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Cultivation-Independent Analysis of Bacteria in IDEXX Quanti-Tray/2000 Fecal Indicator Assays[▽]

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Monitoring microbiological water quality is important for protecting water resources and the health of swimmers. Routine monitoring relies on cultivating fecal indicator bacteria (FIB), frequently using defined substrate technology. Defined substrate technology is designed to specifically enrich for FIB, but a complete understanding of the assay microbiology requires culture-independent analysis of the enrichments. This study aimed to identify bacteria in positive wells of Colilert and Enterolert Quanti-Tray/2000 (IDEXX Laboratories) FIB assays in environmental water samples and to quantify the degree of false-positive results for samples from an urban creek by molecular methods. Pooled *Escherichia coli*- and *Enterococcus*-positive Quanti-Tray/2000 enrichments, either from urban creek dry weather flow or municipal sewage, harbored diverse bacterial populations based on 16S rRNA gene sequences and terminal restriction fragment length polymorphism analyses. Target taxa (coliforms or enterococci) and nontarget taxa (*Vibrio* spp., *Shewanella* spp., *Bacteroidetes*, and *Clostridium* spp.) were identified in pooled and individual positive Colilert and Enterolert wells based on terminal restriction fragments that were in common with those generated *in silico* from clone sequences. False-positive rates of between 4 and 23% occurred for the urban creek samples, based on the absence of target terminal restriction fragments in individual positive wells. This study suggests that increased selective inhibition of nontarget bacteria could improve the accuracy of the Colilert and Enterolert assays.

Quantifying fecal pollution in recreational waters is important for protecting the health of swimmers. Current standards for microbiological water quality in the United States and elsewhere are based on culturable fecal indicator bacteria (FIB), i.e., total coliforms, fecal coliforms, or *Escherichia coli*, and enterococci (21, 53, 57). Various methods are used to quantify FIB, including multiple-tube fermentation, membrane filtration, and defined substrate technologies (9, 16, 45). All rely on temperature, substrate, and selective growth inhibitors to select for FIB (23, 29). The commercially available defined substrate technologies Colilert and Enterolert (IDEXX Laboratories, Westbrook, ME) are accepted by the U.S. Environmental Protection Agency as alternatives to the multiple-tube fermentation and membrane filtration methods for fresh, marine, and estuarine surface waters (54). Specific enzyme-substrate relationships are the basis of both assays (45). In the Colilert assay, total coliforms and *E. coli* are indicated by a yellow or a yellow and a fluorescent metabolite, respectively. Positive Enterolert assays are indicated by a fluorescent metabolite. The manufacturer-supplied tray format is more convenient than multiple-tube fermentation and membrane filter techniques (20, 45). However, there is evidence that the Colilert and Enterolert assays are not specific (2, 6, 17, 43, 51).

False-positive Colilert or Enterolert readings could occur when there is sufficient abundance of nontarget bacteria, i.e., non-FIB, that enzymatically cleave the chromogenic or fluorogenic substrates. Beta-galactosidase activity, which yields the yellow color in Colilert, has been described for nontarget species in the genera *Aeromonas*, *Vibrio*, *Pseudomonas*, and *Flavobacterium* (17, 19, 51). Beta-glucuronidase activity, which yields the fluorescence in Colilert, has been found in *Shigella*, *Salmonella*, and *Yersinia* strains, in *Flavobacteria*, and in some streptococci, clostridia, *Bacteroides* spp., and *Corynebacterium* spp. (17, 35, 45). In the Enterolert assay, several nontarget bacteria were identified that yielded false-positive reactions, such as *Proteus vulgaris*, *Serratia marcescens*, *Sphingomonas* spp., and *Flavobacterium* spp. (2, 9). The basis for defined substrate technology is that nontarget bacterial growth should be excluded, and thus, high concentrations of nontarget bacteria would have to be initially present in a water sample to yield false-positive results (12, 17, 20). However, previous assessments of nontarget bacteria in defined substrate technology assays involved two cultivation steps, the first with the initial assay and the second with bacterial isolation from assay enrichments. Since many environmental bacteria resist cultivation (55), relying on isolate cultivation to characterize assay enrichments could incompletely describe nontarget populations in defined substrate technology assays.

In this study, we used culture-independent methods to identify bacterial taxa in the positively scored wells of Colilert and Enterolert enrichments for selected water samples. Our objectives were to more thoroughly describe bacterial assemblages within the completed assays and to quantify the rates of false positives. The bacterial assemblages in pooled positive Colilert and Enterolert wells of creek water and sewage samples were analyzed for terminal restriction fragment length polymorphism (TRFLP) of genes encoding 16S rRNA. Clone libraries

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based on 16S rRNA genes were developed for the same pooled positive wells. Bacterial community structure and false-positive rates were determined in individual positive Colilert and Enterolert wells of additional creek water samples by quantifying the occurrence of terminal restriction fragments (TRFs) not associated with target coliform and enterococcus bacteria. Taxa that had not been previously associated with IDEXX assays were found, and the apparent growth of nontarget bacteria in IDEXX assay enrichments resulted in quantifiable false-positive rates for these samples.

MATERIALS AND METHODS

Study sites and sampling design. The study site was Arroyo Burro Creek in Santa Barbara, CA, which drains a semiurban watershed and is listed under the Clean Water Act Section 303(d) as impaired for recreation because of high FIB concentrations. A prior study revealed sources of human waste entering the creek via storm drains that flow year-round (47). The watershed is 23 km^2 with 16% urban and suburban land use in the lower watershed and 84% natural land, including mixed forest, chaparral, and rural residential land use, in the upper watershed (13). Arroyo Burro Creek discharges into a brackish lagoon just upstream of a popular beach that is frequently posted with swimming advisories based on weekly FIB monitoring. The creek sampling site, which was just upstream of the lagoon, was furthest downstream in the urban and suburban contaminated reach described previously (47).

The study design was comprised of field sampling and analysis to (i) characterize bacterial assemblages from within creek waters and from a contaminated reference sample (sewage), (ii) characterize bacterial assemblages recovered from Colilert and Enterolert assays of the samples, (iii) identify taxa in source samples and FIB enrichments using clone library analysis, and (iv) quantify the rate of false-positive reactions due to nontarget bacteria in additional samples from the same site. Three creek samples were acquired during dry weather, and one additional wet weather sample was acquired for the clone library development. Accordingly, one creek (CR-1) and one sewage (SEW-1) sample were taken on 24 October 2005, when there had been no rainfall for at least a month preceding. The wet weather creek sample was acquired on 9 November 2005 after a first seasonal rainfall event. The same creek location was sampled again during dry weather on 6 November 2007 (CR-2) and 18 December 2007 (CR-3).

Sampling and FIB quantification. Creek water was sampled (2 liters) approximately 10 cm beneath the creek water surface. Municipal sewage was sampled from the influent of the El Estero Wastewater Treatment Plant (Santa Barbara, CA). Samples were filtered through 22- to 25-μm-pore-size sterile Miracloth (Calbiochem-Novabiochem, La Jolla, CA) to remove large debris and stored on ice until processing in the laboratory (<3 h). Total coliforms, *E. coli*, and *Enterococcus* spp. were quantified using the Colilert and Enterolert Quanti-Tray/2000 (IDEXX Laboratories, Westbrook, ME) according to the manufacturer's instructions. Samples were diluted to either 1:10 (creek water) or 1:100,000 (sewage) in sterile Nanopure water. The results were scored using the IDEXX MPN Generator 3.2, a software tool calculating the most probable number (MPN) based on the statistical analysis proposed by Hurley and Roscoe (28).

DNA extraction from environmental samples and Quanti-Tray wells. DNA was extracted from water and sewage samples, pooled positive Colilert/Enterolert wells, and individual positive Colilert/Enterolert wells. For the creek water and sewage, the samples were vacuum filtered (0.22 μm) to the point of refusal to collect bacteria, and the filters were frozen (−20°C) prior to DNA extraction using an UltraClean water DNA kit (Mo Bio, Carlsbad, CA) according to the manufacturer's instructions. The extracted DNA was ethanol precipitated and resuspended in 50 μl of 0.1× WD5 solution from the kit. The volumes filtered were approximately 1,500 ml (creek samples) and 135 ml (sewage sample).

Individual large (~1.9 ml) Quanti-Tray wells were sampled through the sterilized (70% ethanol swabbed) paper backing by using a sterile syringe and needle or sterile pipette tips. Small wells (~0.19 ml) were not sampled, as the small culture volume was not expected to yield sufficient DNA for analysis. To recover the DNA from the FIB enrichments of CR-1 and SEW-1, yellow/fluorescent Colilert and fluorescent Enterolert Quanti-Tray well contents for a sample were each pooled from 18 to 49 positive wells (28 to 43 ml) and were filtered and extracted using an UltraClean water DNA kit as described above. This DNA was used for TRFLP and clone library analyses.

To recover the DNA from CR-2 and CR-3 FIB enrichments, the contents of individual yellow Colilert, yellow/fluorescent Colilert, and fluorescent Enterolert wells were collected into sterile 2-ml screw-cap microcentrifuge vials (BioSpec Products, Bartlesville, OK). Each sample was centrifuged (10 min at 16,000 × *g*), and the pellet was stored (−20°C) until DNA extraction. DNA extraction was performed following a previously published protocol using bead beating in CTAB (cetyltrimethylammonium bromide) buffer followed by purification using phenol-chloroform (24) but with some modifications. First, the diethyl pyrocarbonate step to remove RNase was not used. Second, the pellet was lysed by mixing three times for 30 s (with 20 s of cooling between intervals) in a BioSpec MiniBeadbeater-8 (BioSpec Products, Bartlesville, OK) after adding 0.5 g of 0.1-mm zirconia/silica beads (BioSpec Products, Bartlesville, OK) plus published reagents. Third, the pellet was extracted again after adding 0.5 ml CTAB buffer, and the aqueous phases of the extractions were pooled. Total nucleic acids were precipitated overnight using 0.1 volume of 3 M sodium acetate (pH 5.5) and 1 volume of isopropanol. After centrifugation (30 min at 16,000 × *g*), the pellet was washed with ice-cold 70% (vol/vol) ethanol, centrifuged again, and air-dried prior to resuspension in 50 μl of sterile 0.1× Tris buffer. The final DNA concentrations were determined with fluorimetry, using a Quant-iT Picogreen double-stranded DNA assay kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

Terminal restriction fragment length polymorphism analysis. The bacterial community structure was investigated using TRFLP and clone library analysis for CR-1, SEW-1, and the wet weather sample and for the pooled positive Quanti-Tray enrichments of CR-1 and SEW-1. The dominant TRFs in the pooled positive wells of the IDEXX assay enrichments were identified by *in silico* restriction digest (as described below). TRFLP analysis was also performed for CR-2 and -3.

The amplification of 16S rRNA genes by PCR and subsequent TRFLP analysis of HhaI restriction digests were based on published protocols (33). PCR was performed using the primers 8F hex (fluorescently labeled forward primer, 5′-AGAGTTTGATCCTGGCTCAG-3′) and 1389R (5′-ACGGGCGGTGTGTAC AAG-3′), using reaction conditions as previously described (33) but with the addition of bovine serum albumin (0.2 mg/ml) and Q-Solution (Qiagen, Valencia, CA) to the PCR master mix. PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA), and 200 to 300 ng of purified DNA was digested with HhaI (New England Biolabs, Ipswich, MA). After inactivation of the restriction enzyme by heating (65°C for 20 min), the lengths of the fluorescently labeled fragments were determined with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA) at the Genomics Technology Support Facility (Michigan State University). TRFs with lengths between 50 and 1,000 bp were aligned and normalized to total peak height with Excel software, using a macro developed by C. Walsh (http://www.wsc.monash.edu.au/~cwalsh/treeflap.xls). Peaks with a relative peak height of less than 1% were discarded (44). The automatic alignment was verified manually.

For CR-1, -2, and -3 and SEW-1, the richness (*S*) and Shannon diversity (*H*) indices were estimated using PRIMER version 6 software (Primer-E Ltd., Plymouth, United Kingdom), based on TRFLP patterns. Hierarchical cluster analysis was performed using TRFLP data based on Bray-Curtis similarities (15). Significant results were analyzed using the similarity profile routine (SIMPROF) (14), which tests for random clustering (8). The SIMPROF test works by ordering similarities from a group of *a priori* unstructured samples from smallest to largest and plotting similarities against their rank. The observed profile is compared with that expected under the null hypothesis of no meaningful structure within that group, using permutation. Repeated application of this test generates a stopping rule for *a posteriori* division of the samples into ever smaller subgroups, as in hierarchical cluster analysis (14).

The *in silico* PCR and Restriction program of the web-based tool Microbial Community Analysis (MiCA) (48) was used to suggest possible phylogenetic affiliations of peaks from the electropherograms in relationship to cloned sequences. A size window of ±3 bp was used to account for the possible differences between real and predicted TRF lengths (36, 39). Only taxa that were found in the clone library analysis (below) were further evaluated.

Clone library analysis and TRF identification. Partial 16S rRNA genes were amplified using primers 8F (not labeled) and 1398R and cloned into the pCR2.1 vector (Invitrogen, Carlsbad CA). Blue/white screening and further processing and sequencing were performed by Agencourt Bioscience (Beverly, MA). The primers used for sequencing were the same as for PCR amplification. Sequencing was performed using the BigDye terminator version 3.1 chemistry, which is optimized for longer reads, uniform peak heights, and robustness. Ninety-six clones were sequenced for each sample, which was expected to yield a sufficient number of different clones for adequate sampling of the IDEXX assay enrichments and for identifying the dominant TRFLP peaks. The occurrence of chimeras was determined using Bellerophon (27), Check_Chimera (34), Pintail (4), and manual comparison of putative chimeric sequences. Excluding sequences of insufficient quality and chimeras, the final numbers of clones analyzed were 62

TABLE 1. Concentrations of fecal indicator bacteria, TRFLP richness and Shannon diversity indices, and clone library rarefaction-based Chao richness estimation for sewage and dry weather creek samples[a]

Expt	Sample	Concn[a] (MPN/100 ml) of:			S^b	H^c	$S_{Chao1}{}^d$
		TC	EC	ENT			
Pooled	SEW-1	2.8×10^7	1.2×10^7	7.4×10^5	16	2.2	124[e]
	CR-1	1.6×10^4	2.8×10^2	2.6×10^2	8	1.4	25
Individual	CR-2	3.4×10^3	1.6×10^2	2.2×10^2	10	1.9	NA
	CR-3	3.2×10^4	2.7×10^2	2.8×10^2	12	1.9	NA

[a] TC, total coliform; EC, *E. coli*; ENT, *Enterococcus* spp.
[b] TRFLP richness index (number of peaks).
[c] TRFLP Shannon diversity index.
[d] Clone library rarefaction-based Chao richness estimation for sewage and dry weather creek samples. NA, not analyzed because no clone library data were available.
[e] Based on rarefaction analysis, diversity was underestimated.

(CR-1), 73 (CR-1, yellow/fluorescent Colilert), 73 (CR-1, fluorescent Enterolert), 56 (SEW-1), 88 (SEW-1, yellow/fluorescent Colilert), and 89 (SEW-1, fluorescent Enterolert). Based on the sequence identity matrix, clones sharing ≥97% identity were grouped into one operational taxonomical unit (OTU). The BLAST algorithm (3) was used to determine the phylogenetic affiliations of all OTUs. Rarefaction analysis and estimation of the clone library richness (S_{Chao1}) were performed using a web interface (http://www.aslo.org/lomethods/free/2004/0114a.html) (31). S_{Chao1} is an abundance-based richness estimator well suited for estimating phylotype richness from prokaryotic libraries of 16S rRNA genes (31).

The clone libraries were used to identify the dominant TRFs from the pooled positive Colilert and Enterolert wells and from the source samples. The MiCA web tool was used for *in silico* restriction by HhaI of all clone library taxa. The resulting *in silico* TRFs, with known phylogenetic affiliations, were then compared to the sample TRFs.

Identification of false-positive wells. To determine the rates of false positives in the Colilert and Enterolert assays, individual positive wells arising from CR-2 and CR-3 were assessed as described above for TRFs associated with nontarget taxa. False-positive wells were defined as those that produced color or fluorescence in the absence of target TRFs.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under accession numbers EF658766 to EF659414.

RESULTS

Fecal indicator bacteria and bacterial community composition in creek and sewage samples. FIB concentrations were highest in the sewage influent sample and much lower in the creek samples (Table 1). The majority of the clones from CR-1 belonged to the *Betaproteobacteria* and, significantly, to the phyla *Bacteroidetes* and *Actinobacteria* (Fig. 1A). Cloned sequences from SEW-1 mostly belonged to the phyla *Bacteroidetes*, *Betaproteobacteria*, *Epsilonproteobacteria*, and *Fusobacteria*, with a minority belonging to the phyla *Gammaproteobacteria* and *Firmicutes* (Fig. 1A). The TRFLP profiles across CR-1, -2, and -3 were fairly similar and differed from that of SEW-1 (Fig. 2A), which was confirmed by hierarchical clustering and SIMPROF analysis (data not shown). Bacterial diversity estimates based on TRFLP patterns and clone library analysis showed the highest diversity in the sewage sample and lower but similar diversities in the creek samples (Table 1).

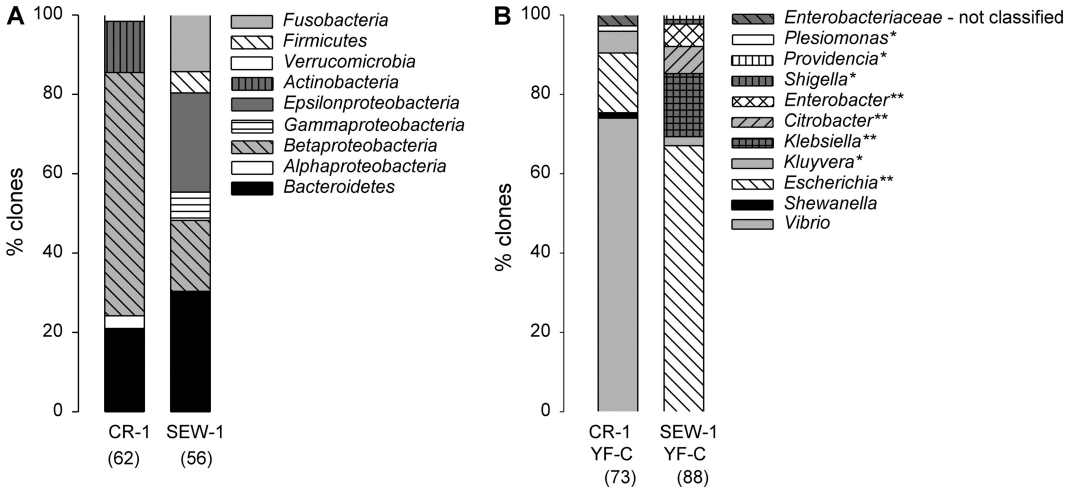

FIG. 1. Composition of the clone libraries, with the total numbers of high-quality nonchimera clones analyzed indicated in parentheses. (A) Creek (CR-1) and sewage (SEW-1) samples. Phylogenetic affiliations indicated are at phylum/class level. (B) Pooled yellow/fluorescent Colilert (YF-C) wells of CR-1 and SEW-1 samples. Phylogenetic affiliations are at genus level, except for clones that could not be classified. Genera belonging to the *Enterobacteriaceae* are indicated with one asterisk; genera belonging to the coliform group within the *Enterobacteriaceae* are indicated with two asterisks.

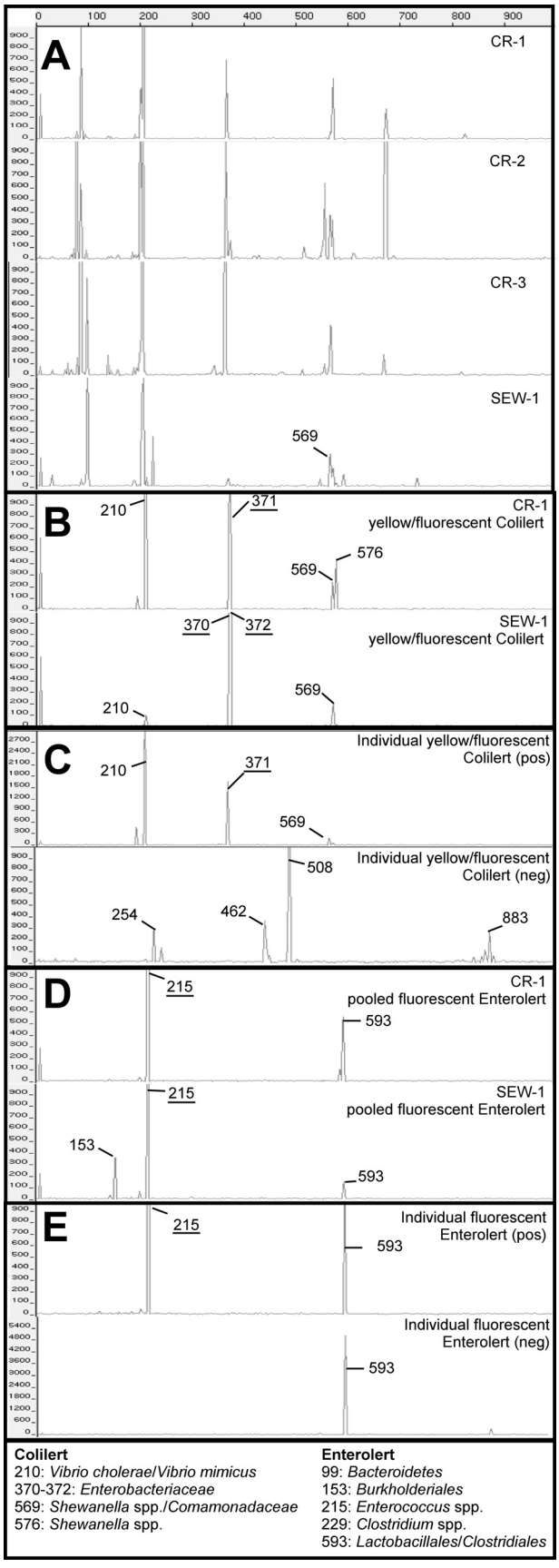

FIG. 2. TRFLP electropherograms for creek and sewage samples (A), pooled yellow/fluorescent Colilert wells (B), representative individual yellow/fluorescent Colilert wells containing (pos) or not containing (neg) the target terminal restriction fragments (TRFs) (C), pooled fluorescent Enterolert wells (D), and representative individual fluorescent Enterolert wells containing (pos) or not containing (neg) the target TRFs (E). The dominant peaks in the Colilert and Enterolert enrichments are indicated by their fragment lengths (bp) and are also shown in panel A when they were detected. TRFs corresponding to the target organisms for Colilert and Enterolert assays are underlined. Lengths (bp) and putative phylogenetic affiliations of the dominant TRFs are listed at the bottom.

Bacterial community composition in pooled positive Quanti-Tray/2000 wells. Multiple TRFLP peaks (≤8) were present in the pooled yellow/fluorescent Colilert and fluorescent Enterolert enrichments (Fig. 2B and D). The most abundant TRFs for each enrichment were similar for CR-1 and SEW-1. No TRFs were shared between environmental samples and IDEXX assay enrichments, except for one (569 bp in SEW-1 and pooled yellow/fluorescent Colilert wells of SEW-1). All clones from the SEW-1 pooled yellow/fluorescent Colilert wells belonged to the *Enterobacteriaceae*, and the majority (67%) were most closely related to *E. coli* (Fig. 1B). However, clones from CR-1 pooled yellow/fluorescent Colilert wells mostly belonged to the genus *Vibrio* (73%) and some to the genus *Shewanella* (1%). All clones from SEW-1 pooled fluorescent Enterolert and CR-1 pooled fluorescent Enterolert wells belonged to the target genus *Enterococcus* (data not shown).

The identities of the major TRFLP peaks in the electropherograms of the pooled Quanti-Tray/2000 enrichments were delineated using the MiCA web tool. The list of putative taxa was culled to include only genera belonging to taxonomic groups found in the enrichment clone libraries. The latter included a library from a wet weather sample in order to enable identification of all TRFs. These taxonomic groups were as follows: for yellow/fluorescent Colilert wells, *Enterobacteriaceae*, *Comamonadaceae*, *Vibrio* spp., and *Shewanella* spp., and for fluorescent Enterolert wells, *Enterococcus* spp., *Bacteroidetes*, *Clostridium* spp., and *Burkholderiales*. Most importantly, the analysis allowed the assignment of TRFs to the target bacterial groups *Enterobacteriaceae* (370 to 372 bp) and *Enterococcus* spp. (215 bp).

Bacterial community composition in individual positive Quanti-Tray/2000 wells. Most individual yellow Colilert wells contained the TRFs of 370 to 372 bp for *Enterobacteriaceae* (Fig. 3A). Only 4 and 2 wells out of 49 for CR-2 and CR-3, respectively, did not contain those TRFs. Other TRFs were typically also present, such as a 210-bp fragment corresponding to *Vibrio* spp., an unidentified 567-bp fragment, a 569-bp fragment corresponding to *Shewanella* spp. and the *Comamonadaceae*, and a 576-bp fragment corresponding to *Shewanella* spp. Most individual yellow/fluorescent Colilert wells also contained the TRFs of 370 to 372 bp for *Enterobacteriaceae* (Fig. 3B). Only 3 of 13 wells for CR-2 and 2 of 14 wells for CR-3 did not contain those TRFs. Individual yellow/fluorescent Colilert wells also contained the 210-, 569-, and 576-bp TRFs. In addition, the unidentified 343-bp TRF occurred frequently in

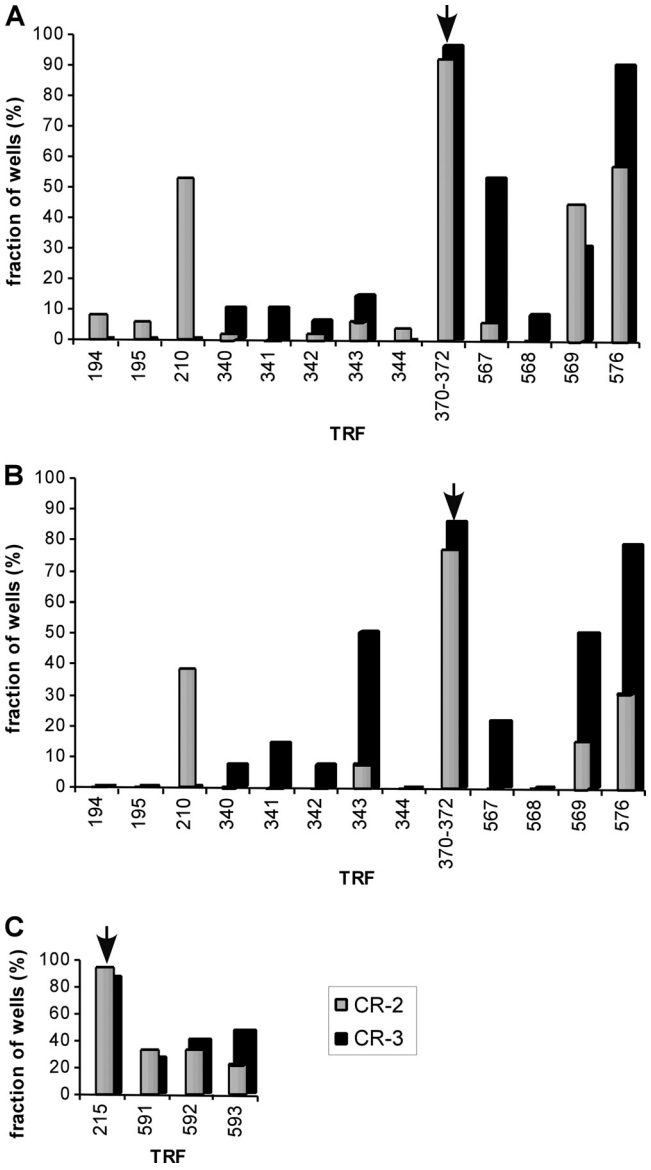

FIG. 3. Distribution of terminal restriction fragments (TRFs) across positively scored individual large Quanti-Tray/2000 wells for CR-2 and CR-3 FIB enrichments. The *x* axis shows TRF bp length. The *y* axis is the percentage of positive wells containing the TRFs for 49 yellow Colilert wells each for CR-2 and CR-3 (A), 13 and 14 yellow/fluorescent Colilert wells for CR-2 and CR-3, respectively (B), and 18 and 16 fluorescent Enterolert wells for CR-2 and CR-3, respectively (C). Samples were diluted 1:10 before addition to the Quanti-Trays. TRFs expected for total coliforms, *E. coli*, or *Enterococcus* spp. are indicated with an arrow.

CR-3 yellow/fluorescent Colilert wells. Finally, most individual fluorescent Enterolert wells contained the 215-bp TRF corresponding to *Enterococcus* spp. (Fig. 3C). Only 1 of 19 CR-2 wells and 3 of 16 CR-3 wells did not contain this TRF. Almost all individual fluorescent Enterolert wells that contained the 215-bp TRF also contained unidentified 591- or 592-bp TRFs and a 593-bp TRF corresponding to unidentified *Lactobacillales* or *Clostridiales*.

False-positive well rates. TRFs of target and nontarget bacteria were usually observed simultaneously in individual positive Colilert and Enterolert wells (Fig. 3). In those cases, the individual wells were deemed true positives. However, in some of the individual wells presumed positive for total coliforms, *E. coli*, or *Enterococcus*, only TRFs associated with nontarget bacteria were detected. Based on the absence of target TRFs in positively scored individual Colilert wells, the false-positive rates for CR-2 and CR-3 water samples under dry weather conditions were 4 to 8% for total coliform, 14 to 23% for *E. coli*, and 6 to 20% for Enterolert (Fig. 3; Table 2).

DISCUSSION

FIB assays are a requisite component of microbiological water quality monitoring, and thus, understanding the specificity of defined substrate technology for FIB quantification is important. Prior studies of defined substrate technology enrichment microbiology relied upon phenotypic profiling of enrichment isolates to reveal false positives (1, 9, 11, 17, 19, 23, 43, 51). In this study, culture-independent bacterial community analyses were used to reduce potential biases due to selective culture media. While TRFLP can have its own biases, e.g., originating during DNA extraction, PCR amplification, enzymatic restriction, and electrophoresis (22, 25, 41), relative changes in community structures are reliably reflected using TRFLP (25, 46). Further, dominant taxa can be detected, even if the dominant taxa are not represented by the dominant TRFs (22, 40). TRFLP biases were minimized in this study by equalizing PCR and restriction template DNA concentrations, using high DNA template concentrations (~250 ng) before restriction, limiting the number of PCR cycles to 28, and using relative fluorescence intensities for data analysis. Finally, the absence of target TRFs in water samples—but the dominance of target TRFs in the positive wells—indicated that positive or negative biases, e.g., due to primer mismatches (49), were unlikely to be significant for the target TRFs.

A variety of nontarget taxa were detected in positive Colilert

TABLE 2. Calculation of false-positive rates based on the number of positive wells before or after subtraction of false-positive large wells

Phylogenetic affiliation[a]	Sample	FP[b]	No. of positive wells (L, S)[c]	FP rate (%)
TC	CR-2	+	49, 22	8
		−	45, 22	
	CR-3	+	49, 48	4
		−	47, 48	
EC	CR-2	+	13, 3	23
		−	10, 3	
	CR-3	+	14, 0	14
		−	12, 0	
ENT	CR-2	+	18, 3	6
		−	17, 3	
	CR-3	+	15, 2	20
		−	12, 2	

[a] TC, total coliform; EC, *E. coli*; ENT, *Enterococcus* spp.
[b] FP, false positive; +, positive wells before subtraction of the number of false-positive large wells; −, positive wells after subtraction of the number of false-positive large wells.
[c] L, large wells; S, small wells.

and Enterolert wells, including taxa that were not detected in previous studies using culture-based techniques. Nontarget TRFs were mainly associated with *Shewanella* spp., *Comamonadaceae*, and *Vibrio* spp. in positive Colilert assays and with unidentified *Lactobacillales* or *Clostridiales* in positive Enterolert assays. *Vibrio* spp. have been recovered from yellow and yellow/fluorescent Colilert wells associated with marine (42, 43) and freshwater (51) samples before, but this study also suggested that *Vibrio* spp. can be among the dominant populations in these wells. Noncoliforms such as *Salmonella* spp. have been isolated from positive Colilert tubes or wells from freshwater samples before, although only in a minority of the samples (17, 43). A larger diversity of noncoliform genera was isolated from tropical freshwaters (11). For Enterolert, growth of members of the class *Flavobacteria* (but no other *Bacteroidetes* bacteria) and the order *Lactobacillales* was previously shown (9), but growth within the orders *Clostridiales* and *Burkholderiales* (in SEW-1 pooled fluorescent Enterolert wells) is newly reported with this study. As both *Enterococcus* spp. and *Clostridiales* are considered fastidious organisms (7, 56), it is reasonable that the Enterolert medium can support the growth of both groups of bacteria. It is known that some *Clostridiales* exhibit significant beta-glucosidase activity (37). However, since enzyme activity can differ substantially, even within a genus or species (37), direct proof of the ability of *Lactobacillales* or *Clostridiales* to cause false-positive results in the Enterolert assay should involve isolation of bacteria.

The relative abundances of target and nontarget TRFs in water samples versus IDEXX assay enrichments suggested growth of nontarget bacteria in the IDEXX assays. The growth of coliforms and *Enterococcus* spp. during incubation in Quanti-Trays increased the relative abundance of the target TRFs from nondetectable (in water samples) to dominant (in individual positive Quanti-Tray wells). The absence of these TRFs in the source water samples is not surprising, given the FIB concentrations in this study and published detection limits for TRFLP ($\sim 10^7$ CFU per 100 ml) (46). Therefore, it is reasonable to infer that where *Vibrio* and *Lactobacillales*/*Clostridiales* were the dominant nontarget TRFs in IDEXX enrichments, the cause was growth.

The increased detection of nontarget organisms in this study compared to their detection in previous studies is probably rooted in the differences between culture-independent versus culture-dependent approaches. Previous culture-based studies have used either general purpose culture media (e.g., tryptic soy agar and R2A agar) or selective media (MacConkey agar, m-ENDO agar, and m-TEC agar) to isolate bacteria from yellow/fluorescent Colilert wells/tubes (10, 17, 19, 43). Selective media chemistry will bias the detection of nontarget organisms, as previously shown for the detection of *Vibrio* spp. in positive Colilert tubes (42). However, even general purpose media are selective and can underestimate culture-independent bacterial diversity (5, 30). Accordingly, the culture media (bile-esculine agar and MacConkey agar) and aerobic incubation conditions used previously for isolation and phenotypic profiling of bacteria growing in fluorescent Enterolert wells (1, 9, 23) were not suitable for detecting either *Clostridiales* or *Burkholderiales*. Still, it is possible that differences between this study and prior studies originate with the samples analyzed. Even within this study, some sample-to-sample variability was observed, as no *Vibrio* spp. were detected in the individual yellow/fluorescent Colilert wells of CR-3.

False-positive wells were identified as yellow Colilert, yellow/fluorescent Colilert, and fluorescent Enterolert wells with no target TRFs present. Consequently, at least one of the nontarget TRFs was associated with bacterial taxa causing the false-positive signals. Since DNA yields from the false-positive wells were sufficiently high for reliable PCR and TRFLP, target TRFs were dominant in other (true) positive wells, and TRFLP is generally very reproducible (25, 46), the absence of target TRFs in some wells was unlikely to be caused by TRFLP biases. The false-positive rates in this study, based on TRFLP, are very similar to those reported previously for total coliforms but slightly higher for *E. coli* (1, 9, 23) and higher for Enterolert (6 to 20% here versus 2.4 to 5.1% previously) (1, 9, 23, 26). The TRF of 370 to 372 bp cannot distinguish between *E. coli* and other coliforms and can also be associated with noncoliform *Enterobacteriaceae*. Therefore, the false-positive rates for *E. coli* and total coliforms based on TRFLP are conservative estimates.

Based on the results from this study, a more general assessment of the impact of false positives on the overestimation of FIB concentrations in environmental water samples is recommended. In this study, a limited number of samples, representative for dry weather flow at one urban creek location, were analyzed in order to support a detailed analysis using clone libraries. Additional research is needed to determine false-positive rates in IDEXX assays for a larger number and wider range of samples (e.g., storm flow). Nonetheless, the results of this study, including the identification of cloned sequences and TRFs, can be applied to detailed false-positive assessments of other locations, samples, and types of FIB assays. Lastly, an implication of our study is that future efforts aimed at reducing false positives in IDEXX assays could constructively focus on reducing the growth of nontarget bacteria, e.g., by testing a variety of selective growth-inhibiting compounds available for members of the *Lactobacillales* and *Clostridiales* and for *Vibrio* spp. (18, 32, 38, 50, 52).

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