Detection and Occurrence of Indicator Organisms and Pathogens

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ABSTRACT: This review summarizes the literature published in 2012 pertaining to the occurrence and detection of fecal indicator bacteria (FIB) and pathogens. It is organized into the following sections: i) methods for the detection and quantification of waterborne fecal indicators and pathogens including polymerase chain reaction (PCR), isothermal amplification, label-free methods, microarrays, immunoassay-based methods, microfluidic-based methods, and advances in sample processing and concentration at the limit of detection (LOD), ii) microbial source tracking (MST) using both genotypic and phenotypic-based methods, iii) environmental occurrence, persistence, and transport of fecal indicators and pathogens.

KEYWORDS: Fecal indicator bacteria, microbial source tracking, PCR, isothermal amplification, microfluidics, label-free, environmental persistence, watersheds, wetlands

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Introduction

This review summarizes some important studies published in 2012 that focus on the environmental occurrence and detection techniques for waterborne fecal indicator organisms and pathogens. New detection techniques and assays were developed this year employing PCR, isothermal amplification, microarrays, label-free methods including biosensors, and microfluidic or immunoassay-based technologies. These techniques and assays have the potential to allow detection and quantification of pathogens in a rapid, specific and sensitive manner. Advances in microbial source tracking techniques for both genotypic and phenotypic-based analyses were also published in 2012. Information presented in these studies can be used to reduce introduction of pathogens into drinking and recreational waters thus reducing the human health effects. Further, there were multiple studies investigating the occurrence, persistence, and transport of fecal indicators in 2012.

Detection Methods

Multiple studies were published in 2012 that focused on the advancement and development of FIB and pathogen detection assays, both molecular and immunological. These assays were developed for endpoint PCR, reverse transcription PCR, quantitative PCR,
Isothermal amplification methods including loop-mediated isothermal amplification (LAMP), and microarrays. New technologies were also developed for the detection of pathogens including biosensors and other label-free based detection methods as well as for microfluidic-based devices. These advances in detection of pathogens are useful to ensure the safety of drinking and recreational waters.

Polymerase chain reaction. Molecular-based detection methods are beneficial because they can provide quantification of pathogens in a more rapid manner compared to culture-based methods. The most commonly used molecular detection method is PCR. Studies focusing on PCR-based assays to detect fecal indicators are summarized below.

Bird feces can be a source of fecal coliforms, enterococci, Escherichia coli (E. coli) and other waterborne pathogens and their indicators in coastal waters. Green et al developed a method to identify the presence of bird fecal contamination using first subtractive hybridization and then qPCR assays (Green et al., 2012). The 16S rRNA gene fragments from gull feces were subtractive hybridized to reveal unique sequences. The sequences belonged to five taxonomic groups – Enterobacteriaceae, Helicobacter, Catellicoccus marimammalium, Fusobacterium and Campylobacter. Three qPCR assays were developed, called GFB, GFC and GFD that were based on designing specific 16SrRNA primers from Fusobacterium spp., C. marimammalium, and Helicobacter spp. All three assays showed specificity to fecal DNA from birds and GFC and GFD were able to detect fecal Enterococcus at dilutions of 30 MPN/100 mL which is within the United States Environmental Protection Agency (USEPA) regulatory limit for recreational waters. The limit of detection for GFC was 0.1 mg gull feces/100 ml corresponding to 2 gull Enterococcus MPN/100ml. The assays could also detect gull fecal contamination in the presence of human wastes (P > 0.10, n=96). For GFD, the LOD was 0.1 mg chicken feces/100 ml corresponding to 87 coliform MPN/100 mL. When the assays were tested against 635 fecal samples from several species, GFB and GFC showed 97% and 94% specificity to gulls while GFD was 100% avian specific and amplified only fecal DNA from gulls, geese, ducks, chickens and other seabirds.

Since bacterial indicators often fail to predict water-borne pathogens, certain viruses are being considered as alternative indicators. One study developed a new method for the detection and quantification of paroviruses in chicken (ChPV) and turkeys (TuPV) in environmental samples (Carratalà et al., 2012). Two nested PCR assays targeting a non-structural region and VP1/VP2 regions and a qPCR assay targeting VP1/VP2 region was developed. 30 chicken fecal samples and 3 turkey, 2 patridge and 7 hen pooled fecal samples from farms in Catalonia were tested for ChPV/TuPV and showed 73%, 72%, 100% and 50% positivity, respectively. The assays showed specificity to only poultry fecal sources when tested against DNA of several non-target species including dog, cat, bovine and porcine sources. Chicken fecal samples had a mean genome copies (GC) of 9.07 X 10^8 /g. Hundred percent of chicken slaughterhouse raw wastewater and 80% of treated
wastewater were positive for the tested viruses. The mean viral concentration was $4.63 \times 10^5$ GC/mL in the slaughterhouse raw wastewater. The assay may be useful to detect poultry fecal contamination in environmental samples.

Another study evaluated and improved the qPCR detection of enteroviruses (EnV) in surface waters in Hawaii (Connell et al., 2012). The efficacy of 18 published primer sets for amplification of various regions of the EnV genome was tested. Only 7 of the 18 primer sets tested were able to detect EnV. These included EQ-1/EQ-2, Primer 1/Primer 3, Primer 2/Primer 3, EV-L/EV-R, EVZ1/EVZ2, EVF/EVR, and ev1qia/ev2qia. The PCR conditions for these primer sets were optimized by changing the annealing temperature, MgCl$_2$ concentrations, primer concentrations and BSA presence/absence. One of these primer set (EQ-1/EQ-2) displayed the highest sensitivity with a detection limit of $10^{-7}$ X. This primer set was used to test 22 sample sites from around the island of Oahu, of which 11 tested positive. This study also evaluated tissue from shellfish at 9 sample sites, 6 of which were found positive. Moreover, there was a positive correlation between 7 of the shellfish sites and water samples, with 3 sites being tested negative and 4 testing positive using both methods.

Isothermal amplification. Isothermal amplification is increasingly described for detection of waterborne pathogens. Advantages of isothermal amplification include: 1) only a water bath is needed for the reaction, 2) the high amplification yields can allow for either endpoint detection with the naked eye or real time detection with less sensitive optics, and 3) an increasing number of studies have shown that isothermal amplification is less influenced by inhibitory substances compared to PCR.

A study by Gallas-Lindemann and coauthors described the development of an isothermal technique for detecting *Toxoplasma gondii* oocysts in water resources (Gallas-Lindemann et al., 2012). Environmental water samples (total 95) were processed by flocculation in aluminum sulfate, purification via a sucrose density gradient, DNA extraction, and loop mediated isothermal amplification (LAMP). DNA from *Toxoplasma* was detected in eight of the tested samples, which were influent and effluent wastewater samples.

Another study by Koloren et al. (2012) described comparing immunofluorescence, nested PCR, and LAMP for the detection of *Cryptosporidium* species in sea and tap waters from the Black Sea (Koloren et al., 2012). Of the three methods, LAMP had the highest rate of detection (65.5% of 12), while the immunofluorescence and nested PCR methods had no better than 61.4% and 31% detection, respectively. Increased detection by LAMP might be due to the reduced influence of potential inhibitors in the sample. In two other studies performed by the same group, isothermal amplification was used for species specific detection of *Alexandrium tamarense* and *A. catenella*, resting cysts in toxic dinoflagellates, from coastal regions (S Nagai and Itakura, 2012). Detection was observed from a single cell using LAMP. The second study explored four
different DNA extraction techniques upstream of isothermal amplification for efficiency and simplicity (Satoshi Nagai et al., 2012). The most effective method, with 100% rate of detection tested on 145 resting cyst samples was also simple in that it only required adding 5% Chelex buffer, 15-20 s homogenization, and boiling for 15 min.

Other isothermal amplification technique have also been described. A study by Fykse and coauthors compared NASBA and qPCR for the detection of *V. cholerae* in ballast waters (Fykse et al., 2012). Using both a 4 hr culturing step to enrich for viable cells, followed by qPCR was the most sensitive method, allow for detection of 1 CFU per 100 mL of ballast water, satisfying IMO-guidelines.

The low power requirements of amplification, compared to PCR, makes it an attractive technique for point of use applications. However, other factors, akin to genetic diagnostics methods, such as multiplexing for multiple targets of interest must also be simpler to both reduce hands on steps, and potential user error. One study, performed by Tourlousse and coauthors described the development of a microfluidic chip for detection of multiple water and foodborne pathogens in parallel (Tourlousse et al., 2012). The simplicity of the microfluidic network allow for sample to be distributed into multiple wells without the use of a valves or any peripheral other than a pipette. The chip was demonstrated for multiple marker genes from *Salmonella, Campylobacter jejuni, Shigella,* and *Vibrio cholerae.* The users described a detection limit of 10–100 genomes per μl in less than 20 min. A complementary study by the same group described using the disposable microfluidic cards in a real-time, wireless, battery operated, point of care device termed Gene-Z (Stedtfeld et al., 2012). The Gene-Z device was demonstrated using *E. coli* and *S. aureus.* Another study by Zhao and coauthors described the development of a device that uses both an immunoassay and NASBA for quantitative detection of waterborne pathogens. In detail, barcode RNA that is captured via the immunoassay with the pathogen of interest, is amplified via NASBA to increase the detectable level of captured RNA. The device was tested with *E. coli* and rotavirus, and a detection limit of $4.7 \times 10^{-4}$ cells per ml was observed for *E. coli.*

**Label-free detection.** Label-free biosensors can detect antigens in real-time and be used at point-of-care diagnosis in a rapid, inexpensive, sensitive and specific manner. Luo and coauthors developed an immunobiosensor using an electrospun nanofiber and conductive magnetic nanoparticles (CMP) that was able to detect *E. coli* O157:H7 (Luo et al., 2012). The membrane made from cellulose nitrate nanofibers held *E. coli* antibodies that could easily capture the pathogen by antigen-antibody binding. The concentration of the pathogen was measured by a sensing platform that read the resistance signals. This biosensor displayed a sensitivity of 67 CFU/mL in 8 minutes. It also has a response range of $10^1 – 10^6$ CFU/mL.

In another study, a circuit model for electrical impedance spectroscopy (EIS) based label-free biosensor was developed that overcame its earlier disadvantages of
being non-specific and giving signals that could not be easily reproducible (Siddiqui et al., 2012). The circuit model is based on experimental data and has the ability to differentiate between positive signals and false-positive signals. This study also employed an ultrananocrystalline diamond (UNCD) microelectrode array (MEA) that significantly improved signal reproducibility and showed increased sensitivity. The UNCD array based biosensor was able to detect *E. coli* K12 using EIS.

Mesoporous titania thin-film substrates were used as biosensors for the detection of *E. coli* O157:H7 (Mura et al., 2012). Silicon wafers were dipped in a solution containing TiCl4/Pluronic F127/H2O/EtOH and then heated to 200°C to prepare the titania thin films. Treating with a solution of (3-aminopropyl) Triethoxysilane (APTES) and then glutaraldehyde (GA) functionalized the films. The FTIR spectrometer provided mid-infrared spectroscopic fingerprints of the pathogens. The functionalized APTES-GA mesoporous films containing the anti-*E. coli* O157:H7-antibody displayed a limit of detection of 1 - 10^2 CFU/mL.

A real-time cell electronic sensing (RT-CES) system was utilized to develop a new Vero cell assay (Xing et al., 2012). This assay was able to identify verotoxin-producing *E. coli* (VTEC) specifically and in a much shorter time span (from 48 hr to 15 hr). The RT-CES system recorded the changes in electrode impedance caused by the interaction of the cells with the electrodes. A parameter called Cell Index (CI) measured the status of a cell based on the electrode impedance. The more cells attached to the electrode, the higher the impedance and therefore the CI. This automated, real-time system for monitoring cytotoxicity of Vero cells was able to successfully identify VTEC in human fecal samples.

Microarray detection. High-throughput detection technologies have the potential to make detection of pathogens simpler because multiple organisms can be tested at once. One such technology is the microarray, which uses many oligonucleotide probes at once, each specific to a particular organism. In one study, a microarray-based detection method was developed as a tool to aid in fecal source tracking as well as whether the fecal source was bird, human or other mammals (Dubinsky et al., 2012). The method utilizes a PhyloChip gene chip with 16S rRNA probes targeting 59,959 bacterial and archaeal taxa. It was determined that *Actinobacteria*, *Bacilli*, and *Gammaproteobacteria* taxa discriminated birds from mammals. Field tests in marine environments determined that when *enterococcus* limits were exceeded, samples contained 78-96% of the fecal indicators for humans and 0-10% for birds.

In another study by Kim et al, a sensitive, specific and high throughput biochip for the detection of enteric viruses including astrovirus, norovirus, adenovirus, and rotavirus (J.-M. Kim et al., 2012). Oligonucleotide probes (total of 551) were developed and the most successful were chosen for implementation into the biochip for simultaneous detection of the five enteric viruses.

In a similar study by Unc et al., the virulence, antibiotic resistance, and heavy metal resistance of the
microbial population in an Ontario watershed was investigated using microarray technology at various times of the year (Unc et al., 2009). Assays were developed for *E. coli*, *Enterococcus* spp., *Clostridium perfringens*, *Salmonella enterica*, and male-specific coliphages (F+) with *E. coli* Famp as a host. Antimicrobial resistance genes were found in all samples with 73 different gene types. The detection of virulence genes changed significantly depending on the season with 17 samples specific to the month of November, and higher levels reported throughout the fall season.

**Imunoassay-based detection.** Multiple studies were conducted in 2012 that utilized immunoassays, typically antibodies, for the detection of species based on the proteins of interest. For example, Jain et al developed a method that is highly sensitive for the detection of *Salmonella typhi* through the use of surface aminated polycarbonate membrane enhanced-ELISA (enzyme-linked immunosorbent assay) (Jain et al., 2012). Isopore polycarbonate black membranes were antibody immobilized for the capture of *S. typhi* while colorimetric detection was used for quantification. A detection limit of 2x10^5 cells bacteria/mL was achieved with an intra-assay precision of 5.3-7.4% for 10^4 and 10^5 cells/mL. The inter-assay precision for the same concentration was 10.3-19.7%.

In another study by Knauer and coauthors, an immunoassay-based microarray flow-through system was developed for the detection of *E. coli* (Knauer et al., 2012). The system utilized surface-enhanced Raman scattering (SERS) using a He-Ne laser to analyze the bacteria. Chip surfaces coated with PEG were covered with colloidal metal nanoparticles to allow the label-free detection by SERS through the immobilization of antibodies. The range of detection achieved was 4.3x10^7 to 4.3x10^5 cells/mL. The limit of detection and limit of quantification were determined to be 4,485 cells/mL and 7,775 cells/mL, respectively.

An immuno-based assay was developed for the detection of five bacterial toxins: cholera, *E. coli* heat-labile, *S. aureus* (enterotoxins A and B and the toxic shock syndrome toxin) (Shlyapnikov et al., 2012). The assay worked in the following way: the toxins were collected on an antibody microarray by electrophoresis, then antigens were captured and labeled with biotinylated antibodies to allow detection, then the labels were scanned to the microarray and images were analyzed. The process was completed in approximately 10 min. A limit of detection of 0.1 to 1 pg/mL was obtained in water samples.

In another study (Zhao et al., 2012), an immuno-based NASBA (nucleic acid sequence-based amplification) chip was developed to alleviate the complicated protocol of typical ELISA by performing the entire process on a single chip. The purpose of this lab on a chip (LOC) assay was to allow simultaneous detection of waterborne pathogens. The device uses antibodies to recognize the different targets (*E. coli* and rotavirus), then the signals are amplified with NASBA and detected with a 96-well ELISA reader. It was determined that a range of detection of 10^-9 to 10^-16 mol was achievable.
Microfluidic-based detection. Microfluidics confers several advantages when it comes to detection of bacteria in different kinds of samples. Economically, minimizing the volume needed in order to test a sample can allow multiple tests to be performed with little expense. Furthermore, microfluidic devices often have the added benefit of portability, allowing for point-of-care diagnostics. Lastly, microfluidics-based assays have the potential for single cell detection without amplification. There have been several advancements in microfluidic-based detection technologies in 2012.

One group (Rane et al., 2012) reported the development of a chip capable of amplification-free single cell detection, that combined digitalization, cell lysis, and detection into a single tool. The group utilized fluorescence resonance energy transfer (FRET) as their method of detection. A peptide nucleic acid (PNA) was created which targets the 16S rRNA of enteric bacteria. This PNA contained a nucleic acid sequence that bonded to the 16S rRNA, the fluorophore Cy5, and the quencher BH3 to absorb the Cy5 fluorescence when the PNA beacon. A single chip contained all the steps needed for single cell detection using this method. The first region digitalized the sample by creating picoliter droplets of the sample encased in oil, which contain about one cell. By encasing and lysing a single cell in a picoliter volume the concentration of 16S rRNA becomes great enough to detect using fluorescence methods. The cells in the droplets were lysed and the PNA beacon was allowed to bind to the 16S rRNA. The sample then traveled to the detection region where the fluorophore was excited and the emission was measured. The group was able to differentiate between droplets containing cells and “empty” ones.

Another study (Packard et al., 2012) also made use of FRET to perform amplification-free detection of microbes. Using a minor-groove binding high energy dye (SYTO-9) and a probe tagged with a low energy dye (HEX-6) the researchers were able to detect the presence of microbes. By using FRET, in situ hybridization background fluorescence from leaky HEX-6 was minimized to be near the SYTO-9, allowing fluorescence. A silicon and glass chip was used to concentrate, lyse, and detect cells. With dielectrophoresis, bacteria was concentrated to a detectable level using SYTO-9 fluorescence from a starting concentration of as little as 10 cells/mL.

Microfluidics have also been incorporated into amplification-based detection methods for bacterial cells in water. A study by Fang and coauthors reported the development of portable chips capable of detecting one or more species of bacteria with minimal time from sample collection to data retrieval (Fang et al., 2012). However, its performance was demonstrated with Mycobacterium tuberculosis. The chip consists of two chambers, a sample preparation chamber separated from the reaction chamber by a screw valve. The screw valve was opened, and cell lysate transferred to the detection chamber via the thermal vapor pressure generated during the incubation. LAMP reaction mix was added to the detection chamber. The researchers used calcein as an indicator with the production of a green color indicating a positive reaction. LAMP in the chip was shown to have a highly stable limit of detection at
270 gene copies/µL with 27 gene copies/µL showing both positive and negative results.

Jin and coauthors developed a single-plex microfluidic chip that utilizes capillary-flow and detection of nucleic acids without amplification (Jin et al., 2012). Magnetic beads tagged with DNA sequences complementary to the targeted sequences and Ru(bpy)$_{2}^{2+}$-doped silica nanoparticle probes were used. A mixture of the sample containing the DNA tagged with magnetic beads and the probe were placed in one of the two inlets and allowed to travel to the well via capillary-flow. Once the sample reached the absorbent pad the flow remained constant and liquid passed through the well. Under the well, a magnet fixed all of the magnetic beads in the well. The limit of detection of nucleic acids using this method was found to be 1pmol due to a large amount of background fluorescence attributable to the material used for the chip.

In a study by Chen and coauthors, a single microfluidic system capable of concentrating targeted bacteria, thermal lysis, gDNA purification, PCR and ligase detection reaction (LDR) was developed (Chen et al., 2012). This multiple module device was tested using two genes, uidA and sipB/C, to detect different E. coli strains. The device used a “zip coded” array section where fluorescence measurements were taken to determine the presence of a particular gene. Detection of the genes was done by on-chip PCR followed by LDR. The LDR barcoded allele-specific amplicons and targeted them to a specific region on the zip coded array. The assay had a detection 100 CFU of E. coli O157:H7 per cell.

**Sample Filtration and Preparation.** Detection via culturing, immunoassay, and genetic diagnostics requires a means to capture the low concentration of pathogens that could be present in drinking and surface water samples. Studies in 2012 described mechanisms for microbial concentration via filtration and chemical flocculation. This review does not cover all sample concentration studies performed in 2012, but attempts to provide some examples of various technologies and methods. A comprehensive review for concentrating viruses can be found elsewhere (Ikner et al., 2012, Julian and Schwab, 2012).

Several filtration based methods were described in 2012. A study by Low and coauthors (Low et al., 2012) explored a series of membrane casting dopes with different formulations and viscosities. It was determined that a faster capillary flow time was achieved by the addition of cellulose acetate. Another study used dead-end ultrafiltration (DEUF), as a field deployable method for recovering bacteria, viruses and parasites from large volumes of water from turbid surface waters (Mull and Hill, 2012). Tests were performed using water with various turbidities and evaluated for recovering MS2 bacteriophage, enterococci, E. coli, Clostridium perfringens, and C. parvum oocysts. The systematic study showed that up to 100 L of turbid surface waters at an average flow rate of 379 mL/min could be filtered using a hollow fiber ultrafilter. The level of turbidity only
influenced recovery efficiency of MS2 and C. perfringens, with average capture efficiencies of 66% and 63%, respectively. A study by Pei and coauthors described the development of a filtration method that combined monolithic affinity filtration and crossflow ultrafiltration (Pei et al., 2012). Tested with MS2, and qRT-PCR, the technique was able to concentrate by a volumetric factor of $10^4$ within 33 min. Overall, a detection limit of 5.6 Genomic Unit per L was observed and the time to results including filtration and PCR was 3 to 4 hr. Studies by Leskinen and coauthors described the development and validation of Portable Multi-use Automated Concentration System (PMACS) for concentrating microbes (Leskinen et al., 2012). The PMACS was used to filter 100 l of sample from cooling towers and surface waters, and was compared with a more conventional collection and dead-end filtration using a volume of 500 ml. The PMACS was shown to lower sample variability over time compared to the conventional filtration method.

**Microbial Source Tracking**

Pollution of surface waters is an unfortunate regularity that can result in unsafe environments for wildlife as well as recreational users. Microbial source tracking (MST) is used to relate the presence of specific microbes with a point or source of contamination, so that resource managers can develop methods for prevention. Over the past several years there has been a growing interest in finding host-specific microbes that can be used to definitively identify the source of contamination. Many studies use nucleic acid approaches to target genes of bacteroidetes as markers of fecal pollution. This section focuses on studies targeting this group, some potential pitfalls, and a few alternatives proposed.

Ahmed and coauthors evaluated the effectiveness of the HF183 marker to detect human fecal pollution (Ahmed, Masters, et al., 2012). This marker targets the 16S gene of Bacteroides and had been reported to be specific to human fecal pollution. The study found that out of 79 fecal and wastewater samples tested, 75 were positive for the HF183 marker. The four samples that were negative were from individual fecal samples (out of 20 total), suggesting that the marker could still be effective in identifying human-associated fecal pollution. However, some chicken, bird, and dog fecal samples were also positive for HF183 marker.

In another study, three clone libraries were developed for the 16S gene of Bacteroidales present in human sewage, bovine feces, or ovine feces (Dorai-Raj et al., 2012). The primer sets used were designed to target the three largest members of the Bacteroidales order; Bacteroides, Porphyromonas, and Prevotella. It was determined that 59% of the cloned 16S genes from the human samples were Prevotella while only 11% were Bacteroides. This was in contrast to the ovine and bovine derived libraries that were 71% and 73% Bacteroides, respectively. During phylogenetic alignment the clones derived from the human sewage clustered separately from any of the ovine or bovine fecal clones, indicating the potential for host specific targets.

Bacteroidetes markers are considered useful in determining the source of pollution in a timely manner.
However, it was recently shown that these markers were present in pristine alpine soils (Vierheilig et al., 2012). The study addressed three *Bacteroidetes* markers; BacR, AllBac, and BacUni. BacR is a marker specific to ruminants and was used as a measure for “pristine” soils. Soil samples that were not positive for BacR were tested for AllBac and BacUni as well, two markers thought to be feces specific. It was found that the concentrations of AllBac and BacUni markers were high in pristine soils, considering the supposed specificity for feces. This work demonstrates the potential danger in relying on only one marker for fecal pollution.

To minimize the risk of using only one marker, a study by McQuaig et al. described the relationship between four different MST markers at two beaches in California (McQuaig et al., 2012). It was proposed that by using a suite of MST trackers, the source of contamination and the quality of the water could be determined. Both culture-dependent and independent methods of detection were employed. Culture tests were used to identify and quantify enterococci and total fecal coliforms, while nucleic acid tests targeted human specific *Bacteroides* (HF183), *Methanobrevibacter smithii*, adenovirus, and HPyVs. At both beaches, the presence of the human specific *Bacteroides* marker was only weakly correlated with fecal coliforms and total coliforms. The *Bacteroides* marker was detected in over half of the samples that were under the regulatory standards for fecal coliforms. It was suggested that while each MST marker is imperfect, combining their use highly increases the predictive power.

While human fecal pollution remains a large problem in areas, MST markers are needed for other origins as well. Gómez-Doñate and coauthors developed a qPCR assay for human, cattle, pig and poultry specific *Bifidobacterium* sp (Gomez-Donate et al., 2012). The method relies on a TaqMan assay, using the same primers to amplify the four different markers, but each assay uses a different probe specific to a certain host. Limits of detection ranged from 35 CFU/ml to 77 CFU/ml. Non-extracted DNA template did not yield positive results indicating the need for preprocessing before running the assay. Species-specific wastewaters were used to test the specificity of each assay. Probes specific to the porcine *Bifidobacterium* strain were positive in the porcine wastewater but not in wastewaters of feces from other animals.

Another study compared a dog specific *Bacteroidales* marker (CanBac-UCD) with a dog mitochondrial DNA (mtDNA) marker for their abilities to act as MST of canine fecal pollution (Tambalo et al., 2012). Both assays showed a limit of quantification of 10 copies per assay and a limit of detection of 1mg of dog feces per 100mL of water. The mtDNA assay had high sensitivity and specificity, 98% and 100% respectively. The CanBac-UCD assay had 91% sensitivity with only 31% specificity indicating that the marker was present in non-dog feces. The CanBac-UCD marker was shown to be present in mule deer, fallow deer, goat, caribou, pig, white-tailed deer, and coyote feces. When using both markers on environmental samples, dog mtDNA and CanBac-UCD did not show any correlation.
Anthropogenic sources (agriculture, pets, sewage) of fecal pollution are not the only areas in need of MST. In one study, a marker for sandhill cranes was developed, as they have been identified as the source of a campylobacteriosis outbreak (Ryu et al., 2012). A 16S clone library from the excreta of sandhill cranes and snow geese was created. The crane clone library was dominated by Bacilli at 57%, with very few Bacteroidetes at 3.2%. Two markers were developed for crane specific fecal detection. The first was based off of a unique clade of Bacteroidetes present in the crane feces. The second was based off of a sequence similar to Catellicoccus marimammalium (94% identity). C. marimammalium had previously been used as a gull specific marker. Using these two markers a qPCR marker called Crane1 was developed. The marker was not detected in any non-avian fecal samples and in only 25 out of 373 non-crane avian fecal samples.

Environmental Occurrence, Persistence, and Transport

The use of fecal indicators for assessment of water quality is important for protecting human and animal health. Several studies conducted in 2012 focused on the occurrence of FIB in a variety of settings including drinking water sources, watersheds, constructed wetlands, and recreational beaches. Other studies focused on the transport, particle association, and live vs. dead evaluation of microbes. Occurrence of fecal indicators in drinking water sources. Fecal contamination of drinking water sources can be devastating to local communities, particularly in developing countries where clean water is rare and health care is scarce. Many homes throughout the world use rainwater cisterns for drinking water sources without or with minimal treatment. In one study, 24 rainwater tanks from Australia residences were assessed for E. coli and enterococci as well as zoonotic pathogens (Ahmed, Hodgers, et al., 2012). It was determined that 63% of tanks contained E. coli concentrations that exceeded drinking water standards. 92% of tanks contained enterococci. There were also high levels of zoonotic pathogens from birds and possums including 21% Campylobacter, 4% Salmonella, and 13% Giardia lamblia.

In another study, 50 drinking water well tubes were analyzed for fecal indicators including E. coli, F+RNA coliphage, Bacteroides, and human associated Bacteroides (Ferguson et al., 2012). Direct detection of other fecal pathogens was also completed to determine whether or not they could be used as fecal indicators. It was found that rotavirus was present in 40% of wells, Shigella in 10%, Vibrio in 10%, pathogenic E. coli in 8%, culturable E. coli in 40%, molecular E. coli in 86%, Bacteroides in 86%, HuBacteroides in 23%, and F+RNA coliphage in 64%. It was concluded that F+RNA coliphage and molecular E. coli have the potential to be the best indicators of fecal contamination.
McMahan and coauthors analyzed rainwater tanks and drinking water wells similar to the previous studies as well as a protected lake for the presence of fecal indicators including *Aeromonas spp.*, *E. coli*, *Clostridium spp.*, *Salmonella*, *Shigella*, H$_2$S producers, and other organisms (McMahan et al., 2012). Water samples were tested using assays for terminal restriction fragment length polymorphisms (TRFLP) as well as traditional culture-based methods. In the lake samples, culturing methods determined that *E. coli*, *Citrobacter freundii*, *Klebsiella pneumonia* (H$_2$S producer), *Hafnia alvei*, and *Aeromonas hydrophila* represented 67% of the organisms that were cultured. The TRFLP method was able to detect two genera that were not detected via culturing including *Rhodothermus* and *Shewanella*. Similar results were obtained in the rainwater cistern and well isolates. These results suggest that H$_2$S producing bacteria could be used as fecal indicators.

**Occurrence of fecal indicators in watersheds.**

In a study by Edge and coauthors, the use of *E. coli* as a benchmark for environmental conditions in 4 agricultural watersheds was investigated using 4 reference sites that were distanced from livestock and human fecal pollution (Edge et al., 2007). Samples were analyzed for *Campylobacter*, *Salmonella*, *E. coli* O157:H7, *Cryptosporidium*, *Giardia*, and *E. coli*. Results showed an average of 1.54 fecal species per water sample for the agricultural sites and 0.75 species per water sample for the reference sites. This suggests a need to adjust the fecal indicator benchmarks to account for background pathogen levels. *E. coli* concentrations were thus derived to be ≤49 CFU/100 mL for background up to 75 CFU/100 mL for agricultural sites.

In a study by Jenkins and coauthors *E. coli*, fecal *enterococci*, *Salmonella*, and *E. coli* O157:H7 were measured from 3 ponds located in an agricultural watershed (Jenkins et al., 2012). Tracer studies were performed to determine the residence time within the ponds and thus the length of time that pathogens could be exposed to UV radiation. At one pond with a high residence time, high levels of *E. coli* were observed upstream and at the inflow to the pond, but levels decreased within the pond and downstream. This suggests that UV radiation exposure can decrease the pathogen concentration in agricultural ponds. The two other ponds analyzed didn’t follow the same characteristics, but were also highly turbid with elevated levels of nitrogen and phosphorus.

Watersheds transitioning from agricultural or forested land to urban land were sampled (via creeks) under dry weather conditions and storm events (Rowny and Stewart, 2012). Fecal indicators including fecal coliforms and *E. coli* were measured using membrane filtration and plate counts. It was determined that average for fecal coliforms during dry weather and storm events was 178 CFU/100 mL and 412 CFU/100 mL, respectively. The average for *E. coli* during dry weather and storm events were 120 CFU/100 mL and 176 CFU/100 mL, respectively. Finding suggest current sampling techniques may inadequately estimate FIB concentrations, particularly if sampled during dry seasons.
Occurrence of fecal indicators in constructed wetlands. Constructed wetlands have been considered sustainable and appealing means of water treatment, particularly for surface-flow runoff and agricultural waste. Recently, constructed wetlands have been shown to reduce both estrogenic and androgenic hormones from dairy wastes (Cai et al., 2012). The hormone removal ability of a constructed wetland in Ireland was analyzed using a solid phase extraction sample cleanup method combined with reporter gene assays with the ability to detect as low as 0.24 ng/L for estrogenic hormones and 9 ng/L for androgenic hormones. It was determined that the constructed wetland was able to remove 95.2% of estrogenic hormones and 92.1% of androgenic hormones.

While natural wetlands can behave like constructed wetlands with pathogenic removal characteristics, they can also be sources of contamination during storm events. In one study by Hogan and coauthors, a number of different wetlands were analyzed for fecal pathogens, including natural and constructed (Hogan et al., 2012). It was determined that both were adequate in removal pathogens but during heavy storm events, the pathogens collected in natural wetlands could be released in the receiving waterways. Hundred percent of samples from all wetlands were positive for *E. coli* and total coliforms, with the highest concentrations present in the constructed wetland, which is designed to remove and store pathogens.

In another study, the potential of constructed wetlands for buffering the receiving waterways from runoff during storm events was analyzed with very high levels of fecal contamination (Mulling et al., 2013). The contaminated water was created by mixing sludge-rich and clarified water from a wastewater treatment plant and injected into the wetland to mimic a storm event with a loading that ranged from 3.5 to 230 mg/L. Results demonstrated a 99% removal of suspended particles in the non-vegetated portion of the pond with pathogen removal of 40-84%. After the contaminated water passed through the vegetated portion of the wetland, pathogen and suspended particles were both reduced over 100%. These findings further validate the use of constructed wetlands for the treatment of surface water runoff and agricultural wastes.

Microbial quality of recreational beaches. The relationship between FIB and human pathogen densities was found to be directly correlated to gull populations (Converse et al., 2012). FIB, fecal source identification genetic markers and pathogens were measured in beach water samples collected from Lake Michigan prior to a 16 day period of gull control. These results were compared to samples of beach water taken following a period of gull control. Using culture and qPCR-based methods, linear regression was used to quantify the effect of a 50% reduction of gulls on beach water FIB densities. Water quality was found to be dramatically improved with a 38% and 29% decrease in *Enterococcus* and *E. coli* concentrations, respectively. Potential human pathogenic bacteria were detected 64% of the time prior to gull control and completely absent following gull control.

In another study, microbiological quality of seawater was used to test for associations between bacterial
titers and correlations with environmental conditions and potential wastewater outflows (Curiel-Ayala et al., 2012). Samples were taken at several public beaches in Mexico in both dry and rainy seasons and were tested for concentrations of Enterococci, C. perfringens and S. aureus. The study found an increase in bacterial titers during the rainy season, and indicated wastewater outflows as a possible source of contamination. Furthermore, S. aureus was found to correlate with amount of swimmers in seawater beaches. It was recommended that all three microorganisms are monitored to ensure a reliable assessment of seawater.

The occurrences of three types of Vibrio species were studied from water samples from recreational beaches in southern California during the peak of the bathing season (Dickinson et al., 2013). Of the samples collected from Doheny State Beach and Avalon Harbor, 40.1% and 18.7%, respectively were positive for V. cholera, 27.3% and 69.8% were positive for V. parahaemolyticus and 1.5% and 5.2% were positive for a V. parahaemolyticus toxin gene. Results indicate that the microbial risk from Vibrios at all locations was below the illness benchmark set by the USEPA of 19 per 1,000 bathers. Microbial risk assessment was determined to be a valuable tool for the management of marine pathogens.

Another study concluded time and location of water sampling on beaches may influence the levels of microbes detected (Enns et al., 2012). Variations in Enterococci measurements were also observed for knee-depth samples versus waist-depth samples, respectively, with 43% and 5% of samples exceeding the 104 CFU/100 mL EPA guideline. Solar radiation, tides and rainfall events were shown to be major environmental factors effecting Enterococci concentrations. Tides and rainfall were recognized as the most likely cause of spatial variation in water samples. Decreases in Enterococci levels were also attributed to higher solar radiation, with the hours outside of 7:00am and 8:00pm have near zero values. The number of swimmers was also found to correlate with S. aureus levels. Furthermore, S. aureus levels were not correlated with Enterococci levels or with other quantified parameters.

Water samples obtained from the surface of a recreational urban lagoon were concentrated and tested for occurrences of Group A rotavirus (RVA), norovirus (NoV) and human adenovirus (HAdV) (Vieira et al., 2012). RVA had the highest rate of detection with 24.4% followed by NoV and HAdV, with 18.8% and 16.7% respectively. Although 95% of the samples were found to be within bacterial parameters for bathing, viruses were found in approximately 50% of those samples. Possible correlations between turbidity and RVA presence and pH and NoV presence were also detected.

Multiple linear regressions of sand and seawater samples were used to determine routes of exposure to bacteria and bacterial water quality (Goodwin et al., 2012). S. aureus was detected in 53% of sand samples and 59% of seawater samples. Methicillin resistant S. aureus (MRSA) was found in relatively low concentration and was detected in 2.7% of sand samples and 1.6% of seawater samples.
The presence of *S. aureus* in seawater was found to be related to water temperature, number of swimmers, *S. aureus* in sand and *Enterococci* in seawater. It was concluded that the presence of *S. aureus* in sand was correlated with water temperature, *S. aureus* in sand, *Enterococci* in seawater and inversely correlated to surf height classification.

Lake water samples from Lake Erie were tested for *Arcobacter*, an emerging waterborne pathogen associated with fecal contamination by humans and animals (Lee et al., 2012). Samples were then concentrated and quantified by real-time PCR targeting the 23S rRNA gene as well as other fecal genetic markers. *Arcobacter* was detected at all tested sites and 75.2% of samples tested positive for the genetic marker. Furthermore, detected *Arcobacter* correlated to the human-specific fecal marker HuBac (*r* = 0.592) and sequences were found to be related to *Arcobacter cryaerophilus*, a known cause of gastrointestinal disease in humans.

Another study investigated the effectiveness of *Bacteroides* quantitative PCR (qPCR) as an early predictor of *E. coli* depends on the contamination concentration and location of freshwater lakes (Mulugeta et al., 2012). At *Bacteroides* concentrations above 235 CFU/100mL, qPCR was found to be less accurate at predicting *E. coli* colony forming units (CFU). *Bacteroides* levels below 4 log cell equivalents predicted *E. coli* levels relevant for beach management decisions 100% of the time. Location specific factors responsible for influencing the correlation between *Bacteroides* and *E. coli* are to be determined. Therefore, *Bacteroides* qPCR is recommended to be used in addition to *E. coli* CFU data to provide correct water quality information.

A model using artificial neural network (ANN) in MATLAB toolbox and two US EPA Virtual Beach (VB) Program-based models (one linear and one non-linear) were tested and compared as possible methods of nowcasting and forecasting *Enterococci* concentration in beach water (Z. Zhang et al., 2012). The ANN model outperformed the two VB models in terms of Root Mean Square Error (RMSE), with a RMSE of 0.803 and an average linear correlation coefficient (LCC) of 0.320. The two VB models performed better than the ANN model in terms of LCC with a 0.354 and 0.521 for the linear and non-linear, respectively. In conjunction with real-time environmental and weather data, the ANN and non-linear VB models can be utilized to now-cast and forecast beach water quality and therefore reduce risk of exposure to contamination.

A negative correlation between rainfall and microbial water quality was observed in beach water samples from No. 1 beach in China (W. Zhang et al., 2013). During dry weather, the detection of *Enterococci* was determined to be lower than fecal coliform bacteria, with maximum values of 61 MPN/100 mL and 1,530 CFU/100 mL, respectively, over a seven day period. Water quality was most affected by pollutants transported to the beach by swimmers during dry periods. During rainy weather, *Enterococci* concentrations reached a maximum of 5,353 MPN/100mL and fecal coliform reached a maximum of 10,090 CFU/100 mL over a seven day period. The
maximum level of indicator bacteria was reached within six hours following rainfall. It was determined that water quality recovery time relied on intensity and duration of rainfall.

**Persistence and transport of fecal indicators.**
In a study conducted using runoff water from a parking lot in North Florida over the course of 25 monitored rainfall-runoff events, the runoff was determined to exceed *E. coli* bacteriological USEPA recreational contact water criteria in 19 of 25 events and exceeded enterococcus criteria in 25 of 25 events (Dickenson and Sansalone, 2012). All 25 events exceeded comparative Australian unrestricted and non-potable residential runoff reuse criteria. Hypochlorite doses, which were used to inactivate the PM-associated bacteria, were effective for the suspended and settleable fractions (15-45 mg/L of HOCl) of PM, but sediment PM-associated coliforms were shielded by the host PM even at hypochlorite doses up to 45 mg/L. Current practices of unmaintained best management practices and drainages appurtenances provided both a temporary sink and source for PM, chemical, and bacterial loads. Conveyance units or unit operation systems designed for PM separation require regular cleaning and management of separated PM so such systems do not function as microbial habitats and PM sources. Such PM management before disinfection, whether by hypochlorite or other disinfection processes, will reduce microbial loadings, disinfection requirements and effluent loads of PM.

In a study investigating recreational water quality criteria, 217 water samples were collected from Pennsylvania streams and analyzed (Duris et al., 2013). It was found that the season of sample collection had an effect on the density and frequency of fecal indicator bacteria (FIB) and *Giardia*, which was likely related to nonpoint sources that are affected by seasonal change. Human source pharmaceuticals (HSPs) accurately reflected differences in the frequency of the protozoan *Giardia*, showing that selected HSPs have utility as a chemical indicator of fecal *Giardia* in similar settings. There was an indication of differences in the source and/or transport characteristics of *exp, stx2, rfbO157*, and *STI* genes, as well as *Giardia* cysts, which occurred most frequently under high discharge, compared to that of other measured virulence genes and *Cryptosporidium* oocysts, which were unaffected by high discharge. Agriculture and forest land use were also concluded less likely to have a larger array of pathogen genes while sites with urban land use had greater densities of *Giardia* and FIB and higher concentrations of HSPs.

A study in NE Scotland was conducted to determine the quality and source of contamination for water samples gathered from drain outlets in agricultural land (Edwards et al., 2012). It was found that the majority of nitrate and suspended sediment originated directly from an agricultural field drainage which contributed the greatest loads of ammonium, phosphate, and fecal indicator organisms even though the stream channel had temporary storage/retention mechanisms and fencing to eliminate cattle access.

Water samples collected from a field that applied Class B biosolids and analyzed for *E. coli* density and community (Esseli et al., 2012). Results revealed that post-
application drainage water collected from biosolids treated fields contained significantly higher *E. coli* densities following heavy rainfall events, as compared to light rainfall events. Class B biosolids were likely contributing to *E. coli* found in tile drainage and ditch water. Non-biosolids signatures in the fingerprinting analyses indicated other environmental sources like wildlife influenced the *E. coli* communities in the tile drainage and ditch water.

Another study investigated the observed nighttime *E. coli* replenishment in near-shore waters, specifically the surf and swash zones (Ge et al., 2012). It was found that *E. coli* was transported from water of intermediate depth, where sediment resuspension occurred intermittently. The nighttime onshore transport was altered or destroyed later in the morning. *E. coli* also accumulated overnight in the knee-deep water and was inactivated by the UV radiation in the sunlight while the rest is transported offshore and eventually carried back to deeper water. Observed high *E. coli* concentration in the submerged sediment (mean value 1,585 CFU/100 mL) beneath the knee-deep water was likely to be a result of early morning deposition of *E. coli* from deeper water than an original source.

A practical methodology to scale up bacteria transport experiments carried out in the laboratory to a practical field situation was developed (Lutterodt et al., 2012). The segment sticking efficiency of the six *E. coli* strains harvested was not a constant but reduced with increasing transport distance which ranged from 3 to 22 m, dependent on the presence of geochemical heterogeneity, the distribution of the unfavorable sticking efficiency, porewater flow velocity, and the decay rate coefficient. It was also found that the fraction of bacteria and segment sticking efficiency was adequately described by power law distribution functions.

The impact on various growth conditions on the transport of *E. coli* was also investigated (Marcus et al., 2012). When measuring *E. coli* grown in a standard laboratory growth medium and a dairy manure extract solution, cells grown in manure extract were more hydrophobic, had a more negative zeta potential, had lower amounts of surface macromolecules, and had lower attachment efficiencies than cells grown in laboratory growth medium. These findings also concluded that using a single isolate and standard growth media was insufficient for simulating transport of *E. coli* from a manure source and more research is needed to adequately model the fate and transport of bacteria grown in more representative growth solutions.

Another study investigated the water quality of an anaerobic swine manure lagoon (McLaughlin et al., 2012). Abundance of *E. coli* with increasing pH, decreasing populations of *Campylobacter* spp., enterococci, *Listeria* spp., and *Salmonella* spp. with increasing temperature, and higher levels of the water-soluble heavy metals Cu, Fe, and Zn with increasing temperature were measured. Levels of bacteria varied during the year but all species, including zoonotic pathogens, were consistently present year-round, suggesting that water samples should be collected at least seasonally for accurate characterization of lagoon water quality.
Another study overcame the challenges in characterizing the risk from waterborne disease in a data-sparse, less developed region by integrating hydrologic simulations, a program of field measurements and a model of indicator organism fate, using data gathered from the upper Sonora River in Mexico (Robles-Morua et al., 2012). The risk from waterborne disease was characterized by estimating distances downstream of wastewater discharge points where an indicator organism, *E. coli*, exceeded a well-established standard related to contact with waters contaminated with wastewater. The model created assessed those risks based on the relative importance of streamflow variations and the uncertainty in *E. coli* removal coefficient parameters.

Another study used membrane filtration (MF) and defined substrate technology (DST) to identify enterococci such as *E. faecalis* and *E. faecium*, the major enterococcus species derived from humans and livestock (Suzuki et al., 2012). Actual river water samples from 5 urban rivers indicated presence of a large number of unspecified enterococcus species, which exist but, in this study, may have been caused by pseudo-positive bacteria in river water. The study also pointed out that although *E. faecium* is a non-predominant species in the intestinal and fecal enterococcal flora, it was one of the major enterococcus species in surface water samples.

In another study, two bio-retention facilities were tested for removal of indicator bacteria (L. Zhang et al., 2012). Generally, the concentration of indicator bacteria in the input flow increased with increasing daily temperature. During most storm events, the concentration of fecal coliforms and *E. coli* was reduced. The capture and destruction of bacteria in bio-retention was investigated at temperatures of 5, 15, 25, and 37 °C resulting in decay coefficients for strain B6914 of 0.11, 0.17, 0.90, and 1.87 per day, respectively. From 5 to 25°C, levels of protozoa and heterotrophic bacteria counts increased which resulted in faster die-off and higher decay coefficients of trapped strain B6914 via predation and competition for limited nutrients. At 37 °C faster metabolism and higher decay rates of strain B6914 cells as a result of the lack of substrates were a significant cause for the rapid decline of B6914 cells, due to the fact that the growth rates of protozoa and heterotrophic bacteria were lower at 37°C than at other temperatures.

**Alternative fecal indicators.** Enteroviruses (EnV) are a valid alternative to be considered for indication of waterborne disease, as they cause many common health issues, both mild, such as diarrhea and gastroenteritis, and severe (especially in immuno-compromised individuals), such as aseptic meningitis, encephalitis, and paralysis. One study investigated the use of bivalves to detect enteroviruses using RT-PCR (Connell et al., 2012). A clear correlation between EnV presence in shellfish tissue samples gathered from Hawaii’s coast and the water (including treated sewage water) was obtained (p-value = 0.0410). Positive results were obtained in 6 of the 9 tissue samples collected suggesting that not only could EnV be used as fecal indicators, but their presence in shellfish could be used as a sample collection technique.

In coastal areas like North Carolina the presence of many recreational and shellfish harvesting estuarine
areas make it hard for routine monitoring of FIB. In one study, samples were collected during 12 dry and 13 wet periods at 10 different sites (Gonzalez et al., 2012). Statistical predictive models were then made for *E. coli*, *enterococci*, and members of the Bacteroidales group. Models accounted for a range of antecedent rainfall, climate, and environmental variables. *E. coli* and *enterococci* concentrations depended upon the 5-day antecedent rainfall, dissolved oxygen, and salinity. The error rate of these predictive models was 3% for *E. coli* and 9% for *enterococci* models concerning recreational areas, and a 0% error rate using the *E. coli* model for shellfish threshold. This developed model was concluded to be a successful means of predicting water quality.

A novel fecal indicator was also investigated called *Acanthamoeba* and validated for use in a Taiwan watershed (Kao et al., 2012). Of the 211 samples collected, the amoeba was detected via PCR in 16.1% of samples with pathogen-associated species detected. The species T4 (associated with pathogenic strains) accounted for 66.7% of positive samples in the Puzih River. Investigators also determined that physical characteristics (like temperature) and microbiological characteristics (like total coliforms) were associated with the occurrence of *Acanthamoeba*.

Another study showed that real time-PCR (RT-PCR) is a more effective and practical approach than the conventional methods, which cannot identify *E. coli* serotype O157:H7 (Chetta et al., 2012). 54 wastewater samples were analyzed using developed primers, and thus detection, of three *E. coli*-specific genes – HNS (DNA-binding transcriptional dual regulator), *Allan* (allantoin permease), and *CadC* (DNA-binding transcriptional activator) – that were highly conserved between 33 serotypes. The primers were tested on gram positive and negative commercial species before application on the samples. This method was concluded to be highly sensitive, allowing a 95% reproducibility of the lowest dilution, tested on 50.6 fg (corresponding to 10 genome equivalent).

In another study, the presence and spatial variability of triazines and their metabolites, an antiepileptic drug, an anti-inflammatory, a nervous stimulant, a lipid regulator, an antibiotic, and three hormones were investigated (Daneshvar et al., 2012). After analyzing samples from the greater Montreal region’s rivers, it was found that these drugs, hormones, and triazine herbicides and their metabolites caused high total organic carbon (TOC) levels. High TOC levels indicated contamination from wastewater treatment plants and combined sewer overflows thus proving these organic compounds could be used as alternate indicators of fecal contamination.

**Viability evaluation.** To allow accurate assessment of microbial quality of water, the difference between viable and non-viable cells must be investigated. Molecular methods, which are growing in popularity due to their rapidity and ease of use, unfortunately are unable to distinguish between live and dead cells. In one study (Kurakawa et al., 2012), a method was produced for the detection of *V. cholerae*, *V. mimicus*, *V. parahaemolyticus*, *V. alginolyticus* and *C. jejuni/ C. coli* using RT-qPCR
which allowed quantification of only viable cells when targeting ribosomal RNA. The 16S rRNA (or 23S rRNA for *V. parahaemolyticus*) sequences were used to design rRNA-targeted primers. It was determined that $10^3$ cells/g stool were detectable by RT-qPCR compared to $10^5$ to $10^6$ cells/g detectable by qPCR.

In another study, two qPCR methods were developed to detect viable oocysts of *Cryptosporidium parvum* in raw surface water and disinfection treated water (Liang and Keeley, 2012). PMA-qPCR targeting the *hsp70* gene and reverse transcription-qPCR heat induced *hsp70* mRNA were compared. Cells were exposed to different concentrations of ammonia and H$_2$O$_2$ to determine if viability was effected. Both methods worked to detect viable *C. parvum* oocysts in distilled water. It was found that targeting for mRNA was superior to targeting *hsp70* in raw water. Detection of mRNA could allow for improved prediction of pathogenic protozoa in drinking water.

A direct viable count-fluorescent in situ hybridization (DVC-FISH) method was also developed to differentiate between viable and non-viable *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* cells (García-Hernández et al., 2012). STH23 and LDE23 rRNA oligonucleotide probes were used to detect both bacteria. The detection limit for this DVC-FISH method was $10^3$ viable cells per gram of sample making a favorable means of detecting viable cells.

A study was conducted that focused on the effect of amplicon length on the ability of PMA-PCR to detect viable cells while ignoring non-viable cells of *S. enterica* and *C. jejuni* (Banihashemi et al., 2012). PMA treatment inhibited the amplification of some heat treated cells when DNA fragments were <200 bp. UV-irradiated seemed to have no effect while PCR of longer amplicons was able to only detect viable cells in the absence of PMA. The detection of viable cells with long-amplicons by the PMA-PCR method was effective following the heat and UV treatment of cells.

A specific PMA-qPCR method was developed for the detection of viable *E. Coli* O157:H7 cells (Li and Chen, 2012). Several genes were found to be specific to *E. coli* O157:H7 and the open reading frame (ORF) Z3276 genetic marker was the only gene that showed no homology to non-O157 genes. ORF Z3276 genetic marker was then used to develop primers and probes. The amplification of PMA-treated cells was inhibited. The method was adapted for a 96-well plate system for multiple sample testing. Viable cells could be detected in a mixture of 8 CFU/g *E. coli* O157:H7 cells in 8x10$^7$ dead cells with an 8 hour enrichment and PMA treatment.

A RT-qPCR was developed to detect and quantify viable *C. jejuni* from fecal samples (Bui et al., 2012) and compared to qPCR. Three primers were used for amplification, one targeting the ciaB gene, the dnaJ gene and the 16S rRNA gene. Viable cells could be detected for up to five days in spiked and naturally contaminated samples. The percentage of *C. jejuni* cells decreased from 100% to 91.3% from day 1 to day 3 using RT-qPCR while the percentage stayed at 100% for the sample amplified with qPCR. The RT-qPCR method was determined to be a more efficient means of the quantification and detection of viable *C. jejuni* cells than qPCR.
A PMA-qPCR assay was also used to distinguish between viable and nonviable cells in bacteria (E. coli and B. subtilis) and viruses (MS2 and murine norovirus [MNV]) in another study (S. Y. Kim and Ko, 2012). The DNA amplification of E. coli displayed a 3.08 log decrease while B. subtilis displayed a 3.48 log decrease. The PMA-qPCR assay was able to distinguish between viable and nonviable cells in Escherichia coli and B. subtilis bacteria and in MS2 virus but not in MNV.

**Particle association and transport of microbes.**

A study conducted in Tampa, Florida examined the relationship among fecal indicator bacteria (fecal coliforms, E. coli, enterococci), pathogens (Salmonella, Cryptosporidium, Giardia and enteric viruses) and human markers (esp gene of E. faecium, human associated Bacteroides marker - HF183, and human polymavirus marker -HPyVs) (Staley et al., 2012). It was determined that submerged aquatic vegetation, sediments and storm water act as reservoirs for FIB and pathogens in water with resuspended sediment contributing the most in significantly increasing levels of FIB in beach water. These results indicate that fecal coliforms have greater correlation with the presence of human pathogens than enterococci. Further, a Bayesian model was developed using physical parameters like temperature and rainfall to predict increased risk of pathogens at or near water bodies.

Another study investigated sand samples from seven beaches along Lake Huron and Lake St. Clair in Michigan for the presence of genes associated with pathogenic E. coli (Bauer and Alm, 2012). Attachment genes (eae and bfp) and toxin producing genes (stx1 and stx2) that are associated with enteropathogenic E. coli (EPEC) and enterohemorrhagic E. coli (EHEC) were investigated by multiplex PCR amplification. The attachment genes were frequently present in the samples with 79% detecting eae and 38% having bfp. The toxin gene stx1 was absent in all the samples whereas stx2 gene was detected in only 1.7% of samples (2 samples). The frequency of eae gene is of concern for possible horizontal gene transfer events and possible emergence of new E. coli pathotypes.

A study by McCarthy and coauthors characterized the variability of total suspended solids (TSS) and E. coli levels within a storm event by examining over 100 events in five different catchment areas(McCarthy et al., 2012). They also studied the effect of antecedent climate, rainfall, storm flow pattern on the variability and transport of TSS and E. coli. The results showed that smaller catchments have significantly higher correlation between TSS and E. coli during the start of an event compared to larger catchments. Also, initial E. coli concentrations were determined more by antecedent climatic conditions, including temperature and vapor pressure, whereas TSS levels were usually influenced by hydrological parameters. Also, the median within-event variability in E. coli concentrations was less than variability of TSS for all the sites. However, the variability between events was higher for E. coli than for TSS. The size of the drainage system also influenced the variability of contaminant concentrations.
The studies presented in this review represent recent advances in research focused on the detection and characteristics on waterborne fecal indicators and pathogens. The techniques and assays described previously have enormous potential for the detection of fecal pathogens in a more rapid, sensitive, and specific manner. Studies on source tracking, occurrence, persistence and transport of microbes allow for better understanding of the sources and behaviors of pathogens. Enhanced detection methods in conjunction with a better understanding of pathogens can greatly reduce the impact of human activities.

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