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Microbial source tracking in impaired watersheds using PhyloChip and machine-learning classification

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ABSTRACT

Sources of fecal indicator bacteria are difficult to identify in watersheds that are impacted by a variety of non-point sources. We developed a molecular source tracking test using the PhyloChip microarray that detects and distinguishes fecal bacteria from humans, birds, ruminants, horses, pigs and dogs with a single test. The multiplexed assay targets 9001 different 25-mer fragments of 16S rRNA genes that are common to the bacterial community of each source type. Both random forests and SourceTracker were tested as discrimination tools, with SourceTracker classification producing superior specificity and sensitivity for all source types. Validation with 12 different mammalian sources in mixtures found 100% correct identification of the dominant source and 84–100% specificity. The test was applied to identify sources of fecal indicator bacteria in the Russian River watershed in California. We found widespread contamination by human sources during the wet season proximal to settlements with antiquated septic infrastructure and during the dry season at beaches during intense recreational activity. The test was more sensitive than common fecal indicator tests that failed to identify potential risks at these sites. Conversely, upstream beaches and numerous creeks with less reliance on onsite wastewater treatment contained no fecal signal from humans or other animals; however these waters did contain high counts of fecal indicator bacteria after rain. Microbial community analysis revealed that increased *E. coli* and enterococci at these locations did not co-occur with common fecal bacteria, but rather co-varied with copiotrophic bacteria that are common in freshwaters with high nutrient and carbon loading, suggesting runoff likely promoted the growth of environmental strains of *E. coli* and enterococci. These results indicate that machine-learning classification of PhyloChip microarray data can outperform conventional single marker tests that are used to assess health risks, and is an effective tool for distinguishing numerous fecal and environmental sources of pathogen indicators.

1. Introduction

Pathogen contamination in inland and coastal waters is a widespread problem in the United States. Under the Clean Water Act, over 10,300 water bodies are considered impaired by pathogens due to high counts of fecal indicator bacteria (FIB) (U.S. Environmental Protection Agency, 2016). In many cases the sources of this pollution are unknown and may originate from diffuse, nonpoint sources such as urban and agricultural runoff, wildlife droppings or even growth in the environment. Nonpoint sources are difficult to pinpoint because impaired waters typically receive drainage from many parcels of land with assorted agricultural and urban land uses that occur throughout a watershed. Correct source identification is needed to accurately assess health risks and implement effective controls for nonpoint discharge of pollutants (U.S. Environmental Protection Agency, 2005).

Direct measurements of harmful fecal pathogens are currently impractical for routine environmental monitoring because the assays are difficult, time-consuming and costly. Instead, quantification of FIB, namely *Escherichia coli* or enterococci, is commonly used as a proxy to assess pathogen risks (Gerba, 2000). Epidemiological studies have established human health standards based on exposure to these FIB recreational waters, and the reliance on these conventional FIB to protect public health presumes high FIB counts are caused by fecal inputs and are associated with higher rates of

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illness (Prüss, 1998; Wade et al., 2003). Conventional FIB tests, however, are not reliably associated with health risks at beaches with nonpoint FIB sources and provide no identification of sources. Because *E. coli* and enterococci are facultative aerobes they can survive and even grow under oxygenated conditions found in surface waters, and FIB tests are prone to false positives caused by extra-enteric, environmental strains of these taxa that are found in sediments, soils, beach sands and decaying vegetation (Byappanahalli et al., 2003, 2012; Hardina and Fujioka, 1991; Imamura et al., 2011; Yamahara et al., 2009). Epidemiological studies support a stronger association between fecal indicators and waterborne diseases when sewage contamination is present, compared to presence of high FIB originating from non-human sources (U.S. Environmental Protection Agency, 2010). In light of these limitations, additional tests are needed to reliably detect potential nonpoint sources and accurately gauge health risk when FIB tests indicate impairment.

Molecular detection of host-specific markers is increasingly used to identify sources of FIB (Field and Samadpour, 2007; McLellan and Eren, 2014). Most approaches measure single genetic markers that are presumed to be unique to a single host. The specificity and sensitivity of single marker tests varies among hosts. Some hosts, such as humans, have single molecular targets that are potentially reliable indicators of human feces (Boehm et al., 2013). Others, such as livestock, dogs and various wildlife have less reliable markers that lack adequate specificity and sensitivity (Boehm et al., 2013), and many wildlife and non-fecal FIB sources have no known fecal markers that are suitable for identification. A toolbox of reliable tests is needed to disentangle the complex mixture of upstream sources that contribute FIB in watersheds with nonpoint source problems (Ahmed et al., 2015; Noble et al., 2006).

High-throughput sequence analysis of microbial community 16S rRNA genes is a promising approach for detecting FIB sources in complex environmental settings (Unno et al., 2010; Dubinsky et al., 2012; Cao et al., 2013; Newton et al., 2013; Ahmed et al., 2015). An advantage of community sequence approaches to source tracking is that source discrimination is based on distinctive combinations of potentially thousands of genetic markers and is not completely dependent on the performance of a single marker. The 16S rRNA gene that is used as the standard to define bacterial communities is universally present in bacterial genomes and typically occurs in multiple copies, supplying a highly abundant target for sensitive detection and discrimination of fecal sources that have distinct microbial communities. PhyloChip DNA microarray analysis has been shown to be a highly sensitive and specific classification method of fecal sources (Dubinsky et al., 2012; Cao et al., 2013). A potential advantage of this method for source tracking compared to next generation sequencing is the replicable detection of rare taxa (down to 0.01% of microbial community; DeSantis et al., 2007), which is important as sources are diluted and degraded within diverse environmental background microbial communities (Probst et al., 2014; Zhou et al., 2015).

Routine application of PhyloChip or other community-based method for real world monitoring requires an automated, statistically robust classification approach to assess the likelihood that individual sources contribute to the overall composition of the sample microbial community. Machine-learning classification methods have recently been adopted for community-based source tracking (Knights et al., 2011b; Smith et al., 2010) but are untested with PhyloChip microarray analysis for microbial source identification in environmental samples. Supervised classification approaches to microbial source tracking use DNA sequence data from complex microbial communities to train models to distinguish different types of samples. These algorithms select subsets of features from typically thousands of species or DNA sequences that are most useful for classification and eliminate uninformative features from the model. Random forests is an ensemble learning method that uses decision trees, and is widely-used technique to classify highly dimensional sequence data because of its ability to analyze large datasets and high accuracy (Knights et al., 2011a; Lee et al., 2005). Several studies have found random forests are the best performing classification method for highly-dimensional microarray and community sequencing datasets compared to other supervised classification methods (Knights et al., 2011a; Lee et al., 2005; Smith et al., 2015). SourceTracker is a more recently developed Bayesian classification tool that uses Gibbs sampling to calculate a posterior probability that microbial DNA from each source is present in the microbial community of the sink, and was shown to outperform random forest for pyrosequencing datasets (Knights et al., 2011b). Recent studies have used SourceTracker to detect fecal sources in next generation sequencing data (Ahmed et al., 2015; Henry et al., 2016; Neave et al., 2014; Newton et al., 2013; Staley et al., 2016); however, no source tracking studies have evaluated the performance of phylogenetic microarrays, including PhyloChip, with SourceTracker or random forest classifiers.

In this study, we analyzed the performance of PhyloChip detection and classification of fecal microbial communities using random forest and SourceTracker. We then applied the best performing method to detect sources of fecal contamination in the Russian River watershed in Northern California, an area that is heavily used for recreation and includes a diverse mix of land use types including urbanized areas, dairy farms and pastureland, and open space. Several communities along the river rely on septic systems, many of which do not meet modern guidelines (North Coast Regional Water Quality Control Board, 2015). The river frequently exceeds FIB water quality limits for *E. coli* and enterococci in both winter and summer months (Butkus, 2011; North Coast Regional Water Quality Control Board, 2012). To support the development of the Russian River Pathogen TMDL, the PhyloChip was used for microbiological source identification in the middle and lower Russian River watershed. The goals of this study were to determine the effectiveness of fecal source identification using PhyloChip data and different machine-learning classifiers, and apply the best performing method to identify sources that impact water quality in the Russian River during periods of high runoff and heavy recreational use.

2. Methods

2.1. Fecal and water sampling

A total of 70 fecal reference samples were collected and analyzed from human waste (sewage, septage, stool), ruminants (cows, elk, deer), cats and dogs, pigs, horses, and birds (gulls, geese, pigeons, cormorants, pelicans, chickens). Each reference fecal sample was collected from different locations throughout California and was a composite of individual droppings from multiple individuals (Dubinsky et al., 2012). An additional 64 challenge samples from Boehm et al. (2013) were analyzed that were created from freshly collected fecal material from 12 sources including: humans (feces), sewage, septage, dogs, pigs, deer, horses, cows, chickens, gulls, pigeons, and geese and contained either a single fecal source (38 singletons) or two fecal sources (26 doubletons) (Table S1). The 38 singleton challenge samples included 24 full strength and fourteen 1:10 strength singletons, which were created by filtering 200 ml and 20 ml of the corresponding singleton slurry, respectively, through polycarbonate membrane filters (Isopore Millipore, 47 mm dia. 0.4 μm pore size). Each of the 26 doubleton samples was created by filtering 200 ml of a corresponding doubleton slurry

created by mixing 90% and 10% (by volume) of the corresponding singleton slurries. More details on the collection and preparation of challenge samples are described elsewhere (Boehm et al., 2013).

Samples from the Russian River were collected from 12 popular swimming beaches along the middle and lower portions of the river extending from Healdsburg to the mouth of the river at Jenner (Table S2). Collections occurred during the summer dry season (August 2011) and the beginning of the wet season (October 2011) at each location between 8:00 a.m. to 1:00 p.m. each day. Wet periods were defined by federal regulation (40 CFR 122.21(g) (7) (ii)) and the USEPA Storm Water Sampling Guidance Document (U.S. Environmental Protection Agency, 1992) as greater than 0.1 inch and at least 72 h from the previously measurable (greater than 0.1 inch rainfall) storm event. Additional river samples were collected at popular beaches in Guerneville and Monte Rio over five consecutive days at 8:00 a.m. each day during the Russian River Jazz & Blues festival (September 22–16, 2011) to assess daily variability during a period of intense recreational activity. Water samples were also collected at 35 different creek sites in the Russian River watershed that encompassed major land cover types including forested areas, agricultural areas, rangeland, sewered residential areas, and non-sewered residential areas with onsite wastewater treatment (Table S2).

Water samples were collected at each site for *E. coli* and enterococci quantification (100 mL) and PhyloChip analysis (300 mL). Samples were transported on ice to the laboratory and immediately processed for analysis. Culturable *E. coli* and enterococci were quantified using the Colilert-24 and Enterolert tests, respectively (IDEXX Laboratories, Westbrook, ME). Samples for PhyloChip analysis were vacuum filtered through sterile polyethersulfone membrane filters (Pall Supor 200, 47 mm dia., 0.2 μm pore size), and filters were archived at −80 °C until DNA extraction.

2.2. DNA extraction and amplification

DNA was extracted from water filters using the DNA-EZ extraction kit (Generite, New Brunswick, NJ). The 16S rRNA gene was amplified from each DNA extract using PCR with bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) for bacteria (Lane, 1991). 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 eight 25 μl reactions that spanned a 48–58 °C gradient in annealing temperatures to minimize PCR bias due to variable template annealing efficiencies and random priming effects (DeSantis et al., 2007). 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.

2.3. PhyloChip analysis

Detailed descriptions of PhyloChip design, validation and laboratory procedures are described elsewhere (DeSantis et al., 2007; Hazen et al., 2010). Purified PCR products were purified then fragmented with DNAaseI; the fragmented products were then labeled with biotin followed by hybridization overnight onto the PhyloChip microarray (Second Genome, South San Francisco, CA); the microarray was then stained and scanned to provide raw PhyloChip data in the form of fluorescent image files. Probe intensities were background-subtracted and scaled to quantitative standards (non-16S spike-ins) and outliers were identified as described in Hazen et al. (2010).

Two approaches were used to analyze the fluorescent image files following array scanning. The first approach for taxonomic description used the standard operational taxonomic unit (OTU) approach described in Dubinsky et al. (2012). In this approach the presence of 59,316 different bacterial OTUs was determined by positive hybridization of multiple probes that correspond to distinguishing 16S rRNA gene polymorphisms (average of 37 probes/OTU). The hybridization score for an OTU was calculated as the mean intensity of the perfectly matching probes exclusive of the maximum and minimum. This approach yields an inventory of detected OTUs that compose the microbial community.

The second analysis approach considered probe quartet data (Probst et al., 2014) and is an advancement of the high performing probe-based analysis described in Cao et al. (2013). The quartet approach uses each of the PhyloChip's 1,015,124 probe that target the sense, anti-sense, and corresponding mismatch probes of each targeted sequence (Probst et al., 2014). This provides more stringent positive match criteria for each targeted sequence than the probe-based analysis described previously (Cao et al., 2013) because it controls for non-specific hybridization and relies on detection of both complimentary DNA strands to increase the performance of the assay.

2.4. Machine learning classification and statistics

Probe-quartet profiles were measured in the 70 fecal reference samples and used to define subsets of 16S rRNA gene sequences that are common among samples of a given source type (>50% of source samples) and rare in other fecal sources (<=1% non-source samples). Fecal sources were categorized into six different source types: humans (stool, sewage, septage), dogs and cats, pigs, ruminants (cows, elk, deer), horses and birds. Out of 121,229 quartets present in the dataset, 9001 were selected as the diagnostic subset for machine learning analysis. Bacterial species that were commonly found in each of these source types by PhyloChip OTU analysis are shown in Fig. S1.

Two machine learning approaches to classification were evaluated: SourceTracker (Knights et al., 2011b) and random forests (Breiman, 2001). Analysis was conducted was conducted in R (version 3.2.3) using SourceTracker (version 0.9.8) (Knights et al., 2011b) and randomForest (version 4.6–12) (Liaw and Wiener, 2002) using default parameters. Quartet data from 70 reference source samples were used to train the algorithm to detect fecal signatures from human (20), dogs and cats (7), pigs (4), ruminants (10), horses (5) and birds (24). The predictive performance of the classifier was evaluated by leave-one-out cross-validation of

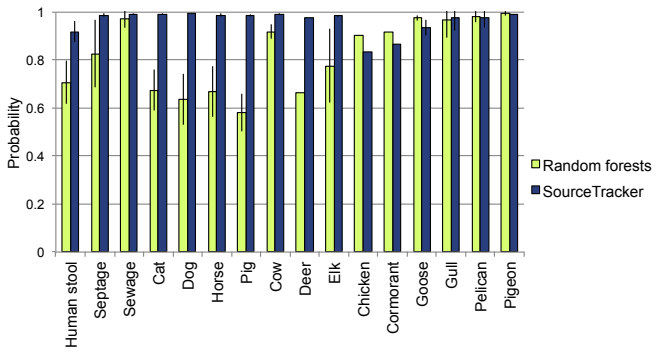

Fig. 1. Cross-validation accuracy using random forest and SourceTracker classification methods. Mean and standard deviation are shown for each fecal source.

training data (Fig. 1). Challenge samples from Boehm et al. (2013) were then evaluated with the superior method (SourceTracker) to evaluate the tradeoff between specificity (true positive rate) and sensitivity (true negative rate) as different categorical probabilities were used as thresholds for calling a source present, and overall accuracy of the test was calculated as area under the receiver operating characteristic (ROC) curve (Fig. S2). Positive likelihood ratios [LR^+ = Sensitivity/(1 − Specificity)] associated with each test result were used to interpret the signal strength using conventional guidelines for diagnostic tests as reviewed by Grimes and Schulz (2005): strong signal ($LR^+ > 10$), moderate signal ($5 \leq LR^+ <= 10$) and nominal signal ($LR^+ < 5$) (Grimes and Schulz, 2005). Using these LR^+ definitions, the categorical probability values from SourceTracker that defined signal strength were >5.3% for strong signal and 3.4–5.3% for moderate signal.

To evaluate the Russian River watershed samples, the algorithm was trained with all fecal samples, 8 equipment blank samples to account for any contamination signal, and 28 background water samples from the Russian River watershed that had FIB counts below USEPA precautionary thresholds (Table S2) in order to account for any influence of background microbial communities on the diagnostic probe sets. By training the model with background water samples, source probes that were cross-reactive with the sink microbial community were reduced in importance for classification, minimizing the influence of background interference on source detection. Water samples were separated based on FIB precautionary thresholds because the goal of the microbial source tracking study was to identify fecal sources that were associated with high FIB counts. In addition, we accounted for the possibility that low FIB samples could have fecal DNA signal by evaluating each candidate background sample with the SourceTracker leave-one-out validation procedure. Only samples with both low FIB counts and no detectable fecal signals were recruited into the background set that was used to train the final model.

Comparisons of overall bacterial community structure were conducted with multivariate statistics in Primer 6 (Clarke and Gorley, 2006). Nonmetric Multidimensional Scaling (NMDS) using the Bray-Curtis distance metric was used to visualize community differences and differences in community structure. Differences among groups were tested by Analysis of Similarity (ANOSIM). ANOSIM R values range from 0 to 1, with values close to 1 indicating strong separation between groups and values close to 0 indicating no significant separation. Similarity Percentage (SIMPER) analysis was used to identify the taxa that were primarily responsible for observed differences in community structure between groups. Culturable FIB counts and PhyloChip results were compared by regression analysis in R (version 3.2.3).

3. Results

3.1. Method evaluation

Random forest and SourceTracker classification tools were cross-validated for all animals in the fecal reference library (Fig. 1). SourceTracker outperformed random forest classification for mammalian sources. Predicted cross-validation probabilities for all mammalian sources were greater than 0.92 using SourceTracker (perfect classification = 1.00), whereas probabilities were mostly less than 0.80 using random forest classification. Avian source prediction was similar between methods with all predicted categorical probabilities above 0.80 and most above 0.92. Based on these results, SourceTracker was chosen as the classification method for all subsequent analyses.

Sensitivity and specificity of PhyloChip quartet analysis with SourceTracker classification was further evaluated with 64 single- or dual-source samples using variable dilutions of 12 different avian and mammalian sources (Boehm et al., 2013). Categorization was highly accurate with an area under the ROC curve of 0.97 (Fig. S2). At the optimal threshold for categorical probability (5.3%), determined by analysis of the ROC curve (Fig. S2), the assay had a detection sensitivity of 100% each source type human waste, dogs, ruminants, birds, horses, pigs) when it was the dominant source in the mixture (Table 1). Specificities ranged from 84 to 100% for each source type (Table 1). The overall sensitivity and specificity of the assay aggregated for all sources were 96% and 90%, respectively, when minor sources were included.

3.2. Russian River fecal signal detection

Field water samples from the Russian River watershed were tested using PhyloChip quartet analysis with SourceTracker detection using the 70 sample training set described in Section 2.1. Frequent occurrence of human fecal signal was detected in the lower portions of the watershed during the wet season (Figs. 2 and 3). All five beaches sampled between Forestville (FAB) and the mouth of the river at Jenner (JBR) contained moderate to strong human fecal signal in the wet season (Fig. 2). The beaches with human fecal signal were adjacent to or downstream from tributaries that flow through residential areas in Forestville, Guerneville and Monte Rio where the Regional Water Quality Control Board has identified high densities of parcels with possible antiquated and/or insufficient onsite wastewater treatment systems. Strong fecal signal from dogs was also detected in the wet season at beaches in Guerneville (JB) and Monte Rio (MRB) (Fig. 2). Moderate ruminant fecal signal was present in the wet season at Russian River beaches directly downstream of the confluence with Mark West Creek (Fig. 3). Mark West Creek drains the Laguna de Santa Rosa watershed, an area with numerous dairies and pasturelands. Ruminant fecal signal was detected at all sites in the southern portion of the Laguna de Santa Rosa watershed in the wet season (Fig. 3).

Community analysis revealed that river samples were enriched in Bacteroidales and Clostridia that are common human gut bacteria (Fig. S3). The most enriched OTUs (SIMPER analysis top 10%) in samples with human fecal signal were human Bacteroidales (genera *Bacteroides*, *Prevotella*) and Lachnospiraceae and Ruminococcaceae in the Clostridiales phylum (genera *Blautia*, *Clostridium*, *Coprococcus*, *Eubacterium*, *Roseburia*, *Ruminococcus*, *Faecalibacterium*). In addition, several OTUs of potentially pathogenic Bacillales (genus *Staphylococcus*) were highly enriched in samples with strong human and signals (Fig. S3).

In the dry season, strong bird signal was detected at the Jenner sampling location (JBR) where the river meets the Pacific Ocean (Fig. 2). This result is consistent with observations of abundant shorebird populations at this site. The widespread detection of human fecal signal observed in the wet season was not observed in the summer likely due to drought conditions that limit runoff from adjacent settlements. There was, however, localized detection of human fecal signal at Johnson's Beach in Guerneville during the Jazz Festival (Fig. S4) that may be caused by leaking bathrooms or

Table 1
Sensitivity and specificity of PhyloChip SourceTracker assay for dominant sources.

	Sensitivity	Specificity	*n*
Human	100	88	22
Bird	100	93	18
Dog	100	100	6
Horse	100	99	4
Pig	100	96	6
Ruminant	100	84	8

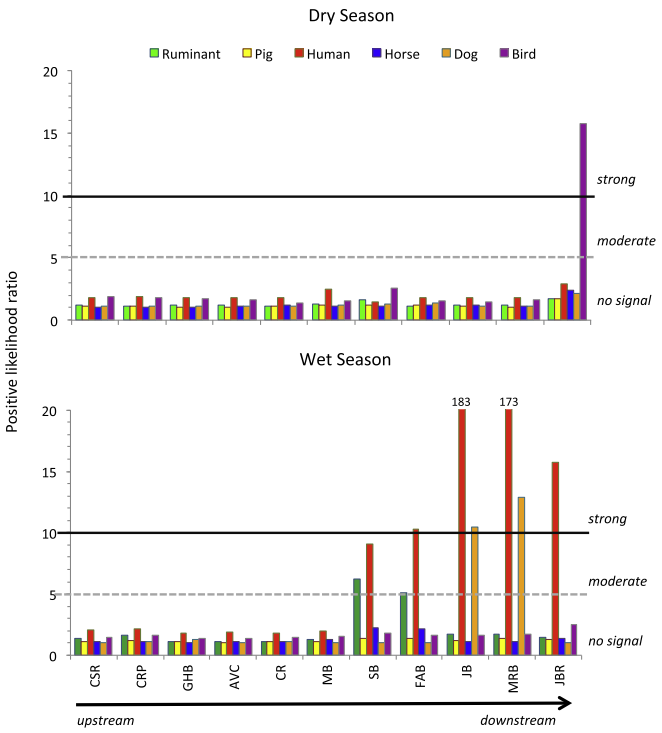

Fig. 2. Dry and wet season fecal signal detected at Russian River beaches. Sites are ordered from upstream to downstream. Positive likelihood ratios were calculated from each SourceTracker test result [Positive Likelihood Ratio = Sensitivity/(1 − Specificity]. Values above the solid line indicate a strong signal (likelihood ratio > 10). Values above the dashed line and below the solid line indicate a moderate signal (likelihood ratio >5 and < 10). No fecal signals were detected in dry season samples except bird signal at the mouth of the river. Strong human and dog, and moderate ruminant signals were detected in the wet season at downstream beaches in the lower watershed.

bather shedding in the river.

DNA signals from humans, dogs and birds were detected at several beaches in the lower Russian River (Fig. 3), however most of these samples did not exceed the most protective U.S. EPA water quality thresholds for *E. coli* [2012 Beach Action Value (BAV) of 190 units/100 mL] and only one exceeded the most protective threshold for enterococci (BAV of 60 units/100 mL) (Table S2). Conversely, most creek samples exceeded *E. coli* and enterococci water quality thresholds in the wet season, however most creek samples with FIB exceedances were unaffiliated with any fecal signal (Table S2).

4. Discussion

PhyloChip quartet analysis and SourceTracker classification is capable of highly accurate identification of fecal sources for microbial source tracking. The sensitivity and specificity of the test was significantly improved compared to the OTU-based classification approach evaluated previously (Dubinsky et al., 2012; Boehm et al., 2013; Cao et al., 2013), and, unlike the previous evaluation of PhyloChip performance compared with other community analysis methods (Cao et al., 2013), this study did not use any exact matches to target sources as reference samples. We found SourceTracker outperformed random forests for classification of PhyloChip data, consistent with previous work that evaluated these methods using 16S rRNA pyrosequencing data (Knights et al., 2011b), Different animals have distinct fecal microbiomes that can be used for microbial source tracking (Dubinsky et al., 2012). Within a source type (e.g. humans) there are unique taxonomic groups of bacteria, or distinctive combinations of several taxonomic groups, that can be used to distinguish one source type from another (Fig. S1). The modified PhyloChip analysis for source detection focuses on 9001 of the most useful 16S rRNA gene targets that distinguish human, ruminant, horse, pig, dog and bird sources based on the composition of their fecal microbial communities. An advantage of the quartet method was the significant reduction of probes needed for fecal identification, approximately 3% of the total probes present on the PhyloChip. For routine screening of fecal sources the next logical step would be to create a smaller, more inexpensive microarray with only probes that are useful for source tracking.

In the Russian River, frequent detection of fecal signal in samples with FIB counts below USEPA precautionary thresholds (Beach Action Values) indicate that PhyloChip quartet analysis is a more sensitive indicator of fecal contamination than conventional FIB tests. PhyloChip categorization primarily relies on molecular detection of Bacteroides and Clostridia sequences that comprise an overwhelming majority (~95%) of the bacteria population in human stool (Eckburg et al., 2005; Segata et al., 2012) (Figs. S1, S3). In contrast, *E. coli* and enterococci each comprise less than 0.2% of human stool bacteria (Eckburg et al., 2005; Segata et al., 2012), and thus may be diluted and degraded to extinction faster than Bacteroides and Clostridia that are 10^2-10^3 times more abundant than FIB used for water quality tests. Furthermore, FIB assays measure only culturable bacteria that grow upon incubation, whereas the DNA-based assay is unaffected by the degree to which different environmental conditions alter bacterial culturability, although the DNA-based microbial signature may overestimate risk if the signature bacteria and associated pathogens are nonviable. The community DNA approach takes advantage of all high-abundance gut bacteria to enhance the sensitivity and specificity of the assay compared to single target tests.

As demonstrated in this study, machine-learning models can be trained with both fecal sources and environmental background samples to account for cross-reactivity with 16S rRNA gene sequences that overlap between fecal and the environmental background. This approach increases the specificity of the assay and minimizes cross-reactivity problems with 16S rRNA gene sequences that are common among fecal and environmental sources. Evidence is emerging that sediments and other environmental habitats can promote survival and growth of *Bacteroides* that are considered host-specific and increasing used for (Drexler et al., 2014; Kim and Wuertz, 2015). Most microbial source tracking tests are validated in controlled laboratory experiments against other fecal sources, but cross-reactivity with environmental sources such as anaerobic sediments and decaying vegetation is less frequently tested. Fecal source identification by PhyloChip minimizes cross-reactivity problems through the parallel detection of thousands of phylogenetically diverse markers, the combination of which are not found in environmental sources. In addition, the machine learning classification approach deemphasizes any cross-reactive sequences that occur among training sets to further improve specificity.

In many samples with high counts of *E. coli* and enterococci there was no detectable fecal signal from any source (Table S2). The highest FIB counts occurred during the wet season in tributaries throughout the watershed (Fig. S5, Table S2), and conventional *E. coli* and enterococci counts were correlated with the average OTU hybridization intensity of *Escherichia* OTUs ($r^2 = 0.73$) and *Enterococcus* OTUs ($r^2 = 0.46$), respectively (Fig. S5). High FIB counts were associated with large shifts in overall microbial community structure (Fig. S5). Nearly all (96%) of the 500 most enriched OTUs in samples with FIB exceedances occurred in the Flavobacteria (*Flavobacterium*), Sphingobacteria (*Pedobacter*), Alphaproteobacteria (*Sphingomonas*), Betaproteobacteria (*Janthinobacterium, Massilia*)

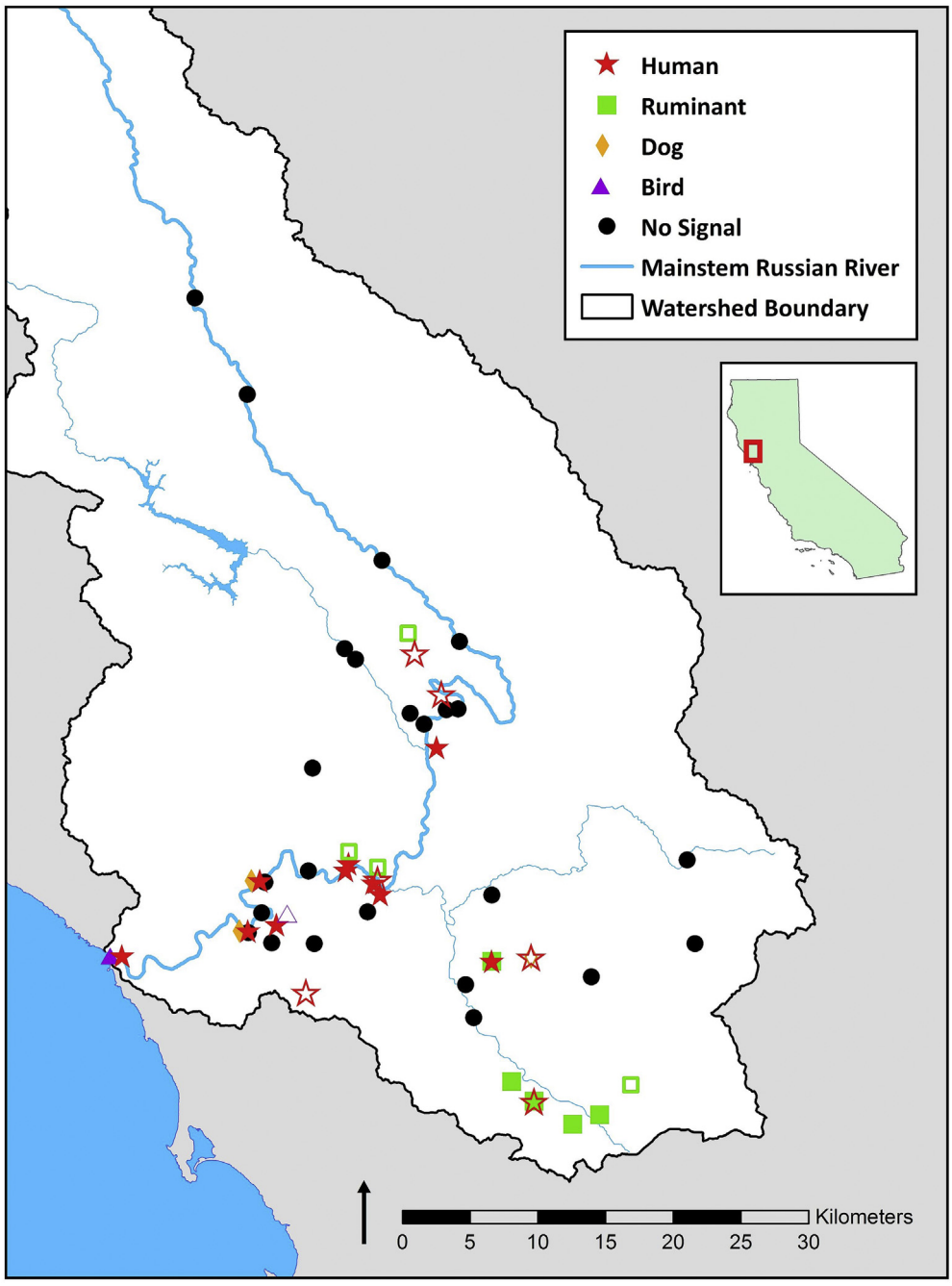

Fig. 3. Detected sources of fecal bacteria in the Russian River watershed in all dry and wet season samples. Closed colored symbols represent strong source signals (likelihood ratio > 10), open symbols represent moderate source signals (likelihood ratio >5 and < 10) and black circles represent no signal (all likelihood ratios < 5). Some locations were sampled at multiple time points. See Table S2 for exact sample locations and times. The tributaries in the southeast portion of the watershed are Mark West Creek and Laguna de Santa Rosa.

and Gammaproteobacteria (*Citrobacter, Erwinia, Klebsiella, Pantoea, Pseudomonas*) (Fig. 4). With the exception of some Enterobacteraceae OTUs (coliforms), none of the OTUs that were most enriched in samples with high FIB counts were features commonly found in any fecal microbial communities (>25% of samples in any one source type). However, the Flavobacteria, Sphingobacteria and Proteobacteria genera that were enriched in high FIB samples are common bacteria in freshwater ecosystems and include taxa that rapidly grow in response to nutrient and carbon enrichment (Kirchman, 2002; Fierer et al., 2007; Newton et al., 2011). These organisms, along with coliforms and enterococci, are typically found attached to suspended particles transported during stormwater events (Jeng et al., 2005; Pachepsky and Shelton, 2011; Fisher et al., 2015). Flavobacteria and Sphingobacteria are often found to be associated with detrital particles and algal blooms (Kirchman, 2002). Betaproteobacteria are also dominant bacteria on organic aggregates and streams with high detrital loading (Fazi et al., 2005; Simon et al., 2002).

The occurrence of abundant coliforms and enterococci in the absence of Bacteroidales and Firmicutes that dominate fecal sources indicates that the majority of FIB in the Russian River watershed do not originate from fecal inputs, but are likely sourced from the

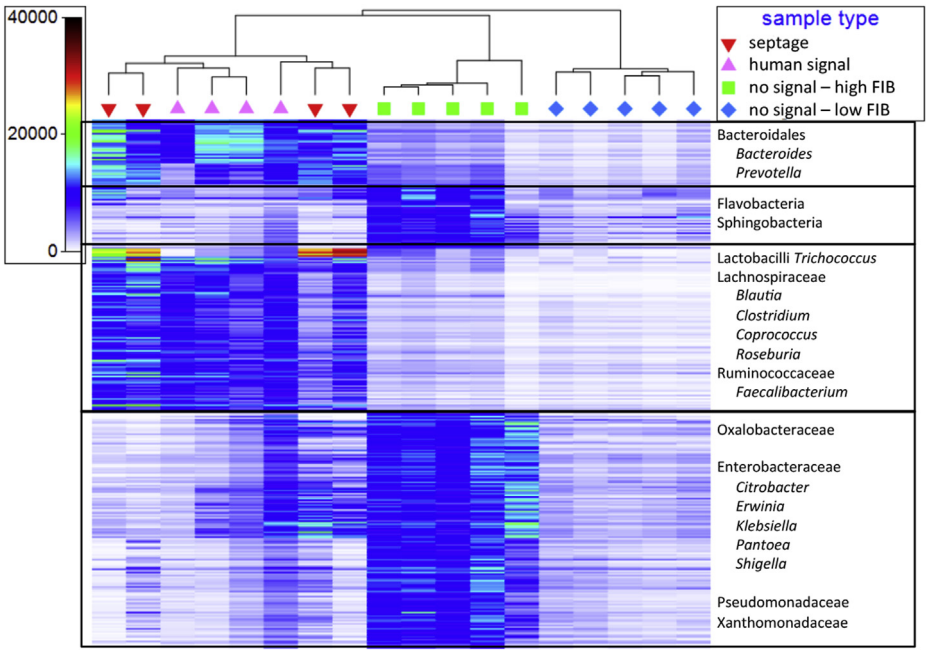

Fig. 4. Most abundant bacterial taxa in samples with either strong fecal signal or no fecal signal with high numbers of FIB. Water samples with the strongest fecal signal (positive likelihood ratio > 100) and no fecal signal (positive likelihood ratio < 5) but high FIB counts (>6000 MPN/100 mL) are shown. Raw septic waste samples and water samples with low FIB counts (<30 MPN/100 mL) and no fecal signal are shown for comparison. Samples with high FIB counts and no fecal signal were dominated by Flavobacteria, Sphingobacteria, Oxalobacteraceae and Gammaproteobacteria (Enterobacteriaceae, Pseudomonadaceae, Xanthomonadaceae).

surrounding environment and growing *in situ*. Co-enrichment of FIB with aquatic bacteria that naturally thrive in nutrient and carbon rich waters suggests FIB are responding to growth-promoting conditions following rainfall. Wet season runoff flushes carbon and nutrients into streams, and may also flush in particle-associated bacteria from stream bank sediments and soils. Both types of widely used fecal indicator bacteria, *E. coli* and enterococci, naturally occur in a variety of environmental habitats that may influence stream and river microbial communities after runoff, including soils, sediments, beach sands, and a variety of aquatic vegetation (Badgley et al., 2010; Byappanahalli et al., 2003, 2012; Hardina and Fujioka, 1991; Ishii et al., 2006; Whitman et al., 2014; Yamahara et al., 2007). These naturalized populations are stable and ubiquitous in many geographic areas and confound the use of *E. coli* and enterococci as indicators of fecal contamination.

Microbial community analysis is likely to be an increasingly valuable tool for identifying both fecal and environmental sources of FIB so long as *E. coli* and enterococci are the standard indicators used to assess recreational water quality and serve as the basis for pathogen TMDLs. Rapidly evolving sequencing technologies and bioinformatics is enabling more comprehensive interrogation of microbial communities for microbial sources tracking. Recent studies have begun to compare molecular community approaches for source tracking in simple laboratory experiments (Cao et al., 2013), but more work is needed to evaluate performance under field conditions with decayed fecal signals in complex microbial backgrounds.

We hypothesize that the closed format of the PhyloChip microarray has an advantage over open format next-generation sequencing for reliably identifying fecal signal within background environmental microbial communities because it avoids problems with non-uniform sample depth, low reproducibility due to random sampling, and sequencing biases (Probst et al., 2014; Zhou et al., 2015). Advantages of the microarray approach for source tracking are its more consistent detection of low abundance taxa, and fixed targeting of an established set of important marker genes from source microbiomes that are always probed for rather than randomly sampled. This yields better reproducibility, better comparability among samples, and less variation in sensitivity due to fluctuations in dominance of background microbial populations (Zhou et al., 2015). We also hypothesize that the PhyloChip method, which is based on 16S rRNA gene amplicon analysis, produces more reproducible and accurate results than methods based on whole genome amplification such as the recently described MST microarray (Li et al., 2015). Whole genome amplification is known to produce non-uniform distortions across phylogenetic clades and different sample types that can negatively affect its performance for fecal source tracking and detection of waterborne pathogens (Probst et al., 2015). Nonetheless, PCR bias and inhibition are always a concern with any PCR-based source tracking method, including all commonly used methods that target 16S rRNA genes such as PhyloChip, quantitative PCR, next generation sequencing and TRFLP. Another limitation of the PhyloChip approach is that the method is currently designed for source detection rather than source apportionment and quantification.

Comparative trials will need to further evaluate the performance and practicality of PhyloChip and other emerging technologies, and explore toolbox approaches that couple these methods with more targeted markers for even better results (Ahmed et al., 2015). Future work should evaluate the performance of these methods as source signals are decayed and diluted in the environment. Source tracking investigations can only benefit from more complete microbial surveillance to disentangle the complexities of non-point source pollution.

5. Conclusions

- A microbial source tracking test based on the PhyloChip microarray can simultaneously detect fecal signals from humans, dogs, birds, ruminants, horses and pigs. The use of a

core set of diagnostic probes with a machine-learning classifier results in accurate identification of these source types. The SourceTracker classifier performed better than the random forest classifier.

- The test is more sensitive than conventional FIB tests for detecting fecal pollution. Human fecal signal was frequently detected in the Russian River near communities with onsite wastewater treatment, even when conventional FIB counts were below water quality limits. Ruminant contamination was also detected in areas near dairy farms and pastureland.
- Concurrent characterization of microbial community structure in the Russian River watershed suggested high FIB counts were often not from fecal sources but due to environmental populations enhanced by runoff.
- Source tracking based on phylogenetic microarrays gives a comprehensive assessment of the bacterial community associated with indicator organisms and can reveal both fecal and environmental causes of FIB impairments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.08.035.

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