

Microbiological evaluation of fecal bacterial composition from surface water through aquifer sand material

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ABSTRACT

When bacterial pathogens from livestock contaminate drinking water supplies, they can cause different forms of gastroenteritis. The objective of this study was to enumerate the concentrations of fecal indicator (*Escherichia coli* and enterococci) in surface water in order to determine removal efficiency by sand filtration. The concentrations of different indicator bacterial species were determined after running tertiary treated water through two tanks containing aquifer material. *Enterococcus faecalis* primers targeting the *ddl* gene and primers for *Enterococcus faecium* were used to identify the two species in the samples. A PCR assay based on the partial sequence of the β -D-glucuronidase gene (*uidA*) for specific detection and differentiation of *E. coli* populations was used to confirm the presence of *E. coli* after a biochemical test. The biochemical test overestimated the percentage of *E. faecium* in our samples, but the PCR assay with the *ddl* gene produced 100% specificity with *Enterococcus faecalis*. The biochemical test was 91.5% specific in identifying *E. coli*. The composition of indicator bacteria in Santa Ana River was dominated by intestinal microflora of humans and animals; filtration by aquifer sand material may reduce the transport of indicator bacteria from surface water to groundwater.

Key words | aquifer material, contamination, drinking water, *Enterococcus*, *Escherichia coli*, fecal bacteria

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INTRODUCTION

The Santa Ana River is the largest river in the Santa Ana Region of southern California and it is a major source of drinking water for over 2 million people that live in Orange County, California. Sources of non-point contaminants into the river may be municipal wastewater, agricultural waste discharges and urban runoffs. About 90% of the volume of the Santa Ana River is treated wastewater, and treated wastewater is known to contain substantial numbers of fecal bacteria such as fecal coliforms at concentrations of 10^6 – 10^7 CFU ml⁻¹ in effluents. This indicates the possibility of pathogens being discharged into receiving waters (Koivunen *et al.* 2001). Different federal, state and private agencies have monitored fecal bacterial composition in the Santa Ana River surface water (Izbicki *et al.* 2003; Rice 2005), but little

has been done to determine the effects of aquifer sand materials on fecal bacterial composition after recharging the river flows into the underlying groundwater basin.

Waters contaminated with human feces are generally regarded as a greater risk to human health as they are more likely to contain human-specific enteric pathogens, including *Salmonella typhi*, *Shigella* spp., hepatitis A and Norwalk-group viruses. Animals can also serve as reservoirs for a variety of enteric pathogens (e.g. various serotypes of *Salmonella*, *Escherichia coli* O157:H7 and *Cryptosporidium* spp.). The presence of *Enterococcus* spp. and *E. coli* at elevated levels indicate that fecal pollution from animal or human sources has occurred, and also the potential presence of pathogenic bacteria such as *Salmonella*, *E. coli*

O157:H7 and *Cryptosporidium*. *Enterococcus* concentrations have, in many cases, been shown to correlate with the incidence of waterborne gastroenteritis (Cabelli 1980; Cabelli *et al.* 1982; Fleisher *et al.* 1993; Kay *et al.* 1994). *Enterococcus faecalis* and *Enterococcus faecium* are increasingly important as opportunistic pathogens in out-patient settings and in acute and long-term care hospitals and nursing homes (Das & Gray 1998; Low *et al.* 2001; Farrell *et al.* 2003; Mutnick *et al.* 2003; Singh *et al.* 2003).

There are many possible sources of *Enterococcus* spp. in environmental waters other than sewage, including animal waste (Devriese *et al.* 1987, 1991; Sinton *et al.* 1993; Harwood *et al.* 2001), invertebrates (Svec *et al.* 2002) and plants (Muller *et al.* 2001). Other sources may be resuspended survivors from sediments (Grant *et al.* 2001; Le Fevre & Lewis 2003) and soils (Fujioka *et al.* 1999). The broad environmental distribution of *Enterococcus* spp. complicates, but does not eliminate, their usefulness as indicators of the risk of waterborne disease for humans (Harwood *et al.* 2004). Environmental water quality studies may benefit from focusing on a subset of *Enterococcus* spp. that are consistently associated with sources of fecal pollution such as domestic sewage, which pose definite human health risks, rather than utilizing the entire genus (Harwood *et al.* 2004). *E. faecalis* and *E. faecium* are potentially good focal species for such studies, as they have been consistently identified as the dominant *Enterococcus* spp. in human feces (Chenoweth & Schaberg 1990; Ruoff *et al.* 1990; Gelsomino *et al.* 2003) and sewage (Manero *et al.* 2002). These bacteria have been widely used as indicators of water quality (US EPA 1989). The detection of *E. coli* in temperate freshwaters usually provides a reliable indication of fecal contamination from humans and warm-blooded animals (Toranzos & McFeters 1997).

The transport of bacterial pathogens from surface water or runoff through the subsurface soil to groundwater is one of the pathways by which bacterial pathogens can enter drinking water wells or groundwater. The objective of this study was to enumerate the concentrations of fecal bacteria in surface water in order to determine removal efficiency by sand filtration. Heterotrophic bacteria, *E. coli* and *Enterococcus* species were enumerated from surface water and from aquifer sand material. Bacteria were further confirmed by biochemical test and molecular methods (Tebbe *et al.*

1992; Martins *et al.* 1993). We studied subsurface material to determine bacterial removal efficiencies by sand material packed in a 1.2 m × 1.2 m × 1.8 m filtration tank with tertiary treated water running through the tanks for 15 days.

METHODS AND MATERIALS

Experimental site and sampling collection

The study was conducted at the Orange County Water District (OCWD) field station in Anaheim, California. The site has three artificial lakes filled with tertiary water from Santa Ana River which was used for percolation studies at the field station in 2004. Santa Ana River (SAR) water at the field station was collected during days 1, 6 and 15 after running tertiary water from SAR through sand materials in the percolation tanks. The water consists of source water (water from the river), process water (water from Liquid Separation Inc. processor, LSI) and filtrate water (water from aquifer sand material after passing through the processor). This process was repeated three times to determine the reliability of data. The study was conducted from April to June 2004 in a filtration tank built with stainless steel outside the field station. Aquifer material was heterogeneous native lake sediment that had been processed through a sand washing plant to remove the majority of silt and clay particles. The material was trucked to the station and packed into the tanks. Samples from aquifer material were collected on day 15 at the end of each experiment. Two LSI processors – an active processor (with magnet) and a passive processor (control, without magnet) – were used as part of this study to monitor water percolation through aquifer sand material. The active processor unit (LSI) works by making the water less likely to hold onto particulates and dissolved solids. This in turn allows micro-filtration or settling tanks to become more efficient and faster in reducing contaminants from water by filtration. The technology was evaluated by the Orange County Water District to determine the rate of water percolation through aquifer material. Source water was obtained from the Santa Ana River water stored in an artificial lake and run through a 2.54 cm PVC pipe into the processors. From each processor, the water runs another 1 m into the filtration tank containing aquifer materials.

Enumeration of fecal bacterial contaminants in water and aquifer material

Water samples were collected in 1-l sterile bottles and transported on ice to the laboratory and processed within 6 h using standard procedures (*Standard Methods* 1995). Various dilutions and volumes were filtered with the goal of achieving 30–300 colonies per dilution. Surface water samples were vortexed and volumes of 100, 10 and 1 ml were filtered in phosphate buffered saline (PBS) water ($0.0425 \text{ g l}^{-1} \text{ KH}_2\text{PO}_4$ and $0.4055 \text{ g l}^{-1} \text{ MgCl}_2$). Tenfold and 100-fold dilutions were also prepared in buffered water, vortexed, and 1 ml of each dilution was filtered in triplicate. Volumes of 1 ml, 10 ml and 100 ml (via membrane filtration) were plated onto tryptic soy agar (TSA) for heterotrophic plate counts (HPC), Enterococcosel media for *Enterococcus* species (Becton Dickinson, Cockeysville, Maryland) and sorbitol-MacConkey agar (SMAC) for *E. coli*, incubated at 37°C for 24 h and colonies were enumerated. At the end of each study period (15 d) soil samples were collected from three columns inserted into the tanks and transported to the laboratory on ice. Columns were opened and divided into three 10 cm sections. Each section was placed inside a separate sterile Ziploc bag. To extract bacteria from aquifer material, 10 g of aquifer sand material was suspended in 100 ml of PBS as mentioned above and shaken in a horizontal shaker for 15 min. Suspended aquifer samples were serially diluted and plated on the media mentioned above. Total HPC and *E. coli* were enumerated as discussed above after incubation for 24 h at 37°C. Enterococci were enumerated using Enterococcosel media and incubated for 22–24 h at 37°C. Fecal indicator levels were reported as colony forming units (CFU) per ml of water or CFU per g of aquifer material.

Identification of *Enterococcus* species and *E. coli* in water samples and aquifer material by biochemical test

Enterococcus characteristics were identified to the species level using the API 20 Strep biochemical test kit (Bio-Merieux, St Louis, Missouri) as described by Harwood *et al.* (2004) and following the direction from the manufacturer. *E. coli* was identified with the same procedure using the API 20E test system. Isolated colonies were selected for Gram

staining and the catalase test, and were inoculated into test tubes containing 5 ml brain-heart infusion broth (BHI; Becton Dickinson), and incubated for 24 h at 35°C. Catalase-negative, Gram-positive cultures were streaked for isolation from BHI broth onto bile esculin agar (Difco Laboratories) and incubated for 24 h at 35°C.

Identification of *Enterococcus* species and *E. coli* in water samples and aquifer material by PCR

E. faecalis primers targeting the *ddl* gene (E1F: ATC AAG TAC AGT TAG TCT and E2R: ACG ATT CAA AGC TAA CTG), which encodes the D-Ala: D-Ala ligase and produces a 941 DNA base pair product upon amplification, and primers for *E. faecium* (EM1A: TTG AGG CAG ACC AGA TTG ACG and EM1B: TAT GAC AGC GAC TCC GAT TCC), which produce a 658 DNA base pair product, were used (Dutka-Malen *et al.* 1995; Cheng *et al.* 1997). The iCycler DNA thermocycler (Bio-Rad, Hercules, California) was used to perform whole cell PCR. Cells were grown overnight on TSA plates. One colony from each culture was transferred with a sterile toothpick to a PCR mix containing 10 pmol of each primer, Ready-To-Go PCR beads from Amersham-Pharmacia Biotech (Piscataway, New Jersey), and sterile distilled water in a final volume of 25 μl , and using the PCR conditions as described by Ibekwe *et al.* (2003). The following PCR programme consisting of a pre-denaturation step of 95°C for 10 min, followed by 40 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min and a final extension step of 72°C for 10 min and 4°C for infinity was used.

A total of 89 isolates from EC media were confirmed as *Enterococcus faecalis* or *E. faecium* after API, with few (12) exceptions. All isolates (89) were PCR amplified and sequencing was done on the isolates that showed conflict between the API test and PCR (12). The 16S rRNA PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, California). Primers for *E. coli* targeting a 0.166-kb region of the *uidA* gene: UAL-1939 (5'-TATGGAATTTCCGCCGATTTT-3') and UAR-2105 (5'-TGTTTGCCTCCCTGCTGCGG-3'), were used for amplification. Primer UAL-1939 is located between bp 1939 and 1958 and primer UAR 2105 is located between bp 2085 and 2104 closer to the carboxyl region of the *uidA* gene of *E. coli*

(Bej *et al.* 1991). PCR was performed with 200 presumptive *E. coli* isolates and 12 known isolates as control. A total of 200 isolates from SMAC media were confirmed as *E. coli* after API, with few (17) exceptions. PCR and sequencing were done on the isolates that showed conflict between the API test and PCR (17).

To confirm the identities of each colony, 16S rRNA gene of selected isolates was amplified in preparation for DNA sequencing. Primers 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R 5'-GGT TAC CTT GTT ACG ACT T-3', were used to extend from each end of the 16S ribosomal RNA gene, producing an amplicon of c.1,500 bp. Amplification of the 16S rRNA was done using the following steps: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and a final extension step of 72°C for 7 min. The resulting PCR products were purified from a 1% agarose gel and cloned into the TOPO TA cloning kit (Invitrogen, Carlsbad, California). Isolation of plasmids was performed using the Qiagen plasmid mini kit (Valencia, California). The 1,500 bp inserts in the TOPO TA kit were sequenced with an Applied Biosystems Prism 377 DNA sequencer using universal M13 forward and reverse primers.

Statistical analysis

Comparisons of bacterial (HPC, *E. coli* and enterococci) plate counts of treatment means at different dates were accomplished with the Tukey's test. All calculations were performed using the general linear model (GLM) procedure of the Statistical Analysis System (SAS Institute 1998) to test the statistical differences ($P = 0.05$) between fecal bacterial species distribution in water samples and in aquifer samples during the three sampling times. Sequence analyses were done using the BLAST database (National Center for Biotechnology Information: www.ncbi.nlm.nih.gov). Sequences of the 16S rRNA gene from the isolates were further aligned with those of reference taxa from Genbank using Clustalx version 1.8 for Windows (Thompson *et al.* 1997). Phylogenetic trees were constructed and checked by bootstrap analysis (1,000 data sets) using the program SEQBOOT. Bootstrap values represented the frequency of resampling that supported a specific branching pattern.

RESULTS

Fecal indicator bacterial levels in source water and aquifer sand material

There were no differences ($P = 0.05$) in the levels of heterotrophic bacteria as determined by plate count in the source water (influent), process water (water that has gone through the active LSI system), control (water that has gone through the passive LSI system) and the filtration effluent (aquifer sand material, Figure 1(a)). Significantly higher levels ($P < 0.001$) of HPC were found in water samples in late May and June than in April and early May. There were significantly ($P = 0.05$) higher numbers of *E. coli* and enterococci in source water in late May and early June than in April. The concentrations of *E. coli* and enterococci in source water were not significantly different from the concentrations found in process effluent where the water had gone through the LSI unit (Figure 1(b),(c)). However, there were significant reductions ($P = 0.05$) in *E. coli* and enterococci during the June experiment after the water had gone through sand materials. This shows that the aquifer sand material had significant impact in reducing the numbers of *E. coli* and enterococci population and may help in the improvement of Santa Ana River water quality that is used in the recharge zone.

When the 30 cm columns that were inserted into the aquifer materials were removed and analysed for heterotrophic bacteria, *E. coli* and enterococci, significantly higher numbers of heterotrophic bacteria were found in the top 10 cm portion of the column during April and May sampling, but these numbers were reversed in June with the highest numbers found in the last 30 cm portion of the column for both filtration tanks (Figure 2(a)). The *E. coli* population was significantly higher in April than in May and June, but significantly lower numbers were found in the 20 cm portion of the column than in the 10 or 30 cm portions (Figure 2(b)). There were no significant differences in *E. coli* concentrations in both filtration tanks in May and June. Also, there were no significant differences ($P = 0.25$) in enterococci population during April and June sampling, but the numbers were significantly higher ($P = 0.001$) in the top 10 cm portion of the columns in May (Figure 2(c)).

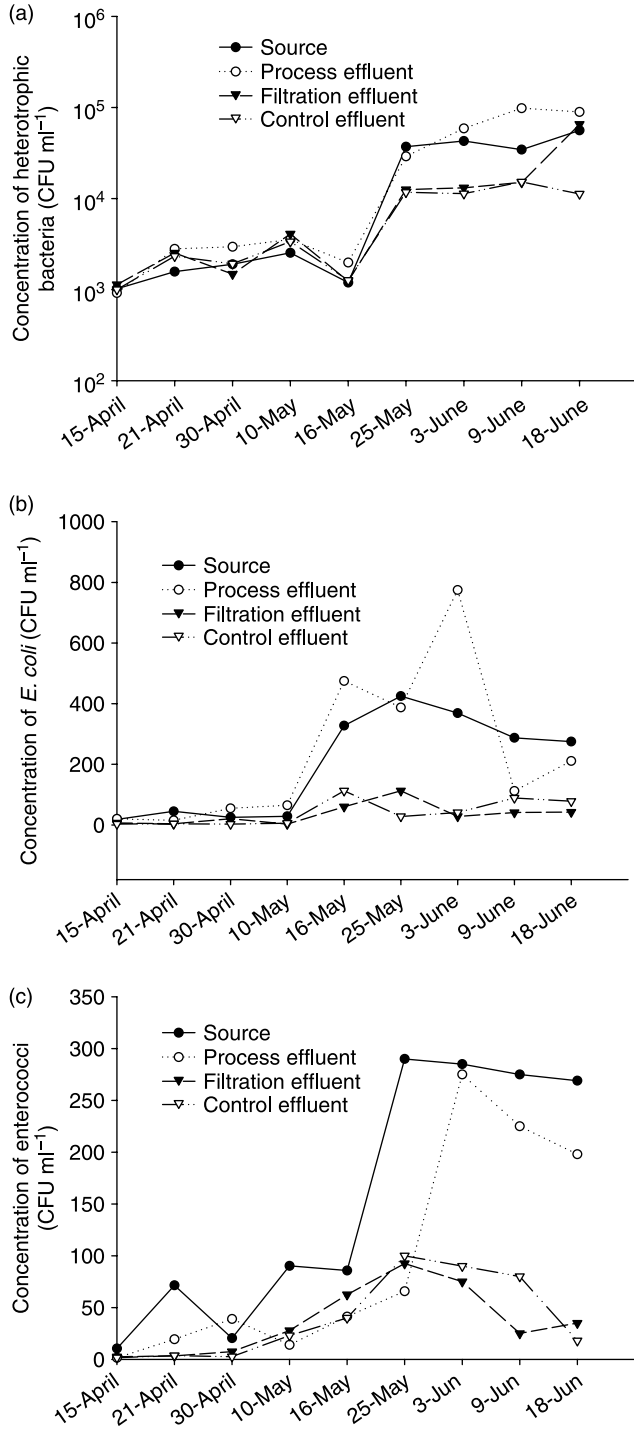


Figure 1 | Levels of (a) heterotrophic bacteria, (b) *E. coli* and (c) enterococci as determined by plate count in the source water, process water, control water after filtration through aquifer material, and the final effluent after water has gone through the processor and filtration through aquifer sand material. All samples were taken from April to June 2004.

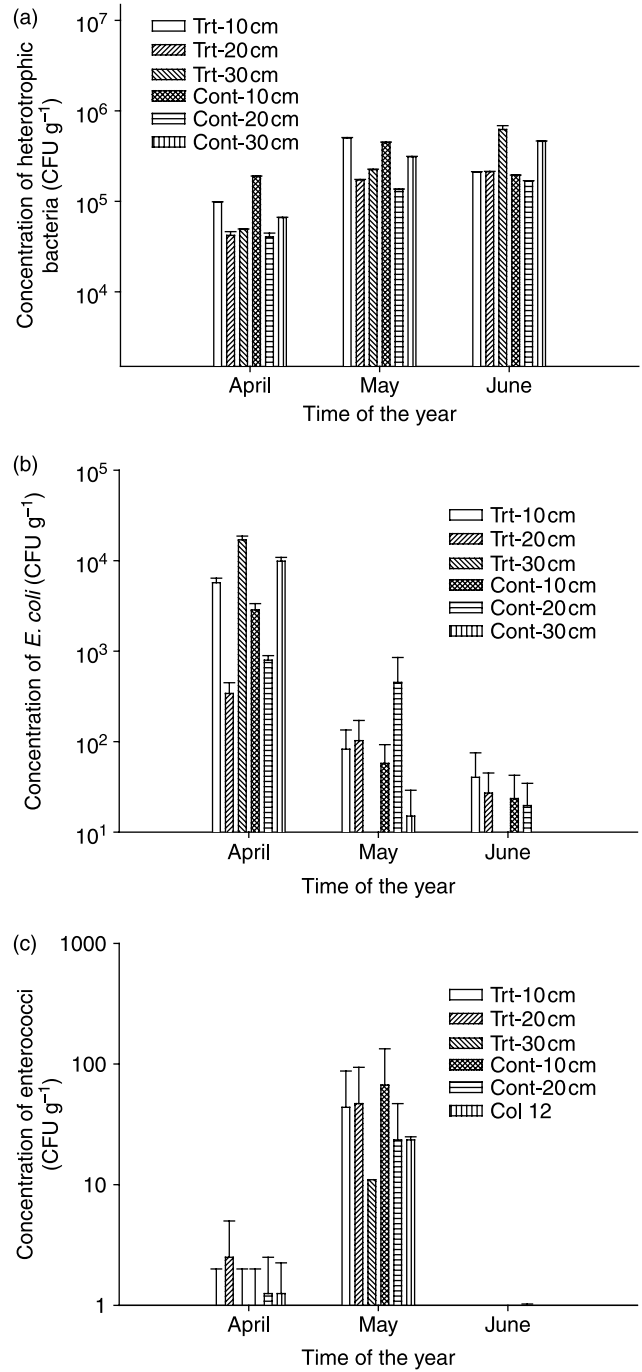


Figure 2 | Concentrations of different bacterial groups from columns in aquifer materials after 15 d in the filtration tank: (a) heterotrophic plate counts, (b) *E. coli* concentrations and (c) enterococci plate counts.

Distribution of *Enterococcus* species and *E. coli* in water and aquifer sand material

After analysing the three types of bacteria, we decided to study the composition of *Enterococcus* species and *E. coli* in the water and column samples. Table 1 shows *Enterococcus* species from different samples collected during the experiment. A total of 89 isolates from EC media were confirmed as *Enterococcus* species after a standard test (*Standard Methods* 1995). All 89 isolates met the standard criteria except 12 which had conflicting results. All 89 isolates including the 12 isolates that showed conflicting results with the API 20 Strep biochemical test system were tested by PCR, and sequencing was done with the 12 isolates to confirm strain identities. *Enterococcus faecalis* was the most frequently identified *Enterococcus* species within the 12 isolates, and all isolates correctly identified by the biochemical test system as *Enterococcus faecalis* were confirmed as the same by PCR. The biochemical test system weakly identified 7 isolates within the 12 as *E. faecium*; however, the expected 658 bp product was amplified from only one of these isolates. PCR analysis with *E. faecalis*-specific primers confirmed three of the isolates as *E. faecalis* and three were not identified as either (Table 1). In general, *Enterococcus faecalis* and *Enterococcus faecium* were the most common species found in both sediment and water samples. Biochemical identification by API 20E assay confirmed 91% of the 200 *E. coli* isolates (Table 2). The 200

isolates were PCR confirmed using the *uidA* gene, including the 17 isolates with conflicting results from the API 20E assay. PCR analysis of the 17 isolates identified 8 isolates as *E. coli* and these were confirmed as such by sequencing (Table 2). The remaining nine isolates were confirmed by sequencing and their identities are shown in Table 2.

To resolve the discrepancy between the biochemical test results for *E. faecium/faecalis* and the PCR confirmation, the 16S rRNA gene of 12 selected isolates was sequenced. Nine PCR-positive isolates were identified as *E. faecalis* and were confirmed by sequencing, and one *E. faecium* was identified by PCR as *E. faecium* and was confirmed by sequencing as such. Therefore, the PCR assay with the *ddl* gene was very specific. Three isolates that were not positively identified by PCR were confirmed by sequencing to be *Enterococcus mundtii* (Table 1). The biochemical test overestimated the percentage of *E. faecium* in our samples.

The neighbour-joining tree of the 16S rRNA clone of the 12 isolates and a clone from ATCC strain were used to generate a tree with sequences retrieved from GenBank (Figure 3). All the clone sequences of the 12 isolates had similarities greater than 98% from the Genbank database. The phylogenetic tree grouped the sequences from this study into separate groups with other bacterial species outside this group. All *E. faecalis* were in a separate group from *E. faecium* and *E. mundtii* which were grouped together. Our study agrees with the work of Harwood *et al.* (2004), which showed *E. faecalis* and *E. faecium* grouping

Table 1 | Sources of *Enterococcus* sp., API 20 strep and PCR results from strains used for sequencing

Sample	Catalase	Gram stain	API results	PCR results	Sequence identification
<i>E. faecalis</i> ATCC strain	–	+	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>
S1-source	–	+	<i>E. faecium</i>	None	<i>E. mundtii</i>
S2 - source	–	+	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>
TI - treatment	–	+	<i>E. faecium</i> (?)	<i>E. faecalis</i>	<i>E. faecalis</i>
C1 - control	–	+	<i>E. faecium</i>	<i>E. faecium</i>	<i>E. faecium</i>
F1 - filtrate	–	+	<i>E. faecium</i> (?)	None	<i>E. mundtii</i>
S3 - source	–	+	<i>E. faecium</i> (?)	<i>E. faecalis</i>	<i>E. faecalis</i>
F2 - filtrate	–	+	<i>E. faecium</i> (?)	none	<i>E. mundtii</i>
S4 - source	–	+	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>
T2 - treatment	–	+	<i>E. faecium</i> (?)	<i>E. faecalis</i>	<i>E. faecalis</i>
S5 - source	–	+	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>
T3 - treatment	–	+	<i>E. faecium</i> (?)	<i>E. faecalis</i>	<i>E. faecalis</i>

Table 2 | Sources of *E. coli*, API 20 E and PCR results from strains

Sample	Gram stain	Shape	API results	PCR results with <i>uidA</i>	Sequence identification
4/20 EHEC 72	–	Rods	+	<i>E. coli</i>	<i>E. coli</i>
4/5 Imperial Gate 2	–	Rods	+?	<i>E. coli</i>	<i>E. coli</i>
4/21 PE-2 -	–	Rods	+?	–	<i>Chryseomonas luteola</i>
4/21 PI-1 -	–	Rods	+?	–	<i>Klebsiella pneumoniae</i>
4/21 S1 - source	–	Rods	+?	<i>E. coli</i>	<i>E. coli</i>
4/23 I2 - source	–	Rods	+?	–	<i>Pasteurella pneumotropica hemolytica</i>
4/23 I1 - source	–	Rods	+?	–	<i>Brucella</i> spp.
4/26 F2 - filtrate	–	Rods	+?	<i>E. coli</i>	<i>E. coli</i>
4/26 S2 - source	–	Rods	+?	<i>E. coli</i>	<i>E. coli</i>
4/26 F1 - filtrate	–	Rods	+?	–	<i>Burkholderia cepacia</i>
4/26 Fe1 Blue - bird feces	–	Rods	+?	<i>E. coli</i>	<i>E. coli</i>
4/26 Fe1 Red - bird feces	–	Rods	+?	–	<i>Citrobacter koseri</i>
4/26 Fe4 - bird feces	–	Rods	+?	–	<i>Enterobacter sakazaki</i>
4/26 Fe6 - bird feces	–	Rods	+?	–	<i>Cedecea davisae</i>
4/28 F1 - filtrate	–	Rods	+?	<i>E. coli</i>	<i>E. coli</i>
4/28 S2 - source	–	Rods	+?	–	<i>Klebsiella pneumoniae</i>
4/30 Fe7 - bird feces	–	Rods	+?	+	<i>E. coli</i>
4/28 P1 treatment	–	Rods	+?	+	<i>E. coli</i>

A total of 200 isolates plus strain 72 as control; 91.5% correctly identified (183).

separately. These authors noted that the *E. faecium* group contains species whose 16S rRNA sequence is 99.3–99.7% similar, and comprises *E. faecium*, *Enterococcus durans*, *Enterococcus hirae* and *E. mundtii* (Leclerc *et al.* 1996). This suggests that *E. faecalis* represents a distinct lineage within the *Enterococcus* sp. (Leclerc *et al.* 1996).

DISCUSSION

The data presented here suggest high levels of indicator bacteria in the river as it continues to the ocean. Our study also suggests that, when surface water is diverted through aquifer sand material, significant ($P = 0.05$) reduction in fecal bacterial population occurs as the water passes through the aquifer sand material by a natural filtration process. Part of the source water (Santa Ana River) used for this study has gone through tertiary treatment and wetlands before flowing into the artificial lakes at the Orange County Water District field station. Water from these lakes is subsequently used for groundwater recharge and discharge. After this process, the water is treated further for domestic use by over 2 million

residents and businesses. Part of the Santa Ana River continues to flow and empties into the Pacific Ocean near Huntington Beach. In southern California, it is well recognized that a major cause of bacterial pollution of coastal waters is urban runoff in rivers/channels and storm drains that discharge into the ocean (Dwight *et al.* 2002; Reeves *et al.* 2004). In a recent paper enumerating enterococci in marine and intertidal sediments (Ferguson *et al.* 2005), high densities of fecal indicator bacteria were reported in Santa Ana River near Huntington Beach. These authors indicated that shoreline waters at Huntington State Beach may be recipients of fecal indicator bacteria originating from intertidal sediments in the Santa Ana River that contain high levels of bacteria. Our data also suggest that the concentration of indicator bacteria in the surface water may be higher as we move into the summer months. This may be due to the higher temperature enhancing the growth of indicator bacteria and also the increased availability of nutrient. Further observations were also made on the filtration tanks containing the aquifer sand materials (Figure 2(b),(c)). *E. coli* and enterococci were significantly ($P = 0.05$) reduced in the filtration tanks. One possible reason

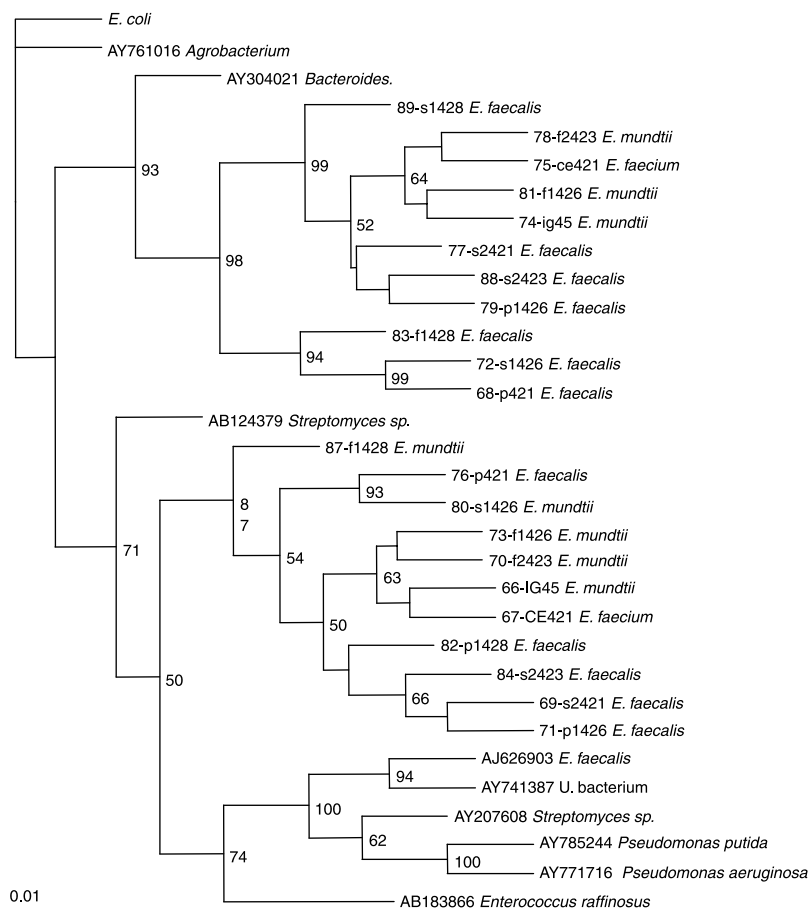


Figure 3 | Neighbour-joining analysis of 16S rRNA gene sequences from putative isolates of *Enterococcus* sp. All isolates recovered from this study are marked in bold. Numbers at the nodes represent values of 1,000 bootstraps replicates that support each node. Bootstrap values are not shown if less than 50%; scale at the nodes is 0.01.

for this may be inactivation of indicator bacteria by UV radiation in the filtration. The inactivation mechanism of solar disinfection is complex and not yet fully understood. The central hypothesis is that UV light produces reactive oxygen species, which can damage nucleic acids, proteins or other life-supporting cell structures (Berney *et al.* 2006). It was also found that broad-spectrum UV light blocks the electron transport chain, inactivates transport systems, interferes with metabolic energy production and can cause a general increase in permeability of the membrane (Eisenstark 1989). Furthermore, direct inhibition of certain enzymes (e.g. catalase) has also been observed (Eisenstark 1998).

In this study we identify *E. faecalis* and *E. faecium* species because they have been consistently shown to be the dominant *Enterococcus* species in human feces (Chenoweth & Schaberg 1990; Ruoff *et al.* 1990; Gelsomino *et al.* 2003), sewage (Manero *et al.* 2002) and environmental samples

(Harwood *et al.* 2004). Therefore, environmental water quality studies may benefit from focusing on a subset of *Enterococcus* species that are consistently associated with sources of fecal pollution such as domestic sewage, which poses definite human health risks, rather than utilizing the entire genus. As seen from our studies, we did not find any other species from our isolates, except the three species reported in Table 1. Ferguson *et al.* (2005) also reported that the predominant *Enterococcus* species found in the Santa Ana River at Huntington Beach were *E. faecalis* and *E. faecium* with other species at a very low level. *E. faecalis* and *E. faecium* are also the predominant *Enterococcus* species in the intestinal microflora of humans and animals and are considered opportunistic pathogens (Willey *et al.* 1999), while *E. mundtii* is associated with plants and soil and is rarely associated with human infection (Pinto *et al.* 1999). In this study, these three environmentally associated species

composed 100% of all isolates tested. Thus, the species composition of enterococci in our samples strongly suggests the intestinal microflora of humans and animals as the major sources of indicator bacteria in the Santa Ana River.

It is also possible that since most of the Santa Ana River water at the lakes is tertiary effluent dominated water, the treatment process may have killed most of the *Enterococcus* species and that what we identified at the site were the isolates from sediment resuspension or fresh input from gaming activities by human and animals near the lakes. It should be noted that the main sources of the tertiary water are publicly owned wastewater treatment plants. It has also been estimated that approximately 500 million litres per day of treated wastewater are discharged by these treatment plants into the river. In some instances, these discharges can constitute up to 90% or more of the flow. The compositions of the discharges are regulated by the Regional Control Board through permits, which include a total coliform discharge limit of 2.2 MPN organisms per 100 ml (Rice 2005).

Characterization and identification of enterococci by using the traditional phenotypic differentiation can be a tedious process requiring numerous tests. Strains are classified based on growth in various media, biochemical reactions in those media, motility and pigmentation. These microorganisms have been used as indicators of fecal pollution for many years and have been especially valuable in the marine environment and recreational waters as indicators of potential health risks and swimming-related gastroenteritis (Cabelli 1983; *Standard Methods* 1998). The identification of the 89 isolates using the traditional phenotypic and biochemical tests required long periods of incubation before the results could be interpreted. Similar problems had been encountered in a clinical and antimicrobial laboratory (Jackson *et al.* 2004). The use of commercial identification kits can help with the problems of time constraints and number of samples to be processed. Therefore, a combination of the traditional methods with PCR where there is conflict can provide 100% accuracy in identification with the *ddl* gene.

CONCLUSION

The compositions of indicator bacteria in Santa Ana River suggest that the intestinal microflora of humans and

animals are the major sources of indicator bacteria in the Santa Ana River. However, after water has gone through the filtration tanks containing aquifer sand materials, there were significant ($P = 0.05$) reductions in *E. coli* and enterococci concentrations in the final effluent water. Therefore, filtration with aquifer material had a significant role in *E. coli* and enterococci population reduction in the final effluent water. Rapid sand filtration of surface water through a natural aquifer followed by other natural processes such as UV irradiation may reduce fecal bacterial populations entering groundwater to an acceptable standard before the water is treated for domestic use. This process may be a cost saving approach especially in developing countries where poor quality of drinking water is the major cause of many waterborne illnesses.

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