for this project.

Results of the routine testing conducted by traditional methods e.g. multiple tube fermentation were compared to the results obtained by the PhyloChip method. For example, in the samples taken from waters adjacent to Campbell Cove, the PhyloChip detected 1524 different types of bacteria using the second-generation PhyloChip. By contrast, only a few species could be identified by traditional culture methods; the PhyloChip method does not require the growth of bacteria in order to reliably detect them. The LBNL staff developed specific primers and genetic probes on the PhyloChip to look for the various types of bacteria of interest. Incorporated into the PhyloChip are error checking and quality control/assurance systems, which are described in detail in the QAPP.

Another objective of the project was to determine whether the analytical capability of the PhyloChip (when adapted to aquatic sampling) could help to differentiate sources of bacterial pollution in receiving waters. If this were possible, then source tracking projects could harness the potential of the PhyloChip to more accurately determine and enumerate the sources of bacteria in

bodies of water such as Tomales Bay, where a TMDL had relied on estimates of contributions of bacteria from various sources. More details of these goals and the results achieved are provided in the remainder of this section, in the Project Evaluation and Effectiveness section beginning on page 13, and in Appendix 5 (see first paper). In particular, the a summary of the results of the part of the research focused on source differentiation conducted as part of this project can be found in the third panel of the poster presentation entitled: *Application of comprehensive bacterial community analysis to discriminate common sources of fecal pollution* (Appendix 5). Also see Appendix 3, line 140

Water quality testing was conducted in other locations, notably during a sizeable sewage spill that occurred in February 2009 off the shoreline of Sausalito, CA. This unfortunate event provided a source of known human sewage contamination with which to verify the PhyloChip's ability to detect and display distinct groupings of bacteria, such that the presence of human sewage could be reliably distinguished from other sources of bacteria e.g. avian, pinniped, bovine etc. For more details on the testing conducted during the 730,000-gallon sewage spill, including a map of the sampling, locations, see Appendix 2, Page 40.

The final aim of the project (Task 5) as it was initially conceived was to design a real-time quantitative polymerase chain reaction (qPCR) test to detect specific bacteria of human origin in recreational waters. Early on in the project it became apparent that the analytical and discriminative capabilities of the third generation PhyloChip greatly exceeded that of the (comparably priced) qPCR test that was originally envisaged by the project proponents. The project team carefully considered an alternative to the PhyloChip that would test for a limited suite of bacteria of interest. However, tests using "simulated qPCR" methodology did not exclude sources of bacteria with an acceptably high degree of statistical confidence. Furthermore, once the complexity of the receiving waters became apparent as revealed by the third-generation PhyloChip, staff realized that the ability of the PhyloChip to resolve and display groupings of source bacteria with a high degree of precision could not be matched by a more limited set of indicators. Project staff consulted advisors at Southern California Coastal Water Research Project and SWRCB staff (Gjerde, Peterson) who agreed that the best approach would be to develop analytical and data display techniques using the PhyloChip, rather than pursuing development of a qPCR method. Therefore this task was modified to provide microbial analytical techniques using the PhyloChip as described in Appendix 4. The Introduction to this section on page 1 provides further background, elucidation and rationale for the decision to emphasize the source tracking capabilities of the PhyloChip rather than pursuing the qPCR methodology.

A related goal of this project was to conduct diffusion chamber testing. Succinctly, this type of test was designed to track the fate of indicator bacteria such as E. coli in the beach environment. Specialized containers holding the bacteria are placed in the beach environment and periodically tested to see if survival or reproduction occurred. The results of this testing are described in Appendix 2, page 40 et seq.

**Roles and Responsibilities**

The project was coordinated and managed by the grantee, the County of Marin's Environmental Health Services Division (County). The project director managed the administrative and financial aspects of the grantee's side of the project with assistance from the County's administrative staff.

In order to develop the tools and to retain the expertise necessary to conduct the "microbial census" of recreational and other water bodies, the County contracted with a laboratory headed by Dr.'s Terry Hazen and Gary Andersen at Lawrence Berkeley National Laboratory, Earth Sciences Division, Ecology Department (LBNL). A scientific coordinator, John Hulls, was retained under contract to the County to act as a liaison between the County and LBNL staffs, and to assist in coordinating field sampling activities. To provide public outreach, County staff established a web site under the domain name phylochip.com and updated this site as developments occurred.

A principal early goal of the project was to adapt the gene chip known as the PhyloChip to water quality sampling purposes. The original version of the PhyloChip was developed by LBNL using funding from the Department of Homeland Security in order to detect and give warning of the presence of pathogenic microorganisms in the air. Central aspects of this project were the transfer of the technology of the existing PhyloChip to aquatic testing and subsequent application of that technology to address public health problems in the recreational water environment.

During the project, the LBNL staff enhanced the capabilities of the PhyloChip through several successive redesigns, each time increasing its scope of analytical capability. The final, third-generation, PhyloChip is capable of detecting and reporting over 59,000 discrete taxa of bacteria. This is almost an order of magnitude greater testing capability than that of the original PhyloChip.

The PhyloChip's design incorporates robust multiple onboard error-checking systems to ensure reliable output data. Affymetrix Corporation of Santa Clara, CA manufactured the PhyloChips used in the project.

Regular meetings were held at the County facilities and LBNL to assess progress and coordinate work on the project. LBNL hired post-doctoral researchers (Cindy Wu, Eric Dubinsky and others) to work on the project and they co-authored some of the scientific papers (see Appendices 3-6).

During the project, staff worked closely with subject matter experts such as the Beach Water Quality Workgroups that meet regularly in Northern and Southern California. Valuable advice and cooperation was also received from the scientists at the Southern California Coastal Water Research Project (SCCRWRP), particularly its Director, Dr. Steve Weisberg. SCCRWRP personnel are listed as co-authors on a paper recently published in Environmental Science. Project scientific personnel were invited to give presentations were given at several conferences including the EPA National Beaches Conference and International Society of Microbial Ecology (see Appendix 5).

**Background & Discussion**

This project began when Marin County environmental health officials became concerned about the accurate detection of risk posed by pathogenic bacteria in its many recreational waters. Of special concern was the detection of pathogens at public bathing beaches, especially at those beaches that lacked a storm drain outfall that could be contributing bacteria from human and urban sources. At the time the project was proposed, little was known about the microbial communities in the recreational water environment, and the PhyloChip was seen as a method to gain understanding of the microbiome in these settings. Secondarily, in Tomales Bay and the tributaries and watersheds that fed into it, there was substantial debate regarding the sources of pathogens in the watershed. In particular, assertions were made by various local groups that the causes of bacterial exceedances of standards were associated with specific sources ranging from defective septic tank systems to wildlife to agriculture. Thus, two primary goals of the project were to evaluate the effectiveness of conventional indicator bacteria in predicting the presence of human waste in recreational and other waters, and to see if it was possible to use the PhyloChip technology to reliably and accurately detect the sources of bacteria in the receiving waters. See Appendix 2, page 48 for further elucidation on this topic.

Since approximately 2001, the County of Marin has tested marine and fresh recreational waters using traditional laboratory methods. In recent years, the process of sampling, testing and reporting results has been accelerated somewhat by using faster methods such as the proprietary Enteroalert and Colilert tests manufactured by Idexx Laboratories. However, even the results from these methods are typically not available for one or two days and then a staff member must drive to the site to post a warning sign, incurring a further delay. The web site is also updated regularly to show the most recent testing results. Wider concerns have been expressed statewide about the accuracy of the traditional "indicator organism paradigm" that relies upon the presence of certain microorganisms and their concentration to assess the safety of recreational waters. Therefore the project sought to determine whether improvements could be made to the current testing and reporting system.

A volunteer staff member working with Marin County's Environmental Health Division was familiar with research performed for Homeland Security by Lawrence Berkeley National Laboratory (LBNL) to conduct a broad census of airborne bacteria to support the detection of airborne bioweapons. This system had the ability to detect nearly 9000 taxa of bacteria in a single test. Subsequent discussions with LBLN determined that the same technology could be adapted to perform a comprehensive microbial community assessment in natural bodies of water. This led to the County of Marin submitting a grant application to California State Water Resources Control Board (SWRCB) under the Proposition 50 Clean Beaches Initiative to use the LBNL technology to conduct a microbial census of selected recreational beaches in Marin, Sonoma and San Francisco. The survey would include selected "problem" beaches identified by the SWRCB "Clean Beaches" task force, who supported the project proposal.

Due to the advanced technical capabilities of LBNL, the County of Marin conducted the main part of the project by contracting with LBNL to develop the PhyloChip microarray and conduct the required water sampling, tests and preparation of scientific papers. Several papers were prepared by LBNL staff, and are attached to this report in the appendices. Scientific liaison work between the

County of Marin and LBNL was carried out under contract to the County, by John R. Hulls. Mr. Hulls also took an active part in field sampling and worked closely with LBNL staff during all phases of the PhyloChip's development as a tool to measure bacteria in recreational waters.

The LBNL technology uses a microarray known as the Phylochip, manufactured by Affymetrix Corporation, to detect specific taxa based on the unique structure of the 16S gene, which is conserved in all bacteria and archaea (another type of microscopic cell with no nucleus). DNA is extracted from the samples, and washed onto the microarray, where it bonds to specific synthetic nucleotide probes, which are then scanned by a laser to detect the presence or absence of specific taxa of bacteria. Rapid development of the technology by LBNL enabled an even more powerful version of the PhyloChip to be used for the Clean Beaches project, capable of detecting 50,000 taxa of bacteria, thus enabling for the first time a rapid method of interrogating essentially entire microbial communities in a single test, which could be performed at a fraction of the cost and time of conventional sequencing methods.

One of the main questions facing communities and regulators was the question of whether the current indicator bacteria test methods were accurately indicating the presence of human fecal matter in recreational waters, especially from non-point sources, such as septic system leakage, or agricultural runoff, as opposed to discharge from major municipal treatment discharges. Thus, a testing program was developed wherein Marin, Sonoma and San Francisco agencies responsible for testing would collect parallel samples over portions of two recreational seasons of their State mandated AB411 programs. The water samples given to the project would be frozen at -80 degrees Celsius for later extraction. Tests were also conducted over complete tidal cycles to determine the impacts of tides in community composition.

In addition, tests were conducted using diffusion chambers. Essentially, a diffusion chamber is a 'cage' for bacteria that is suspended in the receiving waters, which can diffuse through the membranes of the chamber. By testing the community composition over time, it was possible to see the influence of the receiving waters on the community. The tests indicated that receiving water type affects microbial community dynamics over time. The results of this research indicate that creek and bay water exert different effects on the fate of microbial communities derived from waste. Therefore, selection of indicators for monitoring should be based on waste type and persistence of fecal taxa under various receiving waters.

Another major focus of the project was to look at the gut microbiomes of animals and birds that might contribute to the bacterial load in the waters of the test area, as well as potential human sources such as septic tank systems. To that end, samples of waste were collected for large grazing animals (elk/cows/horses) as well as for birds, seals and sea lions. Human sources samples were taken from septic tanks, septic holding tanks and municipal wastewater treatment plants. The sewage contained approximately fifteen thousand discrete taxa. The animal and bird sources contained approximately three thousand taxa. Each group produced a substantial number of unique taxa which could be compared with the results of the AB 411 tests, with special emphasis given to those samples that exceeded the AB 411 standards for recreational use.

The results of these tests are described in detail in the papers contained in the Appendices to this report. A more general overview is provided by the International Symposium of Microbial

Ecologists (ISME) poster sessions of the work in progress, presented in Seattle, WA and also in a recent presentation at the 2011 U.S. EPA National Beach Conference (see Appendix 5, poster *Temporal Dynamics of Cattle and Human Fecal Microbial Communities in Fresh and Marine Waters* and also Appendix 5, poster: *Application of comprehensive bacterial community analysis to discriminate common sources of fecal pollution*. The results show the power of a comprehensive microbial community analysis to discriminate between different sources of pollution, even in complex aquatic and microbial environments. The project was also able to monitor the results of a 750,000 gallon sewage spill which occurred adjacent to one of the test sites. The test results clearly showed the impact of such a spill as measured over time and in comparison to the results of the diffusion chamber tests.

The tests were able to clearly identify and/or eliminate human sewage as a factor in several bathing locations that had proved problematic with AB411 tests. For instance, the Baker Beach tests showed that the outfall from Lobos Creek was strongly influenced by specific taxa associated with humans and birds. In the areas tested in Tomales Bay where it had been thought that there was a strong influence from septic tanks and cattle, none of the taxa for those species were present as a significant percentage of the unique taxa associated with those sources. While samples were not available for all of the bird species associated with Tomales Bay, the avian samples showed a significant influence from gulls.

Muir Beach showed no exceedances of the AB411 beach water quality standards during sampling period. The Park Service was also undertaking a major reconstruction of the upstream wetlands and as part of the process was modifying the topography and drainage flow of the area. Therefore as there were no exceedances, no PhyloChip samples were analyzed as it would have been difficult to draw conclusions about what had caused prior violations of the AB411 water quality standards at this location.

By contrast, PhyloChip samples analyzed from split samples taken at Campbell Cove in Sonoma County clearly indicated that exceedances of the allowable bacterial counts under AB411 were caused by birds, and excluded human sources. PhyloChip analysis of samples taken from Baker Beach indicated that human and bird sources contributed to the exceedances of the AB411 bacterial standards at that location. For more details on the sampling locations see the table and maps on pages 10-14 below.

The Phylochip tests also showed that there are a large number of ‘cosmopolitan’ bacteria that are omnipresent in the environment. These bacteria appeared in stormwater events at all locations over widely differing types of landscapes and land uses. These bacteria, if present in sufficient quantity, are capable of triggering exceedances under the AB 411 tests, even though no bacteria of human origin are present.

The near-universal presence of the cosmopolitan bacteria underscores the point that conventional testing for E-coli and coliform bacteria is incapable of discriminating whether a source of fecal contamination originates from humans or from animal sources, whereas the Phylochip comprehensive microbial community test is capable of detecting 700 taxa that are unique to human sewage. Another example of the power of the Phylochip is evident when it is considered that the AB411 tests look at generic tests for E-coli, enterococcus and coliform, whereas the chip contains

2105 specific probes for coliforms alone. The large number of unique probes for sewage made it possible to accomplish one of the major goals of the project, namely that of determining whether human sewage is present in receiving waters where large numbers of potential sources of bacteria are present that would be detected by conventional tests. In Tomales Bay, ability to discriminate large numbers of unique taxa associated with birds and grazing animals, and to lesser extent pinnipeds, makes it possible to largely eliminate agriculture and septic tank systems as significant contributors to exceedances of AB411 water quality standards. More details of these findings can be found in the papers written by LBNL staff (these papers are included in the appendices). In particular see Appendix 2 beginning on page 37.

The project has already attracted considerable interest, with the result that RWQCB Region 1 is conducting a Phylochip survey of segments of the Russian River, and USGS and the City of Malibu have conducted preliminary surveys of the Malibu Creek lagoon and beaches. In addition, the Phylochip is being used to determine the microbiome of more potential sources of microbial contamination. This work is being conducted in conjunction with SCCWRP, Stanford University and UC Santa Barbara in the State of California Source Identification Pilot Project (SIPP) for source tracking development. Finally, it should be noted that many other uses of the PhyloChip's unique capabilities to address complex microbiomes have been further developed during the course of the project, including work on the human microbiome with UCSF and others, detection of bacterial contamination in NASA spacecraft assembly clean room facilities to prevent outbound contamination of vehicles such as the Mars rovers, and the testing protocols developed for the SWRCB project, which were used to identify the oil-consuming bacteria during the Gulf oil spill.

**TABLE ONE: ITEMS FOR REVIEW**

| Work Item | Items for Review # | Due Date | % Of Work Complete | Date Submitted |
|---|---|---|---|---|
| EXHIBIT A | Scope of Work | | | |
| | 1. GPS information for project site & monitoring locations | 8/30/07 | 100% | 2/16/2011 |
| | 2. Project Assessment & Evaluation Plan (PAEP). | 8/31/07 | 100% | 07/26/07 |
| | 3. Monitoring Plan | 8/31/08 | 100% | 2/16/2011 |
| | 4. Quality Assurance Plan (QAPP) | 8/31/08 | 100% | 2/8/2011 |
| | 5. Copy of final CEQA documentation | 7/30/2007 | 100% | 07/15/07 |
| | 6. Land owner agreements | N/A | N/A | None were needed |
| | 7. Applicable Permits | As needed | N/A | None were needed |
| B | Work to be performed by grantee | | | |
| 1.1<br>1.3<br>2.0 | Develop beach testing protocols<br>Establish Website/ database<br>Conduct standardization tests | August 2008<br>Ongoing task | 100%<br>100%<br>100% | 2/25/10<br>7/15/2007<br>5/10/2011 |
| EXHIBIT B | | | | |
| A. | INVOICING | 5/15/2011 | 100% | 5/10/2011 |
| E. | REPORTS | | | |
| | Progress and Final Reports Complete | | 100% | 6/20/2012 |

## PROJECT MONITORING, INCLUDING TABLE TWO AND LOCATION MAPS

As described in the Introduction, water quality monitoring (for bacteria) was conducted at the locations specified in the table below. Project staff successfully completed the training offered by the SWRCB for the Surface Water Ambient Monitoring Program. The purpose of the monitoring was to obtain water samples that were spilt for analysis. One set was analyzed for traditional bacterial cultures and counts under the AB411 beach monitoring program operated by County Health Department laboratories. The other portion of the sample was frozen for possible retrospective analysis using the PhyloChip method. Thus baseline tests, development of protocols and standardization were accomplished. Details of the testing methods are contained in the Quality Assurance Program Plan prepared for the project. Testing was also carried out across tidal prisms to see what variations were apparent in the microbial communities across tide cycles and time of day.

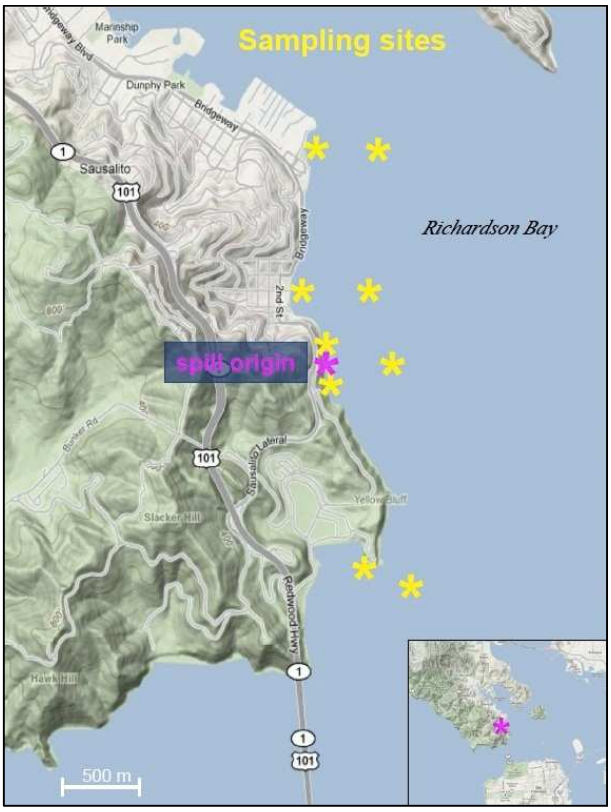
A further purpose of the monitoring activities was to develop the bacterial source tracking capability of the PhyloChip. For an example, see the discussion of the Sausalito sewage spill on page 49 of Appendix 2. Maps depicting the monitoring locations are provided on pages 10-13 of this section.

### Table Two: Monitoring Locations

| Sampling Site Location | Lat/long | Sample type | Parameters sampled | Further references |
|---|---|---|---|---|
| Sausalito, CA (Sewage spill in Marin Co.) | 37.861505 -122.487602 | AB 411-type water quality tests (Idexx Labs materials/methods: Colilert/Enterolert) & PhyloChip<br>These samples were taken during the sewage spill incident of February 2009. | Bacteria (E. coli & enterococci) PhyloChip microarray analysis | Appendix 2 page 42 |
| Muir Beach, CA (Marin Co.) | 37.85932 -122.576394 | Beach water, AB 411 water quality tests (Idexx Labs materials/methods Colilert/Enterolert) & PhyloChip | Bacteria (E. coli & enterococci) PhyloChip microarray analysis | Appendix 2 page 38 |
| Campbell Cove, CA (Sonoma Co.) | 38.304907 -123.057325 | Beach water, AB 411 water quality tests (Idexx Labs materials/methods Colilert/Enterolert) & PhyloChip | Bacteria (E. coli & enterococci) PhyloChip microarray analysis | Appendix 5: 3rd paper, 3rd panel |
| Baker Beach, CA (San Francisco Co.) | 37.792626 -122.484276 | Beach water, AB 411 water quality tests (Idexx Labs materials/methods Colilert/Enterolert) & PhyloChip | Bacteria (E. coli & enterococci) PhyloChip microarray analysis | Appendix 5: 3rd paper, 3rd panel |

**Map 1**
**Sausalito sampling locations: General Vicinity**

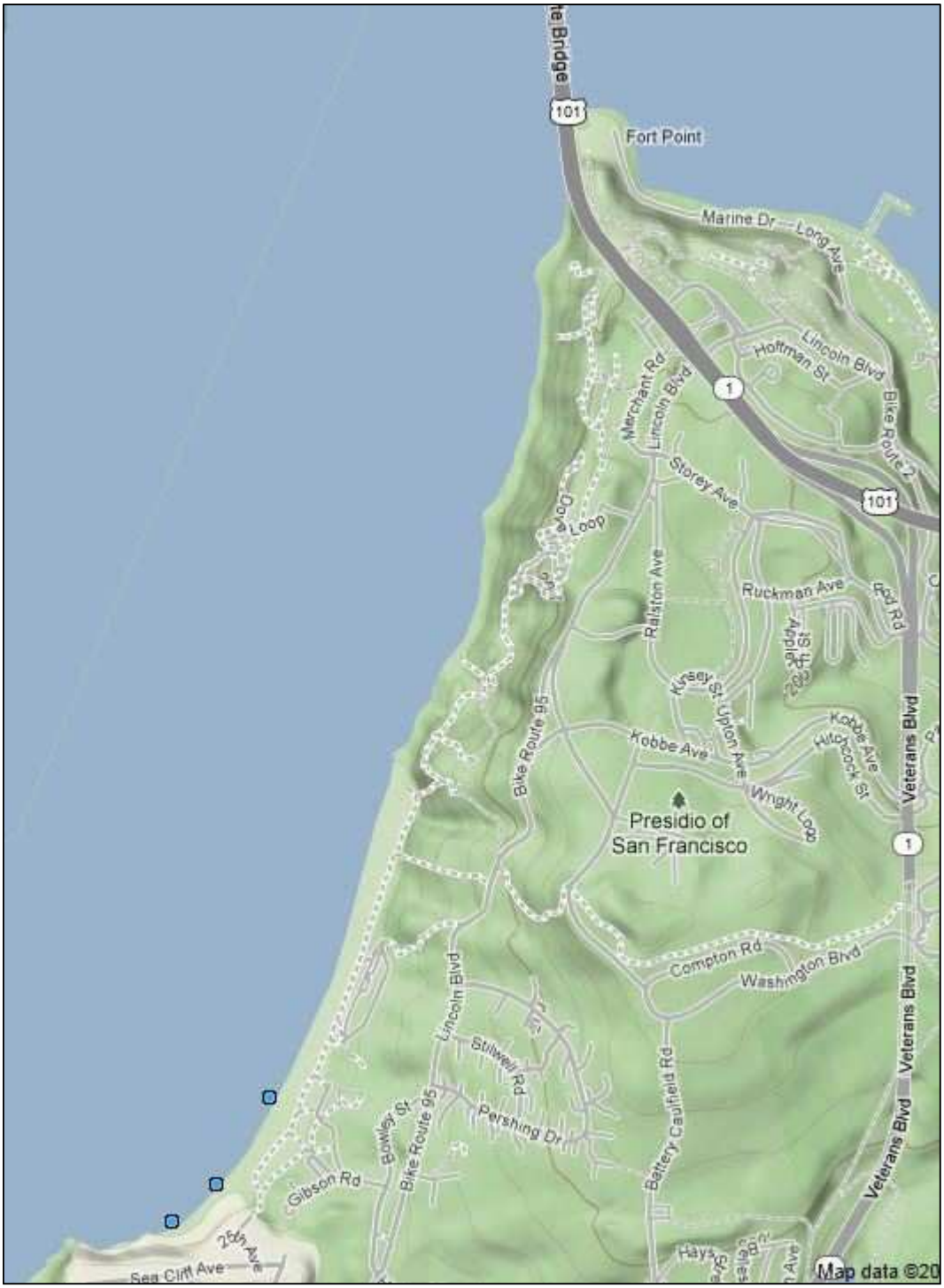

**Map 2**
**Sausalito sampling locations: detailed locations**



**Map 3**
**Muir Beach sampling locations (The North sampling site is shown as point #21)**

**Map 4**
**Campbell Cove (Sonoma County) sampling location (Sampling point shown as #2 in map below)**

**Map 5**
**Baker Beach (City & County of San Francisco) sampling location (see blue dots)**



## PROJECT COSTS

Funding Sources:

1. SWRCB Proposition 50 Nonpoint Source Grant in the amount of $848,000
2. Rathmann Family Foundation Grant of $200,000 to perform additional work

The project's final cost is as follows:

| | |
|---|---|
| Amount invoiced to the SWCRB: | $842,646.77 |
| Amount expended by the Rathmann Family Foundation: | $99,590.33 |
| Total | $942,237.10 |

## PUBLIC OUTREACH

The project web site can be found at www.phylochip.com
This site provides a general overview of the project, periodic updates and a description of the PhyloChip. There are links to Lawrence Berkeley National Laboratory, including the Green Genes database system and to other organizations active in beach water quality monitoring. Educational links are provided for students and teachers interested in the gene probe and chip technology. Also, see the activities listed below.

### Outreach Activities:

Presentations at Scientific Meetings

Andersen, G.L., E.A. Dubinsky, C.Wu and Y. Piceno. A Phylogenetic Microarray Approach to Monitoring and Source Tracking Coastal-Zone Pollution. November 17, 2010, San Diego, CA. Sustainable Approaches to Remediation of Contaminated Land and Contaminated Site Management.

Andersen, G.L., E.A. Dubinsky, C.Wu and Y. Piceno. A Phylogenetic Microarray Approach to Monitoring and Source Tracking Coastal-Zone Pollution. A Phylogenetic Microarray Approach to Monitoring and Source Tracking Coastal-Zone Pollution. August 2, 2010, San Francisco, CA. Annual Meeting of the Society for Industrial Microbiology.

Dubinsky, E.A., L. Esmaili, T.Z. DeSantis, J. Hulls, G.L. Andersen. Bacterial Community Analysis of Fecal Contamination in Coastal California. August 22-27, 2010, Seattle, WA. ISME 13 – 13th International Symposium on Microbial Ecology.

Wu, C. H., E. A. Dubinsky, J. Hulls, S. R. Osman, T. C. Hazen, and G. L. Andersen. Temporal dynamics of cattle and human fecal microbial communities in fresh and marine waters. August 22-27, 2010, Seattle, WA. ISME 13 – 13th International Symposium on Microbial Ecology.

T. Z. DeSantis, S. R. Osman, E. A. Dubinsky, Y. M. Piceno, U. Karaoz, E. L. Brodie, G. L. Andersen. Quantitative Tracking of Microbial Community Dynamics using the Berkeley PhyloChip. May 2010, San Diego, CA. American Society for Microbiology General Meeting.

Andersen, G.L., E.A. Dubinsky, T.Z. DeSantis, L. Esmaili, J. Hulls, C.H. Wu and T.C. Hazen. A comprehensive microbial community approach to water quality research using the PhyloChip. Monterey Bay National Marine Sanctuary Symposium. April 2010. Monterey, CA.

Dubinsky, E.A., C. Wu, J. Hulls, T. Hazen and Gary Andersen. A complete microbial community approach to monitoring and source tracking coastal-zone pollution. September 2010, Oakland, CA. Biennial State of the San Francisco Estuary Conference. September 2009, Oakland, CA.

Esmaili, L., E.A. Dubinsky, J. Hulls, G.L. Andersen. Characterization of Microbial Communities in Animal and Human Waste Sources Near San Francisco Bay. September 2010, Oakland, CA. Biennial State of the San Francisco Estuary Conference. September 2009, Oakland, CA.

Dubinsky, E.A., C. Wu, S. Osman, J. Hulls, T. Hazen, G. Andersen. A complete microbial community approach to monitoring and source tracking coastal-zone pollution. April 2009, Huntington Beach, CA. U.S. EPA National Beach Conference.

Dubinsky, E.A., J. Hulls and G.L. Andersen. An overview of the Marin County/Lawrence Berkeley National Laboratory “Clean Beaches” PhyloChip project. October 2008, Long Beach, CA. Headwaters to Ocean Conference.

Dubinsky, E. A., L. Esmaili, J. Hulls and G.L. Andersen. Indicator Bacterial Communities of Fecal Contaminants in Coastal California. March 15-17, Miami, FL. U.S. EPA National Beach Conference.

Dubinsky, E. A., L. Esmaili, J. Hulls and G.L. Andersen. Discriminating Sources of Fecal Pollution with Phylogenetic Microarrays. May 20-24, New Orleans, LA. American Society for Microbiology General Meeting.

Water Quality Workgroup Meetings

Dubinsky, E.A. Bacterial Community Analysis of Fecal Contamination Sources in Coastal California Nov 17, 2010, Alameda, CA. SWRCB Central/Northern California Ocean and Bay Water Quality Monitoring Group.

Dubinsky, E.A. A whole microbial community approach to water quality research. December 17, 2009, Long Marine Laboratory, Santa Cruz, CA. Cyanobacteria and water quality workshop.

Hulls, J. and E.A. Dubinsky. Update on Phylochip Project: Learning to tell the forest by the phylogenetic trees. November 10, 2009. SWRCB Beach Water Quality Workgroup, Costa Mesa, CA

Dubinsky, E.A. A complete microbial community approach to water quality research October 2009, Santa Rosa, CA. North Coast Regional Water Quality Control Board

Hulls, J. and E.A. Dubinsky. Update on Phylochip Project: Learning to tell the forest by the phylogenetic trees: Initial 3rd generation chip results in Tomales Bay and implications for water quality issues. August 13, 2009, Alameda, CA. Central/Northern California Ocean and Bay Water Quality Monitoring Group.

Community Outreach Activities

Dubinsky, E.A. and C.H. Wu. An Overview of the Clean Beaches Project. March 11, 2009, Point Reyes Station, CA. An informal "science café" for the public.

Hulls, J., An Overview of the Clean Beaches Project. November 17, 2009 Point Reyes National Seashore hosted presentation to Tomales Bay Shellfish Technical Advisory Committee.

Hulls, J., Seeing the Microbiome: From vanLeeuwenhek's Microscope to Lawrence Berkeley's Phylochip. California Environmental Health Association 58th Annual Educational Symposium, Monterey, CA 15 April 2009

## PAEP: ANALYSIS AND CONCLUSIONS

The original PAEP is attached here for reference and is followed by the updated analysis and commentary on the original goals. This section is followed by a discussion of next steps and recommendations for future research.

## *Project Performance Measures for Planning, Research, Monitoring, or Assessment Activities*

### *Beach Monitoring Phylochip Project*

| Project Goals | Desired Outcomes | Output Indicators | Outcome Indicators | Measurement Tools and Methods | Targets |
|---|---|---|---|---|---|
| 1. Determine microbial population of receiving waters at various beach locations in Marin, San Francisco and Sonoma County on a seasonal basis and determine microbial population of potential nonpoint sources. (Septic tank/dairy runoff/storm runoff/specific events) | Identification and cataloging of all microorganisms present based on comparison with 16s gene sequences. Identification of selected pathogens based on specific gene sequences.<br><br>Understanding of diurnal, tidal and seasonal fluctuations in population<br><br>Identification in differences between microbial population of non-point sources and receiving waters | Phylochip provides complete census of all microorganisms for which 16s gene sequence is known.<br><br>Accessible database of microbial ecology for future research, evaluation of pollution control measures, population shifts from environmental changes | Publication in scientific journal<br>Peer Reviewed report (Application of comprehensive bacterial community analysis to discriminate sources of fecal pollution at recreational beaches /Prepared by LBNL) Currently submitted to the Proceedings of the National Academy of Sciences for peer review. | Microarray testing using Lawrence Berkeley National Laboratory "Phylochip technology, with outputs analyzed using "Greengenes" database. | Broad acceptance of microbial census techniques as a research tool for studies in the marine environment, especially evaluation of safety of recreational waters and evaluation of the efficacy of pollution control measures. |
| 2 Based on microbial census of receiving waters and potential non-point sources, select indicator species and develop and validate QPCR test for selected species based on comparison with Phylochip tests and results of existing beach testing protocols. | Comparison of Microarray testing and existing county tests with QPCR tests | Microbial census shows direct presence of pathogens of interest and comparison of census results with standard indicator bacteria and QPCR test results will show relative efficacy of tests as indicators of potential pathogens. | Publication in scientific journal<br>Peer reviewed report (please see reference above). | 1. Phylochip analysis<br><br>2. QPCR keystone tests<br><br>3. Colilert 18<br><br>4. Enterolert<br><br>5. Total Coliform | Broad acceptance of keystone indicator/QPCR testing for potential presence of pathogens in receiving waters where non-point sources predominate. |
| 3. Assess reliability of indicators for beach closure indicator organisms | Reduction in beach closures from false positive indication of presence of pathogens using current indicator species | QPCR keystone species test outputs in comparison with current indicator methodology | Publication in scientific journal<br>Peer reviewed report<br>(Please see reference above). | QPCR keystone species tests | Broad acceptance of keystone indicator/QPCR testing for potential presence of pathogens in receiving waters |

*PAEP Item 1*

In considering the PAEP analysis, it is important to recognize that, much like the analysis of airborne microbiomes for Homeland Security, the Clean Beaches project is the first use of the Phylochip technology to perform a comprehensive microbial community analysis of a geographically distributed microbiome. As in the case of the airborne microbiome, the initial analysis revealed a rich microbial community that was profoundly influenced by the nature of the receiving waters, changing dramatically over time and location. In addition, the project was able to use the 3rd generation of the chip, which is capable of detecting @ 59,000 OTUs of bacteria and archaea, as opposed to the @9000 OTUs capability of the previous generation.

The primary purpose of the project is to "Determine microbial population of receiving waters at various beach locations in Marin, San Francisco and Sonoma County on a seasonal basis and determine microbial population of potential nonpoint sources. (Septic tank/dairy runoff/storm runoff/specific events),"

The desired outcome of this first goal is: Identification and cataloging of all microorganisms present based on comparison with 16s gene sequences. Identification of selected pathogens based on specific gene sequences. Understanding of diurnal, tidal and seasonal fluctuations in population. Identification in differences between microbial population of non-point sources and receiving waters.

The output indicators included the Phylochip census and database which is maintained by LBNL.

The project has been very successful in accomplishing these goals, identifying large, diverse populations in salt and fresh receiving waters, even in samples well within the AB411 limits as defined on Colilert and Enterolert tests currently employed. At this point, thanks to the Rathmann Family Foundation, it became possible to undertake a comprehensive survey of major potential animal contributors to the microbiome of Tomales Bay, including multiple samples of large grazers, including cows, elk, and horses, extensive bird samples and also pinnipeds. All samples were taken from multiple individuals within at least 4 geographically dispersed populations.

Once again, a surprising diversity was found, including large numbers of OTUs that were unique to each species. While the project had initially intended to use bacteroides markers as an indication of source, the increased resolution and sensitivity of the G3 Phylochip made it apparent that the presence of specific sources could be ascertained from the unique OTUs associated with the potential sources. Additionally, the diffusion chamber tests indicated that the potential source microbiomes reacted differently over time, showing a great variation in survival rates and percentage of population based on time, and whether the receiving waters were saline or fresh.

As shown in the LBNL paper which forms the body of this report, the combination of all these results makes it possible to determine potential sources of fecal bacteria from a single test, which is invaluable in evaluation of potential remedial measures.

The target of this project goal was the broad acceptance of microbial census techniques as a research tool for studies in the marine environment, especially evaluation of safety of recreational waters and evaluation of the efficacy of pollution control measures.

The use of the PhyloChip for comprehensive microbial community assessment is gaining widespread interest, not only in the environmental testing field, but in areas as diverse as the NASA Planetary Protection program, where it is used to screen NASA spacecraft assembly rooms, but in the NIH human microbiome project. The development of the protocols under the Clean Beaches project led to the deployment of the PhyloChip technology in the Gulf oil spill response, where it lead to the understanding of the microbiome there and the identification of the oil consuming bacterial population, as recently reported in the scientific press.

*PAEP Item 2*

Based on microbial census of receiving waters and potential non-point sources, select indicator species and develop and validate QPCR test for selected species based on comparison with Phylochip tests and results of existing beach testing protocols.

Outcomes As mentioned in item 1, the sensitivity of the G3 PhyloChip enabled the identification of large numbers of unique OTUs from the potential source microbiomes, including human, large grazers (cattle/elk/horses), birds and pinnipeds. It became apparent from the analysis that the G3 'census' results were comprehensive enough to enable direct identification of the OTUs contributing to an exceedance under AB411 standards, and thus the source microbiomes associated with an exceedance of the standards. This is discussed at length in the source tracking section of this report.

It was decided that detecting the contributors to exceedances would provide the best indicator of potential risk as reliable detection of human sources of fecal contamination is primary in determining risk, where specific pathogen detection based on individual strains of bacteria is likely to miss both detection and the risk potential. Therefore, emphasis was placed on developing the source tracking capabilities of the Phylochip, and methods to analyze the large amount of data produced by the test.

Development of a PCR test was not undertaken, as the research showed that that there are a large number of potential indicators, and that selection should be based on the receiving water environment and potential fecal sources within the watershed to obtain reliable results from a small group of indicator species. However, because of rapid developments in microarray and other detection technology currently underway, it is anticipated such testing will soon be within a range of price and availability such that indicator groups can be readily selected from a CMCA, as demonstrated by the Phylochip assay results.

The additional surveys of gut microbiomes of potential sources, enabled the detection of large numbers of unique bacteria for human, mammalian and avian sources sufficient to identify their

presence or absence in receiving waters (See ISME presentation on source tracking and section in paper on Sausalito sewage spill for examples, where the Phylochip tests are compared with conventional Colilert, Enteroalert and Total Coliform samples. The AB411 test samples were also analyzed with current indicator methods and sensitivity compared, as shown in the paper comprising the body of this report).

**Targets**

Data generated shows potential indicator species, and the microbial census provides a valuable data set for other researchers doing risk analysis. The results have already lead to the Phylochip being selected for participation in the SWRCB Source Indicator Pilot Program, involving SCCWRP, Stanford, and other universities both here and abroad.

*PAEP Item 3*

Task: Assess reliability of current indicators for beach closure indicator organisms

**Desired Outcomes**

Reduction in beach closures from false positive indication of presence of pathogens using current indicator species

The project achieved far more positive results than anticipated in this area. As the research papers show, the Phylochip was able to detect a very wide range of potential indicators that allowed determination of the specific OTUs that were causing a given exceedance.
As the chip had probes for thousands of specific coliforms and enterococci, it was possible to associate those OTUs that would trigger AB411 exceedances with the gut microbiomes of the sources that were analyzed. The tests specifically showed that many OTUs from bird sequences would register on AB411 tests. Of more interest was the detection of large numbers of 'cosmopolitan' bacteria. Tests of receiving waters with no waste influence would show populations of several thousand OTUs. The ability to differentiate between receiving water microbiomes and source microbiomes is a unique outcome of the tests.

In addition, the tests show that in many areas, exceedances caused by rainfall events are not related to any of the specific source microbiomes in the area, even when cattle and septic tanks had been implicated as potential sources by conventional tests In tests sites where there was no pattern of source OTUs detected in non-rain events, there was no apparent specific source in the rain event sequences, indicating that establishment of the background microbial census of cosmopolitan bacteria and potential source microbiomes will be essential to the success of selecting successful remediation measures, especially in dealing with outflows caused by rain events.

The results, especially the Sausalito Sewage spill show that the Phylochip tests were far more sensitive than conventional tests, and detected human presence at lower concentrations than conventional tests. The large number of potential indicators for various species and human

sources such as septic tanks (see ISME abstract and current report for details) make it possible to identify sources, and because of the large number of unique taxa, the Phylochip test is less subject to error than single marker species and general coliform and enterococcus tests. However, results showed that the survival of indicator species is highly dependent on receiving water conditions, and rainy weather conditions produced large numbers of 'cosmopolitan', or generally present bacteria not associated with specific sources, as shown in the report. Further analysis of different environmental conditions will be required to develop a QPCR keystone test with limited species, however, the results show that comprehensive microbial community assessment with the Phylochip is far more sensitive, and less subject to error because of the large number of unique taxa in any given species.

**Targets**

The use of comprehensive microbial community assessment has already gained acceptance (see item 2). Results show that the Phylochip is capable of detecting all of the sequenced OTUs present in an environment , providing the ideal testing method for the development of pathogen TMDL's and the selection of the appropriate remediation measures with the highest possibility of success, as well as assessing the impact of treatment projects and remediation measures.

**LESSONS LEARNED**

The main lesson learned concerns the level of complexity of the microbial ecology in recreational waters, and the richness of potential sources. In the course of the project, we identified over 20,368 bacterial OTU's from mammals and birds as potential sources of fecal bacteria in water samples.

From this we also learned that, especially in the case of non-point sources, it is essential that a comprehensive microbial community assessment be performed before determining remediation measures, as attribution to given sources, be they natural or man-made, cannot be determined from normal surveys.

The lesson learned from subsequent tests with the PhyloChip (City of Malibu study) indicates that specific estuarine conditions can create microbial ecologies that contain numerous conventional indicator bacteria without a known source, indicating that there is a relatively stable natural population that is sustained in a specific environment, such as the estuarine environment of Malibu Lagoon. In this case, despite the presence of a large bird population, claims of avian and septic tank impact impacts were not supported by the PhyloChip tests, which showed no specific source of fecal bacteria. Instead, a complex natural population was implicated as the most likely source.

Both the location of sampling points and the nature of receiving waters are critical to determining sources of microbes. In the case of the Baker Beach samples, those taken at the outlet of Lobos Creek showed clear indications of bird and human sources, whereas, at a sampling point slightly more than 100 yards away, no source indicators rose above general background levels.

The nature of the receiving waters was found to exert a very significant effect on the survival of source bacteria, both in general and sustained over time. Diffusion chamber tests revealed

significant difference in fresh and saltwater populations over time. Thus, accounting for survival in receiving waters is essential to understanding the impact of microbial sources over time.

This study found that there are large populations of bacteria that occur in conjunction with rain events that contain the indicator bacteria tested for by conventional recreational water testing methods. We conjecture is that these sources may result from interchange with marshlands and stream bank reservoirs that would be influenced by changes in water level during rain events.

Sensitivity in detecting low levels of bacteria unique to a particular source greatly increases the confidence of determining a source, (as opposed to a single indicator) because of the large number of discrete taxa associated with a given source, even if they are present only at low levels.

## RECOMMENDATIONS AND FUTURE RESEARCH

The work to date clearly demonstrates the power of the comprehensive microbial community analysis (CMCA) in determining the microbial health and make-up of aquatic environments. It also shows that careful attention must be paid to not only the nature of the receiving waters, but to the selection of indicator species.

The data clearly shows that tests using only one indicator such as enterococcus are incapable of providing reliable indication of the presence of human fecal matter in non-point source situations where birds and wildlife are present, especially since the bird gut microbiome contains many enterococci that are shared with mammals, including humans. The current study shows that it is possible to differentiate human waste from other sources, but more analysis will be required to select the minimum number of taxa to produce reliable results. LBNL is already participating in the State of California Source Identification Pilot Project, providing CMCA's and gut microbiome analysis to build up the library of potential animal source fecal matter. This research should be continued and expanded as it will lead to much more reliable selection of indicator species, especially for downselected probe sets for routine testing.

The Phylochip is already being used in other recreational water testing environments, including Region 1 RWQCB, which is planning to use the results to deal with complicated source issues on the Russian River. This is an entirely freshwater environment, in contrast to the current study, and will greatly increase the understanding of watershed microbiomes.
Further understanding of the microbiome of river systems will provide valuable insight into river management, especially under low-flow conditions. It is strongly recommended that watershed level microbiome studies be continued.

This study also shows that, given the complexity of the microbial aquatic community, there is simply no way of providing a single 'magic bullet' test that will accurately predict risk to recreational water users. This mirrors the results of the Phylochip tests of the airborne microbiome for Homeland security, where the results were used to develop new tests for bioterror organisms without triggering false alarms from natural organisms in what turned out to be a far more complex airborne microbial environment than had been anticipated. This study indicates that the best results will be obtained by calculating the probabilities of a given source being present, based on the percentage of probes for a given source (such as sewage) that 'light up' on a given sample. Calculation of such probabilities is also a fruitful area for further research.

In addition to protection of the environment, the prime goal of recreational water testing is to protect public health. By calculating the probability of a given source being present by the percentage of 'hits' on given sources, it would be possible to greatly improve the accuracy and resolution of Quantitative Microbial Risk Assessment (QMRA) for recreational waters and CMCA testing would be of significant use in determining exposure from various fecal sources to facilitate meaningful QMRA development.

While the CMCA/Phylochip approach is appropriate for research studies, preparation of pathogen TMDL's and source tracking; it also indicates that much smaller sets of probes could be used with low-cost chips for routine monitoring of recreational waters, or tracking of specific sources. The

field of probe and array development is evolving very rapidly, and research in this area could lead to the availability of low-cost, downsized probe sets and the development of accurate, rapid risk assessment tools.

## LIST OF APPENDICES

1. Grant Summary Form

2. Scientific Paper: “Application of Comprehensive Bacterial Community Analysis to Discriminate Sources of Fecal Pollution at Recreational Beaches.”

3. Scientific Paper: “Application of Phylogenetic Microarray Analysis to Discriminate Sources of Fecal Pollution.”

4. Peer Reviewed Article

   “Characterization of Coastal Urban Watershed Bacterial Communities Leads to Alternative Community-Based Indicators.” Cindy H. Wu et al

5. Selected presentations to EPA, ISME

   1) Bacterial Community Analysis of Avian and Mammalian Sources of Fecal Contamination in Coastal California

   2) Temporal Dynamics of Cattle and Human Fecal Microbial Communities in Fresh and Marine Waters.

   3) Application of Comprehensive Bacterial Community Analysis to Discriminate Common Sources of Fecal Pollution

# APPENDICES

1. Appendix 1: Grant Summary Form

# CONTRACT SUMMARY

**Date filled out**: *March 6, 2011*

| | |
|---|---|
| **A) Contract Information** | |
| **1. Contract Number: 07-576-550-2 (as amended)** | |
| **2. Project Title:** *Beach Monitoring Phylochip Project* | |
| **3. Project Purpose – Problem: Characterize Microbial Environment in Beach Water using PhyloChip and assess potential for source tracking applications; Identify potential for improved beach water quality testing.** | |
| **4. Project Goals:** | |
| **a. Short-term Goals:** *Conduct Microbial Census of Recreational Waters and Nonpoint sources* | |
| **b. Long-term Goals: Provide a basis for future development of rapid assessment of recreational waters.** | |
| **5. Project Location: Various watersheds: Muir Beach (Marin), Campbell Cove (Sonoma), Baker Beach (S.F).** | |
| **a. Physical Size of Project:**<br>**Size of watershed –** N/A | **b. Counties** included in the project: Marin County<br>Sonoma County, San Francisco City/County |
| **c. Legislative Districts:** (Assembly and Senate) | **Senate Districts – 2, 3, 8**<br>**Assembly District – 1, 6, 12** |
| **6. Which SWRCB program is funding this contract?** Please put an "X" by the one that applies.<br>X Prop 50 *Coastal Nonpoint Source* | |
| **B) Contract Contact:** Refers to contract project director. | |
| **Name: Philip D. Smith** | **Job Title:** (formerly) *Deputy Director, Environmental Health Services* |
| **Organization:** *County of Marin, Comm. Development* | **Webpage Address:** *www.co.marin.ca.us/ehs* |
| **Address:** *3501 Civic Center Drive, Rm 236*<br>*San Rafael, CA 94903* | |
| **Phone:** *415-499-6907* | **Fax number:** *415-507-4120* |
| **Email:** *pdrsmith@gmail.com* | |
| **C. Contract Time Frame:** Refers to the implementation period of the contract. | |
| **From:** *October 26, 2006* | **To:** *April 1, 2011* |
| **D) Project Partner Information:** Name all agencies/groups involved with project.<br>*Marin County Environmental Health Services*<br>*Lawrence Berkeley National Laboratory*<br>*State Water Resources Control Board* | |

| | |
|---|---|
| *San Francisco Dept. of Public Health; Environmental Health Division*<br>*Sonoma County Public Health Dept., Environmental Health Division*<br>*Southern California Coastal Water Research Project*<br>*University of California, Santa Barbara* | |
| **E) Nutrient and Sediment Load Reduction Projection (if applicable):** | **NA** |

Appendix 2:

Scientific Paper: “Application of Comprehensive Bacterial Community Analysis to Discriminate Sources of Fecal Pollution at Recreational Beaches.”

# Application of comprehensive bacterial community analysis to discriminate sources of fecal pollution at recreational beaches /Prepared by LBNL

**Summary Project Description**

Causes of fecal contamination in recreational waters are often unclear because human, agricultural and wildlife sources co-occur in most watersheds. Most source tracking methods rely on the detection of single biomarkers to identify or exclude individual sources. High-throughput DNA sequence analysis has potential to improve the sensitivity and specificity of source tracking by using the entire phylogenetic diversity of fecal microbial communities to identify sources of contaminants. In this study we used phylogenetic microarray analysis to determine combinations of bacterial taxa that can be used to detect suspected sources of animal and human fecal sources in coastal California. Fresh feces were collected from 42 different populations of birds, pinnipeds (seals, sea lions), cows, horses, elk and human sewage. Human wastes were gathered from wastewater treatment plants, community septic tanks and holding tanks. We analyzed bacterial 16S rRNA gene composition using the PhyloChip microarray, which is capable of quantifying differences in the relative abundance of both rare and abundant bacterial taxa from the entire targeted pool of 16S rRNA gene amplicons for 59,959 different taxa. Indicator species analysis was used to determine unique combinations of bacterial taxa that discriminate sources. Cluster analysis revealed strong differences in community composition among human wastes, birds, pinnipeds and grazers. Bacterial communities from ruminants (cows, elk) clustered closely with little variation among populations, while horses were distinct within the grazers. Actinobacteria, Bacilli and many Gammaproteobacteria taxa discriminated birds from other sources. Many Clostridia and Bacteroidetes taxa discriminated human wastes, grazers and pinnipeds. Hundreds of different taxa that were unique to each source type and could be used for source identification.


Illustration 2: Phylochip Microarray

We found that birds are the most likely source of FIB contamination at Campbell Cove. Neither human, grazer or bird sources are likely to be primarily responsible for high FIB counts observed in Tomales Bay sites or Horseshoe Cove. Both human and bird sources are associated with high FIB at Baker Beach in San Francisco. Lagunitas is impacted by a wide range of human, bird and grazer sources.

Background

Each year more than a third of all beaches in the United States monitored for potential health risks are closed or posted with an advisory at some point. Closures and public health advisories have a major economic impact on coastal communities whose economies are based largely on tourism from beach recreation. Likewise the closure of areas used for shellfish harvesting results in substantial economic losses (Meschke and Boyle 2007).

Most closings and advisories are triggered by water samples that exceed microbial water quality standards for "fecal indicator" bacteria, usually culturable coliforms, *E. coli* or enterococci that are considered a proxy for human health risk in recreational waters. Because the direct measurement of all human pathogens is often impractical and unreliable under field conditions, water monitoring relies on the detection of bacterial indicators that have some demonstrated correlation with human illness in areas mostly impacted by human sewage (Field and Samadpour 2007). These tests are based on antiquated methods from the early 1900s and have several shortcomings that are well documented. Many studies have demonstrated that these fecal indicator bacteria are detected in several environmental sources aside from feces, including soils and sediments, algal wrack and

beach sands. These fecal indicators are therefore not always indicative of fecal inputs, and many water bodies often contain measurable amounts of FIB even where anthropogenic or significant animal inputs are absent. Importantly, fecal indicator bacteria tests provide no information about the source of the bacteria, and thus provide no indication of health risk without additional source tracking data.

Shortcomings of the current FIB monitoring approach combined with widespread development and implementation of Total Maximum Daily Load (TMDL) requirements for microbiological pollution are fueling interest in microbial source tracking (MST) methods. Many approaches to source tracking are under development, most of which rely on single phenotypic or genotypic biomarkers to measure sources. Good MST assays must be specific to the host and have a high sensitivity of detection in the environment. Most importantly the MST signal must remain detectable in the environment at least as long as indicator organisms and pathogens (EPA 2005). Most MST approaches fall short of desired specificity, stability and sensitivity objectives.

Most existing MST tests were developed in an era when it was difficult and expensive to measure the huge diversity of microorganisms that is resident in human and animal guts, and most studies have evaluated the performance of single microbial sequences or biomarkers as the basis of source tracking. A drawback of single targets is that no single gene sequence is known to be 100% specific for any one type of waste (Domingo et al. 2007). Single targets, such as host-specific Bacteroides, can be unreliable because they may vary in presence and abundance among individuals, and primer sets may not be entirely specific to a particular host. In addition, MST based on single targets is entirely dependent on the fate of this one target once it enters receiving waters. Comprehensive sequence analysis potentially overcomes this problem by considering multifactorial lines of evidence that a source is present or absent, and may be more robust to differential persistence and decay due to changing environmental conditions because of a redundancy and diversity in source-specific targets.

Approximately 1000 different microbial taxa are now known to reside in the human gut alone, but the potential for this diversity to be used as a means for identifying sources remains largely unexplored. To date there have been few comparative surveys of microbial community

composition among important sources of fecal contamination. New techniques for high-throughput DNA sequence analysis enable identification of nearly all microbes that occur in a sample. Targeting the whole microbial community for source identification is a fundamentally different approach than traditional molecular methods that are dependent on the detection on one gene sequence under complex environmental conditions. Sequence analysis of entire microbial communities creates an opportunity to discover a multitude to different bacterial species that are unique to fecal and environmental sources that contribute FIB to recreational waters. Rather than relying on the detection of one single marker, a suite of bacterial species can be used as the basis of assays to improve MST specificity and sensitivity. Using new high-throughput tools of sequence analysis, a process of discovery is now available that was not readily available before – comparative analysis of all the entire diversity of bacteria that compose the community of each suspected source to identify unique combinations of bacterial taxa that are exclusive to a particular source.

In this study we used a high-density oligonucleotide microarray to census the 16S rRNA gene diversity in different sources of fecal contamination. The microarray targets 59,995 different 16S rRNA gene polymorphisms that represent every known phylogenetic group of bacteria and archaea. We test the assumption that a variety of common fecal sources select for specific populations of bacteria due to differences in ecological conditions presented by their animal host. We screened a variety of fecal sources of concern in coastal California to identify the microbial groups that are source-specific, and then used these unique taxa to detect influence from these sources in marine samples that exceed water quality limits for fecal indicator bacteria.

## Methods

### *Feces sampling and DNA extraction*

Human fecal wastes and freshly deposited fecal samples from animals were collected at numerous locations throughout California. Human fecal sources included primary influent and effluent and five different municipal wastewater treatment plants, two community septic tanks serving more

than 30 households each, and one composite sample of 10 holding tanks from individual households. All animal samples consisted of composites of feces from at least five different individuals in a single population. Sampled animal populations included cows (4), horses (4), tule elk (4), western and California gulls (9), Canada geese (4), pelican (3), pigeon (2), cormorant (1), sea lion (3), elephant seal (1). Composite fecal samples were homogenized and immediately frozen upon collection and transported to the laboratory. Samples were stored at -80 °C until DNA extraction.

Two DNA extraction methods were employed. The first was a modified Miller method (Miller et al. 1999). Each fecal sample was extracted in triplicate. For each triplicate, 0.5 g of homogenized fecal sample was added to a Lysing Matrix E tube (MP Biomedicals, Solon, OH). 300 µL of Miller phosphate buffer and 300µL of Miller SDS lysis buffer were added and mixed. 600 µL phenol:chloroform:isoamyl alcohol (25:24:1) was then added, and the tubes were bead- beat at 5.5m/s for 45sec in a FastPrep instrument. The tubes were spun at 16,000 x g for 5 min at 4°C. 540 µL of supernatant was transferred to a 2 ml tube and an equal volume of chloroform was added. Tubes were mixed and then spun at 10,000 x g for 5 min 400 µL aqueous phase was transferred to another tube and 2 volumes of Solution S3 (MoBio, Carlsbad, CA) was added and mixed by inversion. The rest of the clean-up procedures followed the instructions in the MoBio Soil DNA extraction kit. Samples were recovered in 60µL Solution S5 and stored at -80°C. The second DNA extraction method was conducted with the DNA EZ extraction kit (Generite, North Brunswick, NJ) per manufacturer's instructions.

*Water sampling and DNA extraction*

Water monitoring samples were collected from the field from locations with both known and unknown sources of contamination. One set of samples was collected during a 10-day period following a 764,000 gallon spill of primary effluent from the Sausalito-Marin City Sanitary District treatment plant in Sausalito, California that occurred in February 2009. Samples were collected at 8 onshore and offshore locations up to 1 km away from the plant daily for three days following the spill, and then once more 10 days after the initial spill during an accidental rupture that occurred during the repair. Water samples were collected in 1L bottles and stored at 4° C until laboratory

processing (within 6 hours of collection). For FIB tests, 20 mL of water was subsampled for FIB tests of total coliforms and *E. coli* (Colilert, IDEXX Laboratories, Westbrook, ME) and Enterococcus (Enterolert, IDEXX Laboratories). For DNA extraction, 250 mL x 3 of each sample was vacuum filtered through Whatman Anodisc membrane filters (47 mm dia., 0.2 µm pore size) and immediately frozen and stored at -80° C until DNA extraction. DNA was extracted from filters using the DNA EZ kit per manufacturer's instructions.

Water samples were also collected in conjunction with the State of California AB411 water quality monitoring program at nine recreational beaches in San Francisco, Marin and Sonoma counties. Marine beaches included Baker Beach (San Francisco Bay, 2 sampling locations), Horseshoe Cove (San Francisco Bay, 3 sampling locations), Muir Beach (Pacific Ocean, 2 sampling locations), Miller Point (Tomales Bay), Lawson's Landing (Tomales Bay), Dillon Beach (Tomales Bay), and Campbell Cove (Bodega Bay). Two freshwater beaches were located along Lagunitas Creek in Marin County and included Inkwells and Green Bridge. Samples were collected weekly at each location throughout the 2009 monitoring season (April-October). Samples were collected in knee deep water in 1L sampling bottles and stored at 4° C until laboratory processing for FIB tests and vacuum filtration as described above. Filters were stored at -80° C until DNA extraction.

*Polymerase Chain Reaction*

The 16S rRNA gene was amplified from each sample using PCR with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') for bacteria and 4Fa (5'- TCCGGTTGATCCTGCCRG-3') and 1492R for archaea. Each PCR reaction contained 1× Ex Taq buffer (Takara Bio Inc., Japan), 0.025 units/µl Ex Taq polymerase, 0.8 mM dNTP mixture, 1.0 µg/µl BSA, and 200 pM each primer and 1 ng genomic DNA (gDNA) as template for fecal samples and 10 ng gDNA for water samples. For the PhyloChip assay each sample was amplified in 8 replicate 25 µl reactions spanning a range of annealing temperatures. PCR conditions were 95°C (3 min), followed by 30 cycles 95°C (30 s), 48-58°C (25 s), 72°C (2 min), followed by a final extension 72°C (10 min). Amplicons from each reaction were pooled for each sample, purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA), and eluted in 50 µL elution buffer.

*PhyloChip Assay Design*

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

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

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

*Preparation of Samples for PhyloChip Assays*

For PhyloChip hybridization, 500 ng of bacterial PCR product were prepared for PhyloChip hybridization. PCR products were fragmented with DNAse I to a range of 50-200 bp as verified by agarose gels. Commercial kits were utilized for DNA preparation: Affymetrix (Santa Clara, CA) WT Double Stranded DNA Terminal Labeling, and Affymetrix GeneChip Hybridization, Wash, and Stain kits were used for PhyloChip analysis. Briefly, fragmented 16S amplicons and non-16S quantitative amplicon reference controls were labeled with biotin in 40 µL reactions containing: 8 µL of 5X TDF buffer, 40 units of TDF, 3.32 nanomoles of GeneChip labeling reagent. After incubating at 37°C for 60 min, 2 µL of 0.5M EDTA was added to terminate the reaction. Labeled DNA was combined with 65µL of 2X MES hybridization buffer, 20.4 µL of DMSO, 2 µL of Affymetrix control oligo B2, and 0.4 µL nuclease free water. Each reaction mixture was injected into the hybridization chamber of an array cartridge and incubated for 16 h in an Affymetrix hybridization oven at 48°C and 60 RPM. Hybridization solution was then removed and the microarrays were stained and scanned according to the manufacturer's instructions.

*PhyloChip Assay Analysis*

Analysis procedures for fluorescent image files are described in detail in the supplemental material of (Hazen et al. 2010). Briefly, each individual array feature occupied approximately 8x8 pixels in the image file corresponding to a single probe 25mer on the surface. Probe intensities were background-subtracted and scaled to quantitative standards (non-16S rRNA gene spike-ins) as previously described (DeSantis et al. 2007). Presence/absence calling of each microbial taxon (operational taxonomic unit – OTU) was based on positive hybridization of multiple probes that correspond to an OTU (average of 37 probes/OTU). Differences in mean hybridization intensity (fluorescence) of an OTU probe set among different PhyloChips reflected differences in the relative abundance of the OTU (DeSantis et al. 2007).

## Results and Discussion

### *Fecal source microbial communities*

A total of 20,368 bacterial OTUs were detected across all fecal samples. Samples clustered by source type indicating different samples of the same source type are more similar to each other than they are to other sources. All birds were distinct from mammal sources (Figure 1). Within the mammals samples clustered into three distinct groups comprising grazing animals (cows, elk, horses), human wastes and pinnipeds. Grazing mammals were further partitioned into two clusters comprising ruminants (cow, elk) and horses. Geese formed a distinct cluster within the birds. There was no clustering among the other bird types (gulls, pelicans, pigeons, cormorants), and clustering patterns were not related to geography.

Illustration 3: Collecting samples: Gale Ranch, Marin County.

Clostridia, lactic acid Bacilli and Bacteroidetes dominated taxonomic (OTU) richness of grazing mammals (Figure 2). These bacteria are known to digest of cellulose and other plant polysaccharides in the ruminant gut. Clostridia, Gammaproteobacteria (mostly coliforms) and

Bacteroidetes dominated taxonomic richness of human wastes. Gulls and pelicans had similar composition of bacteria and were dominated by enteric Gammaproteobacteria and Bacilli. Taxonomic richness in geese was distinct from other types of birds and dominated by Actinobacteria, Alphaproteobacteria and Clostridia. Geese differ from other birds in this study because of their unique diet and digestive system. Geese consume high-fiber feed, such as grass, and contain a well-developed cecum that facilitates their breakdown in the large intestine. Canada geese often forage for plants and insects in the soil, and thus ingest bacteria that are resident in soil or on plant surfaces, and may explain the prominence of Actinobacteria and Alphaproteobacteria in their feces. Clostridia and Gammaproteobacteria dominated the taxonomic richness of pinnipeds.

Based on similarities in community composition (Figure 1), the data were partitioned into four major groups for identifier bacteria analysis: human wastes, birds, grazers and pinnipeds. Source identifier taxa were defined as individual OTUs that were detected in a single source type, but never detected in any samples from other sources. The criteria for identifier bacteria selection were as follows: Identifier bacteria for animal sources needed to be present in at least three different samples of each source type (birds, grazers, pinnipeds). Identifier bacteria for human sources needed to be present in at least 7 samples. More stringent requirements were selected for human sources because far more bacterial taxa were detected in human sources than animal sources, likely due to overrepresentation of human-specific bacteria in the 16S rRNA gene database from human microbiome sequencing projects. The number of OTUs present in at least three different populations was 5385 for birds, 2989 for grazers and 140 for pinnipeds. The number of OTUs present in at least seven different human waste samples was 1962. The number of these OTUs that were unique to each source type was 304 for birds, 213 for grazers, 0 for pinnipeds, and 541 for human wastes (Figure 3).

Diffusion chamber tests, in which samples of human and cattle source bacteria were placed in chambers with membranes permeable to the receiving waters and samples taken over time to determine the impacts of the receiving water on population. Results shown (Figure 4) show that bay and creek waters showed different survival rates and relative abundance over time. These tests, more fully reported in Appendix 4 show that is important to understand the fate of bacteria in receiving waters. Therefore, selection of indicators for monitoring either human contamination or

other fecal contamination should be based on the waste type and persistence of fecal taxa in various receiving waters.

Human identifier bacteria were primarily Bacteroidaceae and Clostridiales OTUs that matched known human fecal bacteria 16S rRNA gene sequences (Figure 3). Human Clostridiales OTUs were mainly found in the families Eubacterium, Faecalibacterium and Ruminococcus. Verrucomicrobia in the family Akkermansia were also indicative of human wastes. These bacteria known to be mucin degraders in the human GI tract.

Bird identifier taxa included several different groups of Bacilli, mainly Lactobacillales, Staphylococcaceae (Figure 3). In addition, bird identifiers included one unclassified family in the Clostridiales, Enterobacteriaceae and Fusobacteriaceae. Bacteroidetes are a minor component in avian microbial communities (Lu et al. 2008). We found several Lactobacilli OTUs that are included in the same subfamily as *Catellicoccus marimammalium* and are closely related to *Enterococcaceae*. Lu et al. (2006) found gull feces were dominated by Bacilli (37% sequences), most of which were closely related to *Catellicoccus marimammalium*.

Grazer identifier taxa included a variety of Clostridia, many of which are known from cattle rumen, consisting of Clostridium, Ruminococcus, unclassified Clostridiales, RF6, RF30, RF39 and SHA-32 (Figure 3). In addition, grazer identifiers included several Bacilli taxa found in the Planococcaceae, and Bacteroidales taxa that were distinct from those found in human wastes (Figure 3).

Pinniped microbial communities were distinct from other fecal sources, but all OTUs found in at least three pinniped samples were also found in at least one other human or animal sample. Thus, there are no OTUs represented on the PhyloChip that can be used to reliably distinguish pinniped feces from other fecal sources tested in this analysis.

***Application to source tracking: Sewage spill monitoring***

The Sausalito-Marin City sewage spill into Richardson Bay was used to test the performance of the fecal identifier bacteria in contaminated waters with a complex microbial background. The PhyloChip was used to determine which bacterial taxa significantly increased in abundance in samples with high FIB counts, and whether these enriched bacteria included the expected human identifier bacteria described above. To determine which taxa were specifically associated with high FIB counts, water samples with FIB concentrations that exceeded any 30-day geometric mean concentration limit were compared to samples that fell below all FIB concentration limits. At all sites, baseline microbial communities were defined by mean abundance of taxa in low FIB samples. Taxa that significantly exceeded baseline ($> \text{mean} + 2\sigma$) were determined in high FIB samples. The presence of source identifier bacteria in this enriched subset was used to determine the association between human, bird or grazer feces and high fecal indicator counts. Results are reported as the percent of expected identifier taxa that were detected in each sample. The expected number of identifier taxa for a given source was calculated as the average number of source-specific identifier taxa detected in any one fecal sample.

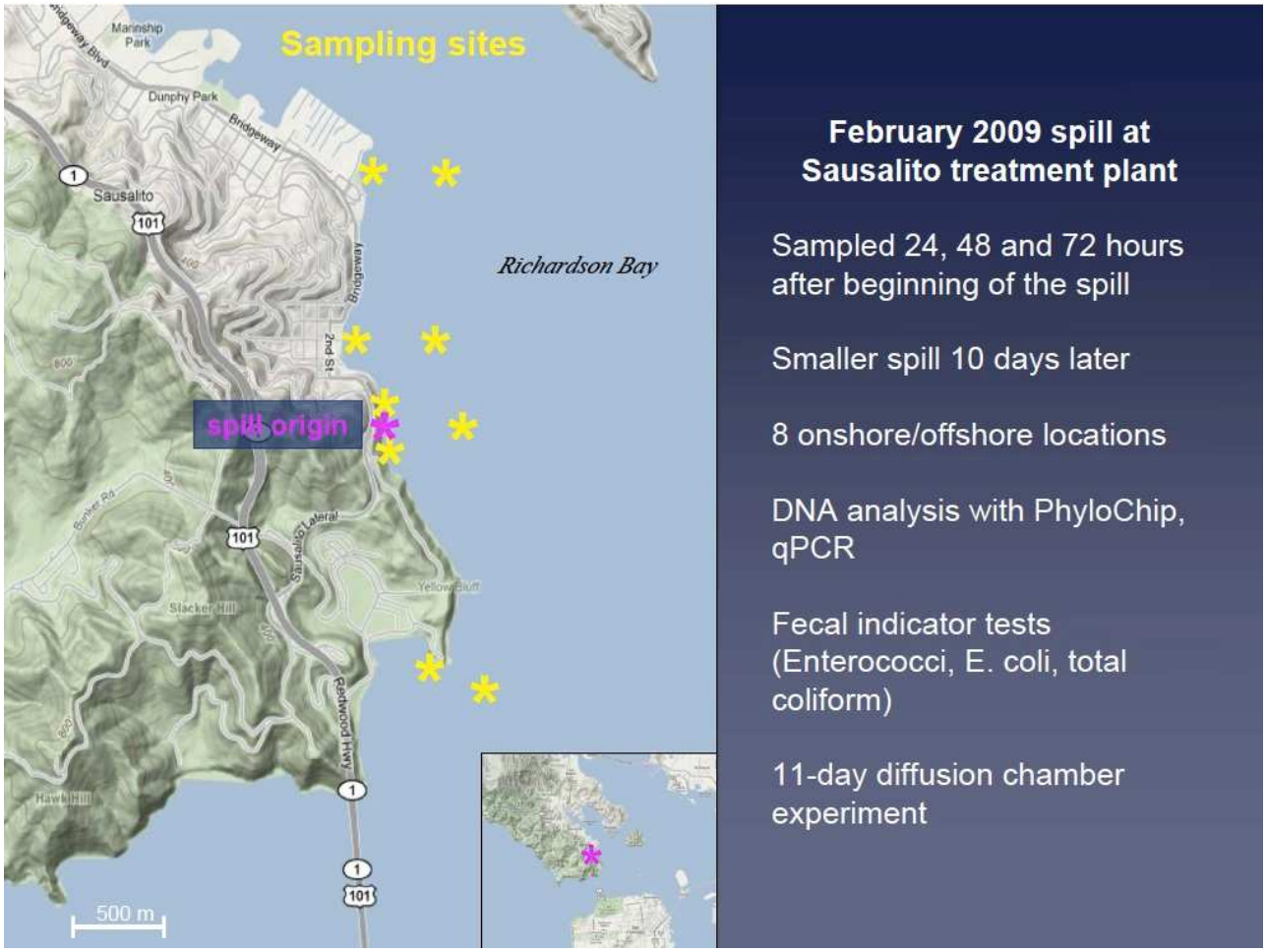



Illustration 4: Sampling sites associated with the Sausalito Sewage Spill

Out of 26 water samples collected in Richardson Bay, three exceeded the 1-day FIB concentration limit and four exceeded 30-day geometric mean limits. These exceedance samples had distinct microbial community compositions from baseline samples that fell below FIB limits (Figure 4). All samples with high FIB counts contained most (78 - 96%) of the expected fecal identifier bacteria for human fecal wastes (Figure 5). The human signal was obvious in samples that were well below 1-day concentration limits and at or below 30-day limits (41 MPN/mL enterococcus, 120 MPN/mL *E. Coli*, 521 MPN/100mL total coliform), indicating the method is sensitive enough to identify the presence of human fecal bacteria at minimum or below concentrations that pose a potential health risk. In addition, the results showed no enrichment of bird or grazer identifier

bacteria in samples with high FIB counts, indicating the method was specific to human waste (Figure 5).

***Source identification of fecal indicator bacteria at AB411 beaches***

Weekly water quality monitoring samples were analyzed from nine different beaches to determine the source of FIB exceedances at each location. PhyloChip analysis was conducted on at least 10 samples from each site and included both FIB exceedance and non-exceedance (baseline) samples that were collected throughout the monitoring season. A total of 125 samples were analyzed (Table 1). The analysis approach was similar to the sewage spill monitoring described above in which FIB samples were analyzed at each site to establish the mean abundance and variance of each OTU under baseline conditions. All OTUs detected in samples with FIB exceedances were compared to their baseline values to determine if they were significantly enriched in relative abundance (> mean + 2σ above baseline). The presence of source identifier bacteria in this enriched subset was used to determine the influence of human, bird and grazer sources.

Community analysis of all detected OTUs revealed that most samples with high FIB deviated in bacterial composition from samples with low FIB (Figure 6). At marine sites, low FIB samples were tightly clustered compared to the two freshwater sites (Figure 6). Thousands of OTUs were significantly enriched in high FIB samples relative to baseline samples at each site, but the strength of the enrichment and shift in bacterial composition varied by site (Figures 6 and 7).

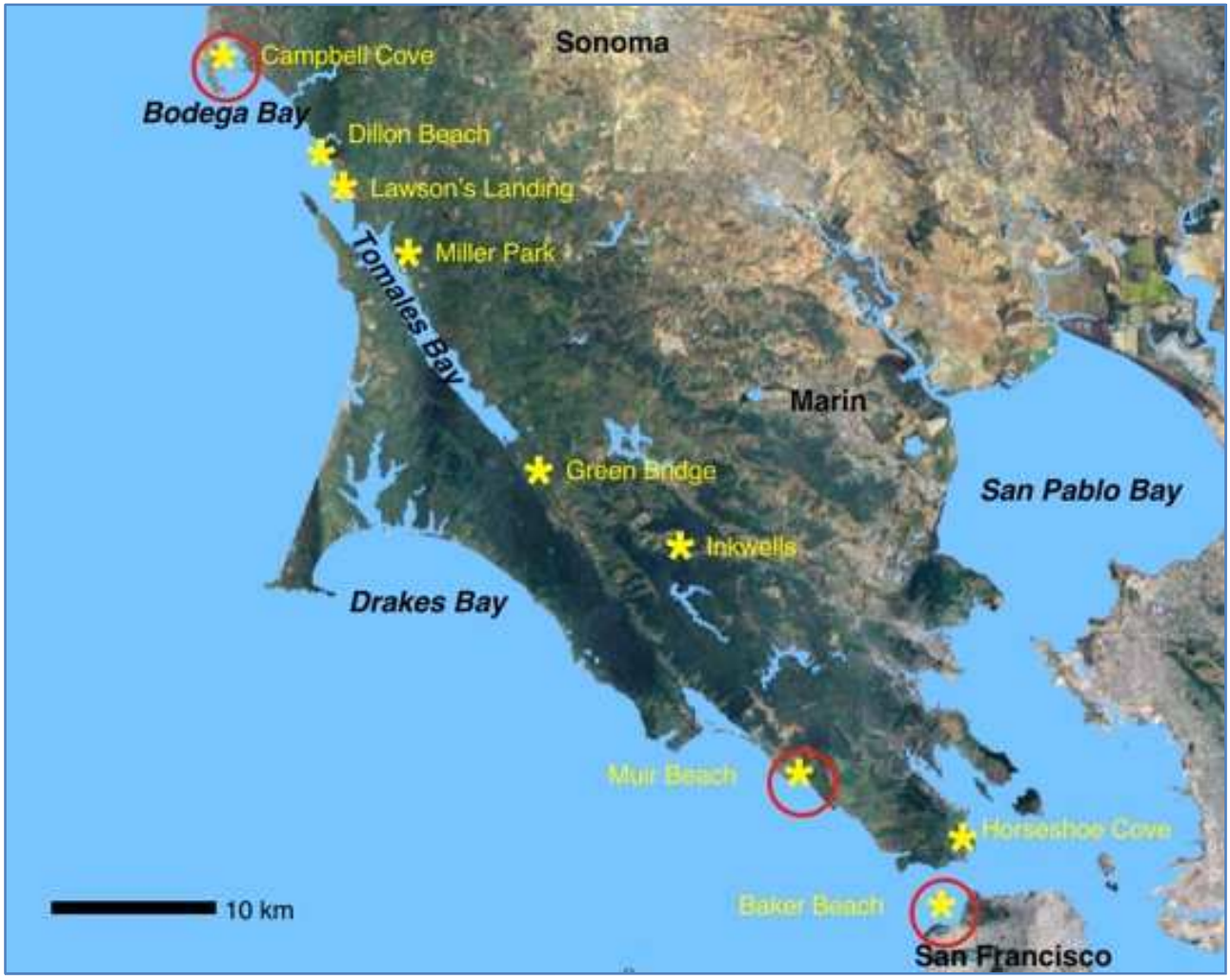



Illustration 5: Selected AB 411 Beach Water Sampling Sites in Marin and Sonoma Counties

Map showing AB411 sites in Marin, Sonoma and San Francisco. Red circles indicate sites identified as 'problem beaches' by SWRCB staff and the Clean Beaches Task Force.

Most exceedances at Campbell Cove in Bodega Bay were associated with enrichment of identifier bacteria associated with birds (Figure 8). Human identifiers were not enriched in any high FIB samples at Campbell Cove (Figure 8). Grazer identifiers were enriched in only one sample that also included at enrichment of bird identifiers. From these results we conclude that birds are a likely source of high FIB at Campbell Cove. These results are consistent with a previous source tracking effort at this beach that used *E. coli* ribotyping to determine that birds and/or marine mammals were the source of FIB. Gulls are frequently observed along the shoreline, and an advisory is currently posted that warns of bird and marine mammal contamination. Interestingly, all exceedances at this Campbell Cove were due to high enterococcus concentrations, while total

coliforms never exceeded 1-day concentration limits during the study. These results are consistent with the findings of Lu et al. (2006) who found Bacilli related to enterococcus dominated gull feces.

At the Tomales Bay sites, all exceedances were caused solely by high enterococcus concentrations, and there was no enrichment of human or bird identifier bacteria in samples with FIB exceedances (Figure 9). Grazer identifier bacteria were not enriched in high FIB samples with the exception of one sample collected at Miller Point. No coliforms exceeded water quality limits. These results indicate that the FIB source is unlikely to be human at the three sites monitored in Tomales Bay. One exceedance sample indicated that grazers may be a potential source, but other exceedance samples showed no evidence of links between grazers and high FIB. The primary source of FIB appears to be from an environmental reservoir that was not characterized in this study. More investigation of other potential sources is needed to pinpoint the FIB sources to Tomales Bay.

Exceedances at Horseshoe Cove were unassociated with human or grazer identifier bacteria (Figure 10). There was moderate enrichment of some (but <20%) of bird identifier taxa. The source of FIB at this site is may include birds or other environmental sources, but is unlikely to be humans or grazers. More investigation of other potential sources is needed to pinpoint the FIB source at this location.

Exceedances at Baker Beach were associated with enrichment in both human and bird identifier bacteria (Figure 11). There was no enrichment in grazer identifier bacteria. The magnitude of exceedances was greater than at other marine sites and included exceedances in both enterococcus and coliforms. The relative importance of human and bird fecal inputs cannot be determined in this study; however the high coliform counts suggest that birds are not the primary cause of exceedances at this site. At Baker Beach samples were collected in two locations. FIB were clearly transported by Lobos Creek because nearly all exceedances occurred at station #15 at the mouth of the creek. Station #16 further west down the beach rarely exceeded FIB limits. The human signal observed at this site is concerning and potential human sources along Lobos Creek need investigated.

Results from Lagunitas Creek (Inkwells, Green Bridge) showed evidence of contamination from human, bird and grazer sources (Figure 12). These freshwater sites frequently exceeded FIB limits, and were in continual violation throughout the late summer. The results indicate that fecal sources to these sites were not consistent, and at times may have been human, bird or grazer in origin. Future studies should investigate the relative contributions of these sources.

## Conclusion

The results of this study show that 16S rRNA gene composition is strongly source-specific and can be used to differentiate sources of fecal contamination in recreational waters. Universal 16S rRNA gene amplification and analysis can serve as a rapid method for identifying sources of fecal indicator bacteria without laborious culturing and fingerprinting methods. We found that birds are the most likely source of FIB contamination at Campbell Cove. Neither human, grazer or bird sources are likely to be primarily responsible for high FIB counts observed in Tomales Bay sites or Horseshoe Cove. Both human and bird sources are associated with high FIB at Baker Beach in San Francisco. Lagunitas Creek is impacted by a wide range of human, bird and grazer sources.

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## LIST OF TABLES



## LIST OF FIGURES

**LIST OF ILLUSTRATIONS**



Cover, Phylochip photos & maps

**TABLE 1.** Fecal indicator counts of samples selected for PhyloChip analysis.

| Beach | County | Date | Enterococcus (MPN/100 mL) | Escolar (MPN/100 mL) | Total Coliform (MPN/100 mL) |
|---|---|---|---|---|---|
| Green Bridge | Marin | 9/24/08 | 20 | 75 | 2247 |
| Green Bridge | Marin | 10/15/08 | 135 | 148 | 629 |
| Green Bridge | Marin | 10/22/08 | 41 | 145 | 789 |
| Green Bridge | Marin | 10/29/08 | 189 | 110 | 8664 |
| Green Bridge | Marin | 4/28/09 | 9 | 52 | 529 |
| Green Bridge | Marin | 5/5/09 | 73 | 228 | 1086 |
| Green Bridge | Marin | 5/12/09 | 9 | 86 | 689 |
| Green Bridge | Marin | 6/9/09 | 30 | 74 | 749 |
| Green Bridge | Marin | 7/14/09 | 9 | 20 | 1785 |
| Green Bridge | Marin | 8/25/09 | 9 | 20 | 1455 |
| Green Bridge | Marin | 9/1/09 | 31 | 20 | 6131 |
| Green Bridge | Marin | 9/8/09 | 75 | 75 | 1106 |
| Green Bridge | Marin | 9/15/09 | 74 | 74 | 11199 |
| Green Bridge | Marin | 9/29/09 | 148 | 185 | 6015 |
| Green Bridge | Marin | 10/6/09 | 20 | 31 | 1279 |
| Green Bridge | Marin | 10/13/09 | 2489 | 2247 | 24192 |
| Green Bridge | Marin | 10/20/09 | 24192 | 5475 | 24192 |
| Green Bridge | Marin | 10/27/09 | 85 | 73 | 1012 |
| Inkwells | Marin | 4/29/09 | 9 | 10 | 816 |
| Inkwells | Marin | 5/6/09 | 134 | 121 | 2098 |
| Inkwells | Marin | 5/13/09 | 63 | 135 | 1674 |
| Inkwells | Marin | 6/10/09 | 41 | 228 | 2282 |
| Inkwells | Marin | 7/15/09 | 52 | 160 | 4106 |
| Inkwells | Marin | 7/29/09 | 86 | 62 | 2359 |
| Inkwells | Marin | 8/26/09 | 30 | 9 | 2755 |
| Inkwells | Marin | 9/9/09 | 160 | 9 | 2603 |
| Inkwells | Marin | 9/30/09 | 530 | 10 | 1483 |
| Inkwells | Marin | 10/7/09 | 108 | 9 | 706 |
| Inkwells | Marin | 10/14/09 | 12997 | 17329 | 24192 |
| Inkwells | Marin | 10/21/09 | 279 | 529 | 4352 |
| Inkwells | Marin | 10/28/09 | 185 | 218 | 2489 |
| Muir Beach North | Marin | 4/29/09 | 10 | 10 | 30 |
| Muir Beach North | Marin | 5/6/09 | 96 | 31 | 288 |
| Muir Beach North | Marin | 5/13/09 | 9 | 9 | 10 |
| Muir Beach North | Marin | 6/17/09 | 10 | 85 | 156 |
| Muir Beach North | Marin | 7/22/09 | 9 | 9 | 9 |
| Muir Beach North | Marin | 8/26/09 | 9 | 20 | 31 |
| Muir Beach North | Marin | 10/7/09 | 9 | 9 | 9 |
| Muir Beach North | Marin | 10/14/09 | 41 | 52 | 249 |
| Muir Beach North | Marin | 10/21/09 | 97 | 20 | 1017 |
| Muir Beach North | Marin | 10/28/09 | 10 | 20 | 250 |
| Horseshoe Cove NE | Marin | 10/15/08 | 9 | 10 | 63 |
| Horseshoe Cove NE | Marin | 7/8/09 | 10 | 9 | 9 |
| Horseshoe Cove NE | Marin | 8/19/09 | 10 | 9 | 530 |
| Horseshoe Cove NE | Marin | 10/21/09 | 9 | 10 | 31 |
| Horseshoe Cove NW | Marin | 10/15/08 | 31 | 31 | 83 |

| | | | | | |
|---|---|---|---|---|---|
| Horseshoe Cove NW | Marin | 4/29/09 | 9 | 9 | 9 |
| Horseshoe Cove NW | Marin | 5/6/09 | 9 | 109 | 109 |
| Horseshoe Cove NW | Marin | 5/13/09 | 9 | 9 | 9 |
| Horseshoe Cove NW | Marin | 6/17/09 | 9 | 10 | 142 |
| Horseshoe Cove NW | Marin | 7/8/09 | 187 | 538 | 624 |
| Horseshoe Cove NW | Marin | 8/19/09 | 148 | 10 | 214 |
| Horseshoe Cove NW | Marin | 10/7/09 | 10 | 9 | 10 |
| Horseshoe Cove NW | Marin | 10/14/09 | 256 | 10 | 473 |
| Horseshoe Cove NW | Marin | 10/21/09 | 327 | 426 | 1414 |
| Horseshoe Cove NW | Marin | 10/28/09 | 9 | 10 | 96 |
| Horseshoe Cove SW | Marin | 10/15/08 | 109 | 1421 | 1607 |
| Horseshoe Cove SW | Marin | 8/5/09 | 10 | 10 | 31 |
| Horseshoe Cove SW | Marin | 8/19/09 | 9 | 10 | 52 |
| Horseshoe Cove SW | Marin | 10/21/09 | 10 | 10 | 20 |
| Dillon Beach | Marin | 4/29/09 | 9 | 9 | 9 |
| Dillon Beach | Marin | 5/6/09 | 448 | 211 | 414 |
| Dillon Beach | Marin | 5/13/09 | 9 | 9 | 9 |
| Dillon Beach | Marin | 6/17/09 | 9 | 9 | 9 |
| Dillon Beach | Marin | 7/22/09 | 9 | 20 | 31 |
| Dillon Beach | Marin | 8/26/09 | 9 | 9 | 10 |
| Dillon Beach | Marin | 10/7/09 | 9 | 9 | 9 |
| Dillon Beach | Marin | 10/14/09 | 9 | 9 | 9 |
| Dillon Beach | Marin | 10/28/09 | 9 | 9 | 9 |
| Lawson's Landing | Marin | 4/29/09 | 9 | 9 | 10 |
| Lawson's Landing | Marin | 5/6/09 | 122 | 41 | 85 |
| Lawson's Landing | Marin | 5/13/09 | 9 | 9 | 9 |
| Lawson's Landing | Marin | 6/17/09 | 9 | 9 | 10 |
| Lawson's Landing | Marin | 7/22/09 | 9 | 9 | 85 |
| Lawson's Landing | Marin | 8/26/09 | 9 | 9 | 86 |
| Lawson's Landing | Marin | 10/7/09 | 10 | 9 | 9 |
| Lawson's Landing | Marin | 10/14/09 | 471 | 135 | 295 |
| Lawson's Landing | Marin | 10/28/09 | 9 | 9 | 31 |
| Miller Point | Marin | 4/29/09 | 10 | 9 | 9 |
| Miller Point | Marin | 5/6/09 | 156 | 10 | 97 |
| Miller Point | Marin | 6/17/09 | 9 | 9 | 9 |
| Miller Point | Marin | 7/22/09 | 9 | 9 | 388 |
| Miller Point | Marin | 8/26/09 | 9 | 9 | 197 |
| Miller Point | Marin | 9/23/09 | 9 | 9 | 146 |
| Miller Point | Marin | 9/30/09 | 243 | 218 | 323 |
| Miller Point | Marin | 10/7/09 | 9 | 9 | 9 |
| Miller Point | Marin | 10/14/09 | 189 | 187 | 473 |
| Miller Point | Marin | 10/28/09 | 9 | 31 | 61 |
| Baker Beach #15 | San Francisco | 10/1/08 | 884 | 473 | 17329 |
| Baker Beach #15 | San Francisco | 10/15/08 | 10 | 31 | 63 |
| Baker Beach #15 | San Francisco | 10/29/08 | 670 | 910 | 24196 |
| Baker Beach #15 | San Francisco | 3/24/09 | 31 | 10 | 2143 |
| Baker Beach #15 | San Francisco | 5/27/09 | 985 | 727 | 14136 |
| Baker Beach #15 | San Francisco | 8/19/09 | 135 | 373 | 24196 |
| Baker Beach #15 | San Francisco | 9/8/09 | 246 | 369 | 12033 |
| Baker Beach #15 | San Francisco | 9/22/09 | 886 | 780 | 24196 |
| Baker Beach #16 | San Francisco | 10/1/08 | 142 | 85 | 269 |
| Baker Beach #16 | San Francisco | 10/15/08 | 298 | 107 | 7701 |

| | | | | | |
|---|---|---|---|---|---|
| Baker Beach #16 | San Francisco | 10/29/08 | 10 | 20 | 52 |
| Baker Beach #16 | San Francisco | 2/10/09 | 20 | 10 | 86 |
| Baker Beach #16 | San Francisco | 3/24/09 | 10 | 10 | 31 |
| Baker Beach #16 | San Francisco | 5/27/09 | 10 | 30 | 41 |
| Baker Beach #16 | San Francisco | 7/14/09 | 10 | 10 | 10 |
| Baker Beach #16 | San Francisco | 8/19/09 | 10 | 52 | 288 |
| Baker Beach #16 | San Francisco | 9/8/09 | 10 | 10 | 31 |
| Baker Beach #16 | San Francisco | 9/22/09 | 10 | 10 | 98 |
| Campbell Cove | Sonoma | 9/15/08 | 74 | 218 | 462 |
| Campbell Cove | Sonoma | 9/29/08 | 631 | 472 | 1,046 |
| Campbell Cove | Sonoma | 10/27/08 | 20 | 201 | 275 |
| Campbell Cove | Sonoma | 11/3/08 | 275 | 1086 | 2481 |
| Campbell Cove | Sonoma | 11/10/08 | 408 | 185 | 275 |
| Campbell Cove | Sonoma | 12/1/08 | 959 | 216 | 216 |
| Campbell Cove | Sonoma | 12/8/08 | 10 | 10 | 52 |
| Campbell Cove | Sonoma | 12/15/08 | 2,723 | 1,259 | 2,909 |
| Campbell Cove | Sonoma | 12/22/08 | 10 | 10 | 10 |
| Campbell Cove | Sonoma | 12/29/08 | 465 | 6,131 | 9,804 |
| Campbell Cove | Sonoma | 1/5/09 | 10 | 10 | 31 |
| Campbell Cove | Sonoma | 1/12/09 | 318 | 305 | 2,603 |
| Campbell Cove | Sonoma | 1/20/09 | 10 | 30 | 30 |
| Campbell Cove | Sonoma | 2/23/09 | 959 | 63 | 211 |
| Campbell Cove | Sonoma | 3/2/09 | 10 | 10 | 10 |
| Campbell Cove | Sonoma | 6/8/09 | 30 | 10 | 10 |
| Campbell Cove | Sonoma | 7/13/09 | 10 | 10 | 10 |
| Campbell Cove | Sonoma | 8/17/09 | 10 | 10 | 10 |
| Campbell Cove | Sonoma | 10/5/09 | 10 | 10 | 10 |

FIGURE 1.

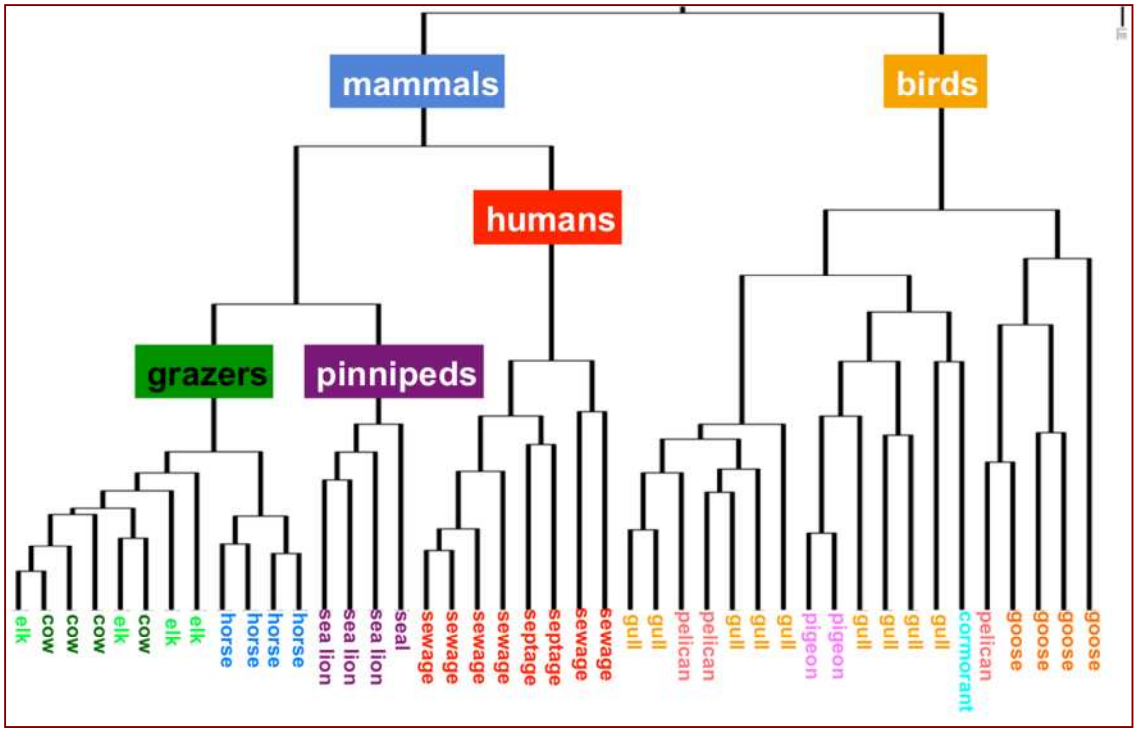



**Figure 1**. Cluster analysis dendrogram of 16S rRNA gene composition showing similarity among microbial communities.

FIGURE 2.

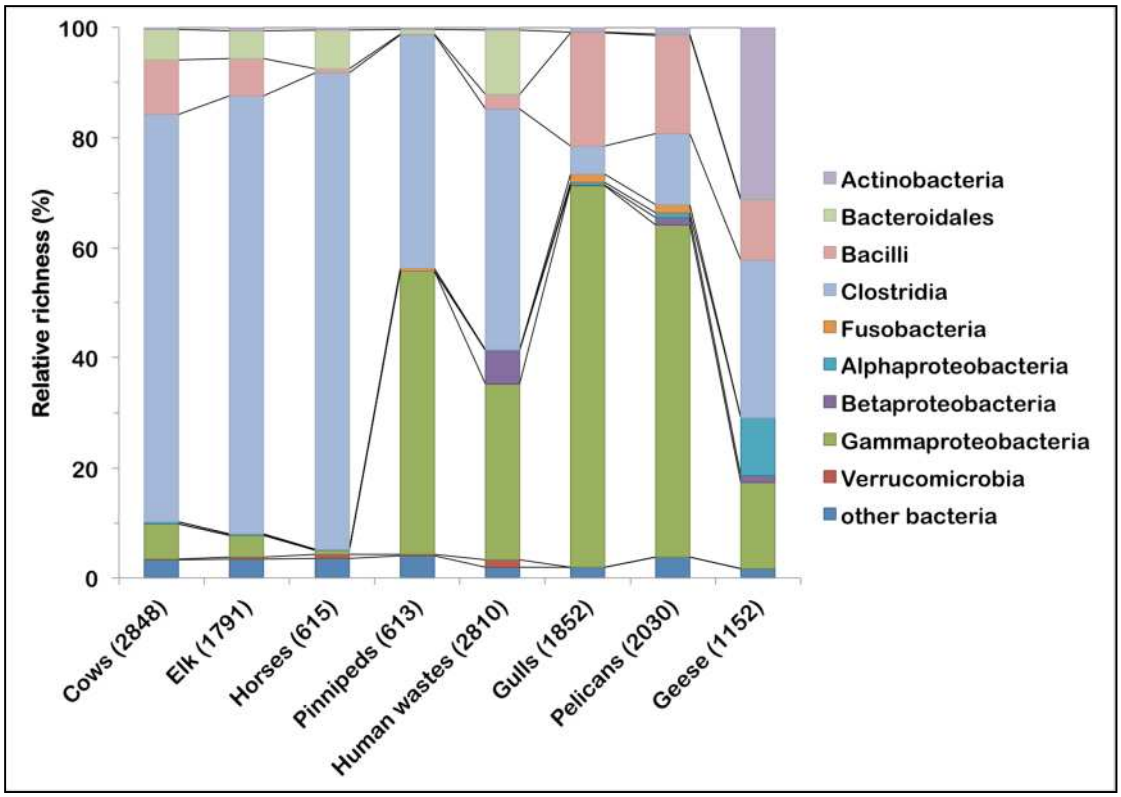
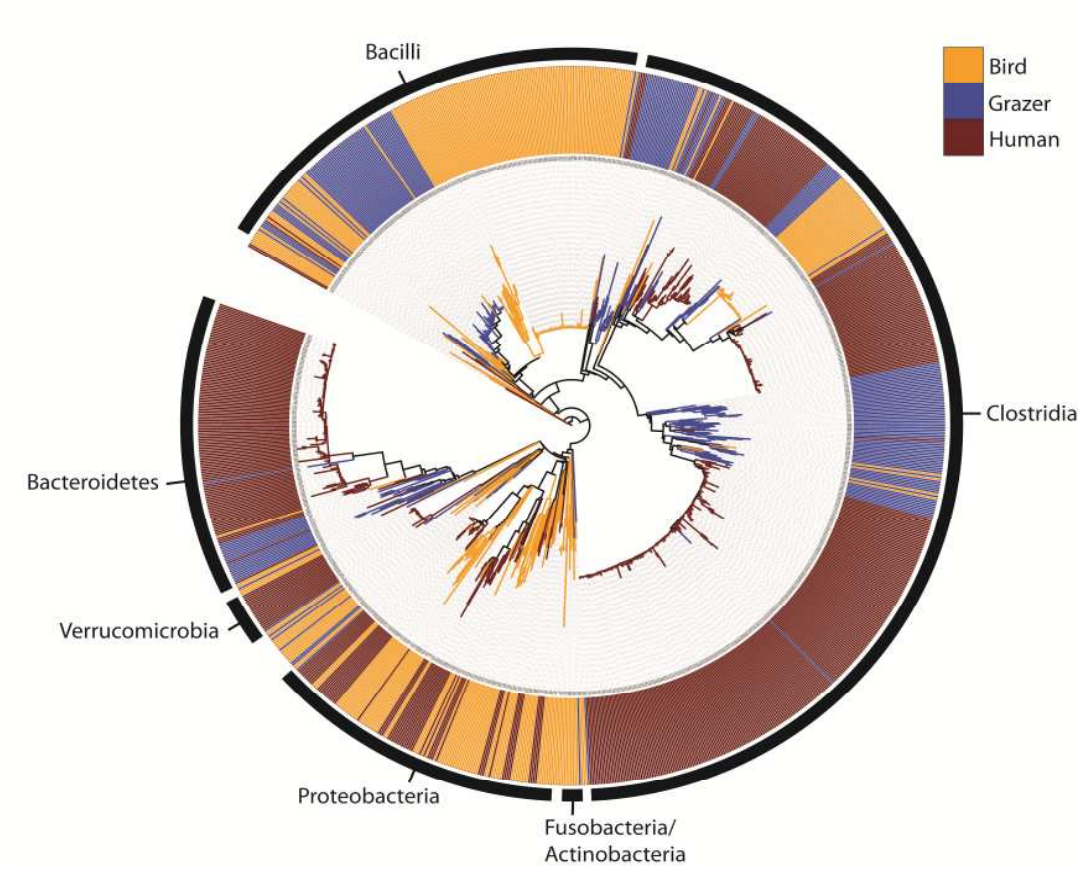



**Figure 2**. Composition of OTUs in each fecal source type. OTUs are shown that were detected in the majority of samples for each source type. Total numbers of OTUs are shown in parentheses. Source type

FIGURE 3.



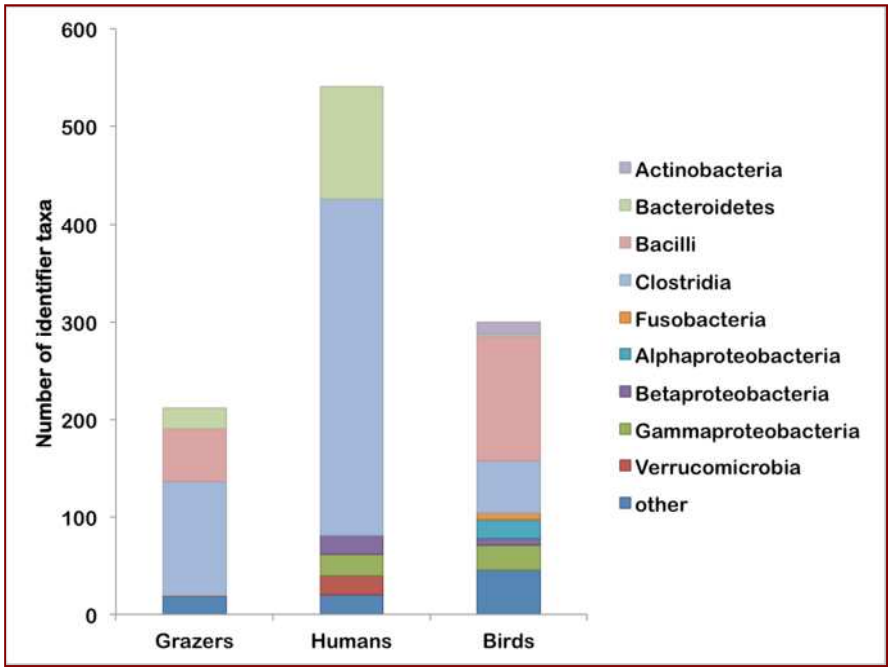




**Figure 3**. Phylogenetic tree (a) and taxonomic composition (b) of source identifier OTUs for human wastes, birds and grazers.

FIGURE 4

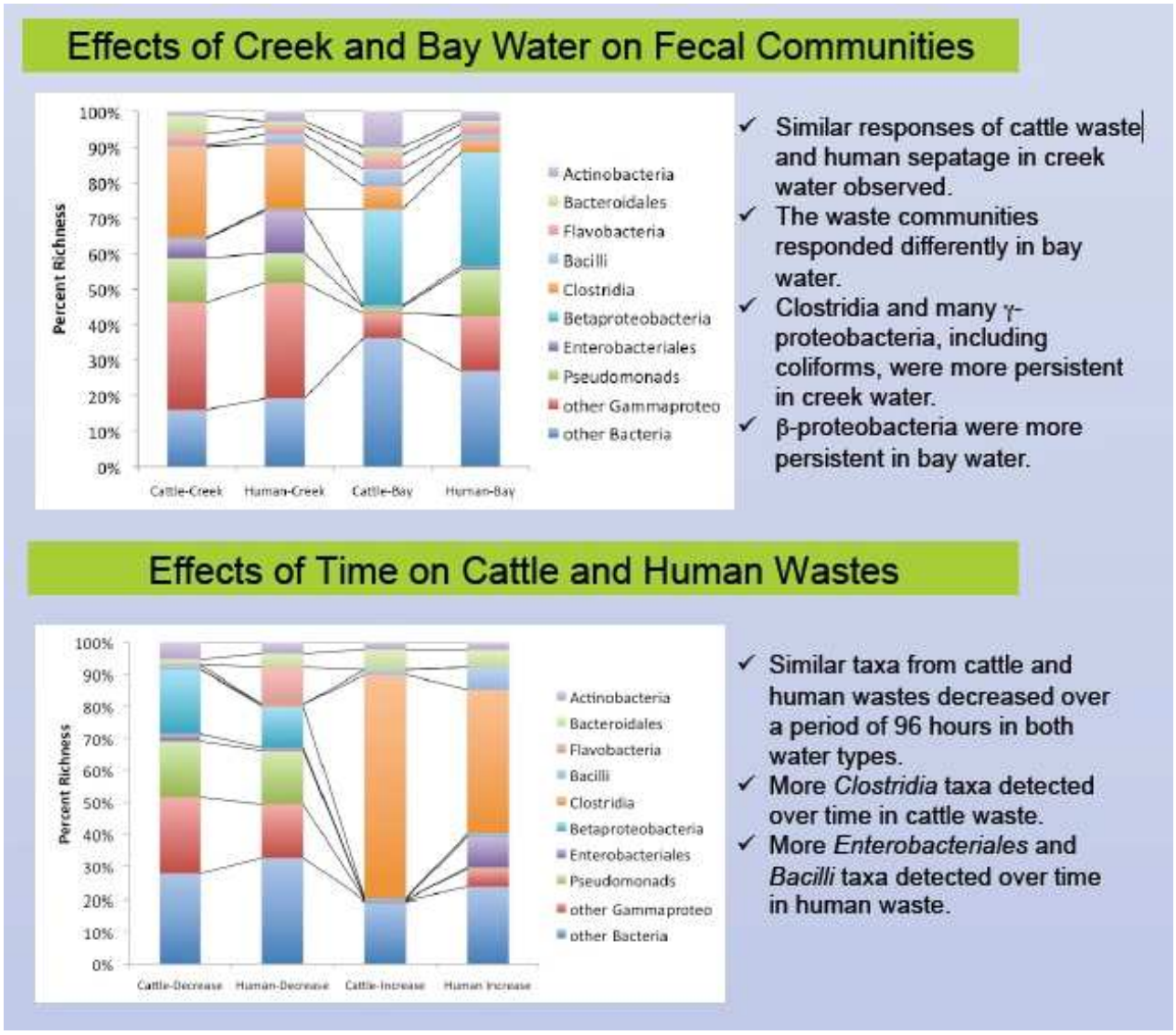

Effects of Creek and Bay Water on Fecal Communities
Percent Richness
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Cattle-Creek
Human-Creek
Cattle-Bay
Human-Bay
Actinobacteria
Bacteroidales
Flavobacteria
Bacilli
Clostridia
Betaproteobacteria
Enterobacteriales
Pseudomonads
other Gammaproteo
other Bacteria
✓ Similar responses of cattle waste and human sepatage in creek water observed.
✓ The waste communities responded differently in bay water.
✓ Clostridia and many γ-proteobacteria, including coliforms, were more persistent in creek water.
✓ β-proteobacteria were more persistent in bay water.
Effects of Time on Cattle and Human Wastes
Percent Richness
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Cattle-Decrease
Human-Decrease
Cattle-Increase
Human Increase
Actinobacteria
Bacteroidales
Flavobacteria
Bacilli
Clostridia
Betaproteobacteria
Enterobacteriales
Pseudomonads
other Gammaproteo
other Bacteria
✓ Similar taxa from cattle and human wastes decreased over a period of 96 hours in both water types.
✓ More *Clostridia* taxa detected over time in cattle waste.
✓ More *Enterobacteriales* and *Bacilli* taxa detected over time in human waste.

Figure 5

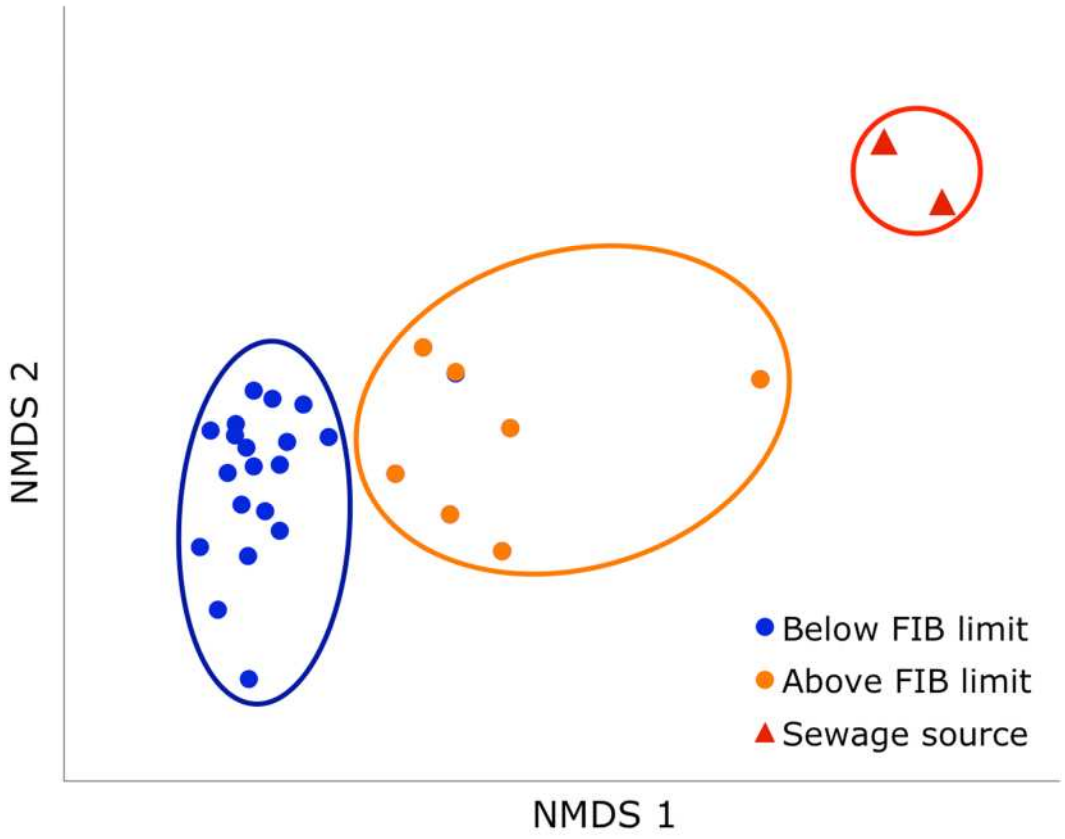



**Figure 6**. Microbial community analysis of water and sewage samples collected following the 2009 sewage spill from the Sausalito Marin-City Sanitary District treatment plant. Ordination was conducted using non-metric multidimensional scaling with the bray-curtis distance metric.

FIGURE 6

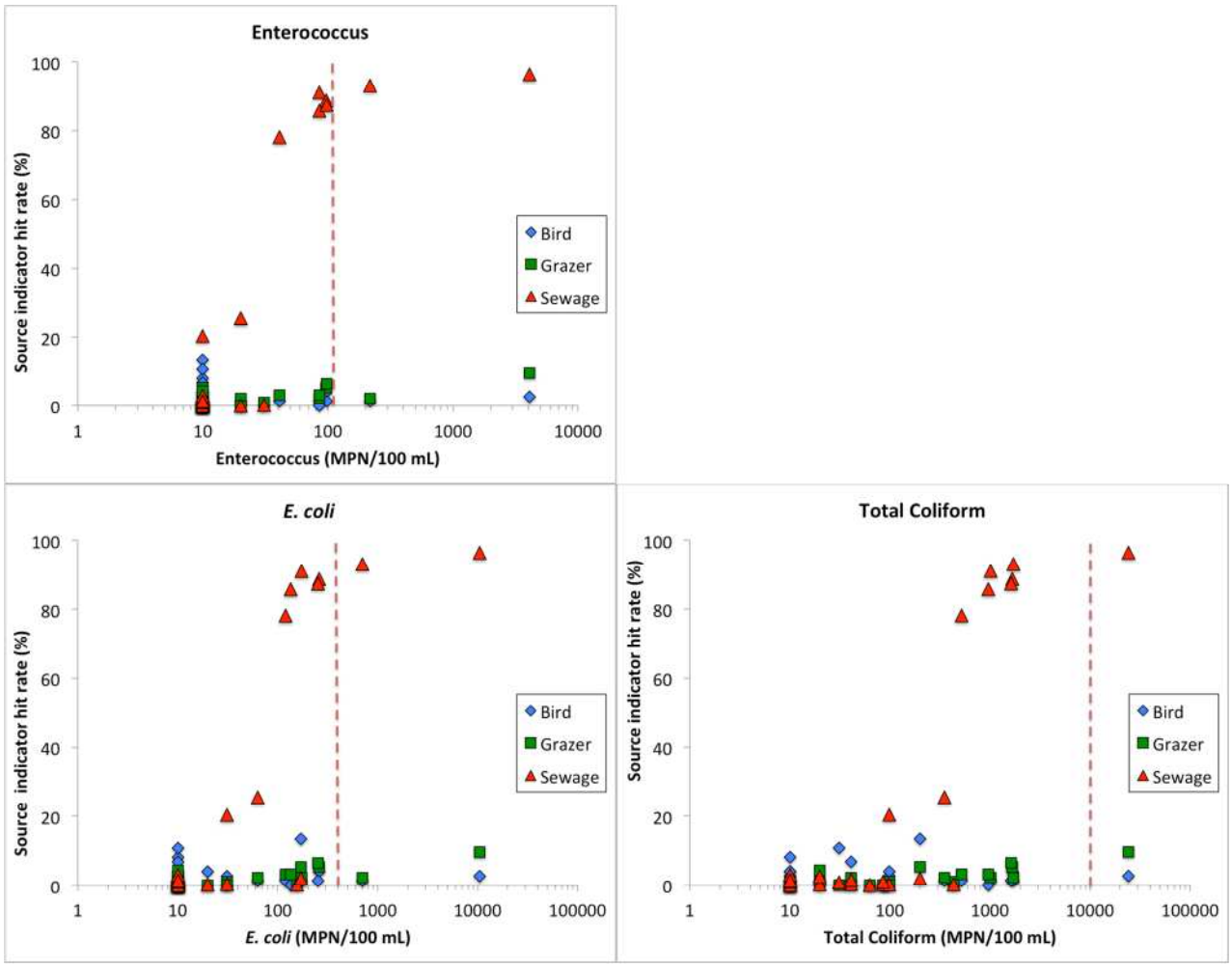



**Figure 6**. Source identification of high FIB samples from February 2009 Sausalito sewage spill monitoring. Results from FIB tests (enterococcus, *E. coli*, total coliform) are plotted against the percent of potential source-identifier taxa that were detected in high abundance (>mean baseline + 2σ) by PhyloChip. Dashed line is the single-day FIB concentration limit for marine water.
FIGURE 6

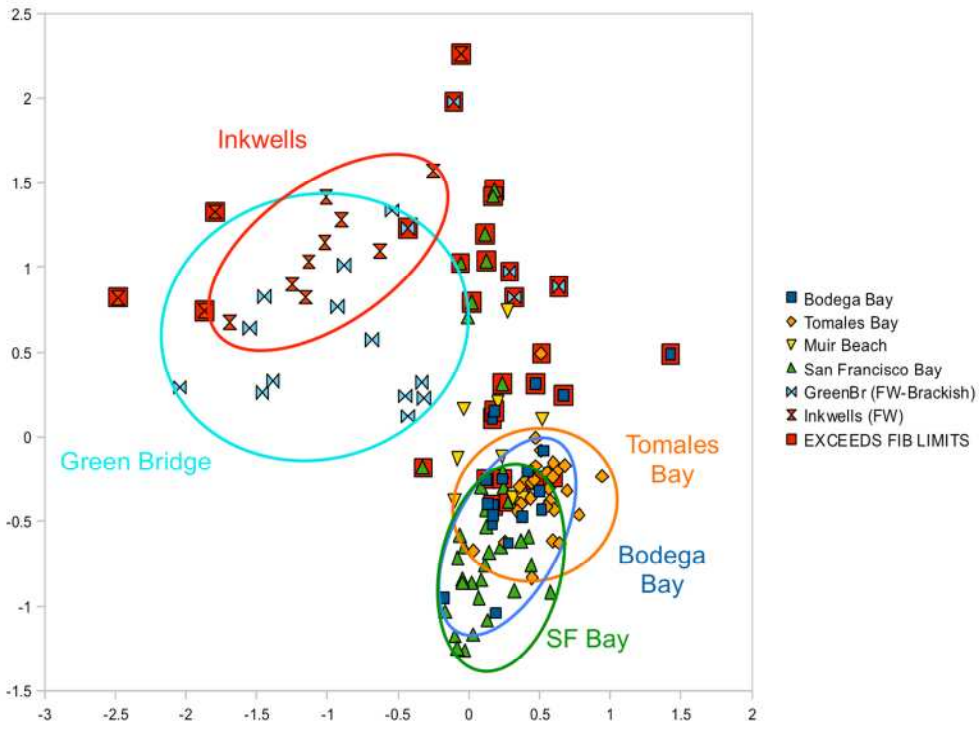



**Figure 7.** Microbial community analysis of AB411 water samples. Ordination was conducted using non-metric multidimensional scaling with the bray-curtis distance metric. Circled clusters of points show the distribution of samples in that were below FIB limits in different bodies of water. Samples that exceeded FIB limits are highlighted with red squares.

FIGURE 8

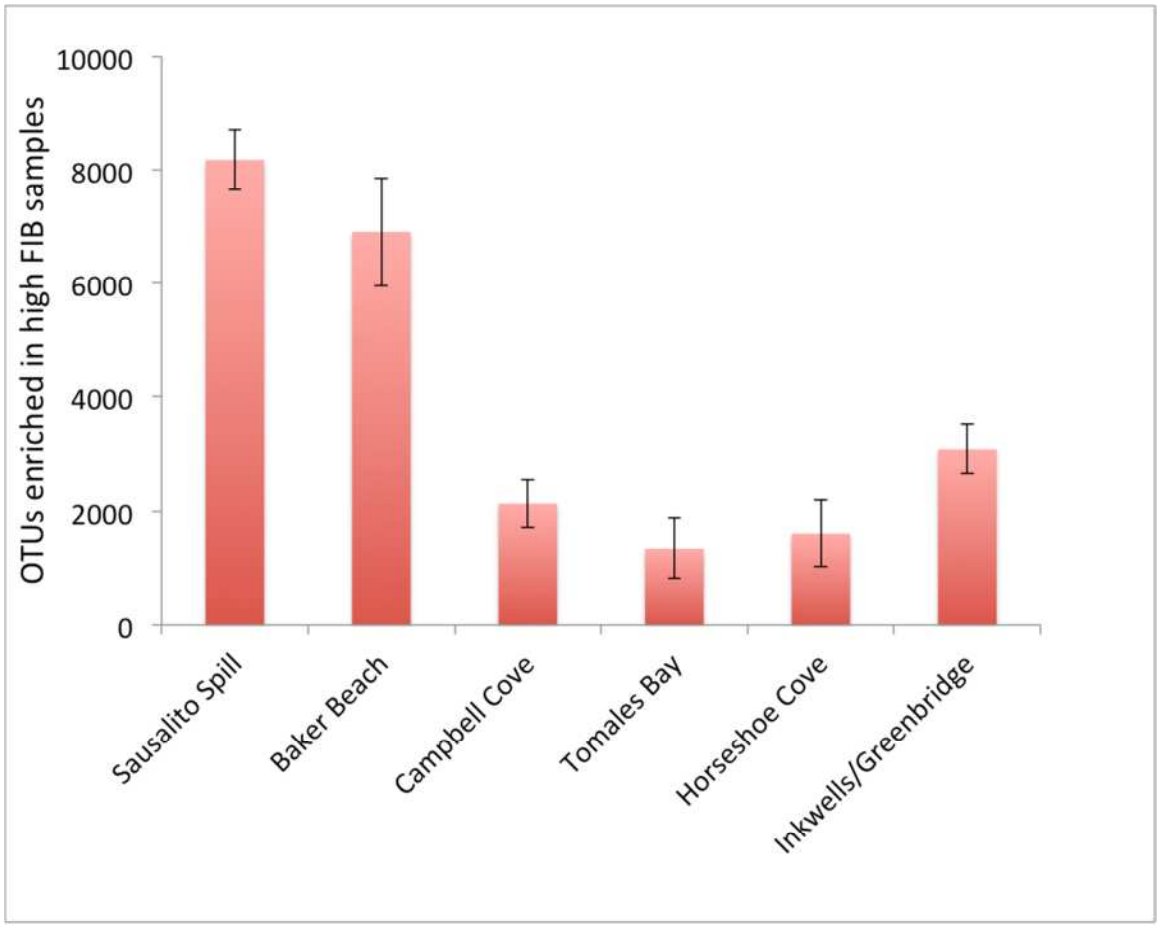



**Figure 8.** Mean number of OTUs that were significantly enriched in high FIB samples. Enriched OTUs exceeded the mean baseline abundance by at least two standard deviations.

FIGURE 9. CAMPBELL COVE

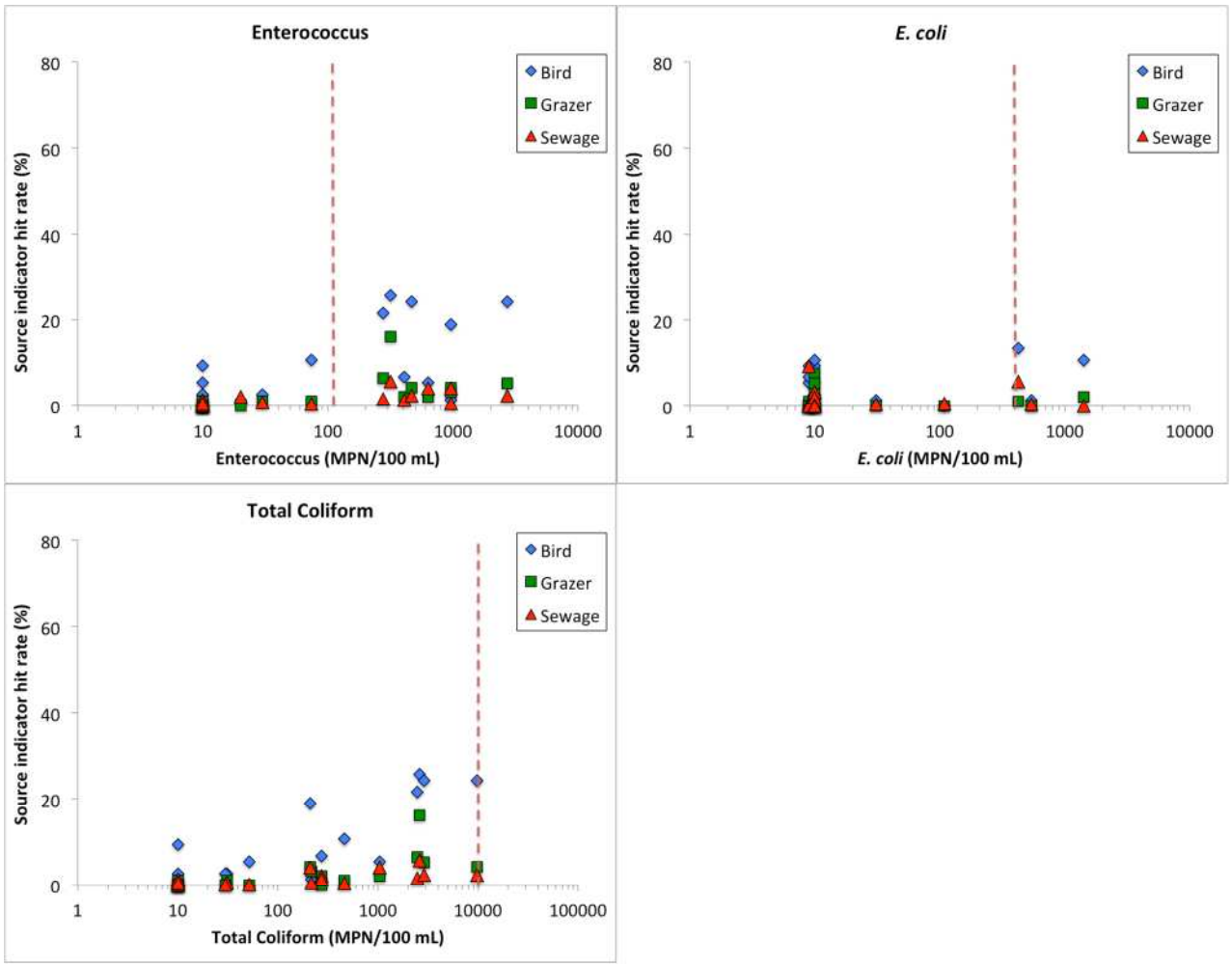



**Figure 9**. Source identification of high FIB samples from AB411 monitoring at Campbell Cove, Bodega Bay. Results from FIB tests are plotted against the percent of potential source-identifier taxa that were detected in high abundance (>mean baseline + 2σ) by PhyloChip. Dashed line is the single-day FIB concentration limit for marine water.

FIGURE 9. TOMALES BAY

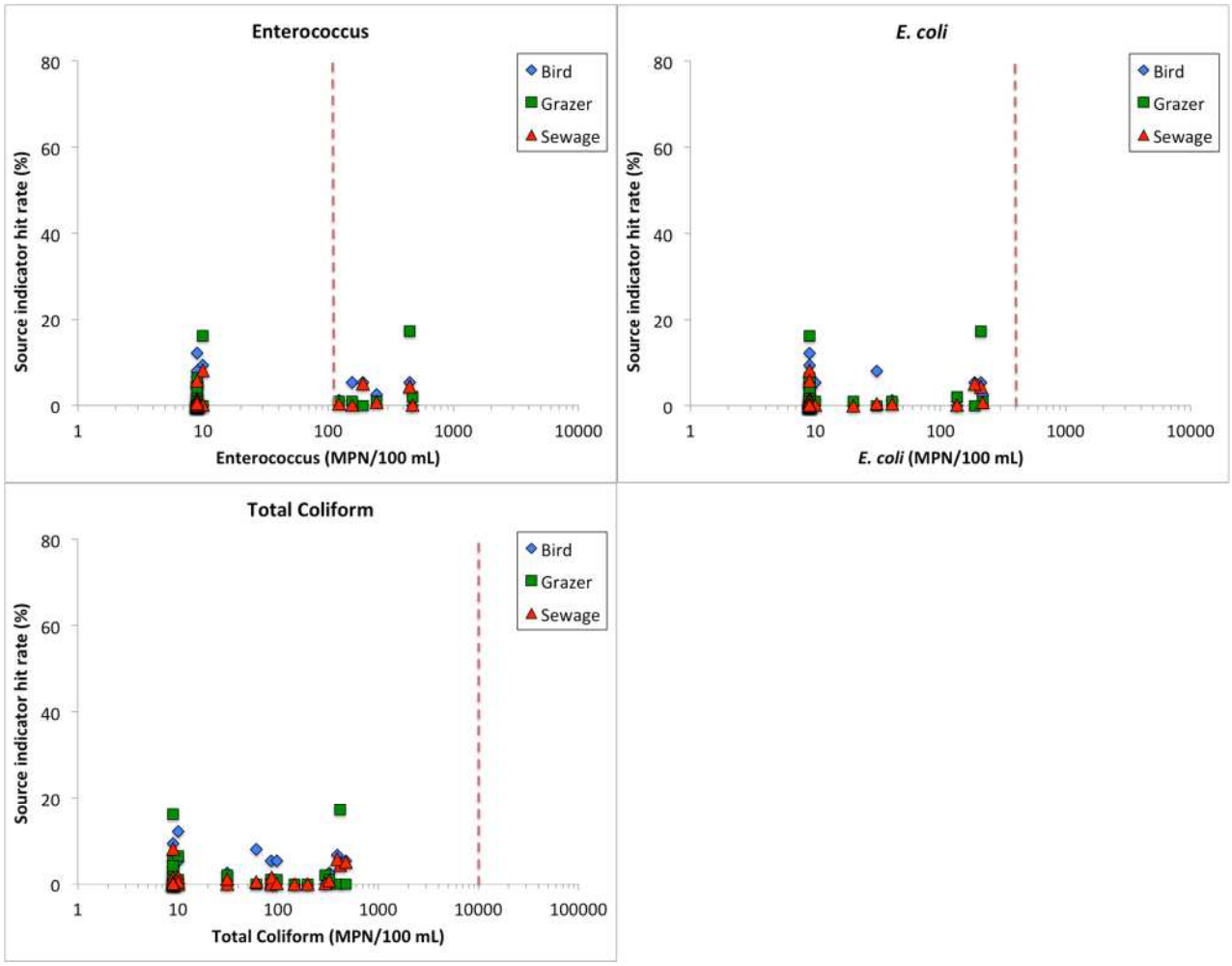



**Figure 10**. Source identification of high FIB samples from AB411 monitoring at Tomales Bay sites (Dillon Beach, Lawson's Landing, Miller Point).

**FIGURE 10**. HORSESHOE COVE

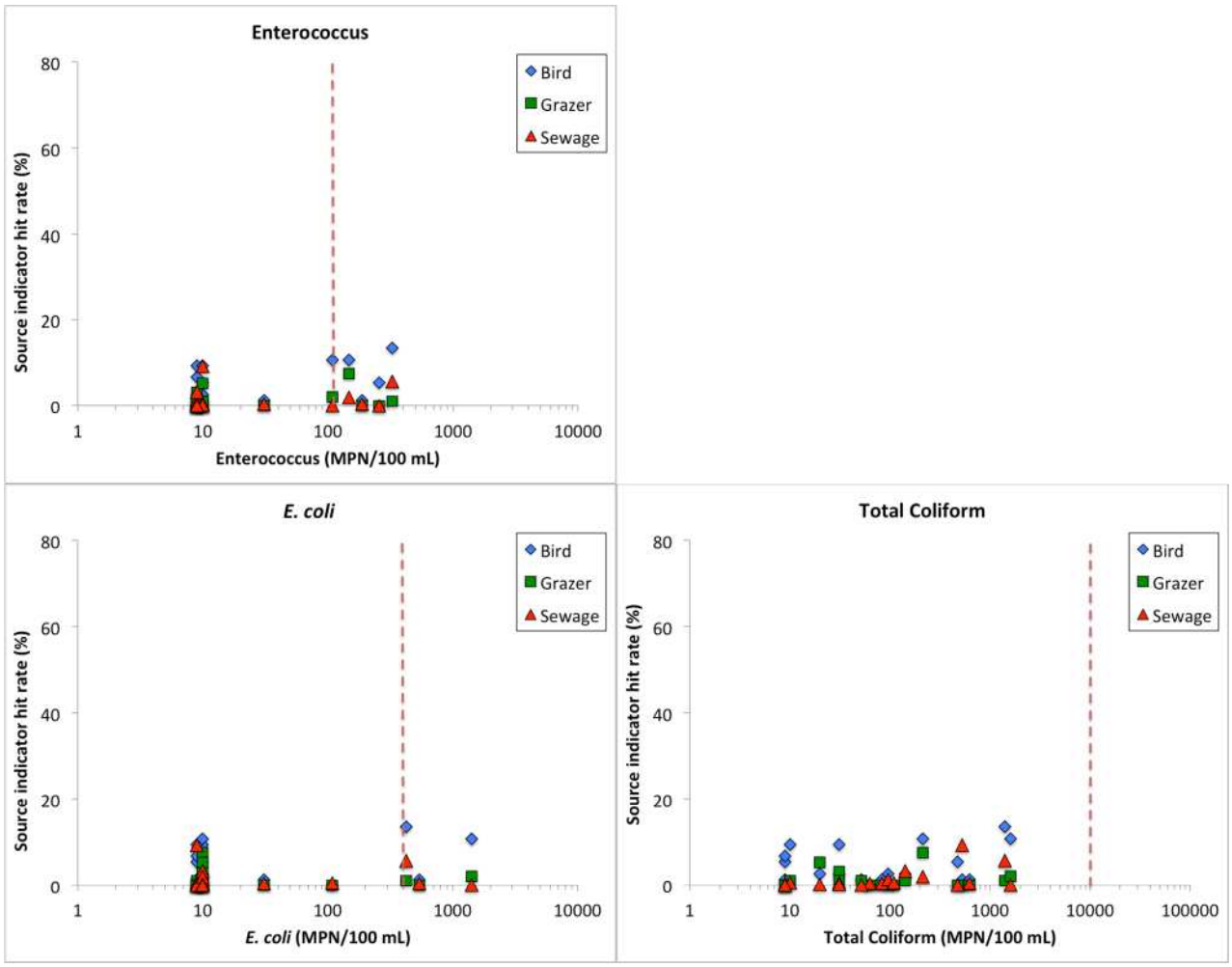



**Figure 11**. Source identification of high FIB samples from AB411 monitoring at Horseshoe Cove sites.

FIGURE 12. BAKER BEACH

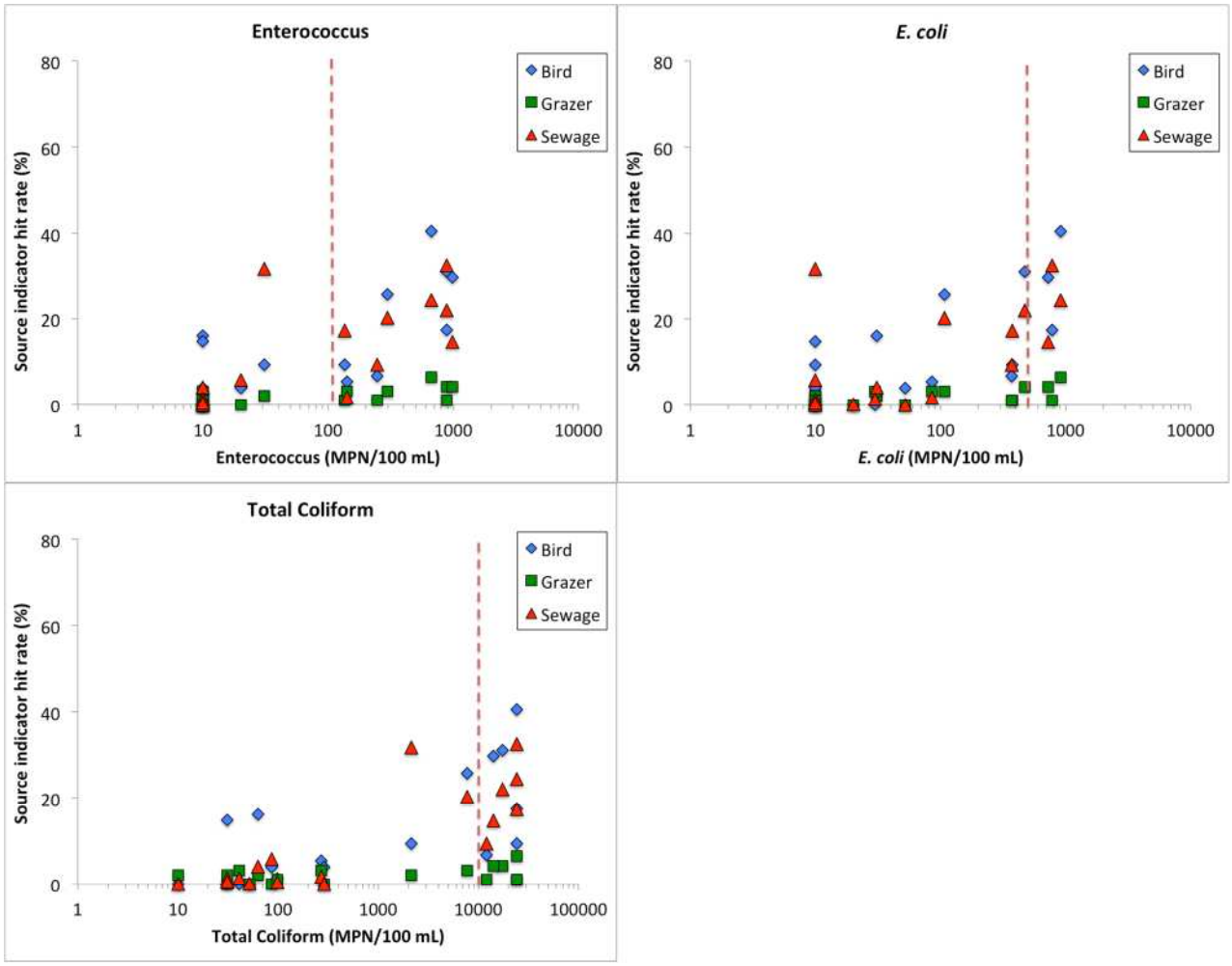



**Figure 12**. Source identification of high FIB samples from AB411 monitoring at Baker Beach in San Francisco.

FIGURE 13. LAGUNITAS CREEK

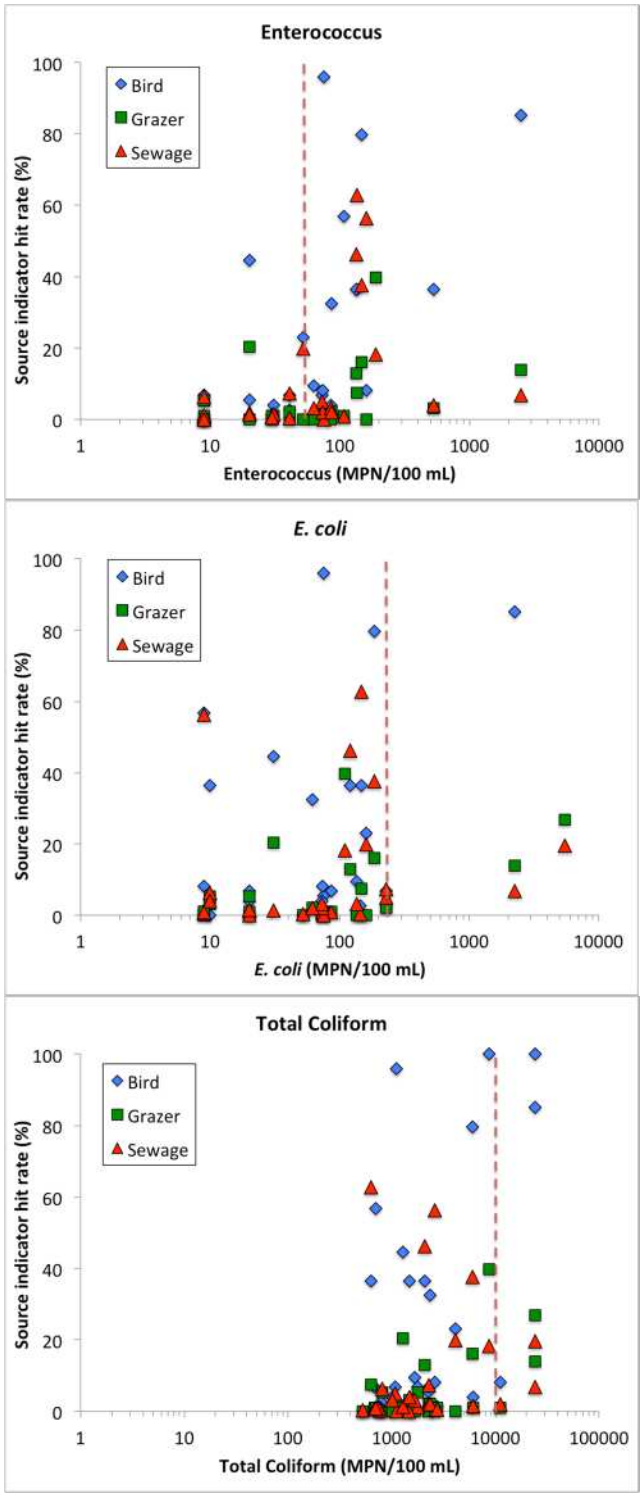



**Figure 13**. Source identification of high FIB samples from AB411 monitoring at Lagunitas Creek sites (Inkwells and Green Bridge).

Appendix 3:

Scientific Paper: “Application of Phylogenetic Microarray Analysis to Discriminate Sources of Fecal Pollution.”

Environmental Science & Technology

Article

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# Application of Phylogenetic Microarray Analysis to Discriminate Sources of Fecal Pollution

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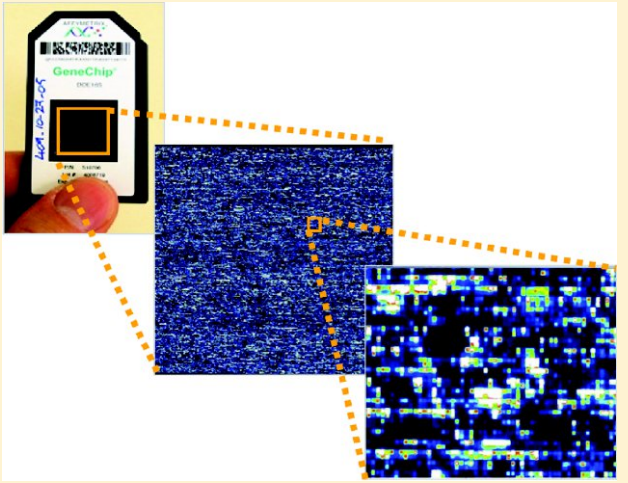
Supporting Information

**ABSTRACT:** Conventional methods for fecal source tracking typically use single biomarkers to systematically identify or exclude sources. High-throughput DNA sequence analysis can potentially identify all sources of microbial contaminants in a single test by measuring the total diversity of fecal microbial communities. In this study, we used phylogenetic microarray analysis to determine the comprehensive suite of bacteria that define major sources of fecal contamination in coastal California. Fecal wastes were collected from 42 different populations of humans, birds, cows, horses, elk, and pinnipeds. We characterized bacterial community composition using a DNA microarray that probes for 16S rRNA genes of 59 316 different bacterial taxa. Cluster analysis revealed strong differences in community composition among fecal wastes from human, birds, pinnipeds, and grazers. Actinobacteria, Bacilli, and many Gammaproteobacteria taxa discriminated birds from mammalian sources. Diverse families within the Clostridia and Bacteroidetes taxa discriminated human wastes, grazers, and pinnipeds from each other. We found 1058 different bacterial taxa that were unique to either human, grazing mammal, or bird fecal wastes. These OTUs can serve as specific identifier taxa for these sources in environmental waters. Two field tests in marine waters demonstrate the capacity of phylogenetic microarray analysis to track multiple sources with one test.




## INTRODUCTION

Beach closures and public health advisories have a major economic impact on coastal communities whose economies are based largely on tourism from beach recreation. Most closings and advisories are triggered by water samples that exceed microbial water quality standards for fecal indicator bacteria (FIB), usually culturable coliforms, *E. coli*, or enterococci that are considered a proxy for human health risk in recreational waters. Because the direct measurement of all human pathogens is often impractical and unreliable under field conditions, water monitoring relies on the detection of bacterial indicators that have some demonstrated correlation with human illness in areas mostly impacted by human sewage.[1,2] Sewage, however, is one of many potential sources of FIB, and monitoring results are often confounded by inputs from a variety of wildlife and nonfecal sources.[1,3−5] FIB are common in most warm-blooded animals, and many studies demonstrate that FIB occur in several environmental sources aside from feces, including soils and sediments, algal wrack, and beach sands.[3−5] Thus, water bodies often contain measurable amounts of FIB even where anthropogenic inputs are absent, and the presence of FIB provides an insufficient indication of health risk without additional source tracking data.

Shortcomings of the current FIB monitoring approach combined with widespread development and implementation of total maximum daily load (TMDL) requirements for microbiological pollution are fueling interest in microbial source tracking (MST) methods.[6,7] Many approaches to source tracking are under development, most of which rely on single phenotypic or genotypic biomarkers to measure sources.[1,6] A limitation of single targets is that no single assay is known to be 100% specific for any one type of waste,[6] and MST based on single targets is entirely dependent on the fate of one biomarker once it enters receiving waters.[8−10]

A huge diversity of microorganisms is resident in human and animal guts. Approximately 1000 different microbial taxa are now known to reside in the human gut alone, but the potential for this diversity to be used as a means for identifying sources remains largely unexplored and there have been few comparative surveys of microbial community composition among important sources of fecal contamination.[11−13] New

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techniques for high-throughput DNA sequence analysis such as high-density microarrays and next-generation sequencing (NGS) technologies like pyrosequencing are enabling comprehensive surveys of diverse microbial communities that occur in a sample. Targeting the whole microbial community for source identification is a fundamentally different approach than traditional molecular methods that are dependent on the detection on one gene sequence under complex environmental conditions.[11,14−16] Sequence analysis of entire microbial communities creates an opportunity to discover a multitude to different bacterial species that are unique to fecal and environmental sources of FIB in recreational waters.

In this study, we used a high-density oligonucleotide microarray to census the 16S rRNA gene diversity in different sources of fecal contamination. The microarray targets 59 316 different 16S rRNA gene polymorphisms that represent most known phyla of bacteria. We test the assumption that different avian and mammalian fecal sources can be distinguished on the basis of their bacterial community composition. We screened a variety of fecal sources of concern in coastal California to identify the microbial groups that are source-specific and then used these unique taxa to detect influence from these sources in marine samples that exceeded water quality limits for fecal indicator bacteria.

## METHODS

**Feces Sampling and DNA Extraction.** Human fecal wastes and freshly deposited droppings from animals were collected at numerous locations throughout California (Supporting Information). Human fecal wastes included primary influent or effluent from eight different municipal wastewater treatment plants, leachate samples from two community septic tanks serving more than 30 households each, and one composite sample of 10 holding tanks from individual households. Sampled animal populations included cows (4), horses (4), tule elk (4), western and California gulls (9), Canada geese (4), pelican (3), pigeon (2), cormorant (1), sea lion (3), elephant seal (1). Each animal sample was a composite of droppings from at least five different individuals from one location and every replicate sample is from a unique population. Individual fecal samples were homogenized and immediately frozen upon collection. Samples were stored at −80 °C until DNA extraction.

Each fecal sample was extracted in triplicate to obtain genomic DNA from the microbial community. Two extraction methods were employed: a CTAB extraction method and a kit extraction. In the CTAB method, 0.5 g of homogenized fecal sample were added to a Lysing Matrix E tube (MP Biomedicals, Solon, OH), and 650 μL TE buffer, 250 μL 10× phosphate buffered saline, and 100 μL 10% SDS were added. The tubes were bead-beat at 5.5 m/s for 25 s in a FastPrep-24 instrument (MP Biomedicals, Solon, OH), 5.5 m/s for 25 s, 10 μL of 20 mg/mL Proteinase K was added and tubes were incubated at 37 °C for 30 min. Following centrifugation at 10 000*g* for 5 min, supernatant was transferred to 2 mL microcentrifuge tube and amended with 80 μL 5 M NaCl and 80 μL 10% CTAB buffer solution. Tubes were heated at 65 °C for 10 min, 700 μL 24:1 chloroform/isopropanol added and then centrifuged at 6000*g* for 5 min. Supernatant was transferred to a clean microcentrifuge tube, amended with 0.8 volumes of isopropanol, gently mixed, and incubated at −20 °C for 1 h. Following centrifugation at 16 000*g* 4 °C for 15 min the supernatant was discarded and the remaining extract washed with ice-cold 70% ethanol. Following centrifugation at 16 000*g* at 4 °C for 5 min, the supernatant was discarded, and the remaining DNA pellet was air-dried and suspended in 30 μL elution buffer. The second DNA extraction method was conducted with the DNA EZ extraction kit (GeneRite, North Brunswick, NJ) per manufacturer's instructions. We extracted a subset of samples with both methods and saw little difference in the measured community profiles, and we saw no similarity patterns in our final results that were explained by extraction method.

**Water Sampling and DNA Extraction.** Water monitoring samples were collected from the field at sites with known sources of contamination. One set of samples was collected during a 10-day period following a 720 000 gallon spill of primary effluent from the Sausalito-Marin City Sanitary District treatment plant in Sausalito, California, that occurred in February 2009. Samples were collected daily for three days following the onset of the spill and then once more 10 days after the initial spill during an accidental rupture that occurred during the repair. Sample locations included eight onshore and offshore sites ranging from directly adjacent to the ruptured pipe at the plant to up to 1 km away from the spill origin. Water samples were collected in 1 L bottles and stored at 4 °C until laboratory processing (within 6 h of collection). For FIB tests, 20 mL of water was subsampled for total coliforms and *E. coli* (Colilert, IDEXX Laboratories, Westbrook, ME) and Enterococcus (Enterolert, IDEXX Laboratories). For DNA extraction, 250 mL × 3 of each sample was vacuum filtered through Whatman Anodisc membrane filters (47 mm dia., 0.2 μm pore size) and immediately frozen and stored at −80 °C until DNA extraction. DNA was extracted from filters using the DNA EZ kit per manufacturer's instructions.

Water samples were also collected in conjunction with the County of Sonoma as part of the State of California AB411 monitoring program at Campbell Cove, Bodega Bay. Samples were collected weekly throughout 2008−2009 in knee-deep water with 1 L sampling bottles and processed in the laboratory as described above. A subset of samples was analyzed based on FIB counts. The analysis focused on nine samples that exceeded 1-day concentration limits and an additional 10 samples scattered throughout the sampling period that fell below FIB limits.

**Polymerase Chain Reaction.** The bacterial 16S rRNA gene was amplified from each sample using PCR with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) for bacteria. Each PCR reaction contained 1× Ex Taq buffer (Takara Bio Inc., Japan), 0.025 units/μL Ex Taq polymerase, 0.8 mM dNTP mixture, 1.0 μg/μL BSA, and 200 pM each primer, and 1 ng genomic DNA (gDNA) as template for fecal samples and 10 ng gDNA for water samples. Each sample was amplified in 8 replicate 25 μL reactions spanning a range of annealing temperatures. PCR conditions were 95 °C (3 min), followed by 30 cycles 95 °C (30 s), 48−58 °C (25 s), 72 °C (2 min), followed by a final extension 72 °C (10 min). Amplicons from each reaction were pooled for each sample, purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA), and eluted in 50 μL elution buffer.

**PhyloChip Assay Description and Analysis.** A complete description of the PhyloChip design and analysis is available in the supplementary methods of Hazen et al.[17] The PhyloChip (Second Genome, San Bruno, CA) was designed to detect most 16S rRNA gene sequences that identify bacteria and archaea. The PhyloChip probes for 59 959 different bacterial and

archaeal taxa that represent 147 phyla, 1123 classes, 1219 orders, and 1464 families according to the placement of its member organisms in the taxonomic outline as maintained by Philip Hugenholtz.[18] The microarray includes 1 016 064 probe features, the majority of which target 16S rRNA gene sequences that are useful for differentiating taxa. Additional probes are for quality management, processing controls, image orientation, and normalization controls.[17]

**PhyloChip Assay Analysis.** For PhyloChip hybridization, we used 500 ng of bacterial PCR product for each microarray. PCR products were fragmented with DNase I to a range of 50−200 bp as verified by agarose gels. Commercial kits were utilized for DNA preparation: Affymetrix (Santa Clara, CA) WT Double Stranded DNA Terminal Labeling, and Affymetrix GeneChip Hybridization, Wash, and Stain kits were used for PhyloChip analysis. Briefly, fragmented 16S amplicons and non-16S quantitative amplicon reference controls were labeled with biotin in 40 $\mu$L reactions containing: 8 $\mu$L of 5X TDF buffer, 40 units of TDF, 3.32 nanomoles of GeneChip labeling reagent. After incubating at 37 °C for 60 min, 2 $\mu$L of 0.5 M EDTA was added to terminate the reaction. Labeled DNA was combined with 65 $\mu$L of 2X MES hybridization buffer, 20.4 $\mu$L of DMSO, 2 $\mu$L of Affymetrix control oligo B2, and 0.4 $\mu$L nuclease free water. Each reaction mixture was injected into the hybridization chamber of an array cartridge and incubated for 16 h in an Affymetrix hybridization oven at 48 °C and 60 rpm. Hybridization solution was removed and the microarrays were stained and scanned according to the manufacturers instructions.

Analysis procedures for fluorescent image files are described in detail in the Supporting Information of Hazen et al.[17] Briefly, each individual array feature occupied approximately 8 × 8 pixels in the image file corresponding to a single probe 25mer on the surface. Probe intensities were background-subtracted and scaled to quantitative standards (non-16S rRNA gene spike-ins) as previously described.[19] Presence/absence calling of each microbial taxon (operational taxonomic unit − OTU) was based on positive hybridization of multiple probes that correspond to an OTU (average of 37 probes/OTU). Differences in mean hybridization intensity (fluorescence) of an OTU probe set among different PhyloChips reflected differences in the relative abundance of the OTU.[19] The PhyloChip data used in this study are available for download at http://greengenes.lbl.gov/Download/Microarray_Data/.

PhyloChip results are output as lists of detected OTUs and their hybridization scores with associated taxonomic information and references to represented sequences in public 16S rRNA gene repositories (greengenes.lbl.gov). Hybridization results were reduced to a community profile from each PhyloChip assay to a format useful for multivariate statistics consisting of log transformed hybridization intensity values for all detected OTUs. Interprofile dissimilarity was calculated with the Bray−Curtis metric, and the resulting distance matrix was analyzed with hierarchical cluster analysis and nonmetric multidimensional scaling (NMDS) ordination using the Primer v.6.1.13 statistical package. Analysis of Similarity (ANOSIM) was used to test the significance of differences in community composition among sample groups.

**Determination of Source Identifier Taxa.** Source identifier taxa were defined as individual OTUs that were detected in a single source type but never detected in any samples from other sources. The criteria for identifier bacteria selection were as follows: Identifier bacteria for animal sources needed to be unique to a single animal type and present in at least three distinct populations. Identifier bacteria for human sources needed to be present in at least 7 of 8 samples. More stringent requirements were selected for human sources because a greater number of bacterial taxa were detected in human sources than animal sources likely due to over-representation of human-specific bacteria in the 16S rRNA gene database from human microbiome sequencing projects.

**Source Identification in Environmental Water Samples.** Application of PhyloChip for source identification in marine waters was tested in two field scenarios with known sources of human and avian contamination. The first was a monitoring study of a 765 000 gallon spill that occurred in Richardson Bay, an arm of San Francisco Bay, off the coast of Sausalito, CA, in February 2009. The PhyloChip was used to determine which bacterial taxa significantly increased in relative abundance in samples with high FIB counts and whether these enriched bacteria included the expected human identifier bacteria described above. To determine which taxa were specifically associated with high FIB counts, water samples with FIB concentrations that exceeded any 30-day geometric mean concentration limit were compared to samples that fell below all FIB concentration limits. Baseline microbial communities were defined by mean abundance of taxa in low FIB samples. Taxa whose relative abundance significantly exceeded baseline (>mean $+2\sigma$) were determined in high FIB samples. The presence of source identifier bacteria in this enriched subset was used to determine the association between fecal sources and FIB exceedances. Results are reported as the percent of expected identifier taxa that were detected in each sample. The expected number of identifier taxa for a given source was defined as the average number of source-specific identifier taxa detected in individual populations of that source. A positive signal for source detection was define as >20% enrichment of expected identifier taxa in a sample. Significant association between the detection of each source type and high FIB exceedances (Enterococcus above regulatory limit) was tested with contingency analysis (JMP 7.0.1).

The second field test occurred at Campbell Cove in Bodega Bay, California, a recreational beach that frequently exceeds FIB water quality limits. Contamination at this beach is not from human sources and is likely due to gull feces.[20] We collected weekly monitoring samples over a 1-year period at this beach in conjunction with the county as part of the California Clean Beaches Initiative. Samples were split for both routine FIB testing and filtration for subsequent PhyloChip analysis. The analysis approach was similar to the sewage spill monitoring described above in which low FIB samples were analyzed at each site to establish the mean abundance and variance of each OTU under baseline (nonexceedance) conditions, and association between the enrichment of source identifier taxa and high FIB counts was tested with contingency analysis.

## RESULTS AND DISCUSSION

**Fecal Source Microbial Communities.** A total of 20 368 bacterial OTUs were detected across all fecal samples. Samples clustered by source type indicating fecal bacterial communities of the same type of source animal were more similar to each other than to those of other sources (Figure 1). The deepest branching clusters separated all mammalian sources from avian sources indicating that microbial community composition is a distinctive characteristic of these two classes of vertebrates. Within the mammals, samples clustered into three distinct


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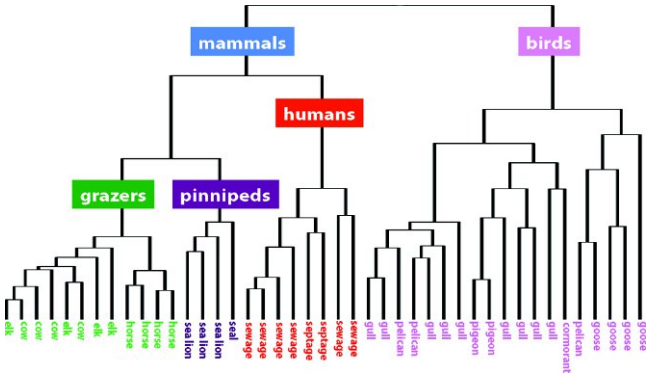


**Figure 1.** Cluster analysis dendrogram of 16S rRNA gene composition showing similarity among microbial communities in fecal sources. Each sample represents a distinct animal population or sewage source and is a composite of individual fecal samples from the population.

groups comprised of grazing animals (cows, elk, horses), human wastes, and pinnipeds (Figure 1). Grazing mammals were further partitioned into two clusters comprising ruminants (cow, elk) and horses. Geese formed a distinct cluster within the birds. There was no obvious clustering among the other bird types (gulls, pelicans, pigeons, cormorants), and clustering patterns among these birds were not related to geography. All sources contained taxonomic groups that encompass *E. coli* and Enterococcus that are used as regulatory fecal indicators.

Clostridia dominated the taxonomic (OTU) richness of the fecal bacteria in mammalian fecal sources (Figure 2). The

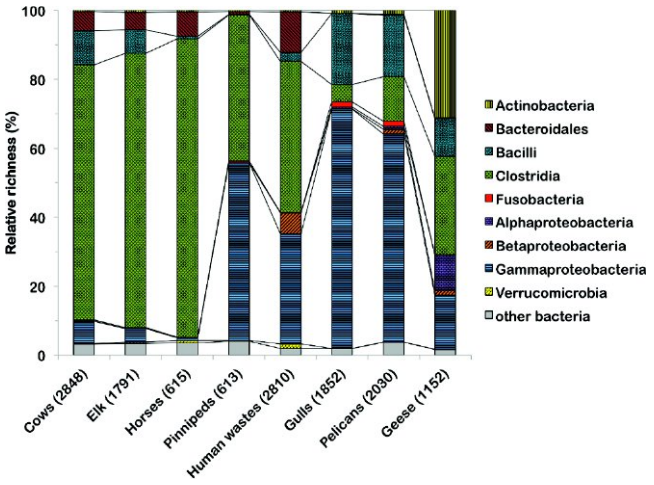


**Figure 2.** Composition of OTUs detected in each fecal source. OTUs are shown that were detected in at least half of the samples for each source animal. Total number of OTUs is shown in parentheses and lists with taxonomic descriptions found in .

remainder of taxonomic diversity in mammals was comprised of mainly Bacteroidales, Gammaproteobacteria, and Bacilli. These results are consistent with previous surveys of other mammalian gut microbial communities.[12,13,21] In contrast to mammals, avian feces contained far less taxa in the Clostridia and Bacteroidales and instead were dominated by Gammaproteobacteria and Bacilli (Figure 2).

Analysis of avian fecal samples revealed that seabirds and pigeons had similar composition of bacteria and were dominated by Gammaproteobacteria (mostly Enterobacteria) and Bacilli (mostly Lactobacillales) (Figure 2). Fecal communities in these birds were also characterized by the presence of Fusobacteriaceae OTUs that were generally absent from mammalian and geese communities. Taxonomic composition in geese was distinct from other types of birds and contained greater numbers of taxa in the Actinobacteria, Alphaproteobacteria, and Clostridia. Geese differ from other birds in this study because of their unique diet and digestive system. Geese consume high-fiber feed, such as grass, and contain a well-developed cecum that facilitates their breakdown in the large intestine.[22] This more rumenlike digestive system facilitates the activity of fermentative Clostridia.[23] Canada geese often forage for plants and insects in the soil, and consequently ingest bacteria that are resident in soil or on plant surfaces, which may explain the prominence of Actinobacteria and Alphaproteobacteria in their feces. Despite these differences between geese and other birds, geese bacterial communities were more similar to other types of birds than they were to any mammalian fecal sources (Figure 1).

Within the mammals, a variety of Clostridia, Bacilli, and Bacteroidetes dominated taxonomic (OTU) richness of grazing mammals (Figure 2). These bacteria are known to digest cellulose and other plant polysaccharides in the ruminant gut. Clostridia, Gammaproteobacteria (mostly coliforms), and Bacteroidetes dominated taxonomic richness of human wastes (Figure 2). Human wastes were further distinguished by the presence of several Betaproteobacteria and Verrucomicrobia. Clostridia and Gammaproteobacteria dominated the taxonomic richness of pinnipeds.

On the basis of similarities in community composition (Figure 1), the data were partitioned into four major groups for identifier bacteria analysis: human wastes, birds, grazers, and pinnipeds. Source identifier taxa were defined as individual OTUs that were detected in a single source type but never detected in any samples from other sources. The number of OTUs that met criteria for selection as source identifier taxa was 304 for birds, 213 for grazers, 0 for pinnipeds, and 541 for human wastes (Figure 3).

Human identifier bacteria were primarily Bacteroidaceae and Clostridiales OTUs that matched known human fecal bacteria 16S rRNA gene sequences (Figure 3). Human Clostridiales OTUs were mainly found in the families Eubacterium, Faecalibacterium and Ruminococcus. Verrucomicrobia in the family Akkermansia were also indicative of human wastes, and are known to be mucin degraders in the human GI tract.

Bird identifier taxa included several different groups of Bacilli, mainly Lactobacillales, and Staphylococcaceae (Figure 3). In addition, bird identifier bacteria included one unclassified family in the Clostridiales, as well as Enterobacteriaceae and Fusobacteriaceae. Bacteroidetes are a minor component in avian microbial communities.[24] We found several Lactobacilli OTUs that are included in the same subfamily as *Catellicoccus marimammalium* and that are closely related to *Enterococcaceae*. Lu et al.[24] found gull feces were dominated by Bacilli (37% sequences), most of which were closely related to *Catellicoccus marimammalium*.

Grazer identifier taxa included a variety of Clostridia, many of which are known from cattle rumen, consisting of Clostridium, Ruminococcus, unclassified Clostridiales, RF6, RF30, RF39, and SHA-32 (Figure 3). In addition, grazer identifiers included several Bacilli taxa found in the Planococcaceae, and Bacteroidales taxa that were distinct from those found in human wastes (Figure 3).


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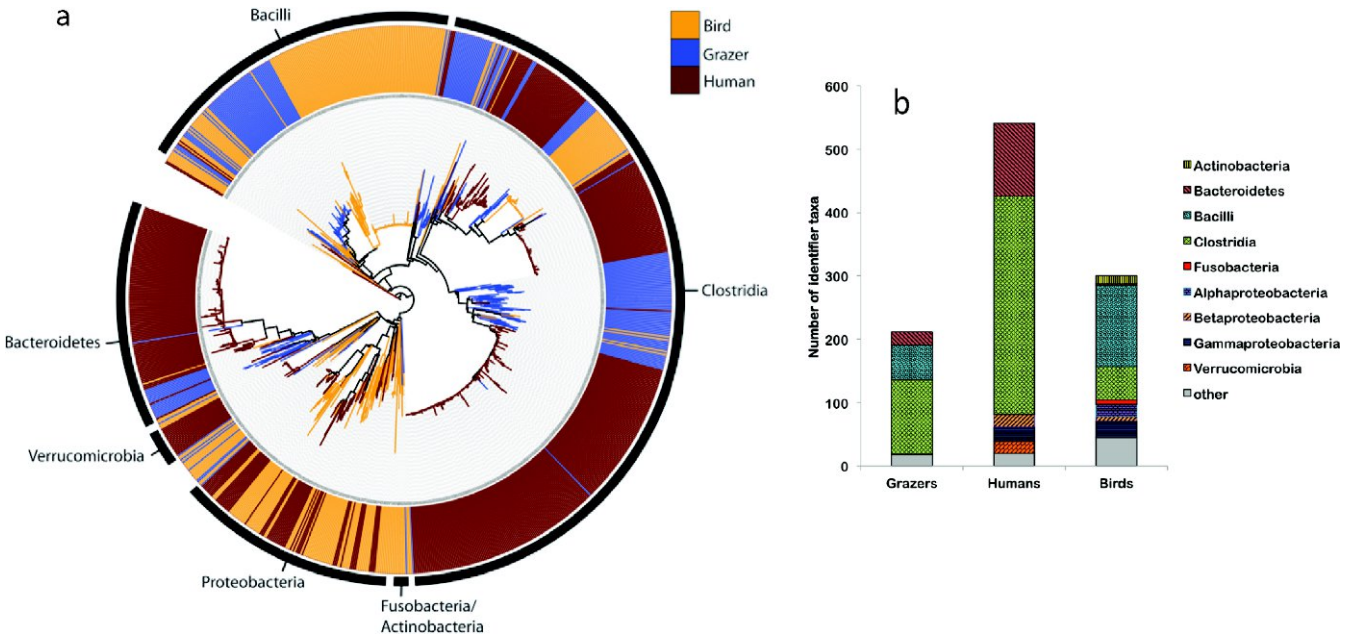


**Figure 3.** Phylogenetic tree (a) and taxonomic composition (b) of source identifier OTUs for human wastes, birds, and grazers. The phylogenetic tree was constructed from using full-length 16S rRNA gene sequences of representative taxa in each OTU using the approximately maximum likelihood algorithm implemented in FastTree,[26] and the tree was displayed using the Interactive Tree of Life tool.[27] The outer bar of the phylogenetic tree (a) represents major bacterial phyla, the next bar is proportional to the number of OTUs for each of the 1053 source identifier OTUs, and the inner circle represents the phylogenetic placement for each OTU with branch lengths proportional to change in 16S rRNA gene sequence. Detailed taxonomic description and reference sequence information for each source identifier OTU is provided in Tables 1−3 of the Supporting Information.

Pinniped microbial communities were distinct from other fecal sources, but all OTUs found in at least three pinniped samples were also found in at least one other human or animal sample. For this reason, this study did not generate identifier taxa for pinnipeds due to the potential for cross-reactivity.

**Source identification field tests.** Application of PhyloChip for source identification in marine waters was tested in two field scenarios with defined sources of human and avian contamination. The first test looked investigated microbial communities in Richardson Bay waters adjacent to a large sewage spill. Out of 26 water samples collected during the spill, two exceeded the 1-day Enterococcus concentration limit for marine water (104 MPN/mL) and an additional five exceeded the 30-day geometric mean limit (35 MPN/mL). These exceedance samples had significantly different microbial community compositions than samples that fell below FIB limits (Figure 4). All samples that were above Enterococcus limits contained most (78−96%) of the expected fecal identifier bacteria for human fecal wastes (Figure 5). In contrast, there was little enrichment of bird or grazer identifier bacteria (0−10%) in samples with high Enterococcus counts. Contingency analysis showed greater than expected numbers of samples with enrichment in human identifier taxa (>20% identifiers enriched) in high enterococcus samples ($P < 0.001$) but insignificant enrichment of grazer or bird identifier taxa ($P > 0.05$). The results show the PhyloChip analysis is sensitive to human fecal signal in marine waters.

The second field test was conducted along the beach of Campbell Cove in Bodega Bay, a site where a previous source tracking investigation found no evidence of human fecal contamination.[20] We analyzed a total of nine samples with high enterococcus counts (>35 MPN/mL) and eleven nonexceedance samples collected over the course of one year. An average

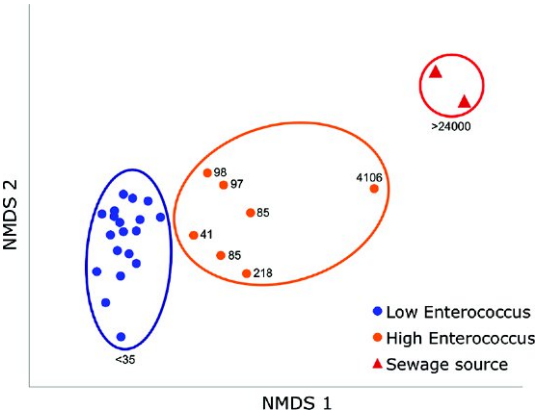


**Figure 4.** Microbial community analysis of 28 water and two sewage samples collected during the 2009 sewage spill from the Sausalito Marin-City Sanitary District treatment plant. Ordination was conducted using nonmetric multidimensional scaling with the Bray−Curtis distance metric. Numerical values are Enterococcus counts (MPN/mL) of individual samples. Microbial community composition in water samples that exceeded regulatory limits for Enterococcus (>35 MPN/mL) was significantly different than in samples with low Enterococcus counts (ANOSIM, $P < 0.05$).

of 1093 out of 6046 detected OTUs were significantly enriched over baseline relative abundances in high enterococcus samples. Several samples with high enterococcus had significant enrichment of identifier bacteria associated with bird feces (Figure 6). Contingency analysis showed greater than expected numbers of samples with enrichment in bird identifier taxa (>20% identifiers enriched) in high enterococcus samples ($P = 0.033$). Neither human nor grazer fecal identifiers were significantly enriched when enterococcus counts were high ($P$


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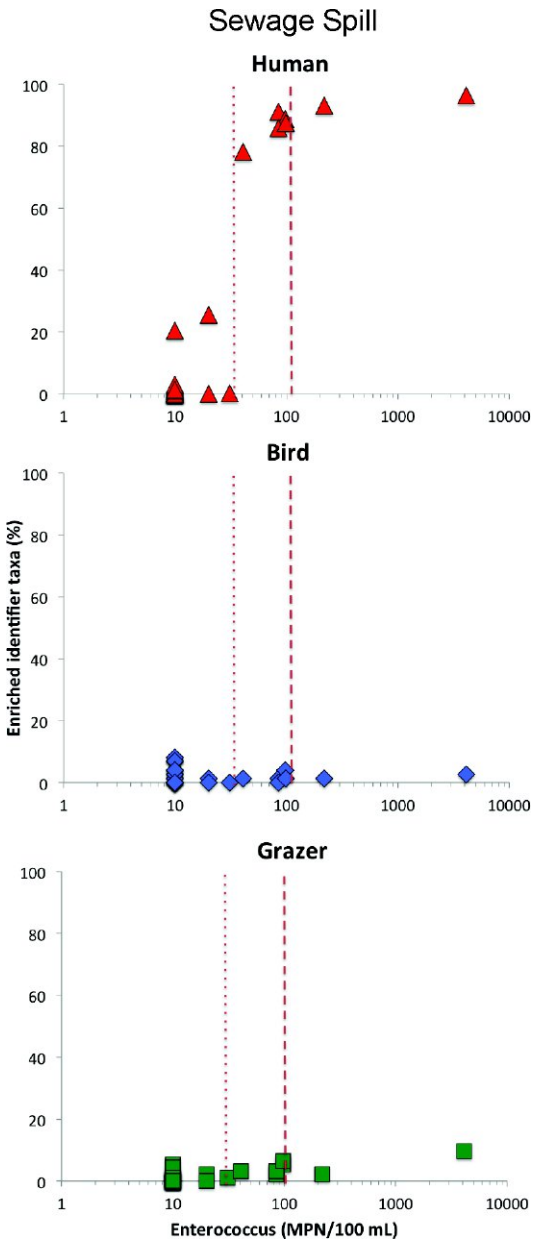


**Figure 5.** Source identification in San Francisco Bay water samples collected during sewage spill monitoring ($N$ = 28). Results from Enterococcus FIB tests are plotted against the percent of source-identifier taxa that were significantly enriched above background (low FIB) conditions. High and low dashed lines show the single-day Enterococcus concentration limit and 30-day geometric mean limits, respectively. Higher than expected numbers of high enterococcus samples were enriched in human identifier taxa ($P$ < 0.001) but not bird or grazer identifier taxa ($P$ > 0.05).

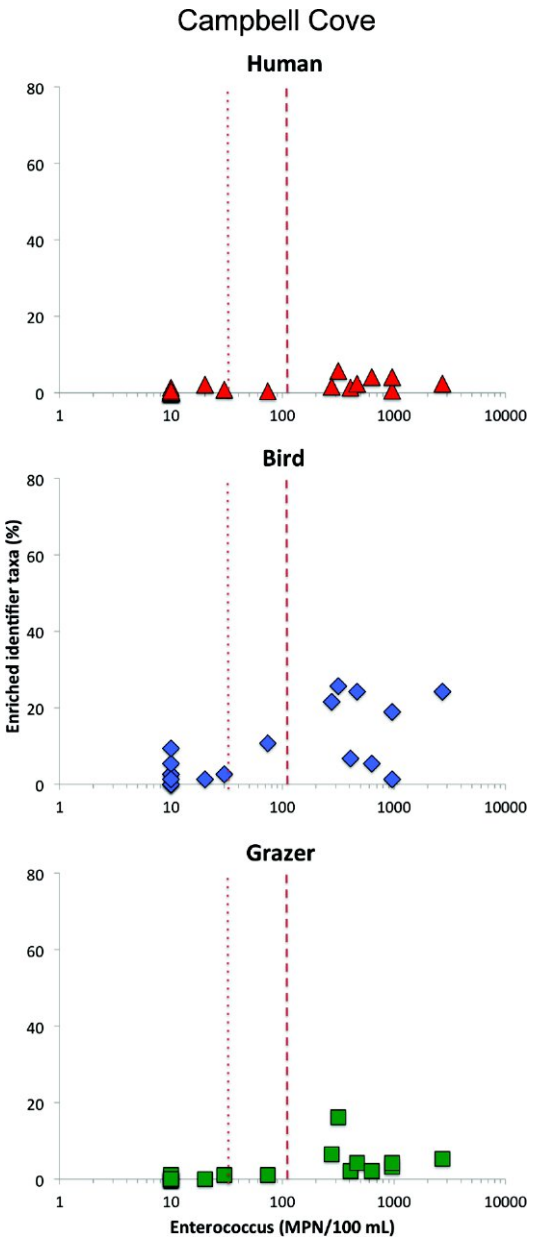


**Figure 6.** Source identification at Campbell Cove, Bodega Bay ($N$ = 19). Results from Enterococcus FIB tests are plotted against the percent of source-identifier taxa that were significantly enriched above background (low FIB) conditions. High and low dashed lines show the single-day Enterococcus concentration limit and 30-day geometric mean limits, respectively. Higher than expected numbers of high enterococcus samples were enriched in bird identifier taxa ($P$ = 0.033) but not human or grazer identifier taxa ($P$ > 0.05).

> 0.05). From these results, we conclude that birds and not human or grazer fecal inputs were associated with high enterococcus counts at Campbell Cove. These results are consistent with the findings of the previous source tracking investigation at this site that used *E. coli* ribotyping to determine gulls and not humans were a fecal source.[20] We also found four high enterococcus samples at Campbell Cove with negligible enrichment in any source identifier taxa indicating additional sources of FIB that were not tested. Further investigation of the phylogenetic inventory of all bacterial taxa from this site, not just fecal identifiers, could help reveal additional fecal or environmental sources of FIB.

The phylogenetic microarray approach to source identification uses simultaneous occurrence of many diverse taxa to determine to detect fecal sources. Future work needs to address how fate and transport influences detection rates of these different taxa once they enter the environment. In the sewage spill example presented in this study, almost all human source identifier taxa were detected in water samples with high FIB. These fecal bacteria were input from a large release of sewage directly into the tested waters, and subject to little aging and decay. This situation is in contrast to the nonpoint source


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situation at Campbell Cove where high FIB samples contained around 20% of the identifier taxa from a known fecal source (gull feces). Inputs of fecal bacteria at Campbell Cove were not necessarily direct into receiving waters but also from shoreline runoff and leaching through beach sands and sediments.[20] As a result, fecal microbial communities were subject to more modification before entering receiving waters compared to direct inputs by the sewage spill. Application of the community identifier approach to source tracking will benefit from adjusting the analysis based on the expected persistence of different taxa.

There are potential advantages and limitations to using a phylogenetic microarray for source identification. An advantage is sensitive detection of taxa with low abundance in the community.[19] As fecal sources are diluted in receiving waters, taxa that are critical for source identification will decrease in relative abundance as they mix with the complex microbial background of the environment. The microarray probes for target sequences from the entire sample of PCR amplicons, and this amplicon pool consists of many billions of 16S rRNA gene sequences. Hybridization the entire pool amplified sequences may offer an advantage over pyrosequencing or other types of next generation sequencing because these methods randomly sequence a relatively small fraction of the amplified PCR product and consequently are not reliable for detecting less abundant members of the community that may be critical for source identification.[25]

A limitation in applying phylogenetic microarrays to MST may be the insufficient number of probes for sources that are underrepresented in 16S rRNA gene databases. For example, few studies have surveyed microbial diversity in pinnipeds, and as a likely consequence we found no unique taxa in pinnipeds using the PhyloChip. More thorough assessments of sequence composition in some source types will be needed to find additional host-specific targets. In addition, cost and complexity can be barriers to widespread adaptation of this technology in its current form. Measuring the full range of 16S rRNA gene sequences in the microbial community is not necessary, however, and a down-selected microarray that targets only the subset of microorganisms that is useful for source identification would simplify analysis and reduce cost.

The results of this study show that 16S rRNA gene composition of the bacterial community can be used to discriminate sources of fecal contamination. Differences in the diversity among fecal sources reveal hundreds of unique taxa that are specific to human, bird and grazer feces. Several different phylogenetic lineages, most of which are not considered in existing MST assays, differentiate these sources and are mainly found in the Clostridia, Bacilli, and Bacteroidetes. Comprehensive interrogation of microbial communities for these diverse identifier taxa has great potential to improve the reliability of source detection in the environment. Phylogenetic microarrays are an effective tool for rapidly measuring the full assortment of microbial taxa that distinguish fecal contaminants and deserve serious consideration for source tracking.

## ■ ASSOCIATED CONTENT

### Supporting Information

List of fecal types and source locations, list of human waste identifier taxa, list of bird identifer taxa, and list of grazer identifier taxa. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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Appendix 4:

Peer Reviewed Article: “Characterization of Coastal Urban Watershed Bacterial Communities Leads to Alternative Community-Based Indicators.”

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PLoS one

# Characterization of Coastal Urban Watershed Bacterial Communities Leads to Alternative Community-Based Indicators

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## Abstract

***Background:*** Microbial communities in aquatic environments are spatially and temporally dynamic due to environmental fluctuations and varied external input sources. A large percentage of the urban watersheds in the United States are affected by fecal pollution, including human pathogens, thus warranting comprehensive monitoring.

***Methodology/Principal Findings:*** Using a high-density microarray (PhyloChip), we examined water column bacterial community DNA extracted from two connecting urban watersheds, elucidating variable and stable bacterial subpopulations over a 3-day period and community composition profiles that were distinct to fecal and non-fecal sources. Two approaches were used for indication of fecal influence. The first approach utilized similarity of 503 operational taxonomic units (OTUs) common to all fecal samples analyzed in this study with the watershed samples as an index of fecal pollution. A majority of the 503 OTUs were found in the phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. The second approach incorporated relative richness of 4 bacterial classes (*Bacilli*, *Bacteroidetes*, *Clostridia* and $\alpha$-*proteobacteria*) found to have the highest variance in fecal and non-fecal samples. The ratio of these 4 classes (BBC:A) from the watershed samples demonstrated a trend where bacterial communities from gut and sewage sources had higher ratios than from sources not impacted by fecal material. This trend was also observed in the 124 bacterial communities from previously published and unpublished sequencing or PhyloChip- analyzed studies.

***Conclusions/Significance:*** This study provided a detailed characterization of bacterial community variability during dry weather across a 3-day period in two urban watersheds. The comparative analysis of watershed community composition resulted in alternative community-based indicators that could be useful for assessing ecosystem health.

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## Introduction

Given that water sustains life, it is not surprising that a large percentage of the world's population lives near coastal regions [1,2]. Coastal urban watersheds in the United States offer aesthetics and recreational value, serve as catchments for storm runoff, establish biological corridors for movements of wildlife, and provide buffers between developed areas and downstream waterways. As human populations increase, so does urbanization and lasting anthropogenic affects on creeks and coastal ecosystems [3]. According to a USEPA report (2007), 45% of streams and rivers, and 32% of bays and estuaries are impaired in the United States. Sources of impairment include pathogens and sewage discharges [4]. The presence of bacterial pollutants warrants comprehensive bacteriological characterization of these water bodies in order for us to understand their fate and transport in the environment.

Since pathogens often come from fecal sources, regulatory agencies require monitoring fecal indicator bacteria (FIB) for water quality assessments. Culture-dependent assays such as total coliform, fecal coliform and enterococci, and culture-independent assays such as quantitative PCR (qPCR) for *Bacteroides* and *Bifidobacterium* spp. [5] have been used as proxies for fecal pollution. However, enumeration of these indicator organisms often does not accurately represent the health of the ecosystem or associated risk [6] as these indicators are ubiquitous, persistent, regenerative [7,8] and have low correlations with pathogen survival [9,10] in the environment. Reliance upon single, even

source-specific, markers of fecal pollution can be ineffective if they are labile or persistent relative to pathogens. The use of multiple indicators for tracking fecal contamination could circumvent the problem of single marker absence or presence and strengthen overall diagnoses of microbiological water quality [6,7,8,9,11].

With the advent of high throughput culture-independent characterization of microbial communities, such as microarray and sequencing approaches [12,13,14,15,16], detailed studies of bacterial community fluctuations due to physical, chemical and biological influences are now feasible. One such phylogenetic microarray, the PhyloChip, targets much of the known diversity within Bacteria and Archaea, and has been employed in a number of complex environments and conditions [17,18,19,20,21,22,23,24,25]. The current version (G2) of the PhyloChip provides the capability of identifying up to 8,741 Bacterial and Archaeal OTUs simultaneously [17], and allows for relative quantification of individual OTUs over a wide dynamic range [18,26]. The highly parallel and reproducible nature of this array allows tracking community dynamics over time and treatment.

Bacterial communities in urban watersheds are sensitive to environmental perturbations and could provide information on impacts of fecal influence and overall ecosystem health. It is important to monitor the conditions of these watersheds because they are intricately tied in with downstream waterways, which could have public health risk and economic implications. Previous studies monitoring FIB most probable numbers (MPN) in urban creeks have found high temporal variability even during dry weather [27,28,29]. In Santa Barbara, California, exfiltration from sewer lines into the storm drain systems has been suspected to cause the observed high densities of FIB and human-specific *Bacteroides* markers (HBM) in urban watersheds that discharge into a recreational beach [29]. Here we analyze whole bacterial communities from the same Sercu et al. [29] samples in order to gain insights regarding the temporal and spatial dynamics of urban watershed bacterial community composition relevant to fecal pollution. Amplified 16S rRNA gene sequences from creek (including storm drains), lagoon and ocean sites in the Lower Mission Creek and Laguna watersheds in Santa Barbara, CA, along with 3 samples of fecal origin, were hybridized onto the PhyloChip for a complete microbial community analysis. Characterization of the whole bacterial community is crucial for understanding fluctuations of various bacterial groups, and could lead to more robust health risk indication by integrating data from multiple bacteria taxa. This work represents the first application of a comprehensive phylogenetic array for the purpose of characterizing urban watershed bacterial communities. Findings from this work suggest that such an approach could be useful for complementing multiple individual tests that are now typically employed to diagnose microbiological water quality related to public health.

## Results

### Resolving community differences by habitats

Samples were categorized into 4 habitat types: fecal, ocean, lagoon, and creek (Figure 1). Comparisons of Bray-Curtis distances of the communities, using Multi-Response Permutation Procedure (MRPP) [30], indicated significant differences between the samples from the different habitat types. Non-metric multidimensional scaling (NMDS) ordination illustrated that the bacterial communities were separated by habitat types for most of the samples, except for M2a and M2b (Figure 2). Salinity measurements at one of the lagoon sites (M2) were low, at ~1 ppt, on days 1 (M2a) and 2 (M2b) (Table S1). On day 3 (M2c), the salinity increased to 5.3 ppt, and a corresponding community composition shift was observed (Figure 2). The bacterial communities of M2a and M2b were more similar to creek samples with low salinity and M2c was more similar to the M4a and M4b lagoon samples, which had higher salinity measurements of 7.3–9.5 ppt. Lagoon sample M4c had lower salinity measurements and the community was more similar to creek samples than to M4a and M4b.

Distributions of detected operational taxonomic units (OTUs) at the class level were compared among all habitat types, shown as relative richness (Figure 3A). The relative richness was normalized to the total number of OTUs detected in all of the samples from the same habitat type. We focused on classes that exhibited high variability of relative richness across the 4 habitats. The top 10 classes with the highest standard deviations were (in descending order): *Clostridia*, *α-proteobacteria*, *Bacilli*, *γ-proteobacteria*, *β-proteobacteria*, *Actinobacteria*, *Flavobacteria*, *Bacteroidetes*, *Cyanobacteria* and *ε-proteobacteria*. Of those classes, only *Clostridia*, *Bacilli*, and *Bacteroidetes* had higher relative richness in fecal samples than in creek, lagoon and ocean samples (Figure 3B). Only *α-proteobacteria* had lower richness in fecal samples than in creek, lagoon, and ocean samples (Figure 3C). The characteristics and potential of these 4 classes as indicators of fecal influence will be discussed further.

### Fecal sample-associated OTUs

In order to define bacteria that were common to all 3 fecal samples used in this study, a set of 503 OTUs, found in all fecal samples but not ubiquitous in the 27 watershed samples, were characterized and defined as fecal sample-associated OTUs (FSAO). The FSAO subpopulation consisted of 43% *Firmicutes* (out of the 503 OTUs), 28% *Proteobacteria*, 9% *Bacteroidetes* and 5% *Actinobacteria* (Figure S1). Of the *Firmicutes* (218 OTUs), 56% were

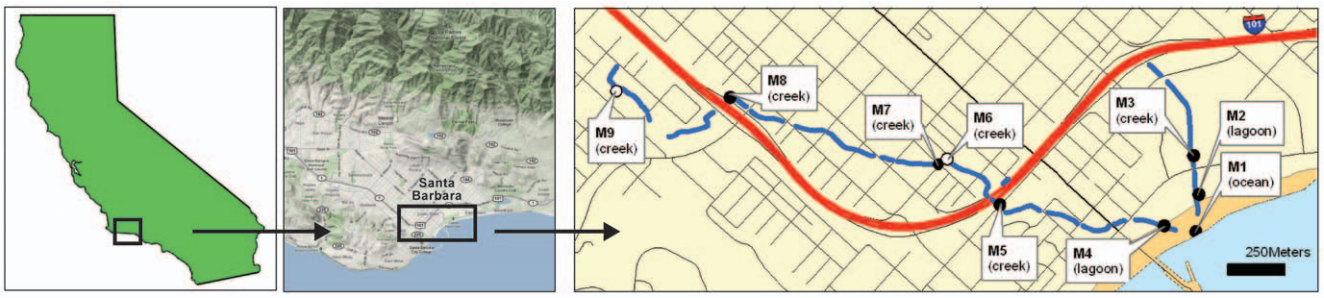


**Figure 1. Sampling sites along Mission (M4–M9) and Laguna Channel (M2 and M3) watersheds.** Samples were delineated into different habitat types: creek (M3, M5–M9, where M6 and M9 were from drains), lagoon (M2 and M4), and ocean (M1). Open circles (○) represent storm drains, and filled circles (●) represent creek, lagoon or ocean sites.
doi:10.1371/journal.pone.0011285.g001

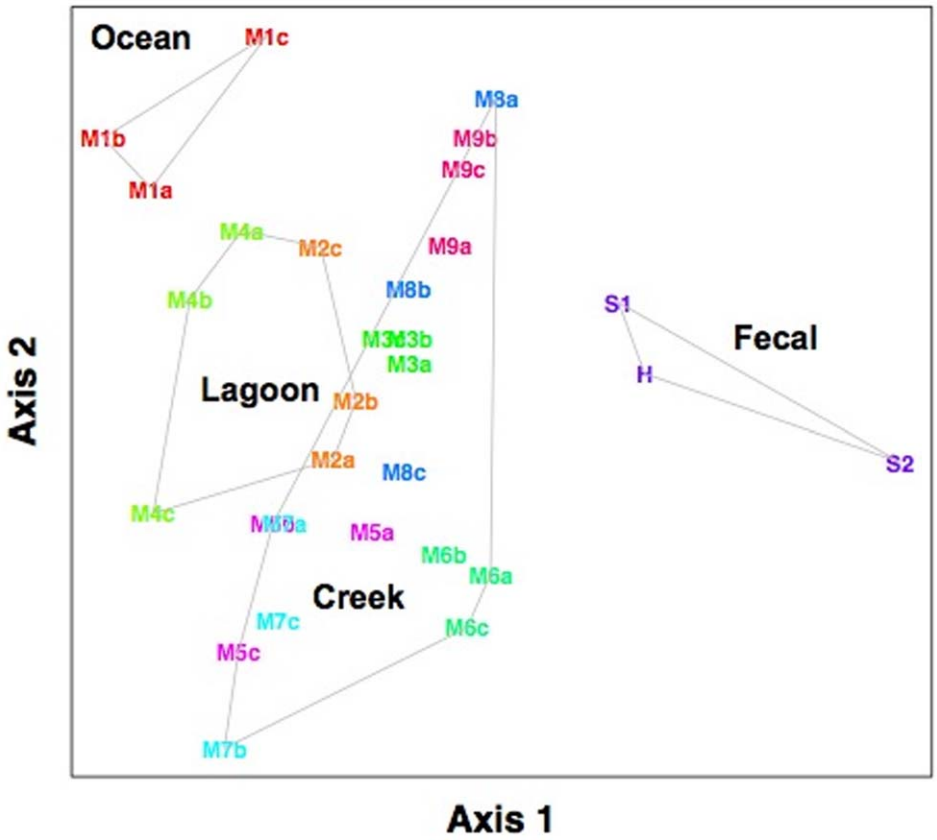


**Figure 2. NMDS plot of PhyloChip community distances.** Bray-Curtis metric was used, and a stress of 8.14 was obtained. Each site is represented by a different color. The grey lines delineate grouping of creek, lagoon, ocean and fecal samples.
doi:10.1371/journal.pone.0011285.g002

from the order *Clostridiales* including the families *Lachnospiraceae*, *Peptostreptococcaceae*, *Peptococcaceae*, *Acidaminococcaceae* and *Clostridiaceae*; 17% were from the order *Bacillales* including *Bacillaceae*, *Halobacillaceae*, and *Staphylococcaceae*; and 17% were from *Lactobacillales* which included the families of *Lactobacillaceae*, *Enterococcaceae* and *Streptococcaceae*. In the *Proteobacteria* phylum (141 OTUs), 30% were from *Enterobacteriales* including *Enterobacteriaceae*; 7% were from *Alteromonadales* including *Alteromonadaceae*, and *Shewanellaceae*; 8% of the OTUs were from the order *Burkholderiales* including *Burkholderiaceae*, *Comamonadaceae*, *Alcaligenaceae*, *Oxalobacteraceae*, and *Ralstoniaceae*. The counts of FSAO for each of the three days are shown in Figure S2. The FSAO counts were highest at M9, M8, M6, M3 and M2 and lowest at M4 and M1. The 3-day average FSAO counts for sites M9, M6, M3, and M2 were significantly different (*t*-test, p-value$<$0.0001) from counts of M4, and M1.

## Variable and stable subpopulations

PhyloChip analysis of subpopulations from each site for which the fluorescence intensities fluctuated the most (variable) and the least (stable) were examined over the course of the three-day sampling period. These variable and stable subpopulations consisted of OTUs from the top and bottom deciles after sorting based on variance of fluorescence intensity over the 3 days. A similarity metric, from the UniFrac [31] distance measure, was illustrated with boxplots for comparison of the median, upper and lower quartiles. Variable subpopulations of M6 were the most similar to the FSAO composition in comparison to the other sites (Figure 4A). Sites M9 and M3 were the second and third most similar to the FSAO. However, the similarity to FSAO for site M9 was not significantly different from that of M6 or M3. A pattern of decreasing similarity from M9, M6 and M3 to immediate downstream sites was illustrated. The majority of FSAO detected in the variable subpopulations was in the orders of *Enterobacteriales* (39 out of 58 FSAO detected in the variable subpopulation) for M6, *Campylobacterales* (6 out of 44) for M9, and *Flavobacteriales* (4 out of 31) for M3. The M9 stable subpopulation was the most similar to the FSAO, and was significantly different from the similarity to FSAO of all other sites (Figure 4B). Many of the FSAO in the M9 stable subpopulation were in the order of *Bacillales* (17 out of 47).

## Ratio of *Bacilli*, *Bacteroidetes* and *Clostridia* to *α-proteobacteria*

Four bacterial classes, which exhibited highly fluctuating relative richness across the habitat types, were further explored as representatives of the fecal bacterial community (Figure 3A). The combined percentage of *Bacilli*, *Bacteroidetes and Clostridia* relative richness was 28.5% of total detected in the fecal samples, whereas in creek, lagoon and ocean they were less than 13.5% (Figure 3B). Almost 15% of the relative richness in creek water, lagoon and ocean samples were *α-proteobacteria*, while the percentage of *α-proteobacteria* found in fecal samples was 7% (Figure 3C). The relative richness ratio of *Bacilli*, *Bacteroidetes and Clostridia* to *α-proteobacteria* (BBC:A) for fecal samples was more than 4-fold higher than the ratios of the other habitat types (Figure 3D). The BBC:A ratio was calculated for each of the samples from the different sites (Figure 5). Site M6 exhibited the highest BBC:A, and sites M1 and M4 had low BBC:A ratios compared to the rest of the sites.

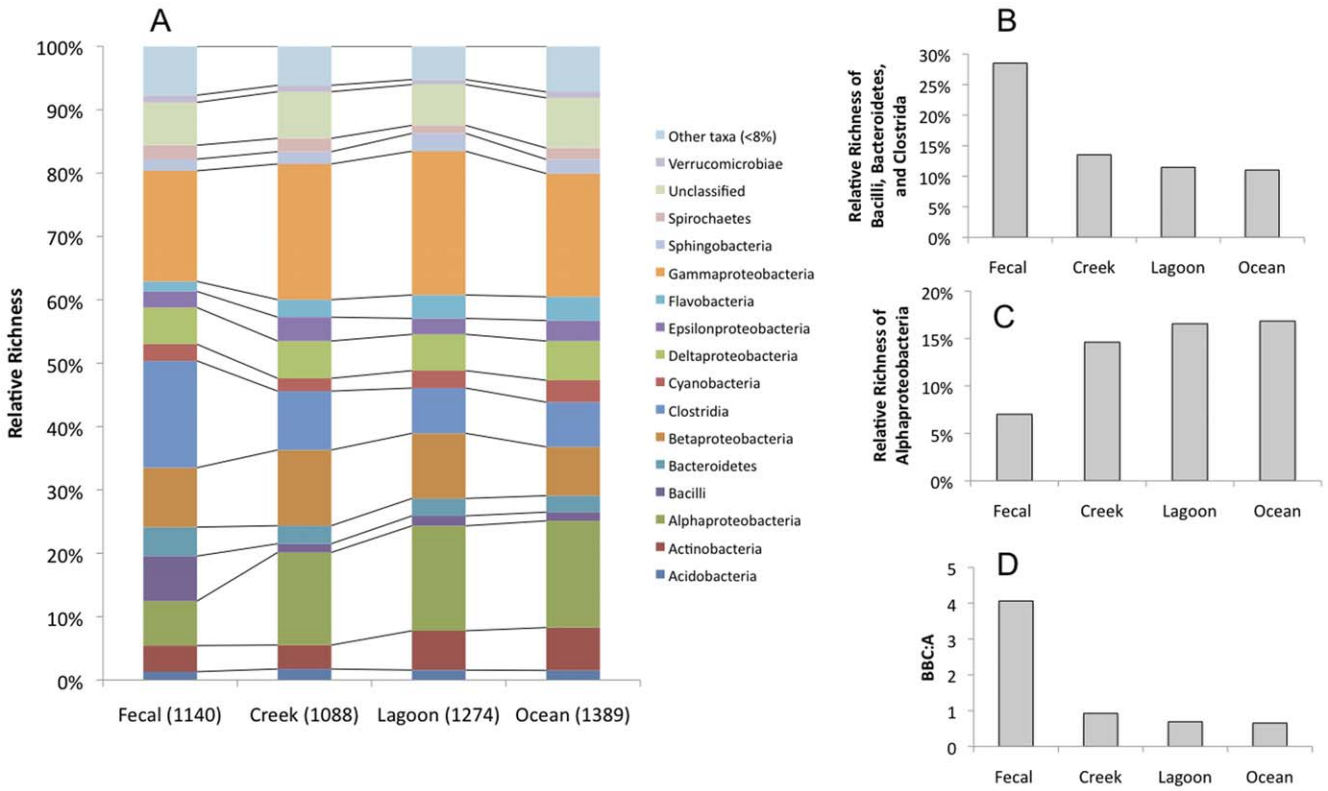


**Figure 3. Bacterial community composition comparison across fecal, lagoon, creek and ocean samples.** (A) Distribution of relative richness at the class level. Number of OTUs in each sample types were divided by the total count for each sample type as indicated in parentheses on the x-axis. (B) Relative richness of *Bacilli*, *Bacteroidetes* and *Clostridia* detected. (C) Relative richness of α-pro*teobacteria* detected. (D) *Bacillus*, *Bacteroidetes*, *Clostridia* to α*-proteobacteria* ratios (BBC:A).
doi:10.1371/journal.pone.0011285.g003

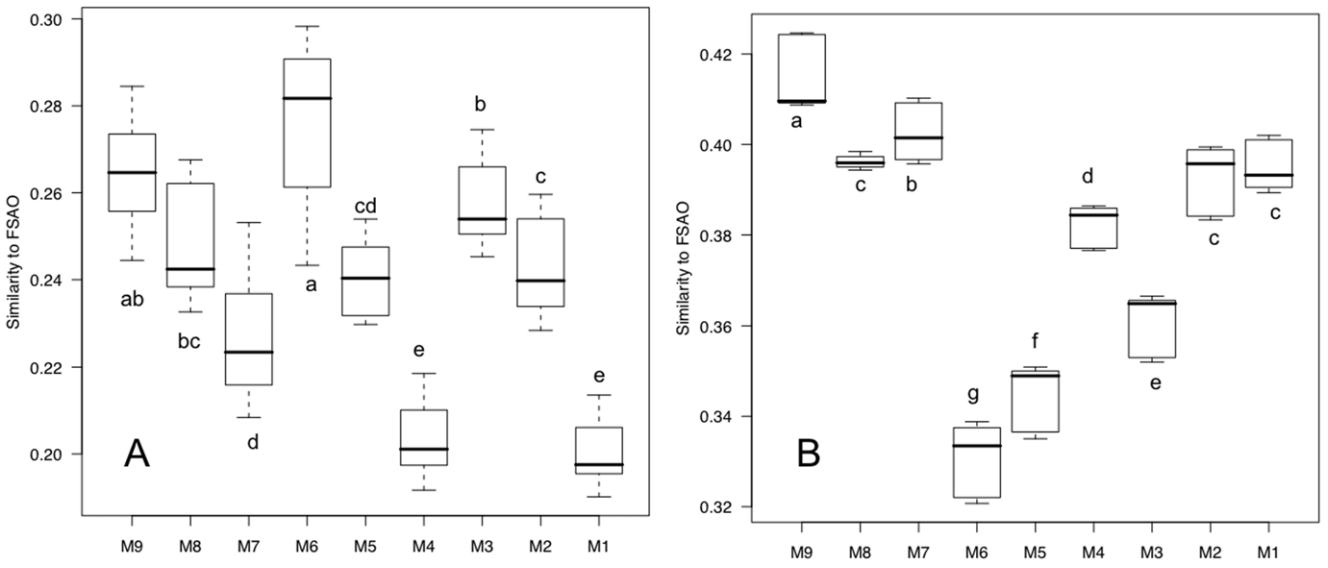


**Figure 4. Boxplots of UniFrac similarity metrics between water and fecal-sample-associated OTUs (FSAO).** (A) Variable subpopulations. (B) Stable populations. Each box represents similarity metrics from all 3 days at each site. Boxplots with different letters indicate significant differences (p-value$<$0.05), compared using the student *t*-test. The samples were arranged from upstream to downstream (left to right) for samples M9-M4, and M3-M2.
doi:10.1371/journal.pone.0011285.g004

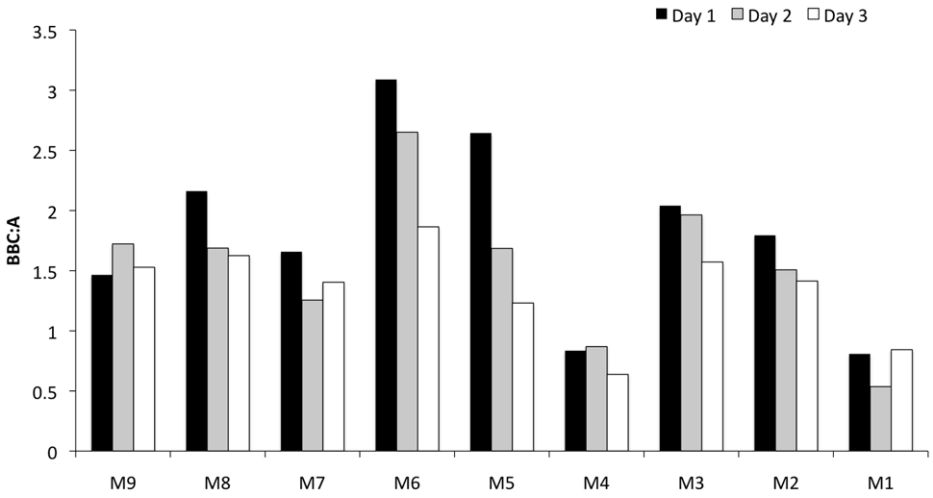


**Figure 5. *Bacillus, Bacteroidetes, Clostridia* to *α-proteobacteria* ratios (BBC:A) from each site.** Ratio from each day is represented by a bar of different color.
doi:10.1371/journal.pone.0011285.g005

## Retrospective comparison of BBC:A ratios from 16S rRNA gene clone library sequencing- and PhyloChip-analyzed samples

The BBC:A ratios of 124 communities characterized by clone-library sequencing and PhyloChip were compared (Figure 6). Detailed descriptions of the communities are included in Table S2. From published sequencing studies, we calculated the BBC:A ratios of bacterial communities from 54 mammalian intestines [32], 5 sewage-associated samples [33,34,35,36,37], and 19 non-fecal samples [23,38,39,40,41,42,43,44,45,46,47,48,49,50,51,52,53]. Likewise, from PhyloChip-analyzed samples, we determined the BBC:A ratios from communities of 11 gut [Brodie et al., unpublished; Marchesi et al., unpublished; Nguyen et al., unpublished; This study] [54], 17 sewage-associated [Conrad et al., unpublished; Sercu et al. unpublished; Wu et al., unpublished], and 18 non-fecal samples [Sercu et al., unpublished; This study] [55]. Anoxic non-fecal samples were included in this comparison as well. For both PhyloChip- and library sequencing-analyzed bacterial communities, gut and sewage-associated samples generally had higher BBC:A ratios than non-fecal samples, except for anoxic non-fecal samples, which had an overlapping range with sewage-associated samples. There were also a few communities that did not follow the general BBC:A ratio trend. The community of a nitrifying-denitrifying activated sludge [35] had much lower BBC:A ratio than the rest of the sequenced sewage-associated communities. Also, beetle posterior hindgut and midgut communities had lower BBC:A ratios than beetle anterior hindgut communities and the other PhyloChip-analyzed gut samples [Nguyen et al., unpublished].

## Discussion

Microbial communities in surface waters are highly responsive to perturbation, shifting with tidal cycles [56], salinity gradients [57,58], dissolved organic matter concentration [59], and chemical stress [60,61,62]. The detection of short-term fluctuations in community composition suggests changes in environmental conditions, nutrients or bacterial sources. An effect of increased salinity due to tidal influence on bacterial composition was observed in this study where the coastal lagoon communities were more similar to creek communities with comparable salinity measurements (Figure 2). Salinity was more strongly correlated to community composition than the other environmental variables measured based on canonical correspondence analysis (data not shown). This result corroborated observations by others [63,64,65]. In addition to being highly sensitive to environmental fluctuations, the response time of community composition shift was within a 24-hour period.

The detection of this rapid community response could be useful for indication of external bacterial inputs, such as from fecal sources. FSAO, derived from the human fecal and untreated sewage samples, were used to represent fecal communities. One caveat is that the OTUs in the FSAO list are specific to the 3 fecal samples used in this study, and do not represent all fecal communities in all environments. However, the prevalent bacterial phyla found in the FSAO are the same as those observed in published studies of human gastrointestinal tract samples [66,67,68,69] and turkey cecal samples [70]. Therefore, community similarity to FSAO could potentially indicate the presence of fecal bacteria. This hypothesis was tested by comparing the community distances between FSAO and variable/stable subpopulations at each of the site (Figure 4A and 4B).

Examination of the variable and stable subpopulations brings to light the bacterial temporal fluctuations across the 3 days. The variable subpopulation represents OTUs with highly fluctuating relative abundances, perhaps due to rapid growth, decay or large sporadic influx of bacterial sources. The stable subpopulation represents OTUs with constant relative abundances. These stable subpopulation OTUs are likely associated with endemic bacteria that are able to grow and persist under the *in situ* environmental conditions or are from consistent external sources.

UniFrac analysis showed that the variable subpopulation of M6 was the most similar to the FSAO (Figure 4A). This suggested intermittent exposure to fecal sources at this site, which was supported by elevated but numerically variable HBM densities and FIB MPN (Figure S3). The prevalence of *Enterobacteriales* in the variable subpopulation falls in line with the high FIB MPN observed at site M6, and further supports the use of similarity of the variable subpopulation with FSAO for demonstrating fecal pollution. Similarity of M9 variable subpopulation to FSAO was not significantly different from that of the M6 (Figure 4A). This

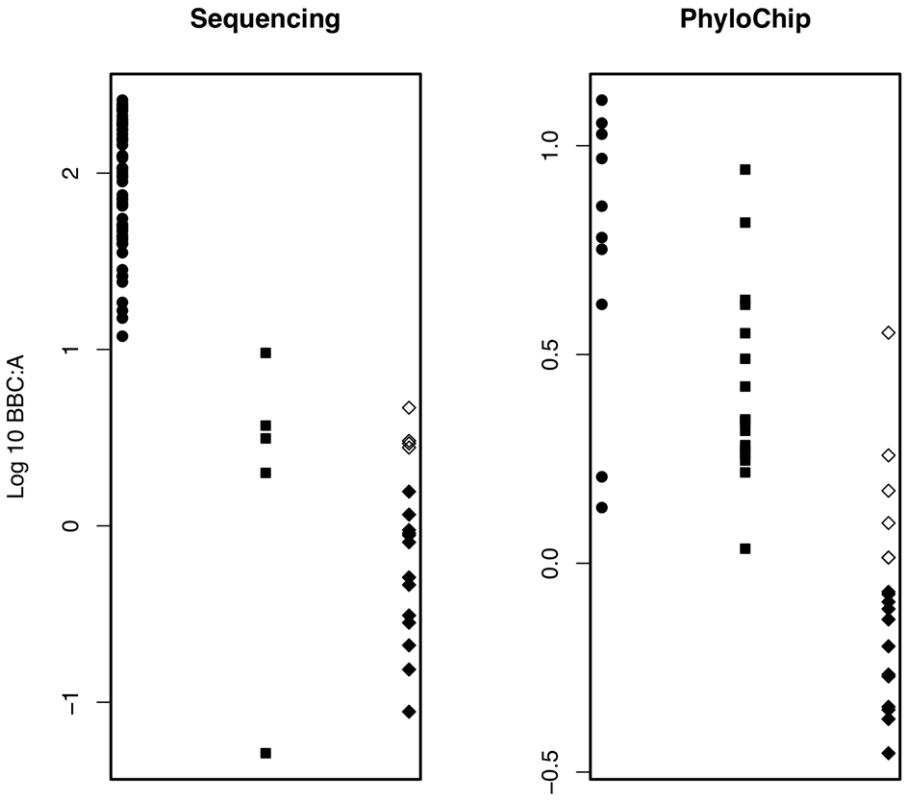


**Figure 6. *Bacillus, Bacteroidetes, Clostridia* to *α-proteobacteria* ratios (BBC:A) of communities analyzed by sequencing or PhyloChip.** Sample types include, gut (●), sewage-associated (■), and non-fecal (♦) associated samples. Unfilled diamond symbols (◇) represent non-fecal samples from anoxic environments.
doi:10.1371/journal.pone.0011285.g006

indicated that there were OTUs in the M9 variable subpopulation that were also found in the FSAO, but they were mostly from the order of *Campylobacterales*, and not represented by FIB or HBM detection. The similarity to FSAO decreased gradually from drains to downstream sites (i.e. M9 to M7 and M6 to M4), illustrating possible fecal community presence at the drains and die-off or dilution effects as the communities flow downstream.

Interestingly, the stable subpopulation at M9 was most similar to FSAO out of all the sites, even though the FIB densities met the California water quality standards on 2 out of the 3 days and no HBM was detected (Figure 4B and Figure S3). The non-detection of HBM at M9 could be due to *Bacteroides* DNA concentration being below the quantitative PCR detection limit of $0.5\times10^{3}$–$10^{4}$ targets $L^{-1}$ [29] or that the fecal source was non-human. The top three families present in the M9 stable subpopulation were *Bacillaceae*, *Staphylococcaceae* and *Lachnospiraceae*. While *Bacillaceae* and *Staphylococcaceae* have been observed in non-aquatic environments [22,26], *Lachnospiraceae* are primarily associated with cow rumen [71], human bowel [67] and anaerobic digesters [72]. Therefore, the data suggested that some of the OTUs detected at M9 could have a fecal, but non-human, origin. However, further confirmatory work is needed to distinguish between a consistent fecal source or bacterial re-growth as the cause for the similarity between M9 stable subpopulation and FSAO.

The FSAO includes OTUs that contain fecal coliforms, which have been demonstrated to re-grow and persist in the environment leading to false-positive water quality diagnoses [6,8,73]. This study further explores the potential of using alternative organisms that are independent of coliforms as fecal indicators by introducing the BBC:A ratio. The ratio excludes coliform bacteria, thus, potentially avoids false-positive results associated with coliforms, and integrates counts for organisms widespread in non-fecal "pristine" environments to assess ecosystem health.

*Bacteroidetes* and *Clostridia* are enriched within the gut microbiota of many mammals [32,66,67,68,69,70], and specific species within these 2 classes have been proposed as fecal indicators [5,10,74]. However, they are also found in anoxic saline aquatic environments [40,45,49], estuaries [38], the deep ocean [41], and high elevation lakes [59]. The class of *Bacilli*, which includes the indicator species *Enterococcus*, is commonly found in fecal samples such as the human gastrointestinal tract [69], turkey intestines [69,70] and aerobic thermophilic swine wastewater bioreactors [75]. All 3 classes are dominant groups found in a chicken fecal metagenomic study [76]. α-pro*teobacteria*, have been found as primary surface colonizers in coastal marine waters [77] and have the ability to thrive under low-nutrient conditions [56]. The BBC:A ratio incorporates the relative richness of OTUs prevalent in these 4 bacterial classes associated with fecal and non-fecal samples to reflect possible fecal inputs, rather than the use of single organism presence or absence. Previous studies have suggested the use of ratios for indicating human or non-human fecal pollution [78], determining fecal age and enteric viral content [79,80], representing the nutrient status of soil ecosystems [81,82], identifying land use in wetland soils [83], and eutrophy in aquatic systems [84].

In order to assess the applicability of the observations from our watersheds to other samples, we calculated the BBC:A ratio from previously published and unpublished studies (Table S2). BBC:A ratios of gut samples analyzed by DNA sequencing or PhyloChip are not completely comparable, mainly due to differences in

sample processing including primers used, PCR conditions and coverage differences across phylogenetic groups on the PhyloChip. However, within communities analyzed by sequencing from different research groups employing varying protocols, the gut, sewage-associated and non-fecal samples exhibited the same BBC:A ratio trend as those communities analyzed by PhyloChip processed with a consistent standardized protocol. The distribution of BBC:A ratios from these studies illustrates that gut and sewage-associated samples have higher BBC:A ratio than non-fecal samples regardless of analysis methods (Figure 6). Anoxic non-fecal polluted environments also have similar ratios of BBC:A as sewage-associated samples (Figure 6). This is most likely an attribute of similar growth conditions favoring both anaerobic and fecal bacteria. The indication of anoxic non-fecal environments is often times pertinent for determining public health risks. Anoxic conditions could lead to eutrophication in both fresh and salt water environments, which changes nutrient cycling, water quality and biodiversity [84]. Eutrophication has led to toxic algal blooms that adversely affect human and wildlife health [85,86].

Kendall rank correlation of FIB, HBM, FSAO and BBC:A ratios from all sites indicated significant positive correlations of BBC:A ratios with HBM, total coliform, enterococcus and FSAO counts, but not with *E. coli* (Table S3). However, many of the samples had reached the total coliform measurement maximum detection limit of 24,196 MPN, therefore, the correlation of total coliform with BBC:A ratio might be misleading. The result also illustrated that even though the BBC:A ratio did not contain fecal coliforms, the fecal pollution pattern was similar to that indicated by the FSAO where coliforms were included. The drain site M6 was the only site where all lines of evidence, i.e. similarity of variable subpopulations to FSAO, FIB, HBM, and BBC:A ratios, pointed to the presence of fecal contamination. At site M1 (ocean), all data indicated a community with the least fecal influence. The data for the rest of the sites (M2, M3, M4, M5, M7, M8 and M9) indicated varying degrees of influence by fecal sources. Also, communities from drains (M6 and M9) were the most similar to organisms found in the fecal samples, although different fecal organisms were detected in the two drains.

Knowledge of who is there and how they change over time and location is the hallmark of an ecosystems approach to studying urban watersheds. We used this concept to track the microbial community dynamics over a three-day period at a location with a history of frequent fecal contamination. In spite of the confounding effect of the movement of water through this watershed, several patterns that correlated with the presence of human fecal contamination were observed. By using the PhyloChip we are able to identify a significantly greater number of bacterial OTUs than is typically examined in coastal watersheds. Comparison of the microbial inventory of the watershed samples with local sewage samples and a human fecal sample led to the identification of specific organisms that were associated with either potential human fecal sources or with the watershed. From this information we observed 503 OTUs that were common to the three fecal samples (FSAO) and the ratios of observed classes of organisms that demonstrated the largest differences between human fecal sources and the receiving waters (BBC:A ratio). Whereas most research for measuring fecal influences on coastal watersheds uses a bottom-up approach to hypothesize that a specific organism is representative of the source, we employed a top-down approach that looked at a large number of potential bacterial contaminants from a majority of the known bacterial diversity to identify a diverse collection of organisms associated with fecal pollution. The advantage of this approach is that we can use the findings of the BBC:A ratio and the FSAO as the basis for additional bottom-up, controlled experiments to examine their applicability at other locations and with other human fecal sources. Using this more detailed microbial community characterization, it may be possible to move away from generic, single indicators to a community-indicator approach for assessing fecal contamination or environments conducive to pathogen growth.

## Materials and Methods

### Ethics statement

The Human Subjects Committee of University of California, Santa Barbara was informed of the anonymous human sample used in this study, and declared that the sample did not meet the definition of a human subject sample, therefore, no approval was necessary for it's use.

### Sample description, collection and extraction

Mission Creek and Laguna Channel flow through an urbanized area of downtown Santa Barbara and discharge at a popular bathing beach. As described previously [29], water column samples from 3 consecutive days (a = day 1, b = day 2, c = day 3), during the dry season (June 2005), were collected from 9 locations (M1–M9) within the Mission Creek and Laguna watersheds in Santa Barbara, California (Figure 1). Samples were delineated into different habitat types: creek (M3, M5–M9, where M6 and M9 were from drains), lagoon (M2 and M4), and ocean (M1). One sample per day was collected at approximately the same time on each of the 3 days. No rain occurred at least 48 hours prior to or during the sampling. The creek flow rate, taken at M5, was 0.016 $m^3s^{-1}$. Both watersheds discharged into the same lagoon at M2 and M4. Surface water flowed from the lagoon into the ocean (M1) at the time of sampling. Three fecal samples, 1 human feces (H), from Santa Barbara, and 2 raw sewage, from the influent at El Estero Wastewater Treatment plant (Santa Barbara, CA) (S1, S2), were also collected. Dissolved oxygen (DO), pH, temperature and salinity were measured along with each sampling [29]. Water samples were filtered in the lab onto 0.22 μm filters on the day of the sampling and stored at −20°C until nucleic acid extractions. DNA was extracted using the UltraClean Water DNA kit (MoBio Laboratories, Inc. Carlsbad, CA, USA), and archived at −20°C. Concentrations of fecal indicator bacteria (FIB) which includes total coliforms, *E. coli*, and *Enterococcus spp*., and quantitative PCR (qPCR) measurements of Human-specific *Bacteroides* Marker (HBM) were reported previously [29].

### 16S rRNA gene amplification for microarray analysis

Genes encoding 16S rRNA were amplified from the gDNA using non-degenerate Bacterial primers 27F and 1492R [87]. Polymerase chain reaction (PCR) was carried out using the *TaKaRa Ex Taq* system (Takara Bio Inc, Otsu, Japan). The amplification protocol was previously described [17].

### Microarray processing, and image data analysis

Microarray analysis was performed using the PhyloChip, an Affymetrix-platform microarray. The protocols were previously reported [17]. Briefly, amplicons were concentrated to a volume less than 40 μl by isopropanol precipitation. The DNA amplicons were then fragmented with DNAse (Invitrogen, Carlsbad, CA, USA), biotin labeled, denatured, and hybridized to the DNA microarray at 48°C overnight (>16 hr). The arrays were subsequently washed and stained. Reagents, conditions, and equipments involved are detailed elsewhere [88]. Arrays were scanned using a GeneArray Scanner (Affymetrix, Santa Clara, CA, USA).

The CEL files obtained from the Affymetrix software that produced information about the fluorescence intensity of each probe were analyzed. The detailed criteria for scoring the probe fluorescence intensities were described elsewhere [17,18,89]. Briefly, a probe set consisted of 11 or more specific 25-mers (probes) that were prevalent in members of a given OTU but were dissimilar from sequences outside the given OTU. Probes with sequences complementing all 25 base pairs of the target sequences were termed perfect match (PM) probes. Each PM probe was matched with a control 25-mer, identical in all positions except the 13$^{th}$ base, termed mismatch (MM) probe. The PM and MM constituted a probe pair that were analyzed together. The probe pairs were scored as positive if the following two criteria were met: 1) the intensity of fluorescence from the PM probe was greater than 1.3 times the intensity from the MM probe, and 2) the difference in intensity (PM minus MM), was at least 500 times greater than the squared noise value. The CEL files from this study are available upon request.

The taxonomic position of each OTU as well as the accompanying NCBI accession numbers of the sequences composing each OTU can be viewed in outline format at: http://greengenes.lbl.gov/Download/Taxonomic_Outlines/G2_chip_SeqDescByOTU_tax_outline.txt.

## PhyloChip data normalization

PhyloChip data normalization was performed using R [90]. To correct for variation associated with quantification of amplicon target (quantification variation), and downstream variation associated with target fragmentation, labeling, hybridization, washing, staining and scanning (microarray technical variation) a two-step normalization procedure was developed. First, for each PhyloChip experiment, a scaling factor best explaining the intensities of the spiked control probes under a multiplicative error model was estimated using a maximum-likelihood procedure [54]. The intensities in each experiment were multiplied with its corresponding optimal scaling factor. Second, the intensities for each experiment were corrected for the variation in total array intensity by dividing the intensities with its corresponding total array intensity separately for Bacteria and Archea. The normalized data is available in Table S4.

## Statistical Analysis

All statistical analyses were carried out in R [90], except for the canonical correspondence analysis (CCA). Bray-Curtis distances were calculated using normalized fluorescence intensity with the *ecodist* package [91]. Non-metric multidimensional scaling (NMDS) and multi response permutation procedure (MRPP) was performed using the *vegan* package. Student *t*-test and Kendall rank correlation from the *stats* package were used to compare samples. A relaxed neighbor-joining tree was generated using *Clearcut* [*92*] and used for UniFrac analysis [31]. Unweighted UniFrac distances, converted to similarity metrics, were calculated for FSAO, variable and stable subpopulations. CCA was carried out using PCOrd [93]. There were no DO, pH and salinity data for sampling days 1 and 2 for site 6, and all 3 days of sampling for site 8. No environmental variables were measured for fecal sample data. Therefore, best-estimate values were inserted based on values measured from the nearest sites on the same day for the CCA. Fecal sample environmental variables were estimated based on reported values in literature.

## PhyloChip derived parameters

Unless otherwise stated, an OTU was considered present when at least 90% of its assigned probe pairs for its corresponding probe set were positive (positive fraction $\geq$0.9). For example, if 10 out of 11 probe pairs are positive, the positive fraction is 0.909 and the OTU is considered present.

Fecal-sample associated OTUs (FSAO) - OTUs that were present in all 3 fecal samples, and in all 27 water samples were tabulated separately. The list of 503 FSAO was derived by removing those OTUs found in all 27 water samples from the OTUs that were present in the fecal samples. The OTUs in each sample which were also found on the list of 503 FSAO were tallied and presented as the FSAO count.

Variable and stable subpopulations - OTUs that were present in at least one of the 3 samples from each site were tabulated and variances of the fluorescence intensities across the 3 days for those OTUs were generated. The OTUs were sorted by variance in descending order. The OTUs in the top deciles (90$^{th}$ percentile) were defined as the variable subpopulation, and OTUs in the bottom deciles (10$^{th}$ percentile) were defined as the stable subpopulation.

The BBC:A ratio of phyloChip samples - The number of OTUs in the classes of *Bacilli* (Bac), *Bacteroidetes* (Bct), *Clostridia* (Cls), and $\alpha$-*proteobacteria* (A) where the positive fraction equal to 1 were tallied. The ratio was calculated using the following formula:

$$BBC : A = \frac{Bac + Bct + Cls}{A}$$

where

$$Bac = \frac{\#OTUs}{520}$$

$$Bct = \frac{\#OTUs}{325}$$

$$Cls = \frac{\#OTUs}{1073}$$

$$A = \frac{\#OTUs}{827}$$

The count for unique OTUs in each of the class was normalized by dividing by the total number of OTUs in each class detectable by the G2 PhyloChip. The denominators were predetermined based on the number of OTUs assigned for each bacterial class on the G2 PhyloChip design.

## The BBC:A ratio of published 16S rRNA gene clone library sequencing samples

Aligned sequences in the Greengenes [94] database were downloaded and re-classified using the PhyloChip (G2) taxonomy on the Greengenes website (http://greengene.lbl.gov). Aligned DNA sequences of various environmental communities were also obtained from [63]. The counts of unique OTUs were tallied for each bacterial class. The BBC:A ratios were calculated using the formulas mentioned above. If no OTU was detected for that class, the count was set to 0.5.

# Supporting Information

**Figure S1** Phylum level profile of 503 fecal sample-associated OTUs (FSAO), and order level profiles of Firmicutes and

Proteobacteria. Pie chart illustrates that the FSAO consist of 43% Firmicutes and 28% Proteobacteria. Most of the Firmicutes OTUs are in the order of Clostridiales, and most of the Proteobacteria OTUs are in Enterobacteriales.
Found at: doi:10.1371/journal.pone.0011285.s001 (0.31 MB TIF)

**Figure S2** Counts of fecal-sample-associated OTUs (FSAO) at each site. Each bar represents one sample from each day. OTUs in each sample which were also found on the list of the 503 FSAO were tallied and presented as the FSAO count.
Found at: doi:10.1371/journal.pone.0011285.s002 (0.15 MB TIF)

**Figure S3** Measurements of Human-specific Bacteroides Marker (HBM), Total Coliform (TC), E. coli (EC), and Enterococcus (ENT) counts. Bars represent HBM values. Lines represent TC, EC and ENT most probable number (MPN).
Found at: doi:10.1371/journal.pone.0011285.s003 (0.49 MB TIF)

**Table S1** Environmental variables measured concurrently with the bacterial community samples. Dissolved oxygen, temperature, salinity and pH were measured at the time of sampling and reported here.
Found at: doi:10.1371/journal.pone.0011285.s004 (0.42 MB TIF)

**Table S2** Description of bacterial communities analyzed by sequencing and PhyloChip used in Figure 6. Gut, sewage-associated and non-fecal samples analyzed by clone-library sequencing and PhyloChip used for the *Bacilli*, *Bacteroidetes*, *Clostridia* to *α-proteobacteria* ratio (BBC:A ratio) are described. All DNA sequences from sequencing samples had a minimum length of 1250 base pairs, except for those with the (*) symbol where the minimum sequence length was 200 base pairs.
Found at: doi:10.1371/journal.pone.0011285.s005 (0.90 MB TIF)

**Table S3** Kendall rank correlation tau coefficient and p-values (in parenthesis). Measurements from all 27 water samples were used. The (*) symbol denotes statistical significance (p-value$<$0.05) differences. Abbreviations: Human Bacteroides Marker (HBM); total coliform (TC); E. coli (EC); enterococcus (ENT); fecal-sample associated OTUs (FSAO); *Bacilli*, *Bacteroidetes*, and *Clostridia* to *α-proteobacteria* ratio (BBC:A).
Found at: doi:10.1371/journal.pone.0011285.s006 (0.09 MB TIF)

**Table S4** Total OTUs detected by PhyloChip for all 30 samples. Positive fraction and normalized fluorescence intensity values are reported.
Found at: doi:10.1371/journal.pone.0011285.s007 (3.87 MB XLS)

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## Author Contributions

Conceived and designed the experiments: CHW PAH GLA. Performed the experiments: CHW BS LCVDW. Analyzed the data: CHW BS JW TZD ELB TCH GLA. Contributed reagents/materials/analysis tools: PAH GLA. Wrote the paper: CHW BS ELB TCH PAH GLA.

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# Appendix 5:

## Selected presentations to EPA, ISME

1) Bacterial Community Analysis of Avian and Mammalian Sources of Fecal Contamination in Coastal California

2) Temporal Dynamics of Cattle and Human Fecal Microbial Communities in Fresh and Marine Waters.

3) Application of Comprehensive Bacterial Community Analysis to Discriminate Common Sources of Fecal Pollution

# Bacterial Community Analysis of Avian and Mammalian Sources of Fecal Contamination in Coastal California

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## Introduction

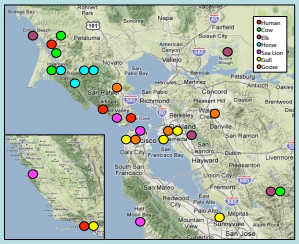
Rising population and aging infrastructure along coastal areas is increasing the frequency of human and agricultural fecal pollution of recreational waters. Concerns over microbiological water quality prompted over 18,000 days of beach closures and advisories in the U.S. last year. Causes of contamination are often unclear because human, agricultural and natural sources co-occur in most watersheds, and common tests for single indicators often fail to convincingly identify or exclude sources. High-throughput sequence analysis has potential to reliably identify fecal sources and resolve contentious water quality issues.

## Objectives

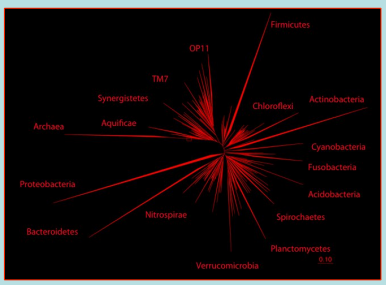
- Characterize bacterial communities in human waste and animal feces that impact water quality in coastal California
- Determine combinations of bacterial taxa that can be used for specific source detection with phylogenetic microarray analysis
- Identify sources of exceedences in water criteria during field tests

## Methods

- Feces collected from California and western gulls, Canada geese, tule elk, California sea lions, elephant seals, cows, horses
- Sampled four populations per animal, minimum five individuals composited per population
- Human wastes: primary sewage, septic and holding tanks.

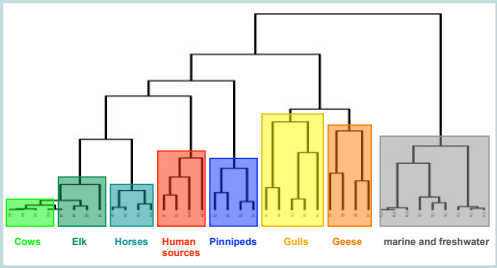



- Analyzed 16S rRNA gene composition using high-density oligonucleotide microarray (PhyloChip)
- Phylogenetic microarray targets 50,443 different bacterial and archaeal sequences



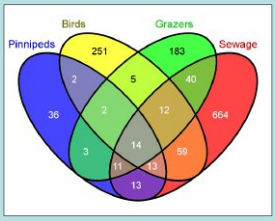

## Results

### Strong clustering of fecal communities by species and diet




- Detected 1548 different bacterial subfamilies in fecal samples
- Human sources form distinct cluster despite type of waste processing
- Little variation among populations of ruminant animals
- Variation among populations of each bird species greater than mammals

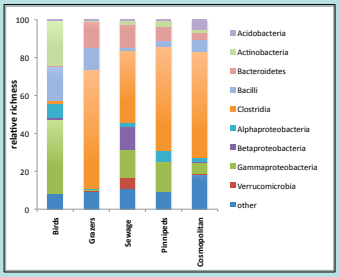
**Shared bacterial sub-families**



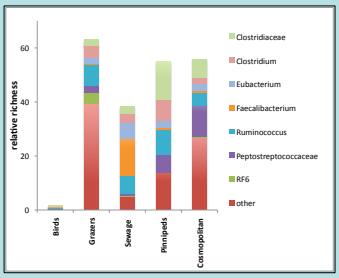

- Birds: many unshared Actinobacteria, Bacilli, Gammaproteobacteria
- Grazers, pinnipeds, sewage: many unshared Clostridia, Bacteroidetes
- Sewage: unshared Beta- and Gammaproteobacteria, Verrucomicrobia
- Cosmopolitan taxa mostly Clostridia (common to all sources)

**Strong potential for source identification based on bacterial community differences**

**Source-specific and cosmopolitan Bacteria**




**Source-specific and cosmopolitan Clostridia**




### Source Tracking Field Test

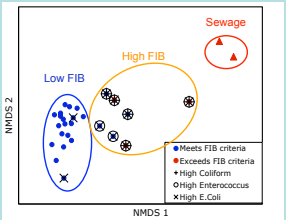
Broken pipe discharged 764,000 gallons of partially-treated sewage into San Francisco Bay

Bay water samples collected for three days after the spill at eight locations within 2 km

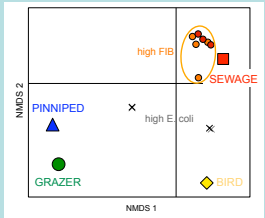
Bacterial community analysis (PhyloChip) compared to counts of culturable fecal indicator bacteria (FIB)

FIB counts were used to determine beach closings and subsequent regulatory action:
*Was the spill responsible for high numbers of FIB measured in some water samples during monitoring?*

**Similarity among bacterial communities**




**Similarity of enriched taxa in high FIB samples to source indicator communities**




Composition of enriched taxa in high FIB samples matched sewage indicator community

Two samples with high *E. coli* did not match indicator community

## Conclusions

- Large differences in bacterial community composition characterize sources of animal and human feces
- Hundreds of taxa are unique to each source and define fecal indicator communities for monitoring
- Phylogenetic community analysis based on variation among gut microbiomes will vastly improve source tracking

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# TEMPORAL DYNAMICS OF CATTLE AND HUMAN FECAL MICROBIAL COMMUNITIES IN FRESH AND MARINE WATERS

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## Introduction

The presence of pathogenic bacteria at recreational beaches has been recognized as an important epidemiological and economic issue. Previous work has demonstrated that indicator species (*Enterococcus* and fecal coliforms) do not necessarily indicate the presence of pathogenic bacteria, and could lead to misguided decisions in beach closures. Urbanization and agricultural runoff have been shown to contribute to non-point source fecal pollution. However, it is unclear to what extent the fecal microbial communities from various sources (e.g. human and cattle) behave differently in the environment.

## Objective

To characterize the survival and persistence of fecal bacteria from different sources under various field conditions in order to quantify risks associated with cattle and human waste.

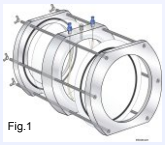


Fig.1

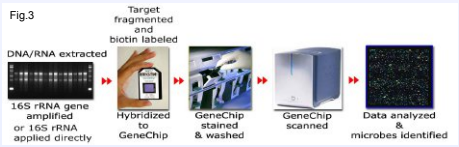



Fig.3

## Experimental Setup & Methods

1. We utilized diffusion chambers with 2um membranes that have 100 ml holding volume. Human septage and cattle waste were injected into diffusion chambers (Fig. 1), and immersed in creek water and bay water (Fig. 2). Samples were taken at 6 time points up to 96 hr. Community compositions were characterized with a high-density microarray (PhyloChip) targeting the 16S rRNA gene of Bacteria and Archaea.

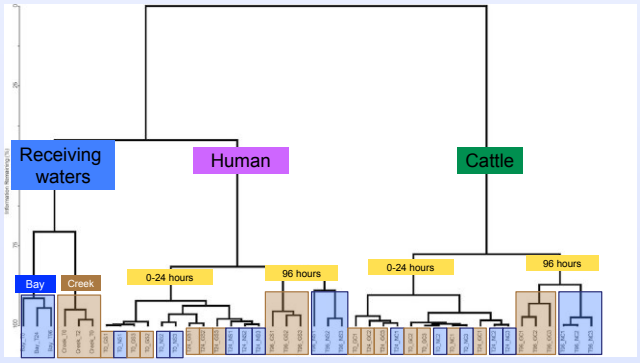
Fig.2

2. Samples were taken at various time points through sampling ports, vacuum filtered onto an inorganic membrane filter and kept at -80°C until sample processing.
3. Nucleic acids were extracted by a modified CTAB method, purified and separated using the AllPrep DNA/RNA Kit (Qiagen).
4. Microbial community composition was analyzed using a Affymetrix platform high-density microarray (G3 PhyloChip), targeting 16S rRNA of ~60,000 microbial taxa (Fig. 3). The 16S rRNA gene was amplified from gDNA extracts using modified universal primers:

| | |
|---|---|
| Bacteria: | 27F (5' AGAGTTTGATCCTGGCTCAG) |
| | 1492R (5' GGTTACCTTGTTACGACTT) |
| Archaea: | 4Fa (5' TCCGGTTGATCCTGCCRG 3') |
| | 1492R (Same as above) |

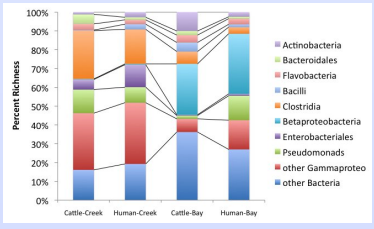
## Results and Discussion

### Microbial Community Composition Similarity



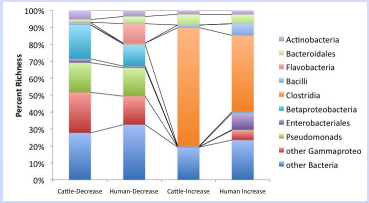

- ✓ Samples clustered by waste type
- ✓ At 0 and 24 hours after incubation, microbial communities were similar in bay or creek water
- ✓ At 96 hours, the microbial communities grouped by receiving water

### Effects of Creek and Bay Water on Fecal Communities



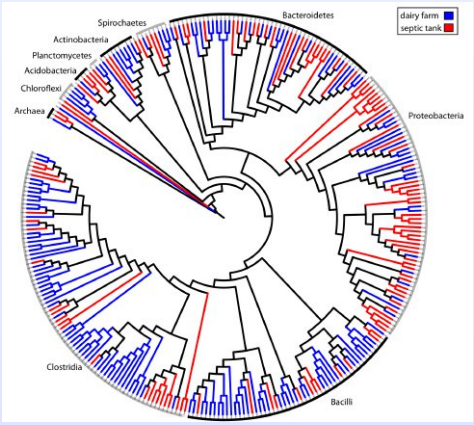

- ✓ Similar responses of cattle waste and human sepatage in creek water observed.
- ✓ The waste communities responded differently in bay water.
- ✓ Clostridia and many γ-proteobacteria, including coliforms, were more persistent in creek water.
- ✓ β-proteobacteria were more persistent in bay water.

### Effects of Time on Cattle and Human Wastes




- ✓ Similar taxa from cattle and human wastes decreased over a period of 96 hours in both water types.
- ✓ More *Clostridia* taxa detected over time in cattle waste.
- ✓ More *Enterobacteriales* and *Bacilli* taxa detected over time in human waste.

### Potential Indicator Taxa




- ✓ Bacteria and archaea that are unique to each waste and absent from receiving waters are presented
- ✓ A total of 13,341 different taxa were detected in waste and receiving water samples
- ✓ Number of potential indicator taxa
  - 165 taxa identified for cattle waste
  - 119 taxa identified for human waste
- ✓ In cattle waste, more unique taxa were *Clostridia*. In human waste, more unique taxa were *Proteobacteria*.

## Conclusions

- ❖ Receiving water type affects microbial community dynamics over time.
- ❖ The results of this research indicate that creek and bay water exert different effects on the fate of waste microbial communities.
- ❖ Therefore, selection of indicators for monitoring should be based on waste type and persistence of fecal taxa under various receiving waters.

## Acknowledgement

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# Application of comprehensive bacterial community analysis to discriminate common sources of fecal pollution

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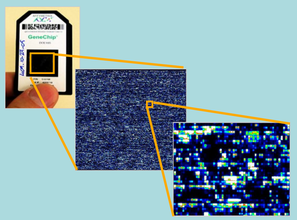
## Introduction

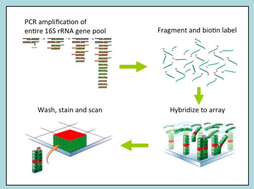
Causes of fecal contamination in recreational waters are often unclear because human, agricultural and wildlife sources of fecal bacteria co-occur in many watersheds. Common tests for single indicators often fail to convincingly identify or exclude sources. High-throughput DNA sequence analysis has potential to reliably track fecal sources by profiling the entire community of microorganisms that differentiate fecal contaminants.

In this study we used the Lawrence Berkeley National Laboratory PhyloChip to discriminate fecal sources based on the detection of over 59,000 different taxonomic groups of bacteria in a single test. We focused on suspected sources of fecal contamination at popular beaches in coastal California (human wastes, birds, cattle and other grazing animals). Measuring nearly all known types of bacteria in fecal samples allowed us to find hundreds of different taxa that were unique to each source type and could be used for reliable source identification. The power of this method was demonstrated in recreational waters with high counts of fecal indicator bacteria.

## Methods

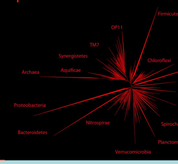
- Comprehensive microbial census with high-density oligonucleotide microarray (PhyloChip)
- Simultaneous detection and quantification of 59,959 different bacterial and archaeal taxa
- Designed from database of all known 16S rRNA gene sequences
- Advantage over sequencing: **reliable detection of rare taxa, profiles entire 16S rRNA gene pool**






### PhyloChip measures most bacterial diversity

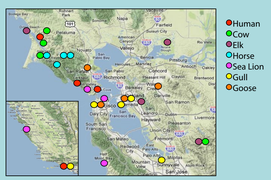
2 Domains
147 Phyla
1,464 Families
10,993 Subfamilies
59,959 OTUs



Includes:
2,105 Enterobacteria (coliforms)
2,272 Bacteroidales
146 Enterococci
12,098 Clostridia
353 Cyanobacteria
643 Archaea

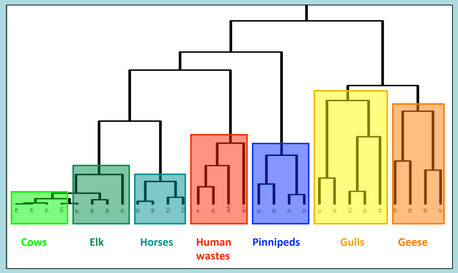
Fecal Samples

- California and western gulls, Canada geese, tule elk, California sea lions, elephant seals, cows, horses
- Sampled four populations per animal, minimum five individuals combined per population
- Human wastes: primary sewage, septic and holding tanks




## Results

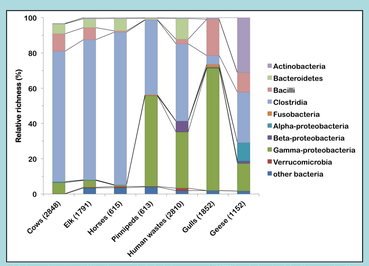
**Strong clustering of bacterial communities by source type**




- Detected 20,368 different bacterial taxa across all fecal samples
- Each source is distinct
- Little variation among ruminant microbial communities
- Bird communities are the most variable but still distinct from other animals

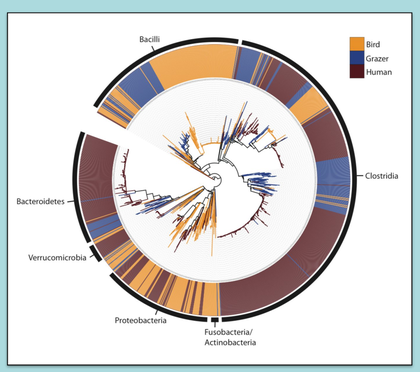
- Taxonomic (OTU) richness of grazing mammals is dominated by Clostridia, lactic acid Bacilli and Bacteroidetes - essential bacteria for digestion of cellulose and other plant polysaccharides.
- Taxonomic richness of human wastes is dominated by Clostridia, Gammaproteobacteria (mostly coliforms) and Bacteroidetes.
- Gulls and geese have distinct richness profiles. Enteric Gammaproteobacteria and Bacilli dominate gulls. Actinobacteria, Alphaproteobacteria and Clostridia more dominant in geese.
- Pinnipeds dominated by Clostridia and Gammaproteobacteria.

**Bacterial community composition of fecal sources**



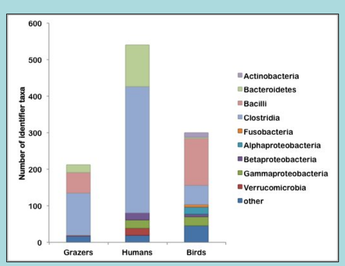

Values in parenthesis are total richness of OTUs detected in the majority of samples for each source

**Hundreds of bacterial taxa unique to each source**




Phylogenetic tree of source identifier taxa for major source types

- Sources can be identified based on hundreds of unique Clostridia, Bacilli, Bacteroidetes and Proteobacteria




Composition of source identifier taxa

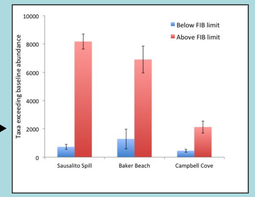
## Application to Source Tracking

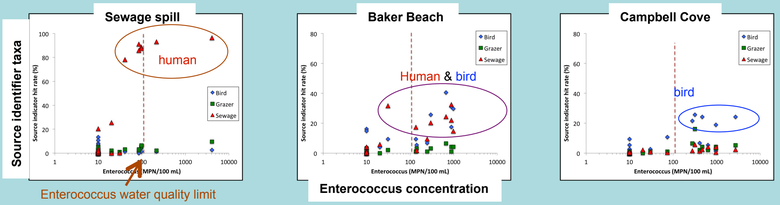
**Sausalito sanitary plant**
764,000 gallon sewage spill in San Francisco Bay

**Baker Beach, San Francisco**
Poor water quality (high FIB) with potential human sources

**Campbell Cove, Bodega Bay**
Poor water quality with no suspected human source


1. Sewage spill monitoring: SF Bay water samples (1 L) from 2 km area for 10 days following spill. Fecal indicator tests for coliforms and enterococcus.
2. Beach monitoring: weekly samples collected for 1 year and analyzed for FIB. Samples preserved on filters and archived at -80°C until DNA extraction. Subset of samples analyzed by PhyloChip based on FIB exceedences.
3. At all sites, baseline microbial communities defined by mean abundance of taxa in non-exceedence samples. Taxa that significantly exceed baseline (> mean + 2σ) determined for each sample.
4. Sources revealed by source identifier taxa that exceed baseline




- Nearly all human fecal identifier taxa found in waters
- Detection of bird or grazer taxa not greater than expected
- Some samples below enterococcus limit clearly impacted by sewage

- Greater than expected numbers of both human and bird identifier taxa
- Mixed sources of FIB: possibly leaking sewer lines, shoreline birds

- Greater than expected number of bird identifier taxa
- No increased detection of human or grazer identifier taxa
- Consistent with previous study that determined birds were a likely source

## Conclusions

- Large differences in bacterial community composition among fecal sources
- Hundreds of taxa are unique to each source and define fecal identifier communities for source tracking
- Tests in high FIB waters demonstrate a single microarray test is capable of identifying or excluding multiple sources
- Reliable source tracking based on a complete census of bacterial taxa has great promise for improving risk assessment

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