Potential Human Pathogenic Bacteria in a Mixed Urban Watershed as Revealed by Pyrosequencing

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Abstract

Current microbial source tracking (MST) methods for water depend on testing for fecal indicator bacterial counts or specific marker gene sequences to identify fecal contamination where potential human pathogenic bacteria could be present. In this study, we applied 454 high-throughput pyrosequencing to identify bacterial pathogen DNA sequences, including those not traditionally monitored by MST and correlated their abundances to specific sources of contamination such as urban runoff and agricultural runoff from concentrated animal feeding operations (CAFOs), recreation park area, waste-water treatment plants, and natural sites with little or no human activities. Samples for pyrosequencing were surface water, and sediment collected from 19 sites. A total of 12,959 16S rRNA gene sequences with average length of ≤400 bp were obtained, and were assigned to corresponding taxonomic ranks using ribosomal database project (RDP), Classifier and Greengenes databases. The percent of total potential pathogens were highest in urban runoff water (7.94%), agricultural runoff sediment (6.52%), and Prado Park sediment (6.00%), respectively. Although the numbers of DNA sequence tags from pyrosequencing were very high for the natural site, corresponding percent potential pathogens were very low (3.78–4.08%). Most of the potential pathogenic bacterial sequences identified were from three major phyla, namely, Proteobacteria, Bacteroidetes, and Firmicutes. The use of deep sequencing may provide improved and faster methods for the identification of pathogen sources in most watersheds so that better risk assessment methods may be developed to enhance public health.

Introduction

Traditionally, fecal indicator bacteria are used as indicators of pathogen levels of water bodies in many localities [1], instead of direct identification of individual pathogens [2]. A large number of bacteria, viruses, fungi, protists, and animalia have been identified as pathogenic for humans [3,4] and a majority (n = 1415) are water-borne [4]. Pathogens in river water can be a problem if sewage is incompletely treated or untreated. This has been reported for many large cities in developing countries where rivers as reported for the Tiete and Pinheiros River, Brazil [3,6] and the Ganges River in India [7,8], are known to carry high loads of fecal bacteria. However, in developed countries, such as, the United States, Canada, and Western Europe, where sophisticated and well managed waste-water treatment facilities are available for the treatment of domestic waste; the presence of pathogenic bacteria may not be as severe as in developing countries. However, in a large, mixed, and complex watershed, there may be significant concentrations of pathogens originating from different sources feeding into the watershed.

In the Santa Ana River Watershed (southern California) there are significant amounts of water contaminants from different sources. The major sources of non-point contaminants into the river are municipal wastewater, agricultural waste discharges from dairy runoff, urban runoffs, and a combination of these sources. Currently, the Santa Ana River is impacted by one of the highest concentrations of dairy cattle in the United States. The watershed is undergoing drastic changes. In general, the varying land uses in the watershed include agriculture, open space, and rapidly growing urban areas [9–11]. In 1993, approximately 340 animal-confinement facilities having over 386,000 animals, mostly dairy cows, operated within the area that is mostly drained by Chino, Cypress, and Cucamonga Creeks. Pollutants in the watershed mainly consist of pathogens and nutrients due to the densely populated areas, agricultural activities, and urban and storm-water runoff in the region. Different federal, state, and private agencies have monitored fecal bacterial composition in the surface water [9–11], but little has been done to determine the main sources of pathogenic bacteria within the water bodies due to the complexity of the watershed. Also, the Santa Ana River is a major source of domestic water supply for over 2 million people that live in Orange County, California. The river is critical for replenishment of Orange County's Groundwater Basin since over 2 million residents in Orange County depend on groundwater for 75% of their water needs [9]. Any factor in the watershed which
degrades the river affects the quality of water for domestic water supply.

For water quality assessment, *E. coli* or enterococci are the main thermotolerant enteric bacteria commonly used to estimate the load of pathogenic bacteria in water and for microbial source tracking. Concerns have been raised about the suitability of *E. coli* or other coliform bacteria in describing the pathogenic potential of a water body [12]. For instance, the prevalence and diversity of *Salmonella* spp. (non-coliform bacteria) and their correlation with fecal pollution indicators and total heterotrophic bacteria counts were investigated in northern Creek rivers. The numbers of *Salmonella* isolates were significantly higher during summer (warm) months than winter (cold months), and the overall counts for all other microorganisms were also higher during warm months [13]. A recent Canadian study revealed a poor correlation between the numbers of coliforms and *Campylobacter* species and suggested genus-specific monitoring techniques as alternative [14]. Data on the occurrence/densities of pathogens and the impacting factors in natural waterways not only provide direct evidence of potential human health risks but also enhance predictions of the fate and transport of pathogens in surface water systems and help identify practices that reduce exposure risks [15–17].

In this study, the diversity and the relative abundance of pathogenic bacteria were analyzed at the genus level based on 454 pyrosequencing of bacterial 16S rRNA gene sequences. This technique has been used successfully to reveal bacterial pathogens in biosolids [18], watershed [19], and sewage-treatment plants [20]. A total of 12,959 sequences were obtained from 40 water and sediment samples, and were assigned to taxonomic ranks based on RDP Classifier and Greengenes. The overall objective of this study was to identify pathogens, including those not traditionally monitored in water and correlate their abundances to specific sources of contamination.

**Materials and Methods**

**Ethics Statement**

Throughout this study, normal operational procedures of the forest service and state park on the creeks and channel were followed. Permits to enter the parks and channels were obtained from the regional parks.

**Study Area and Sample Collection**

This study was conducted in the middle Santa Ana River (MSAR) watershed area that covers ~1,264 km² and lies largely in the southwestern corner of San Bernardino County and northern corner of Riverside County and included a small part of Los Angeles County (i.e., Pomona/Claremont area) [21]. The current population of the watershed, based upon the 2000 census data, is ~1.4 million people [10]. Land use in the MSAR watershed varies between urban and agricultural. Although originally developed as an agricultural area, the watershed is rapidly urbanizing. Open space areas include the National Forest and State Park lands. The principal remaining agricultural area in the watershed was formerly referred to as the Chino Dairy Preserve. This area is located in the south-central part of the Chino Basin sub watershed and contains approximately 200,000 dairy cows in a 77 km² area (although this number is quickly declining as the rate of urban development increases) [10].

The mean annual rainfall for MSAR watershed is ≤380 mm per annum, and predominantly falls between December and April resulting in a base stream flow that is highly variable between seasons [10]. The mean annual stream flow from United states Geological Service (USGS) gauged data from Chino Creek representing urban runoff (S 3-Chino Creek @ Schaefer Ave) was 133.6 m³ s⁻¹ and at Cypress channel representing agricultural runoff (S6– Cypress channel @ Schaefer Ave) was 96.3 m³ s⁻¹. Sampling sites used for this study are shown in Table 1. Locations were selected for sediment and surface water sample analyses based on historical data obtained for the total maximum daily loads (TMDL) for bacterial indicators for the MSAR watershed [10]. All sampling locations, with site names, descriptions, and geographic positioning system (GPS) coordinates are listed in Table 1. Water samples at three waste-water treatment plants (WWTPs) were retrieved from the sampling ports located at the treatment plant site for sample collection (Table 1). The plants discharged tertiary-level-treated water downstream resulting in continuous but variable stream flow throughout the year along Chino Creek. Cypress Channel is more affected by dairy or agricultural runoff, and Chino Creek affected more by WWTPs and urban runoff. The Ice House Canyon (S1; Table 1), which is an open space or natural site, was used mainly as the control site because runoff from this site was mainly from melting snow. Ice House Canyon Creek is located in the San Gabriel Mountains and is a tributary to San Antonio Creek approximately 2.1 km upstream of Mt. Baldy Village. Historical data for Ice House Canyon for fecal coliforms has averaged 9 CFU 100 ml⁻¹ over a five-year period, 2000 to 2005 [10]. Site M1 has the same water quality characteristics as S1, and it is at a lower elevation.

Water samples were collected using sterile Nalgene sampling bottles [22]. All samples were collected in duplicate. For sites that were deep enough to obtain samples, grab samples were collected at ~10–15 cm below the surface of the water. Sites with a shallow flow were sampled using a sterile stainless-steel sampling device. Sediment samples from the 0- to the 10-cm depth were taken from the Creek or river banks using ethanol-disinfected core tubes and stored in Whirl-Pak bags at 4°C until processed; usually within 24 h. Field parameters consisting of electrical conductivity, pH, temperature, turbidity, and dissolved oxygen were taken at each sample location. Sample turbidity was determined using a Hach model 2100P Portable Turbidimeter (Loveland, CO) according to the manufacturer’s instructions and was calibrated daily.

**DNA Extraction and Purification from Sediment and Water Samples**

Total bacterial DNA was extracted from 500 mg of sediment samples and from 250 mg pellet from a concentrated effluent sample prepared from filtered water samples after centrifugation at 3,000 xg for 10 min. DNA was extracted using Power Soil and Water DNA kits (MO BIO, Inc., Solana Beach, CA), according to the manufacturer’s protocol with slight modifications. Extracted DNA (2 μL) was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and run on a 1.0% agarose gel before pyrosequencing.

**Pyrosequencing**

DNA samples from sediment and water were submitted to Core for Applied Genomics and Ecology (University of Nebraska Lincoln, NB) for PCR optimization and pyrosequencing analysis. The V1–V2 region of the 16S rRNA gene was amplified using bar-coded fusion primers with Roche-454 A or B titanium sequencing adapters, followed by a unique 8-base barcode sequence (B) and finally the 5’ ends of primer A-8FM (5’CCATCTCATACCGTCCGTCGTCCGTACGGACATCAGBBBBBB- BBAAGATTTGTACGACGCTCAAG and of primer B-357R (5’-CCTATCGCCGTGTGTGGCCTCGAGCAGGACTGACGGGTB- GBBB- BBBBB CTGCGTGCCTGCGCGTA-3’). All PCR reactions were quality-controlled for amplicon saturation by gel electrophoresis;
Analysis of Pyrosequencing Data

Bacterial pyrosequencing population data were further analyzed by performing multiple sequence alignment techniques using the dist.seqs function in MOTHUR, version 1.9.1 [25]. MOTHUR was also used to assign sequences to operational taxonomic units (OTUs, 97% similarity, using the H-cluster function); Sequences were denoised using the ‘pre.cluster’ command in MOTHUR platform to remove sequences that are likely due to pyrosequencing errors [26,27]. PCR chimeras were filtered out using Chimeras Slayer [28]. Following chimera detection, the RDP Classifier function was used to assign identities to the bacterial pyrotag sequence data [29]. In addition, any sequences shorter than 400 bp in length and/or containing ambiguous base pair reads were removed from the data set.

MOTHUR was used to align the re-sampled data set and create an all-sample distance matrix, as well as assign sequences to OTUs. Overlap was calculated using the Yue-Clayton similarity estimator ($\Theta_{BC}$), a metric that is scored on a scale of 0 to 1, representing absolute dissimilarity to 100 similarities [25,30]. The metagenomic data sets of this study were deposited in Sequence read Archive under the project name SRP028870: Total bacteria from river sediment and runoff water Targeted Locus (Loci) with accession numbers SRX335804 to SRX335812 [http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?study=SRP028870].

Results

Taxonomic Assignment of the Sequences

Between 126–5,109 sequence tags (length >400 bp) were generated for each sample, resulting in 12,959 sequences and 6,462 OTUs in total from all nine sampling sites. To find the potentially pathogenic bacterial sequences from such a large amount of sequences, a reference human pathogenic bacteria list, including the species and genus names, disease caused, and the risk group (RG) was compiled using the number of sequences within each of hazard, 2011. Although it might not be a complete list of all the human pathogenic bacteria, it covers a broad range with RG 0.03 Jukes-Cantor distance of known pathogens [31–33] and NIH Appendix B: Classification of human etiologic agents on the basis of hazard, 2011. Although it might not be a complete list of all the human pathogenic bacteria, it covers a broad range with RG agents per NIH guidelines for human etiologic agents.

The sequences obtained in this study were first assigned to proper taxonomic ranks at the genus level using RDP Classifier in MOTHUR version 1.9.1 [25], and to the species level using taxonomic ranks at the genus level using RDP Classifier in MOTHUR version 1.9.1 [25], and to the species level using

Table 1. Sampling locations for middle Santa Ana River pathogen source evaluation study*.

| Site # | Site locations | Land use | GPS* Location |
|-------|----------------|----------|---------------|
| S1    | Ice House Canyon | Open Space | N34' 15.057 min; W117 37.977 min; 1447 m |
| M1    | Cucamonga Creek @ OCWD** Ponds | Open Space | San Bernardino County Flood Control District |
| S2    | Chino Creek @ Central Ave. | Urban runoff | N33' 58.420 min; W117 41.302 min; 174 m*** |
| S3    | Chino Creek @ Schaefer Ave. | Urban runoff | N34' 00.246 min; W117 43.628 min; 207 m |
| S4    | San Antonio Wash @ County Drive | Urban runoff+Commercial wash out | N30' 01.543 min; W117 43.652 min; 222 m |
| S5    | Chino Creek @ Riverside Drive | Urban runoff | N34' 01.144 min; W117 44.204 min; 207 m |
| S6    | Cypress Channel @ Schaefer Ave. | Agricultural Runoff | N34' 00.262 min; W117 39.766 min; 208 m |
| S7    | Cypress Channel @ Kimball Ave. | Agricultural Runoff | N33' 58.113 min; W117 39.624 min; 177 m |
| S8    | Cypress Channel @ Golf Course | Agricultural Runoff | N33' 57.057 min; W117 39.555 min; 160 m |
| S9    | Big League Dreams storm drain | Urban runoff | N33' 57.364 min; W117 40.788 min; 163 m |
| S11ur | Cucamonga Creek @ Regional Water Recycling Plant #1 | Effluent from WWTP**** | N34' ; 01.853 min; W117 35.946 min; 246 m |
| S11ww | Cucamonga Creek @ Regional Water Recycling Plant #1 | Effluent from WWTP**** | N34' ; 01.853 min; W117 35.946 min; 246 m |
| S12   | Chino Creek @ Pine Ave. | Urban runoff+ wastewater | N33' 56.941 min; W117 39.986 min; 155 m |
| S13   | Inland Empire Utilities Agency Regional Water Recycling Plant #5 | Effluent from WWTP | N33' 57.840 min; W117 40.826 min; 180 m |
| S14   | IEUA Carbon Canyon Waste Reclamation Facility | Effluent from WWTP | N33' 58.799 min; W117 41.655 min; 184 m elevation; |
| ST2   | Santa Ana River @ Prado Dam | Urban Runoff | N33' ; 54.737 min; W117 38.711 min; 141 m |
| C3    | Prado Park outlet | Urban Runoff+ waste discharge | N33' ; 56.402 min; W117 38.763 min; 166 m |
| ST5   | Santa Ana River @ River road | Urban Runoff | N33' ; 55.405 min; W117 35.894 min; 155 m |
| M5    | OCWD (Prado)Wetlands Effluent | Wetland treated (bacteria loaded) OCWD | N33' ; 54.737 min; W117 38.711 min; 141 m |

*Modified from Ibekwe et al. [21].

Sampling from site S10 was discontinued after one sampling due to construction activities on the site.

GPS: geographic positioning system.

OCWD: Orange County Water District.

WWTP: waste water treatment plant.

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and intensity was quantified against standards using GeneTools software (Syngene, Frederick, MD). The resulting DNA amplicon products were quantified using PicoGreen (Invitrogen, Grand Island, NY) before sequencing using Roche-454 GS FLX titanium.
Greengenes. In order to check the correctness of the assignment results of the two methods, sequences from the original FASTA files were extracted, and the individual sequences RDP Classifier were searched using online BLAST (≥99%) search (http://blast.ncbi.nlm.nih.gov/blast) which is considered one of the most reliable sequence searching tools used in taxonomic studies.

**Bacterial Community Composition and Diversity**

The 454 pyrosequence libraries ranged from 126 sequences from sediment samples from urban runoff to 5,109 sequences at the natural site sediment, and contained between 90 OTUs and 1,700 OTUs, respectively, as shown in the rarefaction curve (Fig. 1). Members of at least 26 bacterial phyla were detected with the 454 pyrosequencing technique. Most of the potentially pathogenic bacterial 16S rRNA encoding DNA sequences were identified from the five major phyla shown in Figure 2. *Proteobacteria* (40.73%) and *Bacteroidetes* (10.50%) were encountered most frequently. Sediments collected from sites affected by agricultural activities and the natural site contained the most diverse sequences with sequences representing the five phyla.

**Population of Potential Pathogenic Bacteria**

Using RDP Classifier to identify potential pathogenic bacteria at the genus level, Table 2 shows the number of sequences identified as potentially pathogenic bacteria by 454 pyrosequencing in a mixed watershed. The relative abundance of the 36 genera identified at the nine sites showed *Aeromonas*, *Clostridium*, *Bacillus*, *Pseudomonas*, and *Treponema*, as indicated in Table 2, occurred at all nine sites. However, some of these may be at the RG 1 level or opportunistic pathogens. The most dominant genus in the agricultural sediment was *Bacillus*, although this did not include any of the highly recognized pathogenic *Bacillus* spp. However, *Aeromonas*, *Clostridium* and *Treponema* were the most abundant in the sediment and surface water in the natural sites and Prado Park.

We compared the sequences obtained using pyrosequencing with those of known pathogenic bacteria at the species level, and this information provided a more accurate estimation of the pathogenic populations in the samples. For each known pathogenic bacterial species, a representative 16S rRNA gene sequence was retrieved from Greengenes database (http://greengenes.lbl.gov) to create a reference database. We generated two databases; one with the RDP Classifier to the genus level and the other with the Greengenes to the species levels as recommended [34]. A total of 461 species were obtained and subjected to BLAST search to confirm their true species identity as pathogenic bacteria. Most of our FASTA sequences from 454 pyrosequencing were in agreement to the genus level with RDP Classifier. However, the Greengenes identifications of sequences to the species level were not always in a 100 percent agreement with the blast search, hence we used all the data from BLAST searches as a baseline/standard.

All the sequences that were assigned to the species level as potential pathogenic bacteria from blast search are presented in Table 3. Of the 36 pathogen genus considered (Table 2), 56 species included sequences that were counted as potential
pathogens with RG 2 ratings according to NIH guidelines for human etiologic agents (Table 3) [31–33] are discussed below.

*Mycobacteria, Legionella, Treponema, and Clostridia* were the most common, with different species and were the only pathogen present in most of the sites sampled. The most common *Mycobacteria* spp were the opportunistic pathogens, *M. morikami*, *M. farrinogenes*, *M. brunae*, *M. aurum*, *M. pallens*, and *M. tusdae*. All except *M. brunae* that was found in Chino Creek sediment, which is impacted by urban runoff, were found in natural site sediments. The most common potentially pathogenic *Clostridia* spp. was *C. bartletti* which was isolated from natural site water and in WWTP. Most of our sequences had very high similarity levels with known human pathogens (Table 3). There were many other species of potentially pathogenic bacterial sequences that were recovered from our samples. Most notable were two sequences that were 96% similar to *Brucella microti* and 87–98% similar to *Rickettsia* spp. which are both RG 3 pathogens (Table 3). The *Brucella microti* sequences were recovered from natural site sediments while those of *Rickettsia* spp were recovered from both water and sediment samples from natural sites and WWTP.

**Discussion**

Total potential pathogens (%) were highest in urban runoff water (7.94%), agricultural runoff sediment (6.52%) and Prado Park sediment (6.00%), respectively (Table 2). Although the numbers of sequence tags from 454 were very high for the natural site, percent potential pathogens were very low. The higher percent potential pathogen in urban runoff water is a very serious concern. Most of our urban runoff and agricultural runoff samples contained opportunistic pathogens that are common in soil such as (*Clostridia*, *Mycobacteria*, and *Nocardia* spp.). One human pathogen *Staphylococcus* spp. was found mainly in the natural sites and Prado Park, and *Staphylococcus aureus* sequence in the natural site were confirmed by BLAST searches. However, two sequences of *Legionella pneumophila* were recovered from water samples collected from the natural site. It should be noted that *L. pneumophila* causes about 90% of all reported cases of legionellosis in the United States [35]. Water is the major reservoir for legionellae, and the bacteria are found in freshwater environments worldwide. In another study legionellae were detected in 40% of freshwater environments by culture and in 80% of these samples by PCR [36].

Sequences from *Mycobacterium* genus had a relatively high similarity (96–99%) to the known pathogenic species in this genus. Most of the sequences uncovered in this study were from sediment samples, which was contrary to our expectations because *Mycobacterium* spp are known to be very common in water samples. Two other genus that were found in many samples and with different species representatives were *Aeromonas* and *Treponema*. *Aeromonas* spp were found in water samples from the natural site, WWPTs, and Chino Creek which is impacted by urban runoff. The most common species sequences from the Creek samples was *Aeromonas hydrophila* which is known to be very toxic to many organisms because it produces Aerolysin Cytotoxic Enterotoxin (ACT), a toxin that can cause tissue damage [37]. Five *Treponema* spp sequences were found (Table 2), and this pathogen had one of the most widespread sequences which were found in 6 of 9 sources throughout the watershed. These pathogens are mainly anaerobic, fastidious, highly mobile, and are found in the oral cavity, digestive tract and genital areas of human, animals, and insects [38]. Several species of this pathogen are associated with syphilis in human, human periodontal infection, and bovine digital dermatitis [38]. The diversities and abundances of different genus were quite distinct at the natural sites, indicating potential pathogenic bacteria at this site, despite the lack of inputs from contaminants. This may be dominated by sequences from organisms with many generations.

As the goal of this study was to identify pathogens, including those that are not traditionally monitored, the sequencing depth necessary to identify these pathogens was not known prior to the study. With the goal of directing future monitoring and risk assessment efforts, the sample sites selected were those that were previously used for TMDL evaluation study [10]. The pathogenic bacterial sequences identified in this study include some of the most common bacteria that are very pathogenic and could be used for microbial source tracking such as *Clostridia*, *Rickettsia*, and *Brucella* spp. It should be noted that we did not identify sequences that belong to some of the most pathogenic *Clostridia* spp, such as *C. botulinum*, *C. difficile*, *C. perfringens*, *C. tetani*, and *C. sordelli*. *Clostridium* consists of around 100 species that include common free-living bacteria as well as important pathogens [39]. Only five
sequences that were 93–98% similar to *Rickettsia* spp. and two sequences that were 96–97 similar to *Brucella microti* were identified in this study. These are RG 3 agents that are associated with serious or lethal human disease for which preventive or therapeutic interventions may be available in comparison to RG 4 agents for which preventative or therapeutic interventions are not available. It should be noted that *Rickettsia* spp. are carried by many ticks, fleas, and lice, and cause diseases in humans, such as, **Table 2.** Number of sequence tags assigned to potential pathogenic genus from Santa Ana River watershed as determined by 454 pyrosequencing using RDP Classifier databases.

| Genus                      | Agricultural Runoff – Sediment | Agricultural Runoff – Water | Urban Runoff – Sediment | Urban Runoff – Water | Natural Site – Sediment | Natural Site – Water | Prado Dam - Sediment | Prado Dam - Water | Prado P - Water | *WWT P - Water |
|----------------------------|--------------------------------|----------------------------|-------------------------|----------------------|------------------------|----------------------|----------------------|------------------|----------------|-----------------|
| Acholeplasma               | 0                              | 0                          | 0                       | 0                    | 0                      | 0                    | 0                    | 0                | 0              | 1               |
| Acinetobacter              | 0                              | 0                          | 1                       | 0                    | 1                      | 4                    | 2                    | 1                | 0              | 0               |
| Aeromonas                  | 0                              | 4                          | 1                       | 3                    | 32                     | 20                   | 0                    | 1                | 0              | 0               |
| Aliseewanella              | 0                              | 2                          | 0                       | 1                    | 0                      | 14                   | 0                    | 1                | 0              | 0               |
| Arcobacter                 | 3                              | 2                          | 0                       | 1                    | 3                      | 7                    | 2                    | 0                | 1              | 0               |
| Bacillus                   | 21                             | 0                          | 0                       | 11                   | 1                      | 0                    | 2                    | 0                | 0              | 0               |
| Bartonella                 | 0                              | 0                          | 0                       | 0                    | 0                      | 1                    | 3                    | 1                | 2              | 1               |
| Borrelia                   | 0                              | 0                          | 0                       | 0                    | 0                      | 0                    | 0                    | 0                | 0              | 0               |
| Brucella                   | 0                              | 0                          | 0                       | 0                    | 0                      | 1                    | 0                    | 0                | 0              | 0               |
| Burkholderia               | 1                              | 0                          | 0                       | 0                    | 6                      | 3                    | 2                    | 0                | 0              | 0               |
| Campylobacter              | 0                              | 0                          | 0                       | 0                    | 0                      | 1                    | 0                    | 0                | 0              | 0               |
| Candidatus                 | 0                              | 0                          | 0                       | 0                    | 1                      | 0                    | 4                    | 0                | 0              | 0               |
| Clostridium                | 3                              | 0                          | 0                       | 0                    | 23                     | 8                     | 11                   | 2                | 10             | 0               |
| Corynebacterium            | 0                              | 0                          | 0                       | 0                    | 1                      | 1                    | 1                    | 0                | 0              | 0               |
| Coxiella                   | 0                              | 0                          | 0                       | 0                    | 1                      | 1                    | 1                    | 0                | 0              | 0               |
| Erysiploethrix             | 0                              | 2                          | 1                       | 0                    | 13                     | 3                    | 19                   | 2                | 1              | 0               |
| Escherichia                | 0                              | 0                          | 0                       | 0                    | 3                      | 0                    | 1                    | 0                | 0              | 0               |
| Geodermatophilus           | 2                              | 0                          | 0                       | 0                    | 3                      | 0                    | 0                    | 0                | 0              | 0               |
| Helicobacter               | 0                              | 0                          | 0                       | 0                    | 1                      | 1                    | 6                    | 0                | 1              | 0               |
| Legionella                 | 0                              | 0                          | 0                       | 0                    | 6                      | 4                    | 0                    | 0                | 1              | 0               |
| Leptospira                 | 0                              | 2                          | 0                       | 0                    | 0                      | 6                    | 0                    | 1                | 0              | 0               |
| Moraxella                  | 0                              | 0                          | 0                       | 0                    | 0                      | 1                    | 0                    | 0                | 0              | 0               |
| Mycobacterium              | 2                              | 0                          | 0                       | 0                    | 9                      | 0                    | 0                    | 0                | 0              | 0               |
| Mycoplasma                 | 0                              | 0                          | 0                       | 1                    | 3                      | 0                    | 1                    | 0                | 0              | 0               |
| Neochlamydia               | 0                              | 0                          | 0                       | 0                    | 1                      | 0                    | 0                    | 0                | 0              | 0               |
| Nocardia                   | 0                              | 0                          | 0                       | 0                    | 2                      | 0                    | 1                    | 0                | 0              | 0               |
| Nocardioides               | 0                              | 0                          | 1                       | 0                    | 23                     | 1                    | 1                    | 1                | 0              | 0               |
| Pseudalteromonas           | 0                              | 0                          | 0                       | 0                    | 0                      | 2                    | 0                    | 0                | 0              | 0               |
| Pseudomonas                | 6                              | 11                         | 3                       | 3                    | 38                     | 30                   | 5                    | 2                | 7              | 0               |
| Rickettsia                 | 0                              | 5                          | 0                       | 1                    | 4                      | 4                    | 0                    | 2                | 0              | 0               |
| Rickettsiella              | 0                              | 0                          | 1                       | 0                    | 7                      | 1                    | 0                    | 1                | 0              | 0               |
| Serratia                   | 0                              | 0                          | 0                       | 0                    | 0                      | 0                    | 1                    | 0                | 0              | 0               |
| Shewanella                 | 0                              | 0                          | 0                       | 0                    | 3                      | 1                    | 1                    | 0                | 0              | 0               |
| Staphylococcus             | 0                              | 0                          | 0                       | 0                    | 0                      | 0                    | 0                    | 0                | 0              | 0               |
| Streptomyces               | 1                              | 0                          | 0                       | 0                    | 10                     | 2                    | 1                    | 0                | 0              | 0               |
| Treponema                  | 4                              | 0                          | 0                       | 0                    | 10                     | 1                    | 10                   | 1                | 1              | 0               |
| Total Number of Samples    | 660                            | 744                         | 178                     | 126                  | 5109                    | 3460                 | 1183                  | 806              | 693            |                 |
| Total Potential Pathogens  | 43                             | 29                          | 8                       | 10                   | 206                     | 134                  | 71                    | 18               | 30             |                 |
| Potential Pathogen Percentages | 6.52%                     | 3.90%                       | 4.49%                   | 7.94%                | 4.03%                   | 3.87%                | 6.00%                  | 2.23%            | 4.33%          |                 |

*WWTP: waste water treatment plant.

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**Table 3.** List of potential human pathogenic bacterial sequences identified from different sources within the Santa Ana watershed using 454 pyrosequencing obtained from RDP Classifier data.

| Genus       | Species      | Source*          | Risk group | Diseases#                                                                 | Percent Similarities | Accession #** |
|-------------|--------------|------------------|------------|---------------------------------------------------------------------------|----------------------|---------------|
| Aeromonas   | hydrophila   | N W CCW CNW      | 2          | Gastroentertitis                                                          | 98–99                | NR_043638     |
|             | veronii      | NW               | 2          | Septicemia                                                               | 99                   | NR_044845     |
| Acinetobacter| haemolyticus | PW               | 2          | Bloody diarrheea                                                          | 98                   | NR_026207     |
|             | junii        | NW               | 2          | Septicemia                                                               | 99                   | NR_026208     |
|             | johnsonii    | CNS              | 2          | Nosocomial                                                               | 98                   |               |
| Legionella  | pneumophila  | NW               | 2          | Legionnaires’ Disease, Pontiac fever                                      | 96                   | NR_041742     |
|             | droanskii    | WW, NS           | 2          | Pneumonia                                                                | 97                   | NR_036803     |
|             | brunensis    | NS               | 2          | Pneumonia or flu-like illness,                                             | 93                   | NR_026520     |
|             | impetisoli   | NS               | 2          | Pneumonia or flu-like illness,                                             | 94                   | NR_041321     |
|             | drancourtii  | NW               | 2          | Pneumonia                                                                | 93                   | NR_026335     |
| Bartonella  | chomeli      | NS               | 2          | Cat scratch fever by henselae                                             | 87                   | NR_025736     |
| Brucella    | microti      | NS               | 3          | Brucellosis                                                              | 96                   | NR_042549     |
| Burkholderia| mimosarum    | PS               | 2          | Nonpathogenic                                                            | 98                   | NR_043167     |
| Clostridium | rectum       | NW, PS           | 2          | Gastroenteritis??                                                         | 94–95                | NR_029271     |
|             | cocleatum    | NW               | 2          | Nonpathogenic                                                            | 99                   | NR_026495     |
|             | acidisoli    | NS               | 2          | Nonpathogenic                                                            | 98                   | NR_028898     |
|             | cellobioparum| NW               | 2          | Nonpathogenic                                                            | 98                   | NR_026104     |
|             | bartletii    | NW, WW, NS       | 2          | Nonpathogenic                                                            | 99                   | NR_027573     |
|             | hiranonis    | WW               | 2          | Nonpathogenic                                                            | 99                   | NR_028611     |
|             | irregular    | WW               | 2          | Nonpathogenic                                                            | 92                   | NR_029249     |
|             | cellulovorans| NS, PS           | 2          | Nonpathogenic                                                            | 97                   | NR_027589     |
|             | aciditolans  | PS, NS           | 2          | Nonpathogenic                                                            | 98                   | NR_043557     |
|             | thermobutyricum| PS             | 2          | Nonpathogenic                                                            | 97                   | NR_044849     |
|             | sulfidigenes | NS3              | 2          | Nonpathogenic                                                            | 96–99                | NR_044161     |
|             | clarilavum   | CCS              | 2          | Nonpathogenic                                                            | 90                   | NR_041235     |
|             | citroniae    | NS               | 2          | From unspecified clinical infections (likely nonpatho.)                   | 96                   | NR_043681     |
|             | dispersicum  | NS               | 2          | Bacteraemia                                                              | 95                   | NR_026491     |
| Corynebacterium| appendicis  | NW               | 2          | Appendicitis*                                                            | 98                   | NR_028951     |
|             | callunae     | NS               | 2          | Appendicitis*                                                            | 97                   | NR_037036     |
| Erysipelothrix| inopinata   | CCW, PS, NW      | 2          | Nonpathogenic                                                            | 92–93                | NR_025594     |
| Escherichia | albertii     | NS               | 2          | Diarrheal disease                                                         | 99                   | NR_025569     |
| Helicobacter| brantae      | PS               |            | Nonpathogenic                                                            | 100                  | NR_043799     |
| Leptospira  | meyeri       | NW               | 2          | Unclear role/potentially                                                 | 94–95                | NR_043045     |
|             | wolbachii    | CCW              | 2          | Nonpathogenic                                                            | 99                   | NR_043046     |
|             | alexandri    | NW               | 2          | Pathogenic to animals                                                    | 94                   | NR_043047     |
| Mycobacterium| morioakaense| NS               | 2          | Pneumonia                                                                | 98                   | NR_025526     |
|             | brumae       | CCS              | 2          | Bacteraemia                                                              | 96                   | NR_025233     |
|             | aurum        | NS               | 2          | Bacteraemia, keratitis                                                   | 99                   | NR_029217     |
|             | pallens      | NS               | 2          | Not available                                                             | 99                   | NR_043760     |
|             | tusciae      | NS               | 2          | Lymphnode/chronic fibrosis                                                | 99                   | NR_024903     |
|             | farcinogenes | NS               | 2          | Prosthesis infection                                                      | 99                   | NR_042923     |
| Nocardia    | nova         | NS               | 2          | Nocardiosis                                                              | 99                   | NR_041858     |
| Rickettsia  | montanensis  | NW               | 3          | Nonpathogenic for humans                                                  | 90                   | NR_025920     |
|             | auschimannii | NW               | 3          | Tickborne rikettosis                                                     | 98                   | NR_026042     |
|             | asiatica     | WW               | 3          | Unknown pathogenesis for humans                                           | 87                   | NR_041840     |
can target community composition [45] and all concerned

Another advantage for this technique is high-throughput, which of the PCR-based detection techniques, which may introduce sequencing practice, this technique may overcome the limitation of the PCR-based detection techniques, which may introduce nonspecific amplification and highly relies on the primers selected. Another advantage for this technique is high-throughput, which can target community composition [45] and all concerned pathogens in a single detection. As shown in this study, this technique mainly contained pyrosequencing and bioinformatic analysis, which showed a comprehensive profile of detected bacterial pathogens within the watershed. It serves as a powerful and promising approach to monitor and track human bacterial pathogens. However, it is worthy to note that such molecular technique is difficult to exactly quantify pathogens in terms of cell number in surface water or sediment because of the high complexity to convert gene copy number to cell number.

### Conclusion

The main objective of this study was to identify potential sources of pathogens in the environment (i.e., mixed urban watershed) with respect to human exposure and risk. Based on deep sequencing, we were able to identify sources of potential pathogens belonging to RG 1, RG 2 and RG 3. This presents an added advantage because these pathogens could be further enriched and studied further or quantified using real-time PCR after designing primers at the genus level to focus on the quantification of pathogens with potential risk to human public health. This could lead to better understanding of pathogen loads in the environment and enabling of more effective assessment of their fate and transport in the environment.

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### Author Contributions

Conceived and designed the experiments: AMI. Performed the experiments: AMI ML. Analyzed the data: AMI SM. Contributed reagents/materials/analysis tools: AMI SM. Wrote the paper: AMI. AMI ML SM.

### References

1. Savichtcheva O, Okabe S (2006) Alternative indicators of fecal pollution: relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. Water Res 40: 2463–2476.

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**Table 3. Cont.**

| Genus   | Species       | Source* | Risk group | Diseases#          | Percent Similarities | Accession #** |
|---------|---------------|---------|------------|--------------------|----------------------|---------------|
| canadensis | NS            | 3       | Febrile disease | 87                 | NR_029155            |
| Salmonella | enterica     | NS      | 3          | Tickborne lymphanthropy | 93                  | NR_043755    |
| Serratia | liquefaciens  | ww      | 2          | Gastroenteritis     | 98                   | NR_044373    |
| Staphylococcus | aureus   | NW      | 2          | Gastroenteritis, skin infections | 95                  | NR_042062    |
| Treponema | primitia     | PW, PS, CNS, NS | 2 | Syphilis, yaws | 92                   | NR_041714    |
| denticola | CCW          | 2       | Periodontal disease | 92                 | NR_036899            |
| azotonutricium | PS, CNS | 2       | Nonhuman pathogenic | 90                  | NR_025141    |
| berlinense | NS           | 2       | Nonpathogenic | 85                  | NR_042797    |
| medium    | NS           |         |            | Periodontal disease | 91–92               | NR_037137    |

**N** = Natural site; **W** = Water, **S** = sediment, **CC** = Cypress Channel, **CN** = Chino Creek, **P** = Prado wetland area. e.g. **NS** = Natural site sediment. doi:10.1371/journal.pone.0079490.t003
2. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
3. Taylor LH, Latham SM, Woodhoo EJ (2001) Risk factors for human disease emergence. Philosophical Transactions of the Royal Society B 356: 983–989.
4. Abraham WR, Macedo AJ, Gomes LH, Tavares FC (2007) Occurrence and resistance of pathogenic bacteria along the Tiete River downstream of Sao Paulo in Brazil. Clean 35: 339–347.
5. Hamner S, Tripathi A, Mishra RK, Bouskill N, Broadway SC, et al. (2006) The role of spatial patterns and sewage pollution in incidence of waterborne enteric diseases along the Ganges River in Varanasi, India. Int J Environ Health Res 16: 113–132.
6. Lata P, Ram S, Agrawal M, Shanker R (2009) Enterococci in river: Ganga surface scaterte of Water. A study of species distribution, discrimination of antimicrobial-resistance and virulence markers among species along landscape. BMC Microbiol 9: 1–10.
7. Ibekwe AM, Lesch SM, Bold RM, Leddy MB, Graves AK (2011) Variations of resistances from human and animal sources uncovers multiple environmental and host factors. Proc Natl Acad Sci 107: 18933–18938.
8. Rice BW (2005) Staff report on bacterial indicator total maximum daily loads in environmental water. Appl Environ Microbiol 71: 6935–6940.
9. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75: 7537–7541.
10. Huse SM, Welch DM, Morrison HG, Sogin ML (2010) Ironing out the wrinkles in the rare biosphere through improved OTU clustering. Environ Microbiol 12: 1899–1898.
11. Vandamme P, Puginia P, Benzi G, Van Enterijck R, Vlaes L, et al. (1992) Escherichia coli: the genome of an emerging pathogen. BMC Genomics 10: 352 doi:10.1186/147-5903.
12. Pavli A, Malczow HC (2008) Travel-acquired leptospirosis. J Travel Med 15: 324–327.
13. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
14. St-Pierre K, Levesque S, Frost E, Carrier N, Arbeit RD, et al. (2009) Pathogenic Escherichia coli from human sources. PLoS ONE. 6: e20819.
15. Evans NJ, Brown JM, Murray RD, Getty B, Birles RJ, et al. (2011) Characterization of novel bovine gastrointestinal tract Treponema isolates and comparison to bovine digital dermatitis treponemes. Appl Environ Microbiol 77: 130–147.
16. Bruggemann H, Gottschalk G (2009) Chlamydia: Molecular Biology in the Post-genomic Era. Caister Academic Press. Portland, OR 97213-3786, USA; ISBN 978-1-904455-38-7.
17. Unsworth NB, Stenos J, Graves SR, Faa AG, Cox GE, et al. (2007) Flinders Island spotted fever rickettsioses caused by "Borrelia lonestari" strain of Rickettsia honei, an Eastern Australian. Emerg inf dis 13: 566–573. doi:10.3201/eid1304.060007.
18. Stray JS, Lescot M, Claverie JM, Schiz HC (2009) Brucella microti: the genome sequence of an emerging pathogen. BMC Genomics 10: 352 doi:10.1186/147-5903.
19. Audic S, Lescot M, Claverie JM, Schultz HC (2009) Audic S, Lescot M, Claverie JM, Schlz HC. Assigning rRNA sequence into the new bacterial taxonomy. Appl Environ Microbiol 75: 1857–1862.
20. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
21. Pavli A, Malczow HC (2008) Travel-acquired leptospirosis. J Travel Med 15: 447–453. DOI:10.1111/j.1708-8305.2008.00257.x.
22. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
23. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
24. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
25. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
26. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
27. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
28. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
29. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
30. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
31. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
32. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
33. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
34. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
35. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
36. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
37. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
38. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
39. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
40. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
41. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
42. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
43. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
44. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.
45. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65: 453–467.