Detection and Occurrence of Indicator Organisms and Pathogens

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ABSTRACT: This review summarizes the studies related to detection and occurrence of fecal indicator bacteria (FIB) and pathogens published during 2010. The first section of this review summarizes various detection methods for waterborne pathogens including endpoint, reverse transcriptase, and real-time polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), microarrays, and immunoassays. Relevant literature on sample processing is also included. The second section focuses on phenotypic and genotypic methods for microbial source tracking. The third section summarizes studies related to occurrence, persistence, and transport with emphasis on microbial quality of recreational beaches and watersheds.

KEYWORDS: fecal indicators, watershed, wetlands, transcription PCR, loop-mediated isothermal amplification, LAMP, genotypic, phenotypic, source tracking

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Introduction

During 2010, more than 100 studies related to detection, occurrence, and MST were published. For this review, we selected key studies to highlight the new advancements or factors that may have received less attention in the reviews published in the previous years. Under detection methods, for example, we have included isothermal amplification and label free detection as methods having importance in the area of drinking water and surface water quality. For MST, most of the focus is on approaches that have been published previously but with data related to additional validation/evaluation. For occurrence, persistence, and transport, some of the key studies focusing on modeling of bacterial transport have been included. Overall, more than 75 studies are summarized in three separate sections: Detection Methods, Microbial Source Tracking (MST), and Environmental Occurrence, Persistence and Transport.

Detection Methods

PCR and real time PCR based approaches are approaching maturation for detection of waterborne pathogens. Hence, only selected examples are included here. Emerging approaches such loop-mediated isothermal amplification and on-chip PCR, although not yet applied...
extensively are being evaluated due to their low cost, ruggedness, ease of use, and multiplexing capabilities. Similarly label-free detection is also gaining importance especially because of its potential in real time monitoring. This technique, if successful, could serve the drinking water industry for online monitoring. As always, sample processing is the bottleneck for most of these approaches and key studies related to sample processing are also summarized.

**Polymerase chain reaction.** Assays for Bacteroidales associated with human feces were used to characterize fate and transport of waterborne pathogens (Shanks et al. 2010b). Five end-point PCR assays and ten real-time quantitative PCR assays targeting various genes associated with Bacteroidales were used. Of the five end-point PCR assays, HF183 and HumM19 primer sets proved to be most useful with specificity values of 95%, prevalence in sewage of 100%, and clinical sensitivity greater than 10%. The term clinical sensitivity, defined as the percentage of samples known to contain the target and resulting in a positive amplification, is used here to differentiate it from detection sensitivity. In the case of this study, samples containing $1 \times 10^{-4}$ ng of target DNA per reaction was used to determine the clinical sensitivity. Of the ten quantitative PCR assays tested, the HF183, BsteriF1, and HumM2 primer sets produced results that met the following criteria: a limit of quantification greater than 25 gene copies, a prevalence in sewage samples of greater than 99%, a mean percent coefficient of variation across the range of quantification less than 5%, and a high target abundance of genetic markers. In a similar study from the same group, seven end-point PCR and quantitative PCR assays known to be associated with ruminant or bovine fecal contamination of water targeting Bacteroidales genetic markers were evaluated for suitability in field studies (Shanks et al. 2010a). These assays were tested against DNA extracts from 247 bovine fecal samples and 175 fecal samples from other animals. The results of the assays showed specificity levels between 47.5% and 100% and a varying sensitivity during end-point PCR assay. Because of the varying results in this experiment between the bovine-related assays and sample populations, it was recommended that further investigation was needed for individual populations and sample sources.

A method was developed to detect various enteroviruses using real-time reverse transcription PCR (RT-PCR) (Zhang et al. 2010). The primers were designed based on the non-coding region sequences targeting poliovirus, coxsackievirus, and enterovirus. The detection limit for the real-time RT-PCR method utilizing SYBR-Green was found to be 2.31 gene equivalent copies per µL. The intra- and inter-assay variations of the method were less than 2% and 5%, respectively, and the virus recovery efficiency was 73%. This assay seemed to be an effective method of detecting and quantifying various enteroviruses simultaneously.

In another study, the real-time PCR and RT-PCR assays were used to detect the occurrence of various human enteric viruses in surface water and sewage samples from various areas in Kenya (Kiulia et al. 2010). The enteric viruses infect, replicate in the gastrointestinal tract, and shed through feces into the local waterways. It was
determined that at least one enteric virus was present in nearly all sewage and river samples. Because of the low infectious dose, this is particularly problematic in developing countries. A method for distinguishing between infectious and noninfectious human enteric viruses in water samples was developed. A key strength of the method employed was the use of propidium monoazide in conjunction with reverse transcriptase PCR (Parshionikar et al. 2010). The propidium monoazide penetrates into the damaged capsids of noninfectious viruses and bonds with the viral RNA, rendering it unavailable for amplification during RT-PCR. The heat-inactivated (at 72°C and 37°C) and chemically-inactivated (hypochlorite treatment) virus samples were noninfectious and did not interfere with the detection of the infectious samples. The method could not identify the noninfectious viruses that were inactivated at 19°C. Because the developed method proved adequate in the majority of the cases, it was concluded that it could be used in the environment to differentiate between potentially infectious and noninfectious viruses.

The efficiency between endpoint and real-time RT-PCR were compared for use in detecting and quantifying noroviruses (Anbazhagi & Kamatchiammal 2010). Noroviruses are responsible for acute gastroenteritis caused by contaminated drinking water. Utilizing activated carbon, 100 samples were collected and concentrated for norovirus. The RT-PCR reactions used N1 and E3 primers previously designed for noroviruses. The real-time PCR reactions used a SYBR-Green RT-PCR kit. The results showed that out of 100 samples, 18% were positive with endpoint RT-PCR while 20% were positive with real-time RT-PCR. It was concluded that real-time PCR was more sensitive method of detecting noroviruses than endpoint PCR.

*Methanobacter smithii* is commonly found in the human gastrointestinal system and its presence in the environment is used as a fecal indicator (Johnston et al. 2010). Quantitative PCR was also used to detect and quantify *M. smithii*. Primers were designed to target the *nifH* gene and the results were quantified and compared to a competitive internal positive control in order to measure the effects of possible inhibition. The *nifH* gene was amplified with a detection limit range from 5 genomic copies to $5 \times 10^5$ genomic copies and an efficiency of 95%. The competitive internal positive control showed varying levels of inhibition in 81% of samples.

In another study, a method was developed to detect and quantify the *Mycobacterium* spp. 16S rRNA gene with quantitative PCR (Radomski et al. 2010). Eighteen primer sets were developed using in-silico screening and then tested for clinical sensitivity and specificity. Results indicated that 110F/I571R was the most specific and sensitive primer set. The PCR reaction was optimized to achieve an efficiency of 74.3 ± 1.7%, a specificity of 100%, a clinical sensitivity of 77%, and a limit of detection of 23 copies. It was concluded that this procedure was more specific than previously published methods that targeted the *hsp65* and 16S rRNA genes.

In a study evaluating the use of real-time PCR to detect *Legionella* spp. in spa water, the real-time PCR data was compared with traditional culture based measurements (Guillemet et al. 2010). The results showed that using the
culture method, *Legionella* was detected in 14 out of 101 samples with concentrations ranging from 250 to 3.5 x 10^5 colony forming units (CFU) per liter. Using real time PCR, *Legionella* was detected in 42 out of 101 samples with concentrations ranging from 10^3 to 6.1 x 10^7 genome copies per liter. It was concluded that real time PCR was a suitable method for rapidly screening spa water for *Legionella*. It was also suggested that a positive real time PCR result should be complemented with culture-based method for more accurate confirmation.

A real-time multiplexed PCR assay was developed to detect *Vibrio parahaemolyticus* in water and shellfish from the Gulf of Mexico using the *tlh*, *tdh*, and *trh* genes (Rizvi & Bej 2010). The limit of detection was determined to be 10 CFUs. The results showed that 58% of the oysters tested were positive for *tlh*. Of the *tlh*-positive oysters, 21% were positive for *tdh* and 0.7% were positive for *trh*. These results indicated the presence of pathogenic strains of *V. parahaemolyticus*, as the *tlh* gene is characteristic to *V. parahaemolyticus* while the *tdh* and *trh* genes are associated with pathogenic strains.

**On-chip PCR.** A disposable microfluidic chip with 9 reaction channels made from polydimethylsiloxane and glass was developed for parallel detection of waterborne pathogens (Ramalingam et al. 2010). Capillary forces were exploited to drive the PCR reaction solution into individual reactors and an absorbent pad was used to wick sample overflow. The hydrophobic surface of polydimethylsiloxane was passivated by adding detergent (0.1% Triton X-100) into the reaction solution. A low-cost real time analyzer made with light emitting diode and a charge coupled device camera was used for capturing the amplification signal simultaneously from the 9-reaction channels each having a volume of 5 µL. Virulence genes of 5 major waterborne pathogens were validated on this chip including, *Aeromonas hydrophila*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *E. coli*. The lower limit of detection for *E. coli* *uidA* gene was approximately 51 CFU per mL. Results showed that limit of detection and quantitative values were in high agreement with those obtained using a commercial real-time PCR instrument (RotorGene 3000). Evaporative losses on these unsealed microchannels were also evaluated with respect to the length of bridge channels (channels connecting the input/output wells to the reaction wells). Due to the application of capillary flow of reaction solution, applications of expensive pumps, valves, and liquid handling instruments were eliminated.

**Loop-mediated isothermal amplification.** LAMP is a relatively new amplification technique and is known for being rugged, simple, isothermal, and inexpensive. Because of these characteristics, it has excellent potential for the detection of waterborne pathogens and FIB. LAMP uses four to six primers and a DNA polymerase (*Bst*) with a high specific activity. Because of the specific design of primer and amplification approach, LAMP results in considerably higher DNA yields compared to PCR. The high concentration of DNA produced by LAMP is accompanied with pyrophosphate precipitation, which can be observed at the end of the reaction with the naked eye, or in real-time with a turbidimeter, allowing quantification. Quantification can
also be obtained by the use of DNA-intercalating dyes such as SYBR Green or SYTO-9, along with real-time measurement of the fluorescence (Seyrig et al. 2010).

In 2010, a number of studies applied LAMP to detect waterborne pathogens and indicators. LAMP was used to investigate the effect of warm water treatment on the persistence of Flavobacterium psychrophilum in broth and sterilized groundwater samples (Sugahara et al. 2010). This bacterium was associated with bacterial cold-water disease that causes a high mortality of the ayu (sweet fish) Plecoglossus altivelis in Japan. Results indicated that after 3 days of warm-water treatment (28°C), the pathogenic bacterium was not detectable in any of the samples.

LAMP was compared to PCR to detect two strains of the neurotropic fungus Ochroconis gallopava, previously isolated from hot spring water sampled from a river and several bathtubs in Japanese spas (Yarita et al. 2010). Both LAMP and PCR detected the fungus using primer sets targeting the small subunit, the internal transcribed spacer and the large subunit. None of the methods allowed discrimination between the two strains of O. gallopava. For PCR, this was probably because the amplification product that was obtained from each strain did not have a size large enough to be differentiated on a gel.

LAMP was also used to monitor Giardia cysts and Cryptosporidium oocysts in drinking water samples (Plutzer et al. 2010). In this study, samples consisted of large volumes of water (10 to 100 L) that were first filtered on new polyester microfiber membranes (ARAD, Hungaria Kft., Hungaria). This strategy allowed the detection of one cyst/oocyst per 10 L of drinking water.

**Microarrays.** Microarrays have been applied for high-throughput molecular assays of pathogenic microorganisms, microbial source tracking, and microbial community analysis, among others. A novel functional gene array was designed with 51 probes (two probes per target with probe length of approximately 40 nucleotides) for the detection and serotyping of enterotoxigenic E. coli (Wang et al. 2010c). Probes targeting four virulence genes specific to enterotoxigenic E. coli (elt, estAp, estAh, and estB) and two O-serogroup-specific genes (wzx and wzy) were designed. The rfpB gene was included to differentiate E. coli serogroup O148 from Shigella dysenteriae type 1, as wxz and wzy genes had 99% similarity with these two serotypes. Specificity of this microarray was tested against 223 strains including 50 target references, and 173 other strains (other E. coli O serotypes and closely related species). A limit of detection of 50 ng of genomic DNA (or $10^8$ CFU per mL in pure culture) was obtained. Very low amplification bias was achieved due to the application of random PCR in this study.

A functional gene microarray was validated for waterborne pathogens and indicator organisms (Kostic et al. 2010). The platform was developed by sequence-specific end labeling of probes targeting gyrB gene. Validation was performed with the reference strains and spiked environmental samples. A high specificity with the resolution up to species level and a limit of detection of $10^4$ CFU per mL was achieved. The method was cross-
validated with fluorescence *in situ* hybridization (FISH) and conventional microbiological reference methods.

A low-density 16S rRNA gene microarray was designed for simultaneous detection of multiple protozoans (Lee *et al.* 2010a). The microarray contained 29 oligonucleotide probes targeting eukaryotic small subunit ribosomal RNA gene sequences, covering 6 species and 4 genera of protozoa. In addition, a real-time PCR assay was also designed targeting functional genes and rRNA genes. This microarray was capable of identifying 3 protozoan strains (*Acanthamoeba castellanii*, *C. parvum*, and *G. intestinalis*) with a detection limit of $1 \times 10^3$ target genes or 50 oocysts per assay in the presence of background DNA. In contrast, the limit of detection of the real-time PCR assay (also in the presence of background DNA) was approximately 100 oocysts per assay.

Another low density 16S rRNA gene microarray was designed for detecting waterborne pathogens (Ichijo *et al.* 2010). This microarray contained 11 oligonucleotide test probes targeting 16S rRNA genes of 12 waterborne pathogens. Also, 11 mismatched probes were included with 2 nucleotides mismatches against a corresponding test probe. Specificity of the probes was evaluated by individually hybridizing 1 µg of fluorescently labeled 16S rRNA gene amplicon (generated by PCR) to the microarray. Finally, seven probes, namely AERO, BCEAN, PSHIGE, PSUDO, TSUKA, VFLUV, and VVUPA were selected to target *Aeromonas* spp., *Bacillus cereus*, *Plesiomonas shigelloides*, *Pseudomonas* spp., *Tsukamurella* spp., *V. fluvialis*, *V. vulnificus*, and *V. parahaemolyticus*, respectively.

**Imunoassay-based detection schemes.** A biosensor based on enzyme linked immunosorbent assay (ELISA)-on-a-chip was developed for the detection of *V. parahaemolyticus* (Seo *et al.* 2010). Initially, this sensor had a limit of detection of approximately $6.2 \times 10^3$ cells per mL, which required the prior cultivation of this pathogen, requiring at least a day. Therefore, an immunomagnetic separation method was applied prior to detection, which concentrated the culture by 86-fold. This immunomagnetic separation coupled biosensor was able to detect $10^4$ cells per mL. When the sample specimens were inoculated with 1 to 5 CFUs of *V. parahaemolyticus* cells, cultured between 6-9 h. Finally when enriched, this sensor was able to detect less than 1 CFU. Also this sensor showed negative results for other 23 non-targeted microbial species.

An immunofluorescent aggregation assay was developed to detect *Vibrio cholerae* O1 and O139 directly from estuarine water samples (Wang *et al.* 2010a). Approximately 10 µL of sample (with O1/O139 fluorescent antibody solution) mixed with 100 µL of peptone was placed on a glass slide. After an incubation period of 6 h at 37°C, the slide was imaged by fluorescence microscopy to confirm the aggregation. This immunofluorescent aggregation assay had a limit of detection of $10^3$ CFU per mL. Enrichment broth of 146 estuarine water samples was tested. This assay was compared against conventional culture method and real-time PCR. The percent positive result by the immunofluorescent aggregation assay, conventional culture method, and real-time PCR was 19.9%, 10.3%, and 29.5% respectively.
In another study, a multiplex bead-based assay was designed for the detection of immunoglobulin G antibodies for *G. intestinalis* and *C. parvum* (Priest et al. 2010). Variant-specific surface protein sequences recognized by immunoglobulin G antibodies were identified and applied for their detection from stool samples. More specifically, 3 fragments of variant-specific surface protein from assemblage B strains and α-1 giardin structural antigen for *Giardia* and recombinant 17- and 27-kDa antigens for *Cryptosporidium* were used. Approximately 60% of giardiasis outbreak samples were positive by targeting multiple *Giardia* antigens, while less than 12% non-outbreak samples and cryptosporidiosis outbreak samples were also positive. Approximately 40% of cryptosporidiosis outbreak samples were detected by targeting 17- and 27-kDa antigens, while less than 10% of non-outbreak samples and giardiasis outbreak samples were positive.

Immunoassays were developed on nano-fibers for the detection of *E. coli* O157:H7 and bovine viral diarrhea virus (Luo et al. 2010). Nanofibers were fabricated with nitrocellulose by an electrospinning process, functionalized with antibodies, and directly used for the detection. Nanofibers were treated with oxygen plasma to enhance the flow of solution in nanofibers by capillary action. Antibody attachment and pathogen binding was confirmed by confocal laser scanning microscopy and scanning electron microscopy. The sensor showed a linear response for different *E. coli* O157:H7 concentrations ranging from 0 to $10^4$ CFU per mL. This biosensor was highly rapid (8 min) with a limit of detection of 61 CFU per mL and $10^3$ cell culture infective dose per mL for bacterial and viral samples, respectively.

A graphene-oxide-based sandwich immuno-biosensor was developed for the detection of rotavirus (Jung et al. 2010). Presence of rotavirus was confirmed by observing the fluorescence quenching by fluorescence resonance energy transfer process between the graphene-oxide and gold nanoparticles. The antibodies for rotavirus were immobilized on the graphene-oxide array. Gold nanoparticles were attached with antibodies by using 100-mer single stranded DNA chains. The short DNA chains controlled the distance between antibodies, gold nanoparticles, and the graphene-oxide surface. The fluorescence emission of the graphene-oxide was quenched upon binding of antibody-DNA-gold nanoparticle complex with the rotavirus, enabling the detection of target cells. The detection limit of this sensor was $10^5$ PFU per mL.

A colloidal gold immune-chromatographic sensor was evaluated for detection of *E. coli* O157: H7 (Zhao et al. 2010). Gold nanorod colloid was synthesized by citrate method. These gold nanorods were immobilized with polyclonal antibodies specific to *E. coli* O157: H7. Antibody attachment to gold nanorods was confirmed by UV-visible light absorption spectra and transmission electron microscopy. This sensor was evaluated for 65 strains (36 *E. coli* O157: H7 and 29 non-*E. coli* strains). Specificity of 98.5% and clinical sensitivity of 100% was achieved. Detection limit was approximately $2.3 \times 10^3$ CFU per mL without any prior enrichment step and 2.3 CFU per mL with enrichment step. This sensor was also evaluated with 265 water samples, 340 beef samples, 208 milk...
samples and 120 cake samples after enrichment providing a specificity of 99.2, 97.9, 94.6 and 94.9%, for each sample type respectively. Clinical sensitivity was 100% and a strong agreement was observed with traditional culture method. Detection time was only 10 min.

**Label-free detection methods.** Label-free detection methods are ideal for the detection of pathogenic microorganisms in a rapid, automated, and miniaturized format. In 2010, a number of label-free detection approaches based on different working principles (electrochemical, cantilever, surface-enhanced Raman spectroscopy, and lens-free microscopy) have been explored.

A gold nanoparticle-based electrochemical immune-detection method was developed for the detection of *E. coli* in water (Liu et al. 2010). Based on the sandwich immunoassay, *E. coli* cells were detected by H$_2$O$_2$ electro-reduction current catalyzed by horseradish peroxidase (labeled on anti-*E. coli* antibody) which was measured amperometrically. Detection limit of this sensor was 20 CFU per mL. After including a pre-enrichment step, a limit of detection of 2 CFU per mL was achieved.

In a study, surface-enhanced Raman spectroscopy in combination with gold-active substrates was used to detect seven viruses including, norovirus, adenovirus, parvovirus, rotavirus, coronavirus, paramyxovirus, and herpesvirus (Fan et al. 2010). Surface-enhanced Raman spectroscopy spectral patterns were further analyzed by soft independent modeling of class analogy and principal component analysis, to discriminate these viruses. Soft-independent modeling of class analogy allowed the differentiation of enveloped and non-enveloped viruses with and accuracy greater than 95%. Principal component analysis allowed to specifically segregate the spectral data from different viral strains. This sensor had a limit of detection of 100 viral particles per mL. In addition the results showed that the viral particles needed to be diluted in deionized water to minimize the background signals.

Another immunoassay-based detection platform using surface-enhanced Raman scattering for *E. coli* was proposed (Temur et al. 2010). Two different types of gold nanoparticles (citrate-stabilized gold nanospheres and hexadecyltrimethylammonium bromide-stabilized gold nanorods) were examined in this study. The analytical performance of this sandwich immuno-assay showed linearity for different concentrations of *E. coli* ranging from $10$ to $10^5$ CFU per mL. The total analysis time was 70 min.

A rapid and sensitive assay for *E. coli* O157:H7 was developed by using inductively coupled plasma mass spectrometry (Li et al. 2010). Inductively coupled plasma mass spectrometry was used for trace element analysis (inorganic particles) and provides large dynamic range, low limit of detection, and multiplexing capability. In this case, a monoclonal antibody for *E. coli* O157:H7 was conjugated with gold nanoparticles (10-nm diameter). Detection of gold nanoparticles by inductively coupled plasma mass spectrometry provided quantitative analysis of the bacterial cells. As this technique probes elemental ions (10 nm diameter gold particle will have ~30,000 gold atoms), detection signal could be enhanced by 30,000-fold. Due to the signal amplification property of gold nanoparticles, and the high sensitivity of inductively coupled plasma mass
spectrometry, limit of detection of 500 CFU per mL was obtained. This assay was also tested against non-pathogenic E. coli (DH5r, ATCC35218, and ATCC25922) and showed high specificity for E. coli O157:H7. The total detection time was 40 min.

A micro-cantilever-based biosensor was developed for the quantitative immunoassay of V. cholerae O1 (Sungkanak et al. 2010). Monoclonal anti-V. cholerae O1 antibodies were immobilized on gold-coated cantilevers by self-assembled monolayer preparation method. Resonance frequency shift of the cantilever was measured and correlated with the amount of attached V. cholerae O1 on its surface. A linear relationship between the resonance frequency shift of the cantilever and the log of cell concentration was obtained for cell concentrations ranging from 1×10^3 to 1×10^7 CFU per mL. The limit of detection of this sensor was approximately 1x10^3 CFU per mL with a mass sensitivity of ~146.5 pg/Hz. Specificity of this sensor was tested against 1×10^8 CFU per mL of V. parahemolyticus, which showed a negligible (24 Hz) shift in resonance frequency as compared to 1100 Hz for 1×10^6 CFU per mL of V. cholerae O1.

In another study, a low-cost and portable lens-less holographic microscope was developed to image and detect Giardia lamblia and C. parvum (Mudanyali et al. 2010). This microscope uses a light emitting diode to vertically illuminate the sample. The sample was imaged by a low-cost lens-less complementary metal oxide semiconductor detector, which forms holograms of the cysts/oocysts. These holograms were digitally transferred to a computer for the reconstruction of images. In this experiment, G. lamblia and C. parvum particles were fixed on glass slides with 5 % formalin (pH 7.4) and imaged by the microscope. The field of view of this microscope was ~24 mm^2 with a detection limit of less than 400 particles per mL. As these holograms can be acquired by cell phone cameras and can also be transferred to a central computer for processing, this device has a potential for pathogen diagnosis in resource-limited settings.

Sample processing. For water, large volumes of samples are required due to the presence of very low concentrations of waterborne pathogens and the high abundance of background organisms. Sample enrichment and processing steps are typically used to enhance the sensitivity of molecular methods applied for the detection of waterborne pathogens. A number of studies were published in 2010 on the application of filtration and immunological methods for the enrichment of cells, and for the extraction of the nucleic acids of waterborne microorganisms.

In one study, magnetic nanoparticles coupled with antibodies against the enterobacterial common antigen were applied to enrich E. coli cells from 10 mL water samples (Pappert et al. 2010). E. coli cells were further quantified with a chemiluminescence-based sandwich enzyme-linked immune-sorbent assay. A recovery efficiency of approximately 97% for the sample with 10^6 cells per mL and 89% for the sample with 10^7 cells per mL was achieved. Recovery was reduced to 20% for cell concentrations higher than 5×10^7 cells per mL due to the saturation of the antibody-coupled magnetic nanoparticles. The enrichment step enhanced the limit of detection of E.
coli from 5.0×10⁶ to 2.6×10⁵ cells per mL. The total recovery and detection time was 3 h and 45 min, including only 30 min for the enrichment step.

In another study, a simple method was developed to concentrate a model virus in order to reduce the limit of detection of lateral-flow immunoassays (Mashayekhi et al. 2010). An aqueous two-phase micellar system was generated using the non-ionic surfactant Triton X-114 and phosphate-buffered saline, to concentrate bacteriophage M13. Upon slight increasing of the temperature, a top micelle-poor phase and a bottom micelle-rich phase were formed, due to phase change in the solution. Due to the three interactions, namely repulsive, stearic, and excluded-volume, between M13 particles and micelles, a selective partitioning of M13 was obtained towards the micelle-poor phase. Including this concentration step before lateral-flow immunoassay, the limit of detection was improved by 10-fold from 5×10⁸ to 5×10⁷ PFU per mL.

An integrated flow-through immune-magnetic separation system was developed for the recovery of Giardia cysts and Cryptosporidium oocysts spiked in large volumes (10 L) of tap water, secondary effluent water, and purified water (Ramadan et al. 2010). Periodically arranged rotating magnets along the fluidic channels (containing magnetic particle suspension) were applied to allow the alternate trapping and release of particles during the recovery cycle. Microfluidic channels were fabricated in glass and a low-cost polycarbonate material. The performance of this system was compared with a commercial magnetic bead-based isolation instrument (magnetic tube holder). Experimental protocols of the U.S Environmental Protection Agency Method 1623 were applied to detect the cells after the recovery process. The recovery efficiencies for Giardia cysts from tap water with the flow-through system and commercial system were 90.1% and 90.5% respectively. From secondary effluent samples the recovery efficiencies from the flow-through system and commercial system were 18.5% and 31% respectively. The recovery efficiencies for Cryptosporidium oocysts with the flow-through system and commercial system were 83.3% and 90% respectively, from tap water, and 36% and 38%, respectively, from secondary effluent samples. Lower recovery efficiencies of cells from the secondary effluent samples were due to the presence of high concentration of sand particles, which might have influenced the binding of cells with the functionalized nanoparticles. Recovery efficiencies from all the samples (with the flow-through system) were higher in glass channels in comparison to polycarbonate channels. The non-smooth walls of plastic channels in comparison to smooth walls of glass might have caused the adherence of cells, reducing the recovery efficiency in polycarbonate flow-through channels. Although the recovery efficiency of this flow-through system was comparable or slightly lower than the commercial system, the primary benefit of this method was the rapid and automated recovery of pathogens from high sample volumes.

Filtration performances of four types of membranes, Anodisc® membrane filters, silver membrane filters, and gold-coated polycarbonate track etched membrane were evaluated to assess the concentration of G. lamblia cysts in drinking water (Wigginton & Vikesland...
After the filtration, the cysts were directly labeled with gold nanoparticles conjugated with antibodies and detected with Raman spectroscopy. It was found that the gold-coated polycarbonate track etched membrane was the most efficient in the recovery (~95%) and detection of *G. lamblia* compared to the other applied membranes.

A coagulation-sand filtration process was developed for the removal of recombinant norovirus-like particles (morphologically and antigenically similar to noroviruses) from river water (Shirasaki *et al.* 2010). Performance of this process for the removal of two surrogates for waterborne viruses (Qβ and MS2), were also investigated for the comparison with recombinant norovirus-like particles. Three log orders of removals were observed for recombinant norovirus-like particles with 40 mM aluminum chloride at pH 6.8, or ferric chloride at pH 5.8, respectively. Lower percentage of removal of recombinant norovirus-like particles was observed with alum and ferric chloride at pH 6.8. Removal percentage of MS2 was higher than that of recombinant norovirus-like particles in the coagulation-sand filtration process. Removal percentage of Qβ was similar than that of recombinant norovirus-like particles. However, a high difference in the removal performance of Qβ was observed between the coagulation process and the sand filtration process. Therefore, none of the surrogates were recommended for norovirus removal by coagulation-sand filtration process.

In another study, comparison of the performances of hollow-fiber ultra-filtration and capsule filtration was performed for the concentration of 10 to 1000 *Toxoplasma gondii* oocysts in 1 L of spiked water, fresh surface water, and sea water samples (Shapiro *et al.* 2010). Concentrated oocysts of *T. gondii* were detected by PCR, real-time PCR (TaqMan) and epifluorescent microscopy. Water samples were also spiked with *T. gondii* surrogate microspheres, and detected by flow cytometry and epifluorescence microscopy. Microscopy-based detection was more sensitive than TaqMan and conventional PCR, allowing the detection of oocysts in all the water samples. This study will have implications in the research of transport and fate of oocysts in environmental samples.

The type of nucleic acid extraction methods highly influences the performance of molecular methods commonly applied for the detection of microorganisms (Jothikumar *et al.* 2010). In a study, an efficient RNA extraction buffer (RNAX) was developed for environmental samples for the detection of waterborne viruses such as hepatitis A virus and coxsackievirus B3. Prior to that, river and pond waters were concentrated with the polyethylene glycol precipitation method that also concentrates inhibitors. The RNAX buffer-based technique was optimized for the extraction of RNA from 4.0 mL of virus inoculated sample concentrates by applying a silica column-based protocol. Quantitative recovery of hepatitis A virus and coxsackievirus B3 was determined by reverse transcript quantitative PCR. The RNA obtained by the RNAX buffer-based technique was found to be compatible with reverse transcript quantitative PCR-based detection, with a limit of detection of 0.8 PFU per reaction. A major advantage of this method was the sensitive detection of viruses as the system allowed to process a volume of
sample that was 20-fold larger than the commercial RNA extraction kits.

**Microbial Source Tracking (MST)**

Approaches to MST include library dependent and independent phenotypic and genotypic methods. In 2010, most of the studies used library independent genotypic approaches. However, a few library dependent genotypic approaches using rep-PCR, and phenotypic methods using antibiotic resistance profiling were also used.

**Genotypic MST methods.** The genotypic methods in 2010 included the development of new source tracking bacterial and viral genetic markers that mostly relied on PCR and real-time PCR.

In a study conducted in France (Gourmelon et al. 2010b), a new MST qPCR assay targeting human specific Bifidobacteria phylotype related to *Bifidobacterium adolescentis* was compared with real-time PCR assays targeting bacterial markers such as human-specific *Bacteroidales* (HF183), two pig-specific (Pig-1-Bac and Pig-2-Bac) and one ruminant-specific 16S *Bacteroidales* marker (rum-2-Bac), a pig-specific marker *Lactobacillus amylovorus* (*L. amy*), and viral markers targeted genogroups of F-specific RNA bacteriophages such as human specific genogroups II and III (FRII and FRIII) and animal specific genogroups I and IV (FRI and FRIV) on 33 environmental samples. The *B. adolescentis* real-time PCR assay was found to have a clinical sensitivity of 92% and specificity of 94.5% with concentrations ranging from $5 \times 10^5$ to $1 \times 10^9$ gene copies per g. Markers HF183, Pig-2-Bac, Rum-2-Bac and *L. amylovorus* were found to be more specific in differentiating the sources of fecal contamination.

A human-specific MST study focused on the development and evaluation of a method to detect the *B. fragilis*-specific single-copy gene gyrB, and was compared with a 16S rRNA gene-based Human Bac-1 (Lee and Lee 2010). The host-specific source identification was evaluated using ten fecal samples (from human, pig, cow and dog) gyrB assay showed high human source specificity with 10% cross amplification from pig fecal samples. The 16S rRNA Human Bac-1 assay showed 70% cross amplification with pig, 40% with cow and 30% with dog fecal samples.

Another new human- and bovine- specific (Bac-Human and Bac-Bovine) real-time PCR assays targeting *Bacteroidales* 16S rRNA genetic markers were developed (Lee et al. 2010b). The Bac-Human and Bac-Bovine assays had a limit of detection of 6.5 and 10 copies per respectively. No false-negative reactions were observed for both the assays when tested with 70 fecal samples collected from various animal sources. There were some false-positive reactions for Bac-Human (30% from pigs, 100% from dogs and cats), and for Bac-Bovine (66.6% from deer, and 75% from dogs) indicating a relatively low specificity.

A SYBR green-based real-time PCR assay was developed to target the 16S rRNA gene of a *Brevibacterium* sp. which had 98% nucleotide identity to the 16S rRNA gene of *Brevibacterium avium* to be used as a MST marker specific for poultry fecal contamination (Weidhaas et al. 2010). The marker was detected in 76% of
the poultry-associated fecal samples (n=57) and not in 93% of the non-target fecal samples tested (n=116).

A new quantitative assay was developed to target the bovine polyomavirus (BPyV) VP1 gene as a MST tool (Hundesa et al. 2010). The detection limit of the assay was between 1 and 10 copies of polyomavirus DNA per reaction. This marker was not detected in the non-bovine fecal sources tested, including eight urban sewage samples, five porcine fecal samples, and 10 porcine urine samples. Excretion of these markers in bovine urine samples was studied and out of 26 samples collected from three different farms in Catalonia, Spain, only height were positive, with concentrations ranging from $3.08 \times 10^3$ to $7.58 \times 10^4$ targets per sample. However, BPyV were not detected in any 10 bovine fecal samples tested.

Another viral MST study focused on the development of multiplex real-time assays as follows: 1) VTB1 assay targeting different adenoviruses in pig, sheep, cattle, and human, 2) VTB2 assay targeting different adenoviruses in human, Sheep, cattle, deer, goat, and pig, 3) VTB-3 assay targeting Norovirus GI for human, GII for human and pig, and GIII for sheep and cattle, and 4) VTB-4 assay targeting F+ RNA bacteriophage GI, GII, GIII and GIV (Wolf et al. 2010). The limit of detection of all of these assays was less than 10 DNA plasmid copies per reaction and no cross amplification was observed with any other non-target viruses.

PCR assays targeting human specific HF183 and ruminant specific CF 128 markers were applied to assess the extent of fecal contamination in Dhaka, Bangladesh (Ahmed et al. 2010). Out of the 20 water samples tested, HF183 marker was detected in 70%, and CF128 was detected in 35% of the samples with concentrations ranging from $3.9 \times 10^3$ to $6.3 \times 10^5$ and $9.3 \times 10^4$ to $6.3 \times 10^5$ genomic units per 100 mL of water, respectively.

One study used two MST methods (Bacteroidales host-specific 16S rRNA gene markers measured by real-time PCR and F-specific bacteriophage (FRNAPH) genotyping) to track the sources of fecal contamination in coastal areas around the Guerande-Atlantique peninsula in France (Gourmelon et al. 2010a). In the samples collected from the seven water sources, 44.6% of the samples showed human-specific Bacteroidales marker HF183 in concentrations ranging from 3 to 6.2 log_{10} copies per 100 mL. The presence of human specific FRNAPH genogroup II at these sites suggested human fecal sources to be the possible cause of fecal contamination.

In a study that focused on a library-dependent MST approach, PCR denaturing gradient gel electrophoresis was used to analyze E. coli fingerprints from water samples in order to trace the source of fecal contamination in Ta-An Beach in Taiwan (Shen et al. 2010). The study suggested that the high E. coli counts in these sites were due to the input from pig waste.

Kim et al. (2010) evaluated the potential of Enterococcus spp. to be used for MST purposes by using PCR for esp gene, phylogenetic analysis of partial 16S rRNA sequence, and whole genome analysis by pulsed field gel electrophoresis using a total of 237 samples that included fecal samples from humans and various animals. The esp gene was only detected in human fecal samples, thus showing a specificity of 100%, but the clinical
sensitivity was less than 10% as only three of the 34 human fecal isolates showed the presence of \( \text{esp} \) gene. Based on the principal coordinate analysis (PCoA) to distinguish the 16S rRNA sequences from various animal fecal sources, there was no significant difference between sequences from pig and cow fecal isolates, and no clear difference between sequences from human and cow fecal sample. Results indicated that the whole genome analysis by PFGE cannot be applied as a MST tool.

Statistical tools have been used to develop predictive models by comparing MST markers such as host-specific \textit{Bacteroidetes} (HF134, HF183 for humans, and CF128, and CF193 for cows), \textit{Bifidobacterium adolescentis} (ADO), \textit{Bifidobacterium dentium} (DEN), the gene \textit{esp} of \textit{Enterococcus faecium}, and host-specific mitochondrial DNA associated with humans (Humito), cattle (Bomito), and pigs (Pomito). Based on these models, the highest percentage of correct source identification was 90.1% by combining five molecular markers (HF134, ADO, DEN, Bomito, and Pomito).

**Phenotypic methods.** The phenotypic MST methods used in 2010 relied on antibiotic resistance profiles. A study applied library dependent \( \text{E. coli} \) antibiotic resistance analysis along with genotypic \textit{Bacteroidales} HF183 analysis to study the spatial variation in sources of fecal contamination on Sunnyside Beach and in the Humber River mouth in the Lake Ontario (Edge et al. 2010). The phenotypic approach identified the source of fecal contamination to be from bird feces at beach locations away from the river mouth. Whereas the genotypic method found that fecal contamination at the River and at a beach location closest to the mouth of the river was due to the input from municipal wastewater and urban storm water runoffs.

Another phenotypic MST approach used antibiotic resistance analysis (ARA) in which library was first constructed for of \( \text{E. coli} \) isolates that were collected over a period of four years from animal feces that are known to pollute the Guiwu reservoir at Xuyu, China (Gu et al. 2010). This library was then used to compare the \( \text{E. coli} \) isolates from water samples. Results indicated that the fecal contamination came from multiple sources including human (32.72%), chicken (8.78%), pigs (12.98%), and goats (28.34%). But a genotypic repetitive extragenic palindromic-polymerase chain reaction MST approach also used in this study, indicated the fecal sources to be human (26.49%), chicken (14.74%), and cattle (10.96%). Thus, authors found that both approaches partly direct to different sources of fecal contamination.

In another phenotypic MST study, ARA library was compared with 1,961 isolates of \( \text{E. coli} \) in water samples collected from a segment of Six Runs Creek in North Carolina (Liwinbi et al. 2010). The results indicated that 64% of creek isolates were associated with swine lagoon effluent and 27.1% with cattle manure.

**Environmental Occurrence, Persistence and Transport**

FIB are commonly used to monitor the quality of drinking and recreational waters. Understanding the behavior of FIB and pathogens in the environment is essential for assessing the risks to human health. A number of studies focusing on persistence, occurrence and transport
of FIB and their impact on recreational beaches, watersheds, estuaries, irrigation, and wetlands were published during 2010. Some of these studies also evaluated alternative fecal indicators. These studies are summarized below.

**Persistence and transport of FIB.** The long-term persistence and leaching of *Escherichia coli* was investigated for different soil types sampled from four grassland sites in Ireland: Luvic Stagnosol in Rathangan, Cutanic Luvisol in Elton, Clonroache, and Haplic Cambisol in Oakpark (Brennan et al. 2010). Leachate samples were positive for *E. coli* in 48% of Rathangan, 16% in Elton, 3% in Clonroache, and 2% in Oakpark soil samples. Under natural rainfall conditions *E. coli* was detected in the drainage water from all four soil-types. Peak concentrations of *E. coli* were generally due to high intensity or prolonged rainfalls.

Surface water, groundwater and coral near Key Largo, Florida, were examined for the presence of human sewage by tracking faecal coliform bacteria, enterococci, *Clostridium perfringens*, enteroviruses, and adenoviruses (Futch et al. 2010). The maximum faecal coliform counts found were $10^5$ CFU per 100 mL in coral, 5.5 CFU per 100 mL in surface water and 3.5 CFU per 100 mL in groundwater. The maximum enterococci counts were 700 CFU per 100 mL in coral, 10 CFU per 100 mL in surface water, and 41 CFU per 100 mL in groundwater. For *C. perfringens* the maximum counts were 10 CFU per 100 mL in coral, 5 CFU per 100 mL in surface water, and below detection limit in groundwater. Enterococci were detected more frequently during both summer and spring while faecal coliforms and *C. perfringens* were more frequently detected during spring. Adenoviruses were detected in 49.3% and enteroviruses were detected in only 10.7% of samples analyzed. Both viruses were detected more frequently in the coral than in either of the water types.

In another study, storm-water runoff was characterized for fecal contamination at three coastal sites in North Carolina (Parker et al. 2010). Storm runoff concentration could reach as high as $2.39 \times 10^6$ MPN per 100 mL for total coliforms, $1.20 \times 10^6$ MPN per 100 mL for *E. coli*, and $100 \times 10^5$ MPN per 100 mL for *Enterococcus* spp., but only for specific storms. The *Bacteroides* human-specific marker was present in 7% of samples at Bogue Inlet Pier, in none of the samples at Triple S Pier, and in 44% of the samples at Town Creek Marina. Fecal *Bacteroides* spp. was present in 82% of samples at Bogue Inlet Pier, in 64% of Triple S Pier, and in 100% of samples at Town Creek Marina. Results of this study indicate that storm-water in North Carolina may have high levels of FIB, including sometimes, indicators of human origin.

**Particle association and transport of microbes.**

River plume concentrations of *E. coli* at two beaches in southern Lake Michigan were analyzed by three-dimensional hydrodynamic and transport models (Thupaki et al. 2010). Overall, the vertical turbulent mixing within the water column was the most dominant mode of transport of *E. coli*. Based on sensitivity analysis, solar inactivation had the greatest impact on *E. coli* loss rate. Assessment of the amount of *E. coli* loss rate was carried out by incorporating the effect of solar inactivation and settling (e.g., attenuation of different light energies with the water...
Mathematical models presented in this study may serve as a foundation to describe the association and transport of microbes in surface waters.

Similarly, sources and sinks of \( E. \ coli \) and enterococci were quantitatively estimated, in the Gwangju Creek in Korea, under varying meteorological conditions (Cho et al. 2010a). During wet weather conditions, major increase in FIB was due to storm wash-off from the watershed and re-suspension from the streambed. Maximum total discharge loads from storm wash-off were 79% for \( E. \ coli \) and 77% for enterococci. Total discharge loads from re-suspension from the streambed ranged from 22 to 83% for \( E. \ coli \) and 23 to 84% for enterococci. It was speculated that variation may be due to particle size-distribution of the sediment, which provides an ideal habitat for FIB. Monitoring data was used to develop a spatiotemporal FIB prediction model. The model assessment showed that die-off by sunlight was a major sink of FIB during the daytime in dry weather. This accounted for a reduction of concentration ranging from 65 to 94% for \( E. \ coli \) and 77 to 96% for enterococci. Settling and natural decay played a minor role in FIB loss rates.

Reach-specific modeling analyzed the parameters of streambed \( E. \ coli \) re-suspension in three streams (Cho et al. 2010b). During a high-flow event, an increase in \( E. \ coli \) concentration suggested that a major factor impacting water quality was through the release of \( E. \ coli \) from bottom sediments. In addition, the settling rates were low (1.5x10^4 cells m^2 s^{-2}) indicating that \( E. \ coli \) could be transported far from the initial release site. The transport model with reach-specific parameters resulted in predictions of \( E. \ coli \) re-suspension better than the model with a single set of parameters. \( E. \ coli \) release and transport models commonly use a single set of parameters to generalize the entire stream or reservoir. These models of release and transport commonly generalize parameters for the entire stream or reservoir. This can lead to over or under predicting outputs. This manuscript was proposing another type of modeling where the authors broke up the stream into 3 sections and used the changing parameters for the model.

In another study, the effect of streambed bacterial release on \( E. \ coli \) concentrations was monitored and modeled with a modified Soil and Water Assessment Tool (SWAT) in the Little Cove Creek watershed in southern Pennsylvania (Kim et al. 2010). The concentrations of \( E. \ coli \) were mostly a result of surface runoff, and sediment re-suspension contributed to the persistent concentrations. Uncertainty of \( E. \ coli \) contributions from wildlife was considered as one of the limitations of the model.

Variations in inter-strain attachment have also been studied to better understand the transport of \( E. \ coli \) in natural waters. Attachment efficiencies varied between 0.3 and 1 for the 54 \( E. \ coli \) strains studied (Foppen et al. 2010). A number of factors including sphericity, motility, zeta potential, cell aggregation, lipopolysaccharide composition, and occurrence of 22 genes coding for different outer surface proteins were evaluated. Among the 22 genes studied, Afa had the strongest correlation with attachment efficiency. However, no single factor could statistically explain the variations in attachment efficiency.
Microbial quality of recreational beaches. The microbial water quality after a wet weather event was assessed in terms of FIB for non-human influenced beaches in southern California (Griffith et al. 2010). Of the samples analyzed, 16% violated water quality standards for at least one indicator (total coliform, E. coli, and enterococci) during the wet weather. Factors that affected the frequency of FIB were: watershed size, storm size, and early vs. late season storms. Larger storms and watersheds were observed to have higher frequencies of FIB than smaller storms and smaller watersheds. Early season storms had greater frequency compared to late season storms. In addition, the greatest frequency of FIB occurred within 24 h of rainfall and steadily decreased over a three-day period.

Another study examined the presence of pathogens and FIB at a non-point source subtropical recreational beach (Abdelzaher et al. 2010). The impact of high or low tide during elevated or reduced solar conditions were evaluated for FIB (E. coli, enterococci, and C. perfringens), microbial source tracking markers (human polyomaviruses [HPyVs] and Enterococcus faecium esp gene), and pathogens (Vibrio vulnificus, Staphylococcus aureus, enterovirus, norovirus, hepatitis A virus, Cryptosporidium spp. and Giardia spp.). During a high tide and low solar event, FIB levels exceeded standards and HPyVs, V. vulnificus, Giardia spp., and Cryptosporidium spp. were also detected. Overall elevated microbial levels were observed at high tide and under low solar conditions. At low tide and elevated solar conditions, microorganisms were more susceptible to desiccation and solar inactivation.

The impact of fecal indicator organism density in beach sand was determined by characterizing the sand, hydrologic and beach factors of three beaches of Lake Michigan (Skalbeck et al. 2010). E. coli densities in beach sand increased when the grain diameter decreased and the uniformity increased. Wave movement was the main transport mechanism and groundwater beneath the beaches was not a transport mechanism for E. coli to surface water. The evaluation of beach profiles suggested that the increase in gradient (steeper slopes) would decrease E. coli concentration in beach sands. This would prevent continual wetting which, along with the sand characteristics, may provide a reservoir for replication and persistence of E. coli. Beach management interventions suggested were deep mechanical grooming of fine sand beaches and beach slope assessments.

Hydrogen peroxide (H2O2) naturally occurring in fresh and oceanic waters may affect FIB concentrations. It has been reported that there was an inverse relationship between H2O2 and FIB in seawater (Clark et al. 2010). In this study, H2O2 concentrations in the surf zone were taken on both short and long time scales for bathing beaches in Southern California. Hydrogen peroxide concentrations ranged from 100 to 300 nM with maximum concentrations occurring in the afternoon. Coastal and natural lake water samples showed inhibition of bacteria when concentrations of H2O2 were as low as 100 nM. These results confirm that the presence of H2O2 results in lower concentrations of FIB in seawater. Therefore, this study may have implications in the reductions of FIB in the surf zones of recreational bathing waters.
Dominant non-point sources of enterococci at a marine recreational beach in Miami, Florida was studied using image analysis approach (Wang et al. 2010b). Estimates of average load of enterococci were determined from the number of people and animal visitors at the beach. Shedding loads were roughly computed for humans (\(10^6\) to \(10^7\) CFU/day on week days and \(10^7\) to \(10^8\) CFU/day during weekend days), dogs (\(10^9\) CFU/day on week days and \(10^9\) to \(10^{10}\) CFU/day on weekend days), and seagulls (\(10^3\) to \(10^5\) CFU/day). Limitations of the method was the ability to address non-point source pollution from runoff and regrowth of enterococci in sand and water (Wang et al. 2010b).

Presence of antibiotic resistant bacteria were studied in non-recreational marine beach sand at the Słowiński National Park, Poland (Mudryk et al. 2010). The bacteria from the sand were characterized to have variable but generally low levels of resistance to particular antibiotics. In addition, the highest level of antibiotic resistance bacteria were found in the middle part of the beach and the dune, while the lowest was found at the waterline and in the sea. Amoxicillin, amoxicillin/clavulanic acid, cefaclor, cefuroxime, clindamycin, ciprofloxacin, erythromycin, and penicillin were related to the highest resistance frequency. The majority of the isolated bacteria were resistant to only one antibiotic out of 18 tested in the study. However on human-impacted recreational beaches the majority of the isolated bacteria were resistant to 3 to 8 antibiotics. It was suggested that human activity may have contributed to multiple resistances.

Another study examined the antibiotic resistance of bacteria isolated from recreational beaches with three different levels of organic pollution in southeastern Brazil (de Oliveira et al. 2010). These beaches were rated as poor quality, suitable for swimming and bathing, and excellent quality beaches. For the poor water quality beach, 72% of isolated bacteria were resistant to one antibiotic while 8.3% were resistant to multiple antibiotics. At this beach, for wet sand, the highest frequency of resistance was for penicillin, erythromycin, amoxicillin, vancomycin, rifampicin, and ampicillin. For dry sand, the highest frequency of resistance was for penicillin, vancomycin, and ciprofloxacin. For the beach suitable for swimming and bathing, 70.8% of the isolated bacteria were resistant to one antibiotic while 6.9% had multiple resistances. For wet sand at this beach, the highest resistance was to penicillin, ciprofloxacin, amoxicillin, and ampicillin, while for dry sand it was penicillin and ciprofloxacin. At the beach with excellent water quality, 35.8% of isolated bacteria were resistant to one antibiotic and there were no multiple resistances observed. For both wet and dry sand, the only antibiotic resistance was to penicillin. The level of contamination correlated to the frequency of the bacterial antibiotic resistance from wet and dry sand samples.

**Occurrence of FIB and pathogens.** Studies were conducted to analyze FIB concentrations during rainfall events in several headwater tidal creeks in North Carolina (Stumpf et al. 2010). It was observed that concentrations of FIBs increased during storm events for all the creeks tested. The concentrations of *E. coli* and enterococci increased 30 and 37 times respectively, when...
compared to dry weather concentrations. In another study conducted on the Kalamazoo River, Michigan, FIB concentrations were persistently high with *E. coli* having a significantly higher load compared to *E. feacalis* at downstream locations (Olapade & Weage 2010). Factors for the high concentrations of FIB included direct waste load from animals, waste effluents, the type of stream sediment, and riparian vegetation. Occurrence of *E. coli* was examined by collecting and analyzing samples from six locations at an urban watershed in Houston, Texas (Desai & Rifai 2010). It was observed that *E. coli* concentrations were relatively lower at upstream grassland sites (up to 715 MPN per 100 ml) compared to urbanized downstream sites (up to 7005 MPN per 100 ml). In another study, wetlands were constructed in the San Joaquin Valley, California, in order to reduce the bacterial load from irrigation runoffs. The concentrations of *E. coli* and enterococci were reduced by 76 % and 89 % respectively, between inflow and outflow (Diaz et al. 2010).

A correlation between fecal indicators and pathogens were studied at the Rodrigo de Freitas Lagoon in Rio de Janeiro, Brazil (Gonzalez et al. 2010). The FIBs (coli forms and enterococcus) and pathogens (*Vibrio, Staphylococcus aureus* and *Salmonella*) were measured by traditional culture techniques. It was noted that *Enterococcus* and fecal coliform counts were low in some samples whereas pathogens were constantly detected, with a limit of detection of 1 CFU per 100 mL. Statistical analyses showed that both *Enterococcus* and fecal coliforms positively but non-significantly correlated the presence of pathogens. Therefore, it was concluded these indicators need to be considered together to correlate the occurrence of the pathogens.

**Alternative fecal indicators.** Traditional FIB tracking is known to have several limitations including replication in the environment. Search for alternative indicators is an ongoing effort and a number of emerging and new alternative indicators have been proposed. *Bacteroidales* 16S rRNA gene markers, that is one of the well-known alternative indicators were used in a study conducted on surface waters in California (Schriewer et al. 2010). Surface waters were analyzed for the presence of pathogens (*Salmonella* spp., *Campylobacter* spp., *Vibrio* spp., and protozoal pathogens), FIBs, and universal and human *Bacteroidales* markers using quantitative PCR. Weighted measurement analysis was used to compare the *Bacteroidales* marker to the FIBs for predicting the presence of pathogens. Results indicated that the universal *Bacteroidales* genetic markers performed better than traditional FIBs for predicting pathogens, as they were detected in 100% and 75-99% of the samples respectively. Also, host specific *Bacteroidales* markers gave additional information related to the source of fecal contamination.

Another study compared the decay rates of three *Bacteroidales* 16S rRNA genetic markers to *E. coli* in wastewater under different treatment conditions of artificial sunlight, reduced predation, reduced temperature, and sediment exposure (Dick et al. 2010). The human fecal associated markers were seen to decay at the same rate or faster than *E. coli* in all the treatments. Therefore, human fecal-associated *Bacteroidales* markers could be used along with *E. coli* as indicators for human fecal pollution.
A PhyloChip microarray was an alternative to characterize the extent of fecal contamination in two urban watersheds (Wu et al. 2010). There was a significant difference in occurrence for *Bacilli, Bacteroidetes, Clostridia* and alpha-proteobacteria between fecal and non-fecal sources. These four classes demonstrated a higher ratio in fecal sources compared to non-fecal sources. Sorbitol-fermenting bifidobacteria have also been proposed as good alternative indicators due to the occurrence that differs between levels of fecal contamination in water bodies (Mushi et al. 2010).

*C. perfringens* was evaluated as an alternative fecal indicator by comparing the river sediments in three Italian regions (Mancini et al. 2010). Both microbiological as well as molecular studies were used to characterize *Clostridia*. Based on its genetic sequences, *Clostridia* from the different sediment types could be subdivided into five clusters. The results suggested a correlation between a particular ecological niche (or impact type) and particular species of *Clostridia* present within it.

Enterophages are a group of phages that infect *E. faecalis* and can be found in contaminated water sources. Characterized enterophages as possible alternative fecal indicators have been evaluated, especially for enteric viruses. Initial results indicate that concentration in contaminated water samples was lower than coliphages, coliforms or enterococci across a range of temperatures. This possibly indicated that the bacterial contamination was from an animal source since enterophages are specific to humans (Santiago-Rodriguez et al. 2010). More studies were suggested to fully characterize a particular species of enterophages as a viral indicator.

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