Detection and Occurrence of Indicator Organisms and Pathogens

Farag A. Samhan¹, Maggie R. Kronlein², Umama Fakher³, Cathrine Kronlein², Robert D. Stedtfeld⁴, Syed A. Hashsham⁵

ABSTRACT: This review summarizes the literature pertaining to the occurrence and detection of indicator organisms and pathogens published during 2014. It is organized into the following sections: i) detection and quantification of fecal indicators and waterborne pathogens, ii) microbial source tracking (MST) using genotypic and phenotypic methods, iii) antibiotic resistant bacteria (ARB), iv) live vs. dead cell differentiation methods, and v) next generation sequencing (NGS).

KEYWORDS: Fecal indicator bacteria, microbial source tracking, antibiotic resistance bacteria, propidium monoazide, ethidium monoazide.

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Introduction

This review summarizes studies published in 2014 focusing on the environmental occurrence and detection techniques for waterborne pathogens and fecal indicator organisms. Focus is on new detection techniques and assays that were developed employing immunoassay-based technologies, RT-PCR, next generation sequencing (NGS), and approaches for differentiation of live vs. dead organisms using propidium monoazide and ethidium monoazide. These techniques and assays have the potential to enhance the capabilities for detection and quantification of pathogens, disinfection, and risk management. Advances in microbial source tracking (MST) techniques for both genotypic and phenotypic-based analyses are also summarized. Multiple studies investigating the occurrence, persistence, and transport of antibiotic resistance bacteria are included due to their relevance to waterborne pathogens.

Detection and Quantification of Fecal Indicators and Waterborne Pathogens

Bacterial indicators and bacteriophages for pathogens detection. Cho et al. (2014) presented a functional gene marker-focused strategy for monitoring *E. coli* serotype O157 in water. Sequencing of stx, slt, eae, hlyA, rfb, and fliCh7 genes showed differences at the serotype level. A primer set was developed and evaluated using genomic DNA from 8 isolates of *E. coli* serotype...
O157 and 32 reference strains. The assay was found to be sensitive and specific for *E. coli* serotype O157 in environmental water samples.

Verhougstraete and Rose (2014) reported the measurement of fecal indicator bacteria across mixed-use watershed beaches and characterized environmental parameters related to microbial water quality. The study included testing of *E. coli*, *Enterococci*, *Clostridium perfringens*, F+amp coliphages, and CN-13 coliphage and molecular markers (surface protein of *enterococci*) in four Saginaw Bay beaches (Michigan, USA). It was observed that algal mats and sediments had higher levels of bacteria compared to the water column. The potential for regrowth in sediment and algae was also highlighted. Wind waves and precipitation were reported as factors influencing the occurrence of fecal indicator organisms. *Enterococci* could be identified as human specific fecal indicator organisms using genes associated with surface protein. This study concluded that monitoring of shallow waters for fecal indicators and beach grooming should be included to ensure beach protection.

The prevalence of *E. coli* O157:H7 in surface water downstream and upstream of cattle feedlots was studied using 311 surface water samples in 48 cattle feedlots. Multiplex PCR-based measurements showed that the surface waters exposed to livestock operation discharges were contaminated with more STEC O157:H7 than upstream samples. However, differences were not statistically significant. The control of runoff systems from intensive livestock operations was found to be necessary, among other alternatives, to reduce prevalence of *E. coli* O157. The study concluded that pathogens present in the runoff has the potential to reach recreational waters.

In another study, the incidence of *E. coli* was used to assess suitability of 125 private water supplies that served individual houses in Ireland (O’Dwyer et al. 2014). A logistic regression model was used to predict the probability of *E. coli* contamination using two independent variables - rainfall and aquifer characteristics. The model successfully distinguished the relationship between the independent variables and incidence of contamination. The study concluded that the chance of *E. coli* contamination was higher with increased rainfall, especially in areas with bedrock aquifers. In a similar study, the proportion of illness acquired by foodborne transmission for nine enteric pathogens was investigated by Vally et al. (2014). The proportion of illness acquired by food, environment, water, person, and zoonotic routes was estimated to by 90%. Results supported the notion that norovirus, hepatitis A, non-Shiga toxin pathogenic *E. coli*, and *Shigella* spp. spread primarily from person-to-person, while Shiga toxin-producing *E. coli* has a zoonotic transmission route. In addition, the primary transmission route for *Clostridium perfringens*, *Listeria monocytogenes*, non-typhoidal *Salmonella* spp., and *Campylobacter* spp. were determined to be foodborne.

The use of enterococcus phages as a tool for identifying sewage inputs in the Great Lakes region was investigated (Vijayavel et al., 2014). Enterococcus phages, F+coliphages, *E. coli* and *Enterococci* were quantified in samples from four rivers, four beaches, and three harbors.
Results showed that levels of enterococcus phages were similar to F+ coliphages in all wastewater samples but were absent in samples containing non-human fecal matter. Phages specific to Enterococcus spp. and F+ coliphages were below detection limit in river samples but were detectable in beach and harbor samples. In these samples, slightly higher concentrations of E. coli and Enterococci were detected compared to F+coliphages and Enterococcus phages. The study suggested that phages specific to Enterococcus spp. could serve as sensitive and specific indicators of enteric pathogens.

The microbial pathogens and indicators in sewage effluent and river water were studied during a temporary (4 days) closing of a sewage treatment facility (Grøndahl-Rosado et al., 2014) in SE Norway. Samples of wastewater and river water were taken before and during the shutdown of the facility and analyzed for various microbial indicators and pathogens. Quality of water at the drinking water treatment plant intake (20 km downstream) was not found to be significantly affected, compared with the higher concentrations observed prior to the plant shutdown. It was suggested that this lack in difference was due to heavy rainfall events.

Mookerjee et al. (2014) used a modified technique for determination of coliphages in potable waters. The modified method detected coliphage in 80% of the samples compared to only 55% using standard method. It was concluded that coliforms-free water samples were not necessarily pathogen free. The method was claimed to be easy, rapid, more reliable and accurate for coliphage measurement in water samples.

In a study focusing on the development of low cost system using phenotypic markers, Burnham et al. performed E. coli detection using the release of β-galactosidase (2014). The reaction was carried out in a paper-based format to save reagents and increase sensitivity. The procedure also avoided transport of large volumes of water samples and make the assay suitable for on-site analysis. The modified technique could detect low counts of E. coli (as low as 40 colony-forming units or cfu ml⁻¹) within 8 hr. Specificity of the method was validated using Aeromonas hydrophila, Enterobacter cloacae, E. coli, and Salmonella typhimurium.

The occurrence and abundance of enteric viruses and coliphages were assessed by Rezaeinejad et al. (2014) in highly urbanized catchment water in Singapore. In total, 65 water samples were analyzed for the targeted viruses (adenoviruses, noroviruses, astroviruses and rotaviruses) using qPCR. In parallel, coliphages were detected using single agar layer plaque assay (SAL). The most prevalent viral pathogen was noroviruses - which was detected in 57%, norovirus genogroup II in 48%, and rotavirus in 40% of the samples. The mean counts of somatic and male-specific coliphages were 2.2x10^2 and 1.1x10^2 pfu/100 ml, respectively. The counts of targeted viruses and somatic and male specific coliphages varied from site to site. In addition, male-specific coliphages were positively correlated with norovirus concentrations.
Free-living amoebae (FLA) quantification and pathogens transmission. Several amoebae are pathogenic and some serve as host to bacterial pathogens such as Legionella. A number of studies focused on free-living amoebae (FLA) in different aquatic environments which are summarized below.

The presence of pathogenic FLA and Legionella in various water bodies was investigated by Ji et al. (2014). Water samples (140 total) were collected from surface water (river), intake areas of drinking water treatment plants, and recreational hot spring complexes in central and southern Taiwan and tested. Results showed the abundance of Acanthamoeba T4 and Naegleria australiensis in the hot spring water. It was determined that Vermamoeba vermiformis most likely coexists with Legionella spp. The study recommended more attention be placed on potential legionellosis and amoebae infections from FLA contamination in recreational hot springs and drinking water sources.

Another study by Scheik et al. (2014) investigated the occurrence of FLA and their co-occurrence with Legionella in industrial waters. Water samples (201 samples) including 129 cooling waters, 72 process waters, and 30 cooling lubricants were tested. More than 2/3rd (72.6%) of the samples were positive for FLA. Acanthamoebae were detected in 23.9% of the samples and Vermamoeba vermiformis in 19.4%. One cooling lubricant was positive for Acanthamoeba genotype T4 and Legionella spp. was detected in 34.8% of the water samples. Abundance of Legionella spp. was >1000 cfu/100 ml in 15% of the samples. In total, 81.4% of the samples analyzed by standard methods were positive for both Legionella and FLA. The density and diversity of amoeba were affected slightly by disinfectants. It was concluded that FLA can re-colonize treated waters within a short period and can serve as vehicles for Legionella.

Bichai et al. (2014) investigated the predation and transport of persistent pathogens in granular activated carbon and slow sand filters using Cryptosporidium and Giardia as test organisms. The influent concentrations of Cryptosporidium were 1.3x10^6 and 3.3x10^4 oocysts L^-1 and Giardia was 4.8x10^4 cysts L^-1. The authors developed a model to extrapolate the outcome under different conditions. The transport and survival of the test organisms were low in granular activated carbon compared to slow sand filters. The probability of infection due to internalized (oo)cysts in filtered water estimated risks under environmental scenarios. It was concluded that zooplanktons carrying persistent pathogens may pose health risks when released to the finished water filtered with granular activated carbon.

In another study, Legionella spp. were quantified in reservoirs water in Taiwan (Kao et al., 2014). Approximately 2/3rd (63.2%) of the samples tested were found positive for Legionella spp. Identified Legionella species included L. pneumophila, L. jordanis, and L. drancourtii. Public health concerns due to the presence of these organisms were highlighted.

Delafont et al. (2014) investigated the presence of Non-tuberculous Mycobacteria (NTM) and FLA in a
drinking water network during a one year sampling program. The authors found that NTM are opportunistic pathogens sharing the same ecological niches as FLA. Many studies have demonstrated the ability of these bacteria to colonize and persist within drinking water networks. There was also a strong suspicion that mycobacteria could use amoebae as a vehicle for protection and even replication. Results showed that NTM were detected in 87.6% of the amoebal cultures. *Acanthamoeba*, *Vermamoeba*, *Echinamoeba*, and *Protacanthamoeba* were the main genera found in drinking water networks. It was found that NTM (*M. llatzerense* and *M. chelonae*) can survive and replicate inside FLA. Sequencing of environmental isolates showed frequent association of mycobacteria and FLA.

**Disinfection of pathogens and risk management.** The risk of verotoxigenic *E. coli* (VTEC) in private groundwater sources was quantitatively assessed by Hynds et al. (2014). The study analyzed samples from 262 private domestic wells located in vulnerable areas. The authors developed a quantitative model for prediction of gastrointestinal infections caused by verotoxigenic *E. coli*, which predicted annual estimated rate of 28.3 infections/100,000 well users. This annual rate exceeded the current EU estimate by 40-fold.

Risk factors for hand contamination from environmental interventions were investigated by Devamani et al. (2014). Recontamination of hands after washing with soap was observed within 1 h. Childcare was frequently associated with hand contamination with *Enterococcus* spp. Agricultural jobs were associated with contaminating hands with *E. coli*. Both *Enterococcus* spp. and *E. coli* were associated with food preparation. Other activities such as water access, latrine type, education or diarrhea were not associated higher bacterial counts.

Wang et al. (2014) investigated the survival of *E. coli* O157:H7 in agricultural soils. The study aimed to compare *E. coli* survival ability either in normal or pH modified soils. Results showed that the survival time *tₐ* of *E. coli* O157:H7 was between 7.1—24.7 days. Physical and chemical properties of the soil were the determinants supporting decrease or increase of the *tₐ* values. In soils with lower pH, the *tₐ* values decreased as the soil became more acidic, while in the neutral or alkaline soils the pH values were not affecting *tₐ*. The study concluded that persistence of *E. coli* O157:H7 in soils should be considered when assessing environmental contamination risk.

Real time PCR assays for the detection and enumeration of enterohemorrhagic *E. coli* directly from cattle feces was evaluated by Luedtke et al. (2014). The RT-PCR targeted amplification of ecf1, eae, and stx1–2 genes in *E. coli*. The assay was sensitive enough to detect as low as $1.25 \times 10^3$ CFUs/mL in spiked fecal sample. The procedural requirements for this assay were considered to be simple and offered an acceptable sensitivity for EHEC detection from cattle feces.

A study focusing on disinfection of viruses - *Adenoviridae* and *Polyomaviridae*, by low-pressure monochromatic ultraviolet (UVC) radiation was carried out by Calgua et al. (2014). These viruses were used as markers.
of fecal contamination load in water as well as to track the source of contamination. Previous studies demonstrated that the stability of dsDNA for UVC radiation was higher compared with ssRNA or dsRNA viruses. The authors studied the rate of inactivation for dsDNA and ssRNA viruses under different fluences of UVC. Results showed that JC polyomaviruses (JCPyV) and human adenoviruses 2 (HAdV2) were more stable than MS2 bacteriophages (ssRNA). They also demonstrated that infectivity of viruses could be estimated using mathematical models and qPCR data.

The efficacy of per-acetic acid and hydrogen peroxide as disinfectants were investigated by Sacchetti et al. (2014). Pseudomonas aeruginosa and Stenotrophomonas maltophilia were considered as test organisms for disinfection ability of a microfiltration system. Treatment with peracetic acid greatly reduced the counts of the two test organisms in the dispensed water. However, two days after treatment, counts increased. With hydrogen peroxide, after 40 min contact time, P. aeruginosa could not be detected in 73.7% of the samples. S. maltophilia counts were inversely proportional to contact time.

Microbial Source Tracking (MST) using Genotypic and Phenotypic Methods

Microbial association with different biota and source tracking. Microbial source tracking (MST) is used to link the presence of specific microbes to a point or source of contamination. MST is key to developing approaches for waterborne pathogen prevention by resource managers.

The human markers adenoviruses (HAdV) and JC polyomaviruses (JCPyV) were quantified in parallel with porcine and bovine markers including as porcine adenoviruses (PAdV) and bovine polyomaviruses (BPyV) using real-time PCR (Rusinol et al., 2014). Surface water samples were collected from rivers located in different locations including Brazil, Greece, Hungary, Spain and Sweden. During dry season, water flow in these rivers decreased and secondary effluents discharged into the riverine constituted the main stream. It was noted that low temperatures resulting from ice cover that formed over the river during the winter combined with the absence of solar inactivation enhanced the viral stability. Porcine and bovine markers counts correlated with livestock and agricultural activities. The study claimed to support the applicability of viruses as MST tools.

The effect of biotic factors on persistence of fecal indicator bacteria (FIB) was studied in the Mississippi River using MST (Korajkic et al. 2014). A mesocosm was used at one of the temperate swimming beaches in the Mississippi river to evaluate the effects of ambient sunlight and biotic interactions. The decay rate of culturable FIB was faster in comparison to molecular measures of FIB. Results showed a strong correlation between the decay rate of molecular FIB and human associated genetic markers. However, no correlation was observed between culturable FIB and qPCR measurements. Sunlight was an important factor affecting the decay rate.
The association of human campylobacteriosis with drinking water consumption and agricultural activities was evaluated in a large study (Galanis et al. 2014). The authors compared 2,992 cases of campylobacteriosis with 4,816 cases of enteric diseases reported during from 2005 to 2009. Each case was geocoded and linked to its source, sampling region and socioeconomic status (SES) according to the location of their residence using geographical information systems (GIS) analysis method. The probability of campylobacteriosis infections for the people consuming water from private wells was higher than those using municipal water. In rural settings, the probability of campylobacteriosis was higher particularly during the month of November and in older persons. The risk of campylobacteriosis was correlated with unsafe drinking water consumption and rural environment. The study recommended further studying the microbiological impact of agricultural activities on well water.

In a multi-use catchment, source water Cryptosporidium count, species and rates of infection during rainfall-runoff were investigated by Swaffer et al. (2014). Cryptosporidium counts were positively and significantly correlated with the flowrate and turbidity of rainfall runoff. Cell culture assays measured oocyst infectivity showed an overall source water infectious fraction of 3.1%. Infectious C. parvum and C. hominis were not detected. Using molecular techniques, however, C. parvum was detected in 7% of the samples. Twelve Cryptosporidium species were identified using these techniques and reflected the host animals typically found in remnant vegetation and agricultural areas. The use of molecular techniques to identify Cryptosporidium species and genotypes could explain the diversity of pathogens in water.

Reboredo-Fernández et al. (2014) evaluated the communities of benthic macroinvertebrate as aquatic bio-indicators of Giardia and Cryptosporidium. Thirty two samples of macroinvertebrates were collected from nine rivers in Galicia (NW Spain). Giardia cysts were detected in 3.1% of the samples and Cryptosporidium oocysts were detected in 12.5% of the samples. The results demonstrated that benthic invertebrates could be used as bio-indicators of contamination by these waterborne protozoans.

The application of biosolids and manure as agricultural bio-fertilizers and its effect on the microbial water quality in rural areas in the US were reviewed by Oun et al. (2014). Waterborne disease outbreaks observed in North America were associated with rural drinking water systems. The reported waterborne outbreaks were related to microbial agents (parasites, bacteria and viruses). Rural areas often have a high density of livestock and do not have adequate treatment for wastes. Specifically, livestock wastes from production facilities and biosolids from wastewater facilities are often applied to land without proper treatment. Exposure to manure, biosolids, and leaking septic systems may pose risk of waterborne contaminants for human. The review suggested an approach for risk assessment and best management practice for biosolids and manure application in the USA.

Use of adenosine triphosphate (ATP) was evaluated for detecting microbial contamination in drinking
water (Vang et al., 2014). The ability of ATP assay was tested with different concentrations of wastewater in non-chlorinated drinking water. Different approaches were investigated to improve the performance of the ATP assay in detecting microbial occurrence in drinking water. Compared to regular methods, the proposed assay sensitivity in wastewater and/or surface water was higher than total direct counts. Comparison of results obtained with heterotrophic plate counts at 22°C and at 37°C, and Colilert-18 for E. coli and coliforms, to results obtained with ATP revealed that ATP measurements had lower sensitivity.

**Quality of recreational water.** The adverse health outcomes including gastrointestinal symptoms, skin irritations, eye, ear, nose, and throat infections, and respiratory illness are normally associated with recreational water quality. Many studies indicated that the recreational water associated adverse health outcomes are higher in swimmers compared with non-swimmers. The bacterial indicators such as Enterococci and E. coli can be used to predict the microbial quality of recreational water. The role of filamentous algal species and the interaction of human microbial pathogens with material and nutrients in estuarine environments and their impacts are also covered in this section.

Factors related to water quality, weather, and the environment associated with fecal indicator organism density in beach sand were studied by Heaney et al. (2014). The determinants that influence fecal pollution in beach sand are not totally defined. Results showed that FIB concentrations in beach sand fluctuated over time. Enterococci as CFU and qPCR (results estimated as Calibrator Cell Equivalents or CCE) densities in sand were not correlated together, while fecal indicators in sand correlated. Fecal Bacteroides counts were strongly correlated with FIB density in beach sand and water. The sand–water interface, daily mean counts in water, rainfall density, and wave height together were factors affecting concentrations in beach sand. It was concluded that typical analysis of sand focused on enumerations, combined with chemical and microbial quality data about water, weather data, and environmental interactions may help beach decision makers reduce microbial loads in beach sand.

Detection and enumeration of coliforms and E. coli in recreational water supplies were studied by Fiello et al. (2014). Using 4-methylumbelliferyl-b-Dglucuronide (MUG), only 48% of M-FC positive colonies were E. coli. On the other hand, using API-20E test strips only 23% of the positive results on M-FC media were E. coli. Almost all of the other M-FC blue colonies were found to be Klebsiella, Kluyvera or unidentifiable groups. It can be concluded that full confirmation for M-FC result and accurate reading are important for fecal indicators monitoring.

Enterococci were investigated as FIB in recreational water bodies to assess the risk of different sources mixture of enterococci on human health (Soller et al., 2014). Results showed that risks from mixed sources driven by the proportion of the contamination source had the greatest potential to cause infection. Risk from mixture comprising 30% Enterococci from human sources was nearly half compared to the risk expected from purely
human sources. This risk assessment was based on the age and abundance of Enterococci contamination. The study highlighted the characterization of water quality parameters and its importance for prediction of risk due to human fecal matter.

The role of filamentous algal species to protect bacterial species frequently detected in nearshore public beach was investigated by Beckinghausen et al. (2014). Cladophora spp. is an algal group causing unacceptable water conditions in the Great Lakes region. It was selected to investigate its interactions with E. coli and Salmonella enterica serovar Typhimurium (S. typhimurium). The lake environment and natural sunlight conditions were simulated in a laboratory microcosm. E. coli count decreased by 7-log after 6 hr. In case of algae presence in the microcosm, the same log removal was achieved in 16 hr. It was concluded that, Cladophora spp. protected S. typhimurium resulting in lower log reductions.

The geographic fluctuation and distribution of bacterial pathogens in various matrices at Great Lakes beaches were investigated by Oster et al. (2014). qPCR was used to quantify genes of E. coli O157:H7 (eaeO157), shiga-toxin producing E. coli (stx2), Campylobacter jejuni (mapA), Shigella spp. (ipaH), and an uncharacterized S. enterica - specific sequence (SE). The DNA of these organisms were sequenced in 7 Great Lakes beaches, as well as in algae, water, and sediment. Generally, frequencies of the detected genes were in the order; mapA > stx2 > ipaH > SE > eaeO157. The detected genes showed high variation among beaches with mapA, stx2, and ipaH detection frequencies showing correlations with environmental conditions. The mean of beach seasonal variations of mapA abundance and log10 E. coli counts were correlated. At one of the studied beaches, stx2 gene abundance was positively correlated with daily E. coli counts. The study concluded that pathogenic gene marker quantification might better assist in monitoring the water quality of beaches.

The occurrence of intestinal protozoan parasites (Giardia and Cryptosporidium) in tropical recreational marine water was investigated (Betancourt et al., 2014). Results showed the possibility of infections with parasitic protozoa when persons are in contact with tropical marine waters contaminated with sewage. The risk estimates for Giardia was higher than for Cryptosporidium in the recreational water. The mean risk for cryptosporidiosis and giardiasis for both children and adults were below the U.S. EPA’s upper limit for recreational waters. The study concluded that environmental monitoring of microbial pathogens is essential to predict and control the effects of anthropogenic impacts on marine ecosystems and human health.

The survival of fecal indicator organisms on beaches combined with the role of seaweeds and plastic debris was studied (Quilliam et al, 2014). The aim was achieved by evaluating (i) weather living seaweeds in the littoral zone were colonized with fecal indicator organisms or not, and (ii) quantifying the survival dynamics of waterborne E. coli in microcosms containing senescing seaweeds. Results showed that fecal indicators colonized on Fucus spiralis and senescing seaweeds were supportive of E. coli survival compared with plastic debris. Seaweeds
provided persistence of *E. coli* in the order: *Laminaria saccharina > Chondrus crispus > Ulva lactuca*. This study highlights the importance of seaweed in enhancing fecal indicator survival on bathing beaches especially those casting dense biomass of brown seaweeds.

The occurrence of human microbial pathogens and its interaction with suspended nutritive materials in estuarine environments was studied by Malham et al. (2014). The study identified floc formation of microorganisms, organic matter, and minerals as factors providing supportive environment for survival of pathogenic organisms. Flocs are known to contain and retain nutrients supporting growth and survival. Transfer of pathogens to sensitive areas was also considered a possibility especially during storms. It was noted that pathogens survival could be supported for long periods when flocs are either transported directly to the coastal environment or deposited in the estuary forming cohesive sediments. The study suggested further work for understanding human infectivity with waterborne pathogens and developing the early warning systems for risk assessment purposes.

**Antibiotic Resistant Bacteria (ARB)**

**Antimicrobial susceptibility.** A study by Li et al. (2014) examined antimicrobial resistance in *S. enterica* isolates collected from irrigation pond waters in southern Georgia. In this study, 36 out of 120 samples from five ponds taken over a 24 month period were positive for *Salmonella* spp. Overall, 51 isolates were obtained and tested for antimicrobial susceptibility; out of which 16 were multidrug resistant for ampC. This study also validated a new method for recovering Salmonella isolates from surface waters, reducing the time to results from 5-9 days down to 4 days. Another study by Vincenti and coauthors examined the prevalence and distribution of antibiotic resistant strains of non-fermentative gram-negative bacteria isolated from hospital tap water (Vincenti et al., 2014). A large number (3,268) of water samples were collected between 2004 and 2013 and 149 of the samples were positive for non-fermentative gram-negative bacteria. Use of culture based methods indicated that 55% of isolated strain were resistant to one or more antibiotics. A majority of resistant isolates were from *Pseudomonas aeruginosa* (34.90%), *Pseudomonas fluorescence* (17.45%) *Ralstonia pickettii* (10.74%), and *Stenotrophomonas maltophilia* (10.74%). Out of the drug-resistant isolates, 16.6% were extensively drug resistant.
Antibiotic resistance genes (ARGs). At least three separate studies to examine ARGs in water and other environments using metagenomics approaches were published in 2014. Zhang’s group at the University of Hong Kong examined the fate of antibiotic resistance gene determinants in sewage treatment plants using a metagenomics approach (Yang et al., 2014). They identified 271 ARG subtypes. Previous studies had examined only a handful of ARGs using qPCR. Examination of influent versus effluent samples revealed that the treatment plant in Hong Kong removed 99.82% of ARGs. Another study by Amos and coauthors examined ARGs in river samples upstream and downstream from a WWTP effluent (Amos et al., 2014). Increases in ARG types were observed in many types of antibiotics. For examples, a near 10-fold increase in genes associated with amikacin resistance was observed. Similarly, Nesme and coauthors used metagenomics to examine ARGs in metagnome datasets available from 71 different environmental and fecal samples. ARGs were detected in all samples. Soil samples had the highest diversity of ARGs; however fecal samples from human fecal matter (5.6 to 5.3%), mouse gut (4.6 to 4.3%), and activated sludge (4.3 to 4.2%) had the highest proportion of ARGs. The study also identified genes that were most often observed in soil versus ocean versus fecal environments.

Nucleotide and ARG databases. The emergence of antibiotic resistant infections has also encouraged the development of databases with comprehensive or organism specific annotation of genes and single nucleotide polymorphisms associated with antibiotic resistance, and genes that could be used for potential antimicrobial targets. For example, a study by Jadhav and coauthors performed an exhaustive comparative genomics and protein analysis study to identify potential targets for pathogenic agents in waterborne pathogens (Jadhav et al., 2014). The analysis identified potential targets that could be broadly used in multiple pathogenic agents. Identified candidate genes, which were non-homologous to traditional gut microbiome, included genes related to coding of cytoplasmic proteins and membrane proteins. Potentially, these genes could also be used in a nucleic acid based tool for broad screening for presence of food and waterborne pathogens.

Reviews were also published examining the prevalence of antimicrobial resistant Aeromonas species in aquatic environments (Piotrowska et al., 2014), and highlighting the use of the class 1 integron-integrse (intI1) gene as a marker for anthropogenic pollution (Gillings et al., 2014). The latter review described the use of the intI1 gene as it is a mobile genetic element linked to horizontal gene transfer and genes conferring antibiotic resistance and is found in many bacteria. Gillings et al., lists dozens of studies globally that have found links between anthropogenic pollution and the intI1 gene. The review also presents the use of a novel qPCR primer targeting alleles from clinical isolates.

Live vs Dead Differentiation Methods

Many nucleic acid based tests offer more rapid means for pathogens detection but are unable to differentiate between viable and nonviable cells as
reviewed by Cangelosi et al. (Cangelosi et al., 2014). The need to differentiate live vs. dead cells is gaining increased attention because of the need to use molecular methods for regulatory purposes. Key studies focusing on live vs. dead differentiation are summarized below.

**Propidium monoazide (PMA).** Studies published in 2014 to differentiate viable and non-viable cells using molecular methods (e.g. PCR), focused on the use of propidium monoazide (PMA) with bacteria and protozoa in sea and wastewaters samples. Different studies from multiple authors observed that PMA-qPCR successfully differentiated live versus dead cells.

Conventional methods of PMA treatment prior to quantitative PCR (PMA-qPCR) were used to test viability in a small volume of sample water (0.25 to 1.00 ml) but it was not recommended for testing large sample volumes (Salam et al., 2014). Using blue light emitting diode (LED), a sample of 10 ml marine water was successfully used to demonstrate that amplification of dead *E. faecalis* cells can be reduced in molecular-based amplification methods. However, high concentration of total dissolved solids in seawater can reduce effectiveness of PMA. The study also compared standard membrane filtration and the blue LED PMA-qPCR protocol, but results were not comparable, suggesting that there are different physiological routes inside *E. faecalis* cells in seawater (Salam et al., 2014).

A study by Alonso et al. (2014) used PMA-qPCR to effectively distinguish between viable and nonviable *Giardia* cysts and *Cryptosporidium* oocysts in wastewater. The authors suggested that the key factor of PMA-qPCR is the photo-activation with LEDs instead of large halogen lamps, in which sample is placed on ice to avoid overheating. The study concluded that testing is only suitable for the detection of parasites that are typically present at concentration < 1,000 oo(cysts)/L in tertiary wastewater effluents.

PMA was also used by Eichmiller et al. (2014) to study the decay of genetic markers for fecal bacterial indicators and pathogens in sand from Lake Superior. The overall results showed that culturable FIB and human-specific *Bacteroides* (HF183) had decay rate comparable to the bacterial pathogen markers examined in this study. Authors concluded that the choice of FIB for assessment of fecal contamination in freshwater sand should take into account the pathogen of concern and sand moisture conditions.

Gensberger et al. (2014) used qPCR combined with PMA for detection of coliforms in seawater samples. They found that molecular based assays like qPCR offer a good differential tool for the group *Enterobacteriaceae*. In contrast, other techniques were not adequate for differentiating viable and non-viable cells. Results showed the combination of PMA with qPCR were effective for rapid detection of viable *E. coli*, *Enterococcus spp.* and *P. aeruginosa*.

Another study examined the detection of nonviable and heat-tolerant bacteria in activated sludge (Guo et al., 2014). After heating activated sludge at different temperature and different time intervals, heat tolerance of bacteria were examined by sequencing the V3
region of the 16S ribosomal DNA (rDNA). The authors observed that heating the sample to 100 °C for 60 min was enough to kill all cells. Direct and nested PCR techniques were applied for testing the amplification of DNA from dead cells. Authors concluded that DNA treated with PMA had significantly lower amplification rate than the control.

Another study was performed to examine the use of PMA-qPCR for differentiating viable cells in wastewater. A PMA concentration of 100 μmol/L was added to waste water samples. The results showed 99% of DNA from non-viable cells did not amplify (Li et al., 2014).

**Ethidium monoazide:** The species-specific viability was investigated by Rüger et al. (2014) using *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Staphylococcus aureus* for evaluating ethidium monoazide in mixed culture by flow cytometry. Results supported that flow cytometry was an efficient tool to determine species-specific viability of the above-mentioned organisms. The authors combined both ethidium monoazide and propidium monoazide to evaluate contamination with viable *Campylobacter* cells. They concluded that EMA-qPCR was valuable and faster technique for determining the viability of *Campylobacter* in water samples.

**Next Generation Sequencing (NGS)**

NGS for examining the bacterial presence, diversity, and prevalence of infectious organisms in multiple water types is an emerging and promising technique. Most NGS studies reported are either using 454 or Illumina, characterizing only the 16S rRNA gene or whole genomes in mixed communities.

Hu et al., 2014 carried out a study in Japan using *E. coli* as an indicator of fecal pollution that was isolated from aquatic environment of Yamato River. The results showed that most abundantly found fecal bacteria in the river were of human origin. Another study in Huangpu, China focused on accidental number of pigs found floating on the river surface. This river was the main source of drinking water for the local population in this area. Samples were collected from different parts of the river in between 2013 and 2014. Results showed that 37.6% and 31.5% of the samples were positive for *Cryptosporidium* and *Enterocytozoon bieneusi*, respectively. Monitoring showed that *Cryptosporidium* and *E. bieneusi* occurrence in between March 2013 and May 2014 was fluctuating.

A study carried out using 454 pyrosequencing and Illumina to detect pathogenic bacteria in a full-scale drinking water treatment plant as well as the distribution system (Huang et al., 2014). High bacterial diversity was revealed using 16S rRNA gene. α-Proteobacteria was the most dominant taxonomic class and *P. aeruginosa* was the highly abundant microorganism with approximately 11% of total sequencing reads. Chlorine disinfection eliminated almost all of pathogens found in drinking water except *P. aeruginosa* and *Leptospira interrogans*. High-throughput sequencing results exhibited various pathogenicity islands and virulence proteins in the drinking water samples. The translocases, transposons, Clp proteases and flagellar motor switch proteins were the frequently detected in the samples.
(Huang et al., 2014). When the virulence factors were chlorinated in the case of high-throughput sequencing, both the diversity and abundance increased and thereafter decreased in the distribution pipeline.

Shaw et al. (2014) investigated the control of biofilm in drinking water using NGS. These authors adopted four different water treatment plants practicing: (a) conventional coagulation, (b) magnetic ion exchange contact (MIEX) combined with conventional coagulation, (c) MIEX combined with conventional coagulation and granular activated carbon, and (d) membrane filtration (MF). The test was done at the inlet and one km away from the outlet. The dominance at inlet of all treatment plants except membrane filtration was for δ-proteobacteria. This bacterial count decreased from the inlet to the outlet of the plant by 4%, 24%, and 35% for conventional, MIEX/Conventional and MIEX/Conventional/GAC respectively. The results showed that MF treatment was the most efficient approach to inhibit biofilm growth if combined with highly efficient post-treatment disinfection system.

The studies reviewed here represent some of the advancements made in the detection and characterization of fecal indicator bacteria and pathogens in water. Their focus is on approaches to make the detection methods more rapid, sensitive, and specific. The emerging focus on viable vs. dead, NGS, and antibiotic resistance genes may be useful in a number of niches important to water including waterborne pathogens, microbial source tracking, and recreational water.

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