

GLOBAL WATER PATHOGEN PROJECT

PART TWO. INDICATORS AND MICROBIAL SOURCE TRACKING MARKERS

GENERAL AND HOST- ASSOCIATED BACTERIAL INDICATORS OF FAECAL POLLUTION

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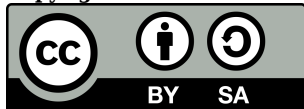
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Summary

Faecal indicator bacteria (FIB) are used worldwide to warn of faecal and sewage contamination and associated human health risk due to an increased probability of the presence of waterborne pathogens. Ideally, FIB are non-pathogenic, and include bacteria such as thermotolerant (faecal) coliforms, *Escherichia coli*, enterococci, *Bifidobacteria Bacteroidales*, and *Clostridium perfringens*. These FIB are widely distributed in the faeces of humans, and most animals. Their levels in sewage and faeces are high enough that they can usually be detected when faecal contamination is present in surface waters. Current use of FIB in regulatory settings is reviewed in this chapter, as well as their ecology, persistence, and density in faeces, sewage, soil/sediments, biosolids and sewage sludge (primary and secondary). Furthermore, the benefits and limitations of using FIB as indicators of sewage and other faecal contamination in developed, developing, and emerging regions with a variety of climates are discussed.

Although FIB have served as useful sentinels of contaminated water for many decades, changing needs in water quality management and better understanding of FIB ecology have revealed several shortcomings, including extended persistence or replication in environmental habitats, and greater survival through wastewater treatment and disinfection systems than some pathogens. The ubiquitous distribution of FIB across different animal pollution sources, which is quite useful for assessing drinking water quality, becomes problematic for many surface water quality applications. The faecal pollution source frequently assumes a greater importance in contaminated surface waters because mitigation strategies and human health risk differ greatly depending upon the particular type of human and/or animal input involved. The field of microbial source tracking (MST) offers a diverse set of methodologies designed to identify human and other faecal contamination sources. This chapter discusses MST methods designed to identify bacteria that are associated with human waste, as well as methods targeting waste from ruminant, porcine, and avian animal groups. In addition, the roles of method standardization, data acceptance criteria, and emerging technologies are explored.

1.0 Introduction to Faecal Indicator Bacteria and Host-Associated Bacteria

Faecal indicator bacteria (FIB) are members of the microbial community of the gastrointestinal tract of most animals (including humans), and can be released into the environment in faeces, sewage, sludge, and other types of waste. The presence of FIB in environmental waters is a warning signal of faecal pollution, indicating the potential presence of pathogens. Ideally, FIB should not be pathogenic to minimize the health risk to analysts (e.g. WHO, 2004); however, some FIB groups are pathogenic (e.g. *E. coli* O157:H7), and many are opportunistic pathogens, such as *Enterococcus faecium* (a member of the enterococci group). However, even high FIB levels do not

always correspond to increased human health risk. FIB are members of bacterial groups or taxa that are ubiquitous in human and other animal faeces, and therefore provide little or no information about specific contamination source(s). In contrast, host-associated bacteria are closely linked to a particular animal group, and therefore can be used to indicate probable contamination sources, which is the basis of the emerging science field of microbial source tracking (MST). This chapter covers FIB and host-associated bacteria and their use for waste and water quality management. Faecal indicator organisms other than bacteria are covered in the chapters entitled "General and host-associated bacteriophage indicators of faecal pollution" and "Human and animal enteric viral markers for tracking the sources of faecal pollution"; while bacterial pathogens are covered in Part Three, Section II.

FIB are highly prevalent in the faeces of humans and most other animals and are easily enumerated by culture methods. High levels are considered to indicate faecal contamination; however, many of these bacteria can survive and even grow in permissive environments with elevated nutrients, shielding from sunlight, and low pressure from predation, e.g. sediments, compost, sewage sludge, biosolids, and soil (Solo-Gabriele et al., 2000; Zaleski et al., 2005). Decades of research have led to the realization that numerous shortcomings are associated with FIB, particularly for surface water quality assessment applications (Harwood et al., 2005).

The distribution of FIB in the gastrointestinal tract of many host species is, however, advantageous for a broad overview of faecal pollution levels in surface waters, and offers minimal impediments to the assessment of solid waste and wastewater treatment. FIB are useful for detecting breaches and inadequate treatment in drinking water distribution systems, as potable water should contain no FIB. However, their suitability for assessing surface water safety for recreational use can sometimes be confounded due to variable human health risks posed by the presence of non-human faecal sources (Soller et al., 2010; 2014). Furthermore, as FIB provide no information about a particular contamination source, they can have limited usefulness for preventing and remediating pollution inputs (Harwood et al., 2014). Host-associated faecal microorganisms, including bacteria, are used in MST applications to provide information about faecal pollution sources in water (i.e. human faeces versus the faeces of different animals).

The objectives of this chapter are to (i) briefly describe the taxonomy, physiology, and ecology of FIB and host-associated bacteria, (ii) review the occurrence and persistence of these bacteria in faeces, wastewater, and sewage sludge, (iii) provide an overview of detection and quantification methods, and (iv) discuss future directions for their use in practice and regulatory settings.

1.1 Description and Taxonomy of Faecal Indicator Bacteria

FIB are a taxonomically and phylogenetically heterogeneous collection of microorganisms which are defined by characteristics that allow for their selective

detection and quantification. Total coliforms, thermotolerant (faecal) coliforms, *E. coli*, and enterococci are used routinely for regulatory purposes throughout the world. Some of the methods approved by regulatory agencies and other standardizing bodies, e.g. the American Public Health Association (Standard Methods), the United States Environmental Protection Agency, and the International Organization for Standardization (ISO) are shown in Table 1. Tables 2 and 3 contain FIB water quality

regulations in various water types based from many countries and organisations, including the European Union, the United States, and the World Health Organization. Several genera of strictly anaerobic faecal bacteria (*Bacteroides*, *Bifidobacterium*, and *Clostridium*) are also inhabitants of the gastrointestinal tract of humans and other warm-blooded animals, and they each have certain characteristics that make them useful indicators of faecal contamination as well.

Table 1. Summary of methods for detecting and quantifying general faecal indicator bacteria

Target Organism or Group of Organisms	Identifiers	Method Type	Examples of Standardized Methods and Test Kits	References
Total coliforms	Growth at 35±0.5°C Lactose fermentation Acid production Negative oxidase enzyme activity β-galactosidase enzyme activity	Presence/Absence Most Probable Number	Standard Methods 0221B; IDEXX Colilert and Quanti-Tray	APHA, 2012
Total coliforms	Growth at 35±0.5°C Lactose fermentation Acid production Negative oxidase enzyme activity β-galactosidase enzyme activity	Membrane Filtration Colony Forming Units (CFUs)	Standard Methods 9222B, 9222C; French Norm NF T90-414	APHA, 2012; AFNOR 1985
Thermotolerant coliforms	Growth at 44.5±0.2°C Lactose fermentation Acid production Negative oxidase enzyme activity β-galactosidase enzyme activity	Presence/Absence Most Probable Number	Standard Methods 9221E; IDEXX Colilert and Quanti-Tray	APHA, 2012
Thermotolerant coliforms	Growth at 44.5±0.2°C Lactose fermentation Acid production Negative oxidase enzyme activity β-galactosidase enzyme activity	Membrane Filtration Colony Forming Units (CFUs)	Standard Methods 9222D and 9222E	APHA, 2012
<i>E. coli</i>	Growth at 44.5°C Lactose fermentation Acid production Negative oxidase enzyme activity β-glucuronidase enzyme activity	Presence/Absence Most Probable Number	ISO 9308-2, 9308-3; IDEXX Colilert; Hach Kit Method 8091; Aquagenx Compartment Bag Test	ISO, 1998; ISO, 2012; Stauber et al. 2014
<i>E. coli</i>	Growth at 44.5°C Lactose fermentation Acid production Negative oxidase enzyme activity β-glucuronidase enzyme activity	Membrane Filtration Colony Forming Units (CFUs)	US EPA Method 1603; ISO 9308-1; Hach Kit (m-ColiBlue24 broth)	USEPA, 2006; ISO, 2014
<i>E. coli</i>	Identification of uidA gene via qPCR Identification of the EC1531 sequence via FISH	Molecular	NRa	Chern et al., 2009; Noble et al., 2010; Langendijk et al. 1995

Enterococci and Faecal streptococci	Growth in azide dextrose media within 48 hours β-D-glucosidase enzyme activity	Culture (MPN)	ISO 7899-1	ISO, 1998
Enterococci and Faecal streptococci	Growth in azide dextrose media within 48 hours β-D-glucosidase enzyme activity	Membrane Filtration Colony Forming Units (CFUs)	Standard Methods 9230B and 9230C; ISO 7899-2; US EPA Method 1600	ISO, 1998; USEPA, 2006; APHA, 2012
Enterococci and Faecal streptococci	Identification of the Enterol1a gene via qPCR	Molecular	US EPA Methods 1609 and 1611	Ludwig and Schleifer 2000; Noble et al.; 2010
<i>Bacteroides</i> spp.	Identification of the Genbac3 gene via qPCR Identification of the sequence between primers Bac32F and Bac708R via endpoint PCR	Molecular	US EPA Method B, EPA-822-R-10-003	Bernhard and Field 2000; Dick and Field, 2004
<i>Bifidobacterium</i> spp.	Identification of colony forming units (CFUs) on BIM-25 media, YN-6, YN-1, Beerens, BFM or HBSA media.	Membrane Filtration Colony Forming Units (CFUs)	NR	Mara and Oragui, 1983; Munoa and Pares, 1988; Beerens, 1990; Nebra and Blanch, 1999
<i>Bifidobacterium</i> spp.	Identification of the <i>Bifidobacterium</i> gene via qPCR Identification of the BIF164 sequence via FISH	Molecular	NR	Gueimonde et al., 2004; Langendijk et al., 1995
<i>Clostridium</i> spp.	Chromogenic CP ChromoSelect Agar Identification of colony forming units (CFUs) on m-CP agar	Presence/Absence Most Probable Number	ISO 6461-1;	ISO, 1986
<i>Clostridium</i> spp.	Chromogenic CP ChromoSelect Agar Identification of colony forming units (CFUs) on m-CP agar	Membrane Filtration Colony Forming Units (CFUs)	ISO 6461-2	ISO, 1986
<i>Clostridium</i> spp.	Identification of the Cperf gene via qPCR Identification of the HIS150 sequence via FISH	Molecular	NR	Sivaganesan et al., 2010; Langendijk et al., 1995

^aNR: Not reported

Table 2. Summary of general faecal indicator bacteria norms, regulations, and standards in wastewater, surface, recreational and marine waters

Area	Regulatory Use	Maximum Limit for Faecal Indicator Bacteria	Guideline, Norm, or Standard	Reference
Global	Wastewater, excreta, greywater use in agriculture and aquaculture	Does not specify a maximum limit for faecal indicator bacteria; instead recommends the use of microbial risk assessment	World Health Organization Guidelines for the Safe Use of Wastewater, Excreta and Greywater	WHO, 2006

Area	Regulatory Use	Maximum Limit for Faecal Indicator Bacteria	Guideline, Norm, or Standard	Reference
Bolivia	Effluent discharge to the environment	Faecal coliforms: 1000 MPN/100mL	Law 1333 – Law of the Environment	MMAyA, 1992
Brazil	Domestic water courses	Class 1 Waters (domestic use with little or no treatment): Discharge of treated effluent not permitted Class 2 Waters (domestic use after conventional treatment; irrigation of horticulture or fruiting plants; primary contact recreation): Total coliforms: <5,000/100mL in 80% of at least 5 monthly samples Faecal coliforms: <1,000/100mL in 80% of at least 5 monthly samples Class 3 Waters (domestic use after conventional treatment; protection of fish and other flora and fauna; use by wildlife for drinking): Total coliforms: <20,000/100mL in 80% of at least 5 monthly samples Faecal coliforms: <4,000/100mL in 80% of at least 5 monthly samples Class 4 Waters (domestic use after heavy treatment; navigation; scenic purposes; industrial use, irrigation and less demanding uses): No faecal indicator limits specified	Regulation/GM/No. 0013: Classifying domestic water courses in order to protect their quality	Brazilian Ministry of Health, 1976
China	Wastewater discharge to the environment	Wastewater from hospitals: Faecal coliforms: 50 MPN/L (Class 1); 1,000 MPN/L (Class 2); 5,000 MPN/L (Class 3) Wastewater from hospitals with tuberculosis units: Faecal coliforms: 100 MPN/L (Class 1); 500 MPN/L (Class 2); 1,000 MPN/L (Class 3)	National Standards of the People's Republic of China: Integrated Wastewater Discharge Standard (GB 8978-1996)	Chinese Environmental Protection Agency, 1996
Ecuador	Wastewater use for irrigation	Unrestricted irrigation (crops consumed raw, sports fields, and public green spaces): Faecal coliforms: 1,000/100mL Restricted irrigation (crops not consumed raw): Faecal coliforms: no limit specified	Norms for the Study and Design of Potable Water Systems and the Deposition of Wastewater for Populations Greater than 1,000 Inhabitants	IEOS, 1992
El Salvador	Wastewater discharged to the environment	Total coliforms: 10,000 MPN/100mL Faecal coliforms: 2,000 MPN/100mL	Salvadoran Norm: Water, Wastewater Discharged to a Receiving Water Body (NSO 13.49.01:09)	CONACYT, 2009
Honduras	Wastewater discharged to the environment	Faecal coliforms: <5,000/100mL *MPN method preferred but membrane filtration accepted	Technical Norm for the Discharge of Wastewater to Receiving Waters and Sanitary Sewers (Agreement No. 058)	ERSAPS, 1996
Japan	Marine and freshwater sources	Category AA Rivers and Lakes: Total coliforms: 50 MPN/100mL Category A Rivers, Lakes, and Coastal Bathing Waters: Total coliforms: 1,000 MPN/100mL Fishery Class 1 Coastal Waters: 70 MPN/100 mL Category B Rivers: Total coliforms: 5,000 MPN/100mL	Environmental Quality Standards Regarding Water Pollution	Japan Environment Agency, 1986
Kenya	Sources of domestic water	<i>E. coli</i> : <1/100mL	Environmental Management and Co-ordination (Water Quality) Regulations	Republic of Kenya, 2006

Area	Regulatory Use	Maximum Limit for Faecal Indicator Bacteria	Guideline, Norm, or Standard	Reference
Kenya	Effluent discharge to the environment	<i>E. coli</i> : <1/100mL Total coliforms: 30/100mL	Environmental Management and Co-ordination (Water Quality) Regulations	Republic of Kenya, 2006
Kenya	Wastewater use in agriculture	Total coliforms: 1,000 MPN/100mL (unrestricted irrigation) 200 MPN/100mL (irrigation of public lawns such as hotel lawns with which the public may have direct contact)	Environmental Management and Co-ordination (Water Quality) Regulations	Republic of Kenya, 2006
Kenya	Recreational waters	Faecal coliforms: <1/100mL Total coliforms: 500/100mL	Environmental Management and Co-ordination (Water Quality) Regulations	Republic of Kenya, 2006
Mexico	Wastewater discharged to the environment and wastewater reuse in agriculture	For discharge to water bodies or to land (irrigation): Faecal coliforms (monthly average): <1,000 MPN/100mL Faecal coliforms (daily average): <2,000 MPN/100mL For discharge to land only (irrigation): Helminth eggs: <1 egg/L (unrestricted irrigation) or <5 eggs/L (restricted irrigation)	Official Norms to Establish the Maximum Permissible Limits for Contaminants in Wastewater Discharged to National Waters (NOM-001-ECOL-1996)	CONAGUA, 1997
Marshall Islands	Sanitation discharge to marine waters	Faecal coliforms: 200/100mL	Marine Water Quality Regulations	Republic of Marshall Islands Environmental Protection Authority, 1992
Palau, Marshall Islands	Marine and freshwater sources	Class AA Waters and Class 1 Groundwater: Total coliform (median of 10 samples): 70/100mL Total coliform: 230/100mL (any one sample) Class A/B Waters and Class 2 Groundwater: Faecal coliform: 200/100mL (geometric mean of 10 samples) Faecal coliform: 400/100mL (any one sample) Class AA/A Waters (Palau): Enterococci: 33/100mL (geometric mean of 5 samples) Enterococci: 60/100mL (any one sample) Class AA and Shellfish Waters (Marshall Islands): Enterococci: 7/100mL (arithmetic mean of 5 samples) Class A Waters (Marshall Islands): Enterococci: 35/100mL (arithmetic mean of 5 samples)	Chapter 2401-11. Marine and Fresh Water Quality Regulations Marine Water Quality Regulations (Marshall Islands)	Republic of Marshall Islands Environmental Protection Authority, 1992; Republic of Palau, 1996
Papua New Guinea	Marine and freshwater sources	Freshwater: Faecal coliforms: 200/100mL (median of 5 samples) Seawater: No regulations for faecal indicator bacteria	Environment (Water Quality Criteria) Regulation	Papua New Guinea Consolidated Legislation, 2006

Area	Regulatory Use	Maximum Limit for Faecal Indicator Bacteria	Guideline, Norm, or Standard	Reference
Sri Lanka	Treated Wastewater	Discharge to Inland Surface Waters: Faecal coliforms: 40 MPN/100mL (max) Discharge on Land for Irrigation: Faecal coliforms: 40 MPN/100mL (max) Discharge to Marine Coastal Areas: Faecal coliforms: 60 MPN/100mL (max)	National Environmental Act, No. 47 of 1980	Sri Lankan Ministry of Environment and Natural Resources, 2008
Turkey	Treated Wastewater	Discharge to Class I Waters: Total coliforms: 100 MPN/100mL Faecal coliforms: 10 MPN/100mL Discharge to Class II Waters: Total coliforms: 2,000 MPN/100mL Faecal coliforms: 200 MPN/100mL Discharge to Class III Waters: Total coliforms: 10,000 MPN/100mL Faecal coliforms: 2,000 MPN/100mL	Regulation for Water Pollution Control. Environment Law No. 2872	Government of Turkey, 1988
UK	Inland Bathing Waters	Classification "Excellent" (95th percentile of log ₁₀ densities): Enterococci: 200 CFU/100mL <i>E. coli</i> : 500 CFU/100mL Classification "Good" (95th percentile of log ₁₀ densities): Enterococci: 400 CFU/100mL <i>E. coli</i> : 1,000 CFU/100mL Classification "Sufficient" (90th percentile of log ₁₀ densities): Enterococci: 330 CFU/100mL <i>E. coli</i> : 900 CFU/100mL	The (Quality of) Bathing Water(s) Regulations	United Kingdom (Scotland), 2008; United Kingdom (England and Wales), 2013
UK	Coastal Bathing Waters	Classification "Excellent" (95th percentile of log ₁₀ densities): Enterococci: 100 CFU/100mL <i>E. coli</i> : 250 CFU/100mL Classification "Good" (95th percentile of log ₁₀ densities): Enterococci: 200 CFU/100mL <i>E. coli</i> : 1,000 CFU/100mL (inland); 500 CFU/100mL Classification "Sufficient" (90th percentile of log ₁₀ densities): Enterococci: 185 CFU/100mL <i>E. coli</i> : 500 CFU/100mL	The (Quality of) Bathing Water(s) Regulations	United Kingdom (Scotland), 2008; United Kingdom (England and Wales), 2013
USA	Surface Water (or groundwater under the direct influence of surface water) for public water supply systems	Cryptosporidium (arithmetic mean of samples from 12 months): 0.075 oocysts/L ^a 1 oocysts/L ^b 3 oocysts/L ^c >3 oocysts/L ^d	National Primary Drinking Water Regulations: Long-Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR)	USEPA, 2006

Area	Regulatory Use	Maximum Limit for Faecal Indicator Bacteria	Guideline, Norm, or Standard	Reference
USA	Recreational Water	Recommendation 1 (for an estimated illness rate of 36/1,000): Enterococci (marine and freshwater): 35 CFU/100mL (geometric mean); 130 CFU/100mL (10% statistical threshold value) <i>E. coli</i> (freshwater only): 126 CFU/100mL (geometric mean); 410 CFU/100mL (10% statistical threshold value)	Recreational Water Quality Criteria (EPA 820-F-12-058)	USEPA, 2012
		Recommendation 2 (for an estimated illness rate of 32/1,000): Enterococci (marine and freshwater): 30 CFU/100mL (geometric mean); 110 CFU/100mL (10% statistical threshold value) <i>E. coli</i> (freshwater only): 100 CFU/100mL (geometric mean); 320 CFU/100mL (10% statistical threshold value)		

^aClassification used to guide the treatment needed for drinking water (type of filtration can be used)

Note: if the system uses filtration AND serves <10,000 people AND the *E. coli* concentration is <10/100mL (in lake/reservoir sources) or <50/100mL (in flowing stream sources),

Cryptosporidium monitoring is not required and any type of filtration can be used;

^brequires filtration for drinking water and 4.0 log₁₀ removal of *Cryptosporidium*;

^crequires filtration for drinking water and 5.0 log₁₀ removal of *Cryptosporidium*;

^drequires filtration for drinking water and 5.5 log₁₀ removal of *Cryptosporidium*;

Table 3. Summary of general faecal indicator bacteria norms, regulations, and standards in drinking water

Area	Regulatory Use	Maximum Limit for Faecal Indicator Bacteria	Guideline, Norm, or Standard	Reference
Global	Drinking water	<i>E. coli</i> (or thermotolerant coliforms): <1/100mL The use of a health-based approach derived from quantitative microbial risk assessment is also recommended in the 4th edition of these guidelines	World Health Organization Drinking Water Quality Guidelines	WHO, 2011
Argentina	Drinking water	<i>E. coli</i> : <1/100mL Total coliforms: 3/100mL	Food Code (Decree No. 2126/71, Regulation for Law 18.284, Chapter XII)	Administracion Nacional de Medicamentos, 2012
Belize	Drinking water	Faecal coliforms: <1/100mL Faecal streptococci: <1/100mL Heterotrophic plate count at 22°C: 100 CFU/mL Heterotrophic plate count at 37°C: 20 CFU/mL	Chapter 211. Belize agricultural health authority (food processing plants) (potable water) (minimum standards) regulations	Belize Agricultural Health Authority, 2001

Bolivia	Drinking water	<p><i>E. coli</i>: <1 CFU/100mL or <5 MPN/100mL</p> <p>Total coliforms: <1 CFU/100mL or <5 MPN/100mL</p>	<p>Bolivian Norm NB 512 - Quality of potable water for human consumption (Norma Bolivia NB 512 - Calidad de agua potable para el consumo humano)</p>	<p>IBNORCA, 2016</p>
Brazil	Drinking water	<p>Entrance of Piped Distribution Network:</p> <p>Total coliforms: <1/100mL</p> <p>Faecal coliforms: <1/100mL</p> <p>Other Locations in Piped Distribution Network:</p> <p>Total coliforms: Absence in 100mL in 95% of samples and <3/100mL in 5% of samples (for systems with treatment); 98% absence and 2% with <3/100mL (systems without treatment)</p> <p>Faecal coliforms: <1/100mL</p> <p>Communal wells and springs (non-piped systems):</p> <p>Total coliforms: Absence in 100mL in 95% of samples and <10/100mL in 5% of samples</p> <p>Faecal coliforms: <1/100mL</p>	<p>Portaria No. 36/MS/GM: Norms and Standards for Potable Water Destined for Human Consumption</p>	<p>Brazilian Ministry of Health, 1990</p>
Chile	Drinking water	<p>Potable Water: Faecal coliforms: Nil/100mL</p> <p>Water in Piped Distribution Network:</p> <p>Total coliforms: Present in 10% of samples when 10 or more samples analyzed per month or present in only one sample if <10 samples analyzed per month; concentrations >5/100mL only allowable in 5% of samples if 20 or more samples analyzed per month or in no more than one sample if <20 samples analyzed per month</p>	<p>Official Chilean Norm 409/1: Drinking Water</p>	<p>INN Chile, 1984</p>
Colombia	Drinking water	<p><i>E. coli</i>: <1/100mL</p> <p>Total coliforms: <1 CFU/100mL or <2 MPN/100mL</p>	<p>Technical Norms for Potable Water Quality. Decree 475-1998.</p>	<p>Colombian Ministry of Health, 1998</p>
Costa Rica	Drinking water	<p>Faecal coliforms: <1/100mL (for water entering the distribution network, water at all points within the distribution network, and for all types of drinking water and ice)</p>	<p>Decree No. 25991-S: Regulations for the Quality of Potable Water</p>	<p>Costa Rican Ministry of Health, 1997</p>

Ecuador	Drinking water	<p>Water Supply Source: Total coliforms: < 50/100mL (requires disinfection only) 50 to 5,000/100mL (requires conventional treatment) 5,000 to 50,000/100mL or if >40% of coliforms are faecal coliforms (requires "more active" treatment) >50,000/100mL (not acceptable for drinking water)</p> <p>Treated Water: Total coliforms: 1 CFU/100mL (monthly arith. mean). Maximum for a single sample is 4 CFU / 100 mL (if <20 samples analyzed per month) or 4 CFU/100mL (in 5% of samples per month if >20 samples analyzed)</p>	Norms for the Study and Design of Potable Water Systems and the Deposition of Wastewater for Populations Greater than 1000 Inhabitants	IEOS, 1992
El Salvador	Drinking water	<p>Total coliforms: <1 CFU/100mL or < 1.1 MPN/100mL Faecal coliforms: <1 CFU/100mL or < 1.1MPN/100mL <i>E. coli</i>: <1 CFU/100mL or < 1.1 MPN/100mL Heterotrophic plate count: <100 CFU/mL</p>	Salvadoran Norm: Water, Potable Water (NSO 13.07.01:08)	CONACYT, 2009
Estonia	Drinking water	<p>Distributed public water supply, containers and tanks: <i>E. coli</i>: <1 CFU/100mL Enterococci: <1 CFU/100mL Bottled into bottles or jerrycans: <i>E. coli</i>: <1 CFU/100mL Enterococci: <1 CFU/100mL <i>Pseudomonas aeruginosa</i>: <1 CFU/100mL Heterotrophic plate count at 22°C: 100 CFU/mL Heterotrophic plate count at 37°C: 20 CFU/mL</p>	Quality and control requirements and analysis methods for drinking water	United Kingdom (Scotland), 2008
Honduras	Drinking water	<p>Recommended Values: Total coliforms: <1/100mL Faecal coliforms: <1/100mL <i>E. coli</i>: not required, but recommended as the "most precise faecal bacterial indicator" to be used in place of or in addition to faecal coliforms Maximum Values Permitted: Total coliforms: 3/100mL (for untreated water entering the distribution network and water within the distribution network; this value is permitted occasionally but not in consecutive samples); 10/100mL (non-piped water supply; not permitted in repeated samples) Faecal coliforms: <1/100mL</p>	Technical Norm for the Quality of Potable Water (Agreement No. 084): Annex 1	Honduran Ministry of Health, 1995

Israel	Drinking water	Total coliforms: 3/100mL Faecal coliforms: <1/100mL Faecal streptococcus: <1/100mL Heterotrophic plate count: 1,000/mL <i>E. coli</i> : <1/100mL	Regulations Concerning the Sanitary Quality of Drinking Water	Israeli Ministry of Health, 1991
Mexico	Drinking water	Total coliforms: <1/100mL For systems serving <50,000 inhabitants: Total coliforms: None detected in 95% of samples collected over a period of 12 months	Official Norms for the Quality of Water in Mexico (NOM-127- SSA1-1994)	COFREPRIS, 1994
Palau	Public water supply systems	Total coliform (presence/absence): No more than 1 positive sample (100 mL) per month (if <40 samples per month), or no more than 5.0% positive samples per month (if >40 samples per month) Faecal coliform or <i>E. coli</i> : <1/100mL	Chapter 2401-51. Public Water Supply System Regulations	Republic of Palau, 1996
Singapore	Piped drinking water	<i>E. coli</i> (or thermotolerant coliforms): <1/100mL)	Environmental Public Health Act (Chapter 95): Environmental Public Health (Quality of Piped Drinking Water) Regulations	Singapore National Environment Agency, 2008
Tanzania	Piped water supplies (non- chlorinated)	Excellent Classification: Total coliforms:<1/100mL <i>E. coli</i> (faecal coliforms): <1/100mL Satisfactory Classification: Total coliforms: 1 to 3/100mL <i>E. coli</i> (faecal coliforms): <1/100mL Suspicious Classification: Total coliforms: 4 to 10/100mL <i>E. coli</i> (faecal coliforms): <1/100mL Unsatisfactory Classification: Total coliforms: >10/100mL <i>E. coli</i> (faecal coliforms): >0/100mL	Regulations for the Environmental Management Act (Water Quality Standards, Cap. 191)	Tanzania Minister of State, 2005
UK	Drinking Water (at the tap)	At the Consumer's Tap (from Directive 98/83/EC): Enterococci: <1/100mL <i>E. coli</i> : <1/100mL	Water Supply (Water Quality) Regulations; implementation of Council Directive 98/83/EC	United Kingdom (Scotland), 2001; United Kingdom (Northern Ireland), 2007; United Kingdom (England and Wales), 2010
UK	Drinking Water (service reservoirs, treatment works)	Service Reservoirs and Treatment Works: Coliform bacteria: <1/100mL (95% of samples) <i>E. coli</i> : <1/100mL	Water Supply (Water Quality) Regulations; implementation of Council Directive 98/83/EC	United Kingdom (Scotland), 2001; United Kingdom (Northern Ireland), 2007; United Kingdom (England and Wales), 2010
UK	Drinking Water (water supply point)	Water Supply Point: Coliform bacteria: <1/100mL <i>Clostridium perfringens</i> : <1/100mL	Water Supply (Water Quality) Regulations; implementation of Council Directive 98/83/EC	United Kingdom (Scotland), 2001; United Kingdom (Northern Ireland), 2007; United Kingdom (England and Wales), 2010

USA	Drinking Water	<p>Total coliforms: <1/100mL (no more than 5.0% positive of ≥40 samples/month or no more than 1 sample positive of <40 samples/month) <i>E. coli</i>: <1/100mL (the situations below also represent non-compliance) Any positive <i>E. coli</i> repeat sample Repeat sample positive for <i>E. coli</i> following positive total coliform routine sample or vice versa Failure to take repeat samples following an <i>E. coli</i> positive routine sample or the failure to test for <i>E. coli</i> following a positive repeat sample for total coliform</p>	National Primary Drinking Water Regulations: Revisions to the Total Coliform Rule	USEPA, 2006
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1.1.1 Coliforms

The term coliform represents a large group of bacterial species that are not rigidly defined by taxonomy, but rather by their ability to ferment lactose with gas and acid production, or their ability to use particular enzymes to break down carbohydrates. Coliforms are facultative anaerobic, Gram-negative, rod-shaped, non-spore forming, oxidase-negative bacteria that are resistant to bile salts and belong to the family Enterobacteriaceae. Dominant genera include *Citrobacter*, *Escherichia*, *Enterobacter*, and *Klebsiella*. Coliforms are shed in the faeces of humans and other animals at daily rates exceeding one billion bacteria per individual. They are most common in warm-blooded animals, but have also been detected in the faeces of some cold-blooded animals including alligators (Johnston et al., 2010), turtles (Harwood et al., 1999), and fish (Sousa et al., 2011). Furthermore, some coliform species and strains (particularly *Klebsiella* spp.) can originate from riparian soils, beach sands, as well as marine or freshwater sediments, and can proliferate in the environment under certain conditions (Sadowsky and Whitman, 2011). For over a century, coliform enumeration was accomplished exclusively by cultivation methods. Because the selectivity of these methods is influenced by a number of factors such as ability to utilize a defined growth substrate (carbon and energy source), response to inhibitors of non-coliforms, incubation temperature, and detection of by-products (e.g. acids, gas, enzymes) that produce a colorimetric reaction, these methods are subject to both false-positive and false-negative errors (see Applications for details).

Thermotolerant coliforms (also known as faecal coliforms) are a subset of the total coliform group capable of growth at elevated temperatures (~ 44.5°C). *E. coli* is generally distinguished from other thermotolerant coliforms by production of the enzyme beta-glucuronidase, the subject of the MUG test. Standardized methods are

used in practice and in regulatory settings to quantify total coliforms, thermotolerant coliforms, and *E. coli* in water samples. In general, total coliforms are most commonly used as indicators for groundwater, drinking water supply, and potable water impairment, while thermotolerant coliforms and *E. coli* are more commonly used as indicators for shellfish and recreational water quality testing. The drawback of growth under permissive environmental conditions is shared by thermotolerant coliforms and *E. coli* (Solo-Gabriele et al., 2000; Vanden Heuval et al., 2010).

1.1.1.1 Total coliforms

Because of their ubiquitous occurrence in the environment, total coliforms can no longer be considered indicators of faecal pollution. Total coliforms have been historically defined by phenotype as bacteria that ferment lactose to produce gas and acid within 48 h at 35°C (APHA, 2012). A more recently-developed methodology defines them as bacteria that possess the enzyme β-galactosidase, which cleaves lactose or the synthetic chromogenic substrate used for the assay (Sadowsky and Whitman, 2011). It is important to note that some coliforms are not capable of producing gas and acid from lactose fermentation; also, some species of bacteria that do not ferment lactose at 35°C possess the gene for β-galactosidase, and coliform bacteria that possess the gene may not always express it (Sadowsky and Whitman, 2011; Pisciotta et al., 2002).

1.1.1.2 Thermotolerant coliforms

Thermotolerant coliforms are operationally defined as the subset of total coliforms that are capable of growth within 24 h at 44.5°C with either gas and acid production or activity by the β-galactosidase enzyme (Sadowsky and Whitman, 2011). The group consists primarily of *E. coli* and some *Klebsiella* spp., with the former usually accounting for

the majority of thermotolerant coliforms in faecal sources. However, members of related bacterial genera such as *Enterobacter* and *Citrobacter* may come from faecal or non-faecal sources, and are also capable of growth at 44.5°C (Figueras et al., 1994). Many countries have adopted the use of coliforms or *E. coli* for regulating surface water quality (Table 2). Thermotolerant coliforms are widely distributed in human and other animal faeces.

1.1.1.3 *Escherichia coli*

E. coli is a thermotolerant member of the coliform group (also known as faecal coliform). It is usually motile via flagella. *E. coli* are easily cultivated in the laboratory, and phenotypic identification relies on lactose fermentation, while generating acid and gas byproducts, and the reduction of nitrate to nitrite. Most *E. coli* strains produce indole from tryptophan and do not use citrate as a sole carbon source (Sadowsky and Whitman, 2011). Most strains also produce the enzyme β -glucuronidase (WHO, 2011), an important differential characteristic of many types of culture media, although up to 10% of environmental strains are β -glucuronidase negative. *E. coli* is ubiquitous in the normal intestinal community and faeces of most animals, so it cannot be used to distinguish pollution by human waste or domestic wastewater from pollution originating from other animal sources. While most strains of *E. coli* are not pathogenic, some strains can cause potentially fatal illnesses, many of which are foodborne. For example, enterotoxigenic and enteropathogenic *E. coli* are major causative agents of diarrhea, particularly in developing countries. Enteroinvasive *E. coli* is a causative agent of dysentery, and enterohemorrhagic *E. coli* causes hemorrhagic colitis and hemolytic uremic syndrome (Levine, 1987). For more about disease-causing strains of *E. coli*, refer to Part Three, Section II: Bacteria.

1.1.1.4 Enterococci and faecal Streptococci

Enterococci and faecal streptococci are phenotypically defined as fermentative, Gram-positive, catalase-negative cocci that form characteristic colonies on certain selective-differential media containing sodium azide, which is inhibitory to Gram-negative bacteria. Their carbon and energy metabolism is predominantly fermentative, therefore they do not require oxygen, but they are not harmed by it. The faecal streptococci designation and the genus *Streptococcus* originally included the phenotypically-defined enterococci group; however, when differences at the DNA level were recognized in the 1980s, a new genus, termed *Enterococcus*, was designated (Murray, 1990). Most members of the genus *Enterococcus* can grow under relatively non-permissive conditions (e.g. at 10°C and 45°C, and in 6.5% NaCl), and therefore, can be differentiated phenotypically from faecal streptococci belonging to the genus *Streptococcus* (e.g. *Streptococcus bovis*). Note that the term enterococci is defined phenotypically, while the genus *Enterococcus* is defined phylogenetically (DNA-based). In practice, the terms are used interchangeably, sometimes leading to confusion.

It is not possible to differentiate among sources of faecal contamination based on the speciation of faecal

streptococci or enterococci (APHA, 2012). Faecal streptococci are less numerous than coliforms in human faeces, which in theory could make them a less sensitive indicator of faecal contamination than coliforms, however in practice this is generally not an issue. The ratio of faecal coliforms to faecal streptococci (FC/FS ratio) was previously proposed to differentiate sources of faecal pollution; however, it was later shown that this approach was not valid. Differences in inactivation rates of these FIB groups, the potential for growth in the environment, and variability between host groups were major drawbacks for source determination (Howell et al., 1996). As a result, the use of the FC/FS ratio is no longer an acceptable method and was removed from the American Public Health Association Standard Methods for the Examination of Water and Wastewater as of 1998 (Meays et al., 2004).

1.1.2 Anaerobic faecal bacteria

Several groups of anaerobic faecal bacteria, including *Bifidobacteria*, *Clostridia*, and *Bacteroidales*, are also used as FIB, in practice and research, though to a lesser extent than coliforms and enterococci. Limited use in regulatory settings is often hindered due to the requirement for anaerobic incubation (Table 2). *Bifidobacteria* are Gram-positive, rod-shaped, non-spore-forming, catalase-negative, obligate anaerobes that belong to the genus *Bifidobacterium*. They have been found in the faeces of humans, pigs, cattle, sheep, and dogs, and also in the human oral cavity and reproductive system (Wilson, 2005). *Bifidobacteria* can ferment different types of sugars and hydrolyze a variety of polysaccharides, proteins, and peptides, and they produce acid from glucose (Wilson, 2005).

1.1.2.1 *Bacteroidales*

Bacteroidales is an order of obligately anaerobic bacteria. Some species are readily cultured from human and other animal digestive tracts and faeces (Coyne and Comstock, 2008); but many phylotypes are known only by their DNA sequences (McLellan and Eren, 2014). Some populations within this order are highly host-associated, and occupy strict niches within the digestive tract of a select animal groups (Coyne and Comstock, 2008). *Bacteroides*, a genus within the order *Bacteroidales*, includes bacterial species that are pleomorphic (variable shape and size), anaerobic, non-spore-forming, generally non-motile, and rod-shaped (Wilson, 2005). They are one of the most abundant species in the human large intestine, with approximately 10 billion cells in each gram of human faeces (Madigan and Martinko, 2006). Most *Bacteroides* spp. are commensal organisms, but some can be opportunistic pathogens (e.g. *Bacteroides fragilis*) (Wexler, 2012). The genetic marker GenBac for the 16S rRNA gene of the *Bacteroidetes* (Dick and Field, 2004; Shanks et al., 2012) is used in practice as a general faecal indicator, and due to close host-associations of some *Bacteroides* species, other markers are used in MST applications to characterize faecal contamination from humans or other animals (see following section on host-associated bacteria).

1.1.2.2 *Clostridium*

Clostridium spp. are obligately anaerobic, endospore-forming, Gram-positive, rod-shaped bacteria that are generally motile. The most common species isolated from the human gastrointestinal system include *C. perfringens*, *C. ramosum*, *C. innocuum*, *C. paraputrificum*, *C. sporogenes*, *C. tertium*, *C. bifermentans*, and *C. butyricum*. Sulfite-reducing clostridia are non-motile, and are normally present in faeces, although typically at lower concentrations compared to *E. coli*. These clostridia can ferment lactose and produce gas. Their spores can tolerate temperatures of 75°C for 15 min, allowing them to typically survive longer than coliforms in water, and they are more resistant to disinfection mechanisms than vegetative cells. Important factors to consider with the use of *Clostridium* spp. as a faecal indicator, are that their spores are extremely persistent in the environment, and that some species are excreted by <35% of human hosts (Ashbolt et al., 2001). Nevertheless, within the past few decades, researchers report that *C. perfringens* can be a useful conservative tracer of faecal pollution from humans and carnivorous animals, because it rarely appears in the

excreta of herbivorous animals (Hill et al., 1996; Vierheilig et al., 2013).

1.2 Description of MST Methods

The basic premise underlying MST is that some faecal microorganisms are strongly associated with the gastrointestinal tract of a particular host species (e.g. human) or a larger taxonomic group of closely related species (e.g. ruminant animals such as cattle, goats, sheep, and deer). To date, there is a wide range of technologies reported to identify these host-associated microorganisms ranging from canine scent detection to next generation sequencing (Boehm et al., 2013). The most widely used technologies utilize the polymerase chain reaction (PCR) (Stewart et al., 2013). By combining the concept of host-associated bacteria with PCR, a central MST hypothesis emerges suggesting that host-associated genetic markers measured by PCR can act as metrics of faecal contamination from a particular animal group. The following section describes well-established, PCR-based methods targeting genetic markers that are closely associated with human, ruminant, porcine, and avian faecal pollution sources (Table 4).

Table 4. Summary of selected host-associated bacterial indicator (MST) methods

Animal Group	Target Organism	Common Target Name	Specific Gene Target	Chemistry	Reference
Human	<i>Bacteroidales</i>	HF183	16S rRNA <i>Bacteroides-Prevotella</i> group	End-point	Bernhard and Field, 2000
Human	<i>Bacteroidales</i>	HF183	16S rRNA <i>Bacteroides-Prevotella</i> group	SYBR	Seurinck et al., 2005
Human	<i>Bacteroidales</i>	HF183	16S rRNA <i>Bacteroides-Prevotella</i> group	TaqMan	Haugland et al., 2010
Human	<i>Bacteroidales</i>	BacH	16S rRNA <i>Bacteroidetes</i>	TaqMan	Reischer et al., 2007
Human	<i>Bacteroidales</i>	Bac-Hum UCD	16S rRNA <i>Bacteroidales</i>	Taqman	Kildare et al., 2007
Human	<i>Bacteroidales</i>	HumM2	Hypothetical protein	TaqMan	Shanks et al., 2009
Human	<i>Bacteroidales</i>	<i>B. thetaiotamicron</i>	1,6-alpha mannanase of <i>B. thetaiotamicron</i> <i>nifH</i> (nitrogenase)	TaqMan	Yampara-Iquise et al., 2008
Human	Methanogens	<i>nifH</i>	gene of <i>Methanobrevibacter</i> <i>smithii</i>	End-point	Ufnar et al., 2006
Human	Methanogens	<i>nifH</i>	<i>nifH</i> (nitrogenase) gene of <i>Methanobrevibacter</i> <i>smithii</i>	TaqMan	Johnston et al., 2010
Human	<i>Bifidobacteria</i>	<i>Bifidobacteria</i>	16S rRNA <i>B.</i> <i>adolescentis</i>	End-point	Bonjoch et al., 2004
Human	<i>Bifidobacteria</i>	<i>Bifidobacteria</i>	16S rRNA <i>B.</i> <i>adolescentis</i>	TaqMan	Gourmelon et al., 2010

Animal Group	Target Organism	Common Target Name	Specific Gene Target	Chemistry	Reference
Human	<i>Enterococcus</i>	<i>esp</i>	<i>esp</i> (enterococcus surface protein) from <i>E. faecium</i>	End-point	Scott et al., 2005
Human	<i>Enterococcus</i>	<i>esp</i>	<i>esp</i> (enterococcus surface protein) from <i>E. faecium</i>	SYBR	Ahmed et al., 2008
Ruminant	<i>Bacteroidales</i>	CF193	16S rRNA <i>Bacteroides-Prevotella</i> group	End-point	Bernhard and Field, 2000
Ruminant	<i>Bacteroidales</i>	Rum2Bac	16S rRNA <i>Bacteroidales</i>	TaqMan	Mieszkin et al., 2010
Ruminant	<i>Bacteroidales</i>	BacR	16S rRNA <i>Bacteroidetes</i>	TaqMan	Reischer et al., 2006
Ruminant	<i>Bacteroidales</i>	CowM2	Energy metabolism genes from <i>Bacteroidales</i> -like organisms	End-point	Shanks et al., 2006
Ruminant	<i>Bacteroidales</i>	CowM2	Secretory protein from <i>Bacteroidales</i> -like organisms	TaqMan	Shanks et al., 2008
Ruminant	<i>Bacteroidales</i>	CowM3	Energy metabolism genes from <i>Bacteroidales</i> -like organisms	End-point	Shanks et al., 2006
Ruminant	<i>Bacteroidales</i>	CowM3	Secretory protein from <i>Bacteroidales</i> -like organisms	TaqMan	Shanks et al., 2008
Porcine	<i>Bacteroidales</i>	PF163	16S rRNA <i>Prevotella</i> group	End-Point	Dick et al., 2005
Porcine	<i>Bacteroidales</i>	Pig2Bac	16S rRNA <i>Bacteroidales</i>	TaqMan	Mieszkin et al., 2009
Avian	<i>Helicobacter</i>	GFD	16S rRNA <i>Helicobacter</i> spp.	SYBR	Green et al., 2012
Avian	<i>Brevibacterium</i>	LA35	16S rRNA <i>Brevibacterium</i> spp.	SYBR	Weidhaas et al., 2010
Avian	<i>Catelicoccus</i>	Gull2	16S rRNA <i>Catelicoccus</i> spp.	TaqMan	Ryu et al., 2012

1.2.1 Human-associated MST methods

The presence of human faecal pollution from sewage outfalls, urban run-off, combined sewer overflows, faulty septic systems, and illicit dumping remains a public health risk worldwide. Technologies that can discriminate human faecal waste from other animal sources can provide water quality managers and health officials with valuable information to mitigate impaired waters. Because human waste has the potential to introduce a number of harmful pathogens into environmental waters, there is a wide range of MST technologies available to characterize this source of pollution. Human-associated MST methods presented below target bacterial genetic markers from *Bacteroidales*, methanogens, *Bifidobacterium* spp., and *Enterococcus* taxonomic groups.

1.2.1.1 *Bacteroidales*

Genetic markers from *Bacteroides*, a genus within the *Bacteroidales* order are described in this section. The most widely used human-associated MST methods target the 16S rRNA gene cluster associated with *Bacteroides doreii*, called HF183 (Haugland et al., 2010). Since the publication of an end-point PCR assay using primers HF183/708R in 2000 (Bernhard and Field, 2000), the method has been modified for SYBR Green and TaqMan real-time quantitative PCR (qPCR) chemistries (Haugland et al., 2010; Seurinck et al., 2005). The widespread use of the HF183/BFDrev TaqMan qPCR technology and performance in multiple validation studies (Boehm et al., 2013) led a team of scientists to develop an improved method, HF183/BacR287 (Green et al., 2014). In head-to-head experiments (HF183/BFDrev versus HF183/BacR287),

HF183/BacR287 was reported to exhibit increased precision and an improved limit of detection in sewage samples (Green et al., 2014). Other qPCR assays targeting *B. doreii* are available including BacH (Reischer et al., 2007) and BacHum-UCD (Kildare et al., 2007). Not all *Bacteroides* spp. human-associated MST methods target 16S rRNA genes. Some scientists assert that chromosomal genes directly involved in bacterium-host interactions harbor sufficient genetic variation for use as MST genetic markers (Shanks et al., 2006, 2009; Yampara-Iquise et al., 2008). Two popular qPCR TaqMan assays target the *B. thetaiotamicron* 1,6-alpha mannanase gene (Yampara-Iquise et al., 2008) and a *Bacteroides*-like hypothetical protein (HumM2) (Shanks et al., 2009).

1.2.1.2 Methanogens

Methanobrevibacter smithii is the only *Methanobrevibacter* species reported to specifically colonize the human intestinal tract (Miller et al., 1984). Two assays are available that target the *nifH* gene including end-point PCR (Ufnar et al., 2006) and qPCR (Johnston et al., 2010) procedures.

1.2.1.3 *Bifidobacterium*

Bifidobacteria are an anaerobic group of microorganisms that are abundant in the gastrointestinal tract of humans and other animals (Bahaka et al., 1993; Matsuki et al., 1999). A multiplex end-point PCR assay targeting 16S rRNA genes from *B. adolescentis* (ADO) and *B. dentium* (DEN) are available (Bonjoch et al., 2004). In addition, a TaqMan qPCR assay is reported (Gourmelon et al., 2010).

1.2.1.4 *Enterococcus*

Like the bacterial groups described above, enterococci are inhabitants of the gastrointestinal tract of humans and many other animals. Some species of enterococci (e.g. *E. faecium*) are reported to be more closely associated with human gastrointestinal tracts and therefore are a potential target for the development of MST methods. An end-point PCR assay targeting the *Enterococcus* surface protein (*esp*) is available (Scott et al., 2005). This PCR method was later adapted to a SYBR Green qPCR chemistry (Ahmed et al., 2008).

1.2.2 Ruminant-associated MST methods

Ruminants are mammals that are able to digest plant-based food via fermentation using a specialized four-compartment stomach. There are roughly 150 known species of ruminants worldwide including domestic and wild species such as cattle, goats, sheep, and deer. It is estimated that exposure to waterborne pathogens originating from some ruminant faecal waste, such as cattle, can have a similar public health risk compared to human faecal pollution sources (Soller et al., 2010). As a result, scientists have developed a number of MST methods designed to identify ruminant faecal waste. Selected methods presented below all target microorganisms from the *Bacteroidales* order. In 2000, the CF193 end-point PCR

method was developed targeting 16S rRNA genes from the *Bacteroides-Prevotella* group (Bernhard and Field, 2000). Several years later, two TaqMan qPCR methods were reported including Rum2Bac (Mieszkin et al., 2010) and BacR (Reischer et al., 2006) both targeting *Bacteroidales* 16S rRNA genes. The large number of domesticated cattle worldwide combined with high volume waste production (average adult cow produces 50-80 pounds of waste/day) (Kellogg et al., 2000) suggests that faecal pollution from this ruminant animal group, in particular, can be a significant public health risk. As a result, researchers have developed cattle-associated TaqMan qPCR methods including CowM2 and CowM3, which target chromosomal genes from *Bacteroidales*-like organisms (Shanks et al., 2006, 2008).

1.2.3 Porcine-associated MST methods

Increased swine farming operations represent another potential risk to nearby environmental waters in many countries worldwide. When swine faecal waste is introduced to water, it can pose a risk to human health due to the presence of a variety of human pathogens. To help characterize the impact of swine agricultural practices, scientists have developed several MST methods designed to identify porcine faecal pollution. Available methods target the 16S rRNA genes from *Prevotella* spp. from the *Bacteroidales* order including the PF163 end-point assay (Dick et al., 2005) and the qPCR Pig2Bac (Mieszkin et al., 2009).

1.2.4 Avian-associated MST methods

Faecal contamination from avian species (e.g. poultry, gulls, Canada geese, ducks, and other birds) can also negatively impact water quality. Avian faeces can contain high concentrations of general faecal indicators such as faecal coliforms, enterococci, and *E. coli*. Bacterial pathogens such as *Salmonella* and *Campylobacter* frequently occur in avian faeces, although exposure to poultry waste has been estimated to be somewhat lower risk than exposure to human and cattle sources (Soller et al., 2010). Several avian-associated MST methods are available, although there is currently no known assay that can detect pollution from all bird species. Methods presented below target 16S rRNA genes from *Helicobacter* spp. (GFD) (Green et al., 2012), *Catelicoccus* spp. (Gull4) (Ryu et al., 2012), and *Brevibacterium* spp. (LA35) (Weidhaas et al., 2010).

2.0 Detection Technologies

Common FIB and MST method technologies can be organized into two groups: cultivation methods and molecular methods. Cultivation methods measure the ability of select bacteria to grow under specific conditions and/or express certain enzymes in the presence of a growth medium, which may be selective and/or differential. Molecular methods detect and/or estimate the concentration of genetic markers, typically the 16S rRNA gene, a highly conserved region of bacterial genomes.

2.1 Cultivation Methods

FIB cultivation methods rely on the growth of target microorganism under selective conditions. Selective media contains ingredients that inhibit the growth of non-target microorganisms, while differential media contains ingredients that discriminate microorganisms based on a particular metabolic characteristic. Some media also include ingredients to measure the activity of enzymes used by FIB to break down certain carbohydrates into sugars (e.g. detection of *E. coli* based on the activity of β -glucuronidase for the IDEXX Quanti-Tray method).

The most basic approach for measuring FIB via cultivation methods is the presence-absence test which, if done in replicate with serial dilutions, can be used to estimate the density of FIB in a sample based on most probable number (MPN) statistics. Another cultivation method is the direct count method, where samples are either applied directly to nutrient agar or filtered through a membrane which is then placed on nutrient agar. Colony forming units (CFUs) are counted and expressed as a concentration per unit volume. Cultivation methods are available for the detection and enumeration of coliforms, *E. coli*, and enterococci, and are used in a wide variety of regulatory settings for water quality management. Standardized cultivation methods for the enumeration of clostridia are also available. *Bifidobacteria* and *Bacteroides* spp. can also be cultivated, but these methods are seldom used for regulatory purposes.

2.1.1 Presence-absence and endpoint dilution (multiple tube) methods

The multiple tube method consists of a series of presence-absence tests performed on replicates of a single sample at one or more sample dilutions. Some tubes (wells) should show positive growth (which may be observed as turbidity, gas production, or color change from acid production or enzyme activity), while other tubes (wells) will be negative. The average density of bacteria in the original sample is then estimated using the MPN method. Compared to the direct count (membrane filtration method), the MPN method is more labor intensive and less precise; it also tends to overestimate the actual concentrations, especially when a small number of dilutions and replicates are used. Standardized methods for the detection of FIB using presence-absence or quantification using endpoint dilution (multiple tube) methods with MPN statistics are described in APHA (APHA, 2012), ISO (ISO, 1986a, 1986b, 1998, 2000), ASTM (ASTM, 2000), AOAC (AOAC, 1995), the U.S. EPA (USEPA, 2006a, 2006b).

2.1.2 Direct count (membrane filtration and plating) methods

For direct count methods, 100mL water samples are passed through a membrane, which is transferred to an agar medium and incubated. Discrete colonies with the desired characteristics are then counted after incubation. One of the major challenges of the membrane filtration method is that samples with high turbidity often clog the

membrane potentially biasing findings. Nevertheless, the membrane filtration method can be more accurate and precise than the multiple tube method. FIB concentrations are expressed as CFU/volume of sample. Standard methods for the detection of FIB using membrane filtration or direct count techniques are described in APHA (APHA, 2012), ISO (ISO, 1986b, 2000), ASTM (ASTM, 2000), AOAC (AOAC, 1995), the U.S. EPA (USEPA, 2006a, 2006b).

2.1.3 Indirect measurements of FIB

Other techniques that measure water quality parameters such as turbidity (Cinque et al., 2004) or H₂S concentration (Luyt et al., 2012) have been used to indirectly infer the presence of faecal pollution in water. These tests do not measure FIB directly, but may be useful for assessing water quality in remote locations or in the wake of natural disasters, when laboratories are non-existent or non-functional. In one study, authors reported the successful application of a field H₂S test procedure for field use (Chuang et al., 2011).

2.2 Molecular Methods

Molecular methods refer to protocols used in genetics, microbiology, biochemistry, or other related fields to study biologically important molecules such as DNA, RNA, and proteins. Protocols typically include a biological sample collection step followed by molecule isolation and characterization. This section will describe PCR and qPCR molecular methods used to measure FIB and host-associated DNA gene sequences harbored by faecal bacteria.

2.2.1 PCR

PCR is a technique used to amplify a small amount of DNA target originating from a faecal microorganism that is closely associated with the presence of faecal material (FIB) or waste from a particular animal group (host-associated indicator). A PCR amplification generates millions of copies of the targeted DNA in a matter of hours. The massive number of DNA copies generated by PCR can then be visualized by agarose gel electrophoresis or any other suitable nucleic acid visualization technology. PCR can also be used for RNA targets, such as RNA viruses, using reverse-transcriptase PCR to convert RNA to complementary DNA (cDNA). The presence or absence of a particular DNA or RNA target is used as evidence to infer the existence of faecal pollution from any source (e.g. Bacteroidales, *Enterococcus*) or from a specific animal group such as human, ruminant, cattle, swine, or avian (host-associated bacteria genetic marker). PCR can be extremely precise, target a specific sequence from a complex mixture of DNA molecules, and provide results in several hours making it ideal for the rapid detection of faecal-associated DNA targets in animal waste and polluted ambient water environments.

PCR is able to amplify a DNA target by mimicking bacterial cell DNA replication in a plastic microtube. Please refer elsewhere for a complete description of the PCR

principles (Snyder et al., 1997). Briefly, total DNA isolated from a test sample (sewage, faeces, ambient water, etc) is mixed with a heat-stable DNA polymerase, nucleotides, primers, and cations in a buffer solution. PCR amplification is carried out in a series of repeated temperature changes (cycles) in a thermal cycler instrument designed to rapidly heat and cool the reaction mixture. As PCR amplification progresses, the new DNA molecules manufactured serve as template for DNA synthesis in the next cycle, setting in motion a chain reaction where the original DNA target is exponentially amplified. Determination of the presence or absence of faecal contamination in an environmental sample provides water quality managers with valuable information; however, the ability to quantify the concentration of the DNA target can offer additional insights about water impairment patterns and pollution sources.

2.2.2 Quantitative real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) is based on PCR where the accumulation of newly synthesized DNA target is measured over the course of amplification. There are two common chemistries employed to detect PCR products in real-time including the use of non-specific fluorescent dyes

that intercalate with double stranded DNA (e.g. SYBR), and the addition of a sequence specific DNA probe labelled with a fluorescent reporter molecule that emits energy upon hybridization to a target sequence (e.g. TaqMan). For a detailed description of qPCR principles, please refer to (Bustin, 2006). Briefly, the qPCR process is similar to PCR with the addition of either a fluorescent intercalating dye (SYBR) or labelled probe (TaqMan). Reactions are conducted in a special thermal cycler equipped with a sensor designed to measure the fluorescence emitted from a fluorophore associated with each newly synthesized PCR product. qPCR is based on the theoretical premise that there is a log-linear relationship between the starting amount of DNA target in the reaction and the measured fluorescence value. The concentration of nucleic acid in a sample is determined by comparison to a standard curve.

3.0 Occurrence in Faecal Pollution Sources

3.1 Data on Faecal Indicator Bacteria

Typical densities of FIB in human faeces, untreated sewage and sewage sludge are summarized in Table 5. Table 6 contains typical densities found in faecal waste from a variety of other animals.

Table 5. Summary of faecal indicator bacteria abundance in common human pollution sources by cultivation methods (Colony forming units, CFUs)

FIB Group	Pollution Source	Typical Range of Concentrations (CFU/100mL or per wet g)
Thermotolerant Coliforms	Faeces (per wet g)	1.0 E+06 to 1.0 E+09
Thermotolerant Coliforms	Untreated Sewage (per 100mL)	1.0 E+06 to 1.0 E+08
Thermotolerant Coliforms	Sewage Sludge (per wet g)	1.0 E+04 to 1.0 E+09
<i>E. coli</i>	Faeces (per wet g)	1.0 E+06 to 1.0 E+09
<i>E. coli</i>	Untreated Sewage (per 100mL)	1.0 E+07 to 1.0 E+08
<i>E. coli</i>	Sewage Sludge (per wet g)	1.0 E+04 to 1.0 E+08
Enterococci and Faecal Streptococci	Faeces (per wet g)	1.0 E+05 to 1.0 E+08
Enterococci and Faecal Streptococci	Untreated Sewage (per 100mL)	1.0 E+05 to 1.0 E+07
Enterococci and Faecal Streptococci	Sewage Sludge (per wet g)	1.0 E+05 to 1.0 E+07
<i>Bacteroides</i> spp.	Faeces (per wet g)	1.0 E+08 to 1.0 E+10
<i>Bacteroides</i> spp.	Untreated Sewage (per 100mL)	1.0 E+09
<i>Bifidobacterium</i> spp.	Faeces (per wet g)	1.0 E+08 to 1.0 E+10
<i>Bifidobacterium</i> spp.	Untreated Sewage (per 100mL)	1.0 E+06 to 1.0 E+09
<i>Clostridium</i> spp.	Faeces (per wet g)	1.0 E+03
<i>Clostridium</i> spp.	Untreated Sewage (per 100mL)	1.0 E+04 to 1.0 E+06
<i>Clostridium</i> spp.	Sewage Sludge (per wet g)	1.0 E+05 to 1.0 E+07

Sources: (Geldreich, 1978; Feachem et al., 1983; Wang et al., 1996; Ashbolt et al., 2001; Rose et al., 2004; Morrison et al., 2008; Boutilier et al., 2009; Sidhu and Toze, 2009; Silkie and Nelson, 2009; Pillai et al., 2011; WHO, 2011; Zimmer et al., 2012; Akiba et al., 2015)

Table 6. Summary of typical faecal indicator bacteria concentrations in agricultural and pet animal waste

Pollution Source	Excretion Rate (wet g/day)	Moisture Content (%)	Target Organism or Group of Organisms	Average Concentration ^a (per wet gram)	Average Daily FIB Excretion Rate (per wet gram)
Chicken Faeces	182	71.6	Thermotolerant coliforms	1.3 E+06	2.37 E+08
Chicken Faeces	182	71.6	Faecal streptococci	3.4 E+06	6.19 E+08
Chicken Faeces	182	71.6	<i>C. perfringens</i>	2.5 E+02	4.55 E+04
Cow Faeces	23,600	83.3	Thermotolerant coliforms	2.3 E+05	5.43 E+09
Cow Faeces	23,600	83.3	Faecal streptococci	1.3 E+06	3.07 E+10
Cow Faeces	23,600	83.3	<i>C. perfringens</i>	2.0 E+02	4.72 E+06
Duck Faeces	336	61	Thermotolerant coliforms	3.3 E+07	1.11 E+10
Duck Faeces	336	61	Faecal streptococci	5.4 E+07	1.81 E+10
Horse Faeces	20,000	NR ^b	Thermotolerant coliforms	1.26 E+04	2.52 E+08
Horse Faeces	20,000	NR	Faecal streptococci	6.3 E+06	1.26 E+11
Horse Faeces	20,000	NR	<i>C. perfringens</i>	<1	<2.0 E+04
Sheep Faeces	1,130	74.4	Thermotolerant coliforms	1.6 E+07	1.81 E+10
Sheep Faeces	1,130	74.4	Faecal streptococci	3.8 E+07	4.29 E+10
Sheep Faeces	1,130	74.4	<i>C. perfringens</i>	1.99 E+05	2.25 E+08
Swine Faeces	2,700	66.7	Thermotolerant coliforms	3.3 E+06	8.91 E+09
Swine Faeces	2,700	66.7	Faecal streptococci	8.4 E+07	2.27 E+11
Swine Faeces	2,700	66.7	<i>C. perfringens</i>	3.98 E+03	1.07 E+07
Turkey Faeces	448	62	Thermotolerant coliforms	2.9 E+05	1.3 E+08
Turkey Faeces	448	62	Faecal streptococci	2.8 E+06	1.25 E+09
Cat Faeces	Not applicable	NR	Thermotolerant coliforms	7.9 E+06	NR
Cat Faeces	Not applicable	NR	Faecal streptococci	2.7 E+07	NR
Cat Faeces	Not applicable	NR	<i>C. perfringens</i>	2.51 E+07	NR
Dog Faeces	413	NR	Thermotolerant coliforms	2.3 E+07	9.5 E+09
Dog Faeces	413	NR	Faecal streptococci	9.8 E+08	4.05 E+11
Dog Faeces	413	NR	<i>C. perfringens</i>	2.51 E+08	1.04 E+11

Adapted from (Geldreich, 1978; Ashbolt et al., 2001); ^aCFU: Colony forming unit; ^bNR: Not reported

3.1.1 Human excreta

Bacteroides spp. and *Bifidobacterium* spp. are typically present in human faeces in higher quantities compared to *Clostridium* spp., enterococci, *E. coli* and other coliforms. Enterococci, *E. coli*, and other members of the coliform group are reported to only account for 7% of the total bacterial ribosomal RNA in human faecal samples (Guarner and Malagelada, 2003).

FIB concentrations in human faeces are highly variable among individuals, and can vary across geographic regions due to many factors, including dietary differences. For

example, the densities of *Bacteroides* spp., *Bifidobacterium* spp., *E. coli*, and members of the family *Enterobacteriaceae* are significantly lower in vegans than they are for people with omnivorous diets (Zimmer et al., 2012). The relative proportions of FIB bacteria populations in human faeces can also vary based on health. For example, Khachatryan and colleagues reported significantly higher proportions of *Bacteroides* in faecal samples from a subset of patients with Crohn's disease and familial Mediterranean fever relative to healthy patients (Khachatryan et al., 2008). Larsen and co-workers (2010) found that the proportions of *Clostridia* in faecal samples from patients with type 2 diabetes were significantly lower than they were in samples from a control

group (Larsen et al., 2010), while another research group reported higher overall microbial diversity with lower quantities of *Bifidobacterium* spp. in faecal samples from children with autism relative to a control group (De Angelis et al., 2013).

Human urine should not contain FIB, although coliforms (including *E. coli*), *Clostridia*, and faecal streptococci have been detected in urine collection tanks from source-separated sewage systems. Cross-contamination with faecal matter has been implicated in contamination levels estimated at 9.1 mg faeces/L urine, with densities of faecal streptococci as high as 105/mL (Hoglund et al., 1998; Schonning et al., 2002).

3.1.2 Untreated sewage

Sewage contains human waste that has been diluted with flushing water. Depending on the region, sewage may also contain greywater from sinks, showers, and laundry (washing clothes). Because of this, the relative densities of FIB can vary greatly depending on the nature of the facilities and residences discharging to the local sewer collection system. In a study of six wastewater facilities in the United States receiving mostly domestic wastewater (Harwood et al., 2005), concentrations of total coliforms in untreated sewage (geometric mean: 3.3×10^7 CFU/100mL) were greater than concentrations of thermotolerant coliforms (geometric mean: 3.4×10^6 CFU/100mL), which were greater than concentrations of enterococci (geometric mean: 9.4×10^5 CFU/100mL); *C. perfringens* was only detected sporadically at quantities that were two or more orders of magnitude lower than coliforms. A study of 166 wastewater facilities in Brazil (Oliveira and von Sperling, 2011) revealed greater concentrations of thermotolerant coliforms in untreated sewage (geometric mean values ranged from 2.6×10^7 to 2.0×10^8 MPN/100mL). Similarly, high concentrations of thermotolerant coliforms have been reported in Bolivian wastewater (3.5×10^7 MPN/100mL) (Zabalaga et al., 2007). However, thermotolerant coliform concentrations reported in untreated sewage from the treatment plants serving 15 cities in India (4.0×10^5 to 9.2×10^6 MPN/100mL) (Sato et al., 2006) were more comparable to the values reported in the United States by Rose et al. (Harwood et al., 2005).

Concentrations of obligately anaerobic FIB *Bacteroides* spp. and *Bifidobacterium* spp. in untreated sewage are not reported as frequently in the literature; however, the concentration of *Bifidobacterium* spp. in untreated sewage (based on cultivation on HBSA medium (Mara and Oragui,

1983) has been reported as 4.0×10^6 CFU/100mL (Ottoson, 2009).

3.2 Data on Host-Associated MST Methods

The occurrence of host-associated bacterial MST genetic markers in target and non-target pollution sources is typically reported as sensitivity (target sources), specificity (non-target sources), and for qPCR methods, it is common to also include genetic marker concentrations (gene copies per volume, mass, or cell count). Sensitivity is routinely expressed as the following: sensitivity = TPC/(TBC+TNI), where TPC represents the total number of samples that tested positive correctly and TNI denotes the total number of samples that tested incorrectly. Specificity is typically defined as the total number of samples that test negative correctly (TNC) divided by the sum of TNC and the total number of samples that tested positive incorrectly (TPI) or TNC/(TNC+TPI). Occurrence data are generated by systematic testing of reference samples from known pollution sources usually collected in close proximity to the research laboratory performing MST experiments. A rapidly growing interest in the application of MST methods has led to testing reference samples collected from a broader range of geographic locations. This section seeks to organize and report MST genetic marker occurrence data reported from reference sample collections across the globe.

3.2.1 Occurrence of host-associated MST genetic markers in common pollution types

A useful MST method should measure a genetic marker that is widely dispersed across the target population of interest that is absent or occurs at a significantly lower concentration in non-target pollution sources present in the study area. The occurrence of host-associated MST genetic markers [sensitivity and concentration (gene copies per volume, mass, or cell count)] has been reported in more than 20 countries to date providing valuable information for researchers and water quality managers. Human-associated MST genetic marker occurrence data is organized by pollution type including sewage (Table 7), faecal (Table 8), and onsite sources (Table 9). Other non-human host-associated occurrence data is shown for ruminant, porcine, and avian MST methods (Table 10 and 11). Summarized data are presented by MST methodology and geographic origin of reference pollution source materials. Only studies reporting genetic marker concentrations in gene copies are shown. For a more detailed description of MST method genetic marker occurrence, please refer to Appendix A.

Table 7. Summary of human-associated MST method target occurrence in sewage

Area	Common Target Name	Number of samples	Sensitivity ^a	Gene Copy Concentration (Mean or Range) per 100mL	Reference
				SYBR	
Australia	HF183	32	100%	NR ^b	Ahmed et al., 2009
Australia	HF183	99	100%	8.0 E+03 gene copies/100mL	Ahmed et al., 2015
Belgium	HF183	4	100%	5.9 E+09 to 3.1 E+10 gene copies/100mL	Seurinck et al., 2005
India	HF183	5	100%	47 (\pm 0.47 log ₁₀) gene copies/ng of total DNA	Odagiri et al., 2015
USA	HF183	16	100%	NR	Layton et al., 2013
USA	HF183	10	100%	4.0 E+08 to 2.5 E+10 gene copies/100mL	Van De Werfhorst et al., 2011
Australia	<i>esp</i>	16	100%	9.8 E+03 to 3.8 E+04 gene copies/100mL	Ahmed et al., 2008
Australia	<i>esp</i>	10	100%	NR	Ahmed et al., 2009
TaqMan					
India	HF183	5	100%	195 (\pm 0.72 log ₁₀) gene copies/ng of total DNA	Odagiri et al., 2015
USA	HF183	20	85 to 100%	NR	Layton et al., 2013
USA	HF183	14	100%	630 gene copies/ng of total DNA	Haugland et al., 2010
Austria	BacH	20	100%	1.4 E+10 to 9.1 E+10 gene copies/g	Reischer et al., 2007
India	BacH	5	40%	107 (\pm 0.35 log ₁₀) gene copies/ng of total DNA	Odagiri et al., 2015
USA	BacH	4	50 to 100%	NR	Layton et al., 2013
USA	BacHum-UCD	24	92%	NR	Layton et al., 2013
USA	BacHum-UCD	10	100%	6.0 E+08 to 8.5 E+10 gene copies/100mL	Van De Werfhorst et al., 2011
USA	BacHum-UCD	14	100%	NR	Kildare et al., 2007
USA	BacHum-UCD	12	100%	7.9 E+08 gene copies/100mL	Silkie and Nelson, 2009
USA	BacHum-UCD	5	100%	178 (\pm 0.75 log ₁₀) gene copies/ng of total DNA	Odagiri et al., 2015
India	HumM2	54	100%	63 to 3.16 E+03 gene copies/ng of DNA	Shanks et al., 2010
USA	HumM2	24	46 to 83%	NR	Layton et al., 2013
USA	HumM2	20	100%	631 gene copies/ng of total DNA	Shanks et al., 2009
France	<i>B. adolescentis</i>	8	100%	1.0 E+04 to 7.9 E+06 gene copies/100mL	Gourmelon et al., 2010
USA	1,6-alpha mannanase	4	75 to 100%	NR	Layton et al., 2013
USA	1,6-alpha mannanase	20	100%	13.4 to 457 gene copies/ng of total DNA	Yampara-Iquise et al., 2008
USA	1,6-alpha mannanase	54	100%	1.82 E+07 gene copies/100mL	Srinivasan et al., 2011

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Area	Common Target Name	Number of samples	Sensitivity ^a	Gene Copy Concentration (Mean or Range) per 100mL	Reference
USA	<i>nifH</i>	20	20 to 55%	NR	Layton et al., 2013
USA	<i>nifH</i>	16	100%	12 to 3.8 E+03 gene copies/100mL	Johnston et al., 2010
End-point^c					
Australia	HF183	45	100%	NR	Ahmed et al., 2008
Canada	HF183	8	100%	NR	Fremaux et al., 2009
Canada	HF183	102	74%	NR	Edge et al., 2013
France	HF183	5	100%	NR	Gourmelon et al., 2007
Spain	HF183	40	50%	NR	Balleste et al., 2010
USA	HF183	3	100%	NR	Bernhard and Field, 2000
USA	HF183	28	57%	NR	Layton et al., 2013
USA	HF183	54	100%	NR	Shanks et al., 2010
USA	HF183	16	75%	NR	Toledo-Hernandez et al., 2013
USA	HF183	39	100%	NR	McQuaig et al., 2009
USA	HF183	48	100%	NR	Harwood et al., 2009
France	<i>nifH</i>	8	100%	1.0 E+04 to 7.9 E+06 gene copies/100mL	Gourmelon et al., 2010
USA	<i>nifH</i>	39	100%	NR	McQuaig et al., 2009
USA	<i>nifH</i>	19	100%	NR	Harwood et al., 2009
USA	<i>nifH</i>	27	93%	NR	Ufnar et al., 2006
USA	<i>nifH</i>	20	20 to 55%	NR	Layton et al., 2013
Spain	<i>B. adolescentis</i>	45	95.6%	NR	Balleste et al., 2010
Spain	<i>B. adolescentis</i>	12	100%	NR	Bonjoch et al., 2004
Spain, France, Sweden, UK, Cyprus, USA	<i>B. adolescentis</i>	114	92.7%	NR	Blanch et al., 2006
USA	<i>B. adolescentis</i>	3	66.6%	NR	Bachoon et al., 2010
Australia	<i>esp</i>	Not known	100%	NR	Neave et al., 2014
Spain	<i>esp</i>	13	77%	NR	Balleste et al., 2010
USA	<i>esp</i>	26	92%	NR	Layton et al., 2009
USA	<i>esp</i>	55	100%	NR	Reischer et al., 2006

Area	Common Target Name	Number of samples	Sensitivity ^a	Gene Copy Concentration (Mean or Range) per 100mL	Reference
USA	<i>esp</i>	3	100%	NR	Korajkic et al., 2009
USA	<i>esp</i>	20	55%	NR	Masago et al., 2011

^aSensitivity is routinely expressed as the following: sensitivity = TPC/(TBC+TNI), where TPC represents the total number of samples that tested positive correctly and TNI denotes the total number of samples that tested incorrectly. Specificity is typically defined as the total number of samples that test negative correctly (TNC) divided by the sum of TNC and the total number of samples that tested positive incorrectly (TPI) or TNC/(TNC+TPI);

^bNR: Not reported; ^cendpoint is a non-quantitative method

Table 8. Summary of human-associated MST method target occurrence in faeces

Area	Common Target Name	Number of samples	Sensitivity ^a	Gene Copy Concentration (Mean or Range)	Reference
SYBR					
Belgium	HF183	7	85.7%	8.4 E+05 to 7.2 E+09 gene copies/g	Seurinck et al., 2005
Bangladesh	HF183	15	87%	1.2 E+05 to 3.9 E+07 gene copies/g	Ahmed et al., 2010
India	HF183	30	86.7%	9 (\pm 1.64 log ₁₀) gene copies/ng of total DNA	Odagiri et al., 2015
USA	HF183	16	100%	NR ^b	Layton et al., 2013
USA	HF183	8	62.5%	4.9 E+03 to 5.3 E+08 gene copies/g	Van De Werfhorst et al., 2011
TaqMan					
India		30	16.7%	204 (\pm 1.71 log ₁₀) gene copies/ng of total DNA	Odagiri et al., 2015
USA	HF183	20	100%	NR	Layton et al., 2013
USA	HF183	16	100%	1.47 E+03 (\pm 0.07 log ₁₀) gene copies/ng of total DNA	Haugland et al., 2010
Austria	BacH	21	95%	6.6 E+09 to 9.1 E+10 gene copies/g	Johnston et al., 2010
Austria	BacH	4	100%	NR	Reischer et al., 2013
India	BacH	30	13.3%	251 (\pm 0.97 log ₁₀) gene copies/ng of total DNA	Odagiri et al., 2015
Multiple Countries ^c	BacH	61	77%	1 to 1.0 E+07 copies/reaction	Reischer et al., 2013
USA	BacH	4	100%	NR	Layton et al., 2013
Multiple Countries ^d	BacHum-UCD	61	87%	1 to 6.0 E+06 gene copies/reaction	Reischer et al., 2013
India	BacHum-UCD	30	40%	288 (\pm 1.61 log ₁₀) gene copies/ng of total DNA	Odagiri et al., 2015
USA	BacHum-UCD	24	100%	NR	Layton et al., 2013

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Area	Common Target Name	Number of samples	Sensitivity^a	Gene Copy Concentration (Mean or Range)	Reference
USA	BacHum-UCD	8	100%	6.4 E+04 to 5.1 E+08 gene copies/g	Van De Werfhorst et al., 2011
USA	BacHum-UCD	18	66.7%	NR	Kildare et al., 2007
India	HumM2	30	40%	37 ($\pm 0.67 \log_{10}$) gene copies/ng of total DNA	Van De Werfhorst et al., 2011
USA	HumM2	24	100%	NR	Layton et al., 2013
USA	HumM2	16	100%	NR	Shanks et al., 2009
USA	HumM2	16	100%	2.6 E+03 ($\pm 0.05 \log_{10}$) gene copies/ng of total DNA	Shanks et al., 2010
USA	1,6-alpha mannanase	4	100%	NR	Layton et al., 2013
USA	1,6-alpha mannanase	10	100%	6.88 E+02 to 1.07 E+09 gene copies/g	Yampara-Iquise et al., 2008
USA	<i>nifH</i>	20	95%	NR	Layton et al., 2013
France	B.adolescentis	10	90%	5 E+05 to 1.0 E+09 gene copies/g	Gourmelon et al., 2010
End-point^e					
Canada	HF183	54	94%	NR	Fremaux et al., 2009
France	HF183	44	97.7%	NR	Gourmelon et al., 2007
USA	HF183	13	84%	NR	Bernhard and Field, 2000
USA	HF183	28	96%	NR	Layton et al., 2013
USA	HF183	16	37.5	NR	Shanks et al., 2010
USA	<i>nifH</i>	70	29%	NR	Ufnar et al., 2006
USA	<i>esp</i>	12	83.3%	NR	Layton et al., 2009

^aSensitivity is routinely expressed as the following: sensitivity = TPC/(TBC+TNI), where TPC represents the total number of samples that tested positive correctly and TNI denotes the total number of samples that tested incorrectly. Specificity is typically defined as the total number of samples that test negative correctly (TNC) divided by the sum of TNC and the total number of samples that tested positive incorrectly (TPI) or TNC/(TNC+TPI);

^bNR: Not reported; ^cArgentina, Austria, Ethiopia, Germany, Hungary, Korea, Nepal, Netherlands, Romania, Spain, Sweden, Tanzania, Uganda, UK; ^dAustria, Argentina, Australia, Ethiopia, Germany, Hungary, Korea, Nepal, Netherlands, Romania, Spain, Sweden, Tanzania, Uganda, UK, USA; ^eendpoint is a non-quantitative method

Table 9. Summary of human-associated MST method target occurrence in on-site* pollution sources in USA

Common Target Name	Number of Samples	Sensitivity ^a	Gene Copy Concentration (Mean or Range)	Reference
SYBR				
HF183	16	94 to 100%	NR ^b	Layton et al., 2013
HF183	3	66.6%	9.8 E+08 to 4.9 E+09 gene copies/100mL	Van De Werfhorst et al., 2011
Taqman				
HF183	20	100%	NR	Layton et al., 2013
BacH	4	75 to 100%	NR	Layton et al., 2013
BacHum-UCD	24	100%	NR	Layton et al., 2013
BacHum-UCD	3	100%	4.2 E+05 to 6.5 E+09 gene copies/100mL	Van De Werfhorst et al., 2011
HumM2	24	54 to 96%	NR	Layton et al., 2013
1,6-alpha mannanase	4	100%	NR	Layton et al., 2013
<i>nifH</i>	20	65 to 85%	NR	Layton et al., 2013
End-point				
HF183	28	71%	NR	Layton et al., 2013
HF183	16	100%	NR	McQuaig et al., 2009
HF183	80	100%	NR	Harwood et al., 2009
<i>nifH</i>	16	93.7%	NR	McQuaig et al., 2009
<i>nifH</i>	25	100%	NR	Harwood et al., 2009
<i>E. faecium esp</i>	10	80%	NR	Scott et al., 2005
<i>E. faecium esp</i>	6	100%	NR	Masago et al., 2011

^aSensitivity is routinely expressed as the following: sensitivity = TPC/(TBC+TNI), where TPC represents the total number of samples that tested positive correctly and TNI denotes the total number of samples that tested incorrectly. Specificity is typically defined as the total number of samples that test negative correctly (TNC) divided by the sum of TNC and the total number of samples that tested positive incorrectly (TPI) or TNC/(TNC+TPI);

^bNR: Not reported. In Australia, HF183 also found in 100% of sewage samples (n=12) by end point chemistry (Ahmed et al., 2008)

Table 10. Summary of reported non-human-associated MST gene target occurrence in Ruminant faecal and agricultural pollution sources^a

Area	Common Target Name	Number of Samples	Sensitivity ^b	Gene Copy Concentration (Mean or Range)	Reference
Taqman					
Austria	BacR	57	100%	4.10 E+09 gene copies/g wet faeces	Reischer et al., 2006
Multiple Countries ^c	BacR	79	90%	0 to 1.0 E+07 gene copies/reaction	Reischer et al., 2013
Canada	BacR	26	94.4%	1.94 E+08 gene copies/g	Ridley et al., 2014
France	BacR	20	100%	1.0 E+10 ($\pm 0.30 \log_{10}$) gene copies/g of wet faeces	Mieszkin et al., 2009
Israel	BacR	NR	100%	NR ^d	Ohad et al., 2015
USA	BacR	NR	100%	1.48 E+06 to 4.37 E+07 gene copies/group	Raith et al., 2013
Canada	CowM2	18	88.9%	1.44 E+06 gene copies/g	Ridley et al., 2014
India	CowM2	10	50%	10 to 158 gene copies/ng of total DNA	Odagiri et al., 2015
Israel	CowM2	NR	50%	NR	Ohad et al., 2015
USA	CowM2	60	100%	NR	Shanks et al., 2008
USA	CowM2	Not known	100%	6.31 E+04 to 3.02 E+05 gene copies/group	Raith et al., 2013
Australia	CowM3	20	80%	NR	Ahmed et al., 2013
Australia	CowM3	20	100% ^a	NR	Ahmed et al., 2013
Israel	CowM3	NR	93%	NR	Ohad et al., 2015
USA	CowM3	60	98%	NR	Shanks et al., 2008
USA	CowM3	Not known	100%	3.3 E+04 to 7.76 E+05 gene copies/group	Raith et al., 2013
France	Rum2Bac	20	97%	1.6 E+08 ($\pm 0.50 \log_{10}$) to 2.5 E+08 ($\pm 0.13 \log_{10}$) gene copies/g	Mieszkin et al., 2010
France	Rum2Bac	10	90% ^a	1.0 E+07 ($\pm 0.05 \log_{10}$) gene copies/g	Mieszkin et al., 2010
USA	Rum2Bac	NR	100%	2.24 E+05 copies/ group	Raith et al., 2013
End-point^e					
France	CF193	44	95.4%	NR	Gourmelon et al., 2007
Spain	CF193	19	0%	NR	Balleste et al., 2010
USA	CF193	6	100%	NR	Bernhard and Field, 2000

Area	Common Target Name	Number of Samples	Sensitivity ^b	Gene Copy Concentration (Mean or Range)	Reference
USA	CF193	247 from 11 herds	68%	NR	Shanks et al., 2010
USA	CF193	NR	67%	NR	Raith et al., 2013
USA	CowM2	184	80%	NR	Shanks et al., 2006
USA	CowM2	247 from 11 herds	0 to 100%	NR	Shanks et al., 2010
USA	CowM3	148	91%	NR	Shanks et al., 2006
USA	CowM3	247 from 11 herds	0 to 100%	10 gene copies/ng of total DNA	Shanks et al., 2010

^aRepresents any agricultural waste management practice such as lagoons, litter, etc.; ^bSensitivity is routinely expressed as the following: sensitivity = TPC/(TBC+TNI), where TPC represents the total number of samples that tested positive correctly and TNI denotes the total number of samples that tested incorrectly. Specificity is typically defined as the total number of samples that test negative correctly (TNC) divided by the sum of TNC and the total number of samples that tested positive incorrectly (TPI) or TNC/(TNC+TPI); ^cAustria, Argentina, Australia, Ethiopia, Germany, Hungary, Korea, Nepal, Netherlands, Romania, Spain, Sweden, Tanzania, Uganda, UK; ^dNR: Not reported; ^eendpoint is a non-quantitative method

Table 11. Summary of reported non-human-associated MST gene target occurrence in Porcine faecal and agricultural pollution sources^a

Area	Common Gene Name	Number of Samples	Sensitivity ^b	Gene Copy Concentration (Mean or Range)	Reference
Taqman					
France	Pig2Bac	25	100%	3.16 E+08 (±0.60 log ₁₀) gene copies/g wet faeces	Mieszkin et al., 2009
France	Pig2Bac	53	100% ^a	3.98 E+02 (± 0.40 log ₁₀) to 1.99 E+05 (± 0.60 log ₁₀) gene copies/g	Mieszkin et al., 2009
Israel	Pig2Bac	NR ^c	100%	NR	Ohad et al., 2015
USA	Pig2Bac	20	100%	NR	Boehm et al., 2013
End-point^d					
France	PF163	25	100%	NR	Gourmelon et al., 2007
France	PF163	10	100%	NR	Gourmelon et al., 2007
USA	PF163	30	100%	NR	Toledo-Hernandez et al., 2013
USA	PF163	2	100%	NR	Dick et al., 2005
USA	PF163	97	89.3%	NR	Lamendella et al., 2009

Area	Common Gene Name	Number of Samples	Sensitivity ^b	Gene Copy Concentration (Mean or Range)	Reference
USA	PF163	6	100% ^a	NR	Lamendella et al., 2009
USA	PF163	50	100%	NR	Fremaux et al., 2009

^aRepresents any agricultural waste management practice such as lagoons, litter, etc.; ^bSensitivity is routinely expressed as the following: sensitivity = TPC/(TBC+TNI), where TPC represents the total number of samples that tested positive correctly and TNI denotes the total number of samples that tested incorrectly. Specificity is typically defined as the total number of samples that test negative correctly (TNC) divided by the sum of TNC and the total number of samples that tested positive incorrectly (TPI) or TNC/(TNC+TPI); ^cNR: Not reported; ^dendpoint is a non-quantitative method

Table 12. Summary of reported non-human-associated MST gene target occurrence in Avian faecal and agricultural pollution sources^a

Area	Common Target Name	Number of Samples	Sensitivity ^b	Gene Copy Concentration (Mean or Range)	Reference
SYBR					
Australia	GFD	36	58%	1.9 to 7.20 E+03 gene copies/ng of total DNA	Ahmed et al., 2016
USA	GFD	10	30%	1.10 E+01 to 6.4 E+03 gene copies/ng of total DNA	Ahmed et al., 2016
USA	LA35	26	54%	2.80 E+04 gene copies/g	Weidhaas et al., 2010
USA	LA35	17	100% ^a	1.5 E+07 to 3.70 E+09 gene copies/g	Weidhaas et al., 2010
USA	LA35	186	22.6%	3.12 E+03 gene copies/g	Ryu et al., 2014
USA	LA35	40	97.5% ^a	1.0 E+07 gene copies/g	Ryu et al., 2014
End-point					
USA	Gull4	255	86.7%	E+05 copies/ng of total DNA	Ryu et al., 2012

^aRepresents any agricultural waste management practice such as lagoons, litter, etc.; ^bSensitivity is routinely expressed as the following: sensitivity = TPC/(TBC+TNI), where TPC represents the total number of samples that tested positive correctly and TNI denotes the total number of samples that tested incorrectly. Specificity is typically defined as the total number of samples that test negative correctly (TNC) divided by the sum of TNC and the total number of samples that tested positive incorrectly (TPI) or TNC/(TNC+TPI).

3.2.2 Occurrence of host-associated MST genetic markers in non-target pollution sources

It is important to characterize the potential for false-positives when interpreting MST findings. False positives

typically result from the occurrence of a host-associated genetic marker in a non-target pollution source. For example, a human-associated MST genetic marker could also be present in chicken waste leading to reduced confidence in human faecal pollution characterization. This

could be problematic if the study area of interest is impacted by both human and chicken faecal pollution sources. As a result, a considerable amount of research has been conducted to characterize the occurrence of MST genetic markers in non-target faecal waste sources (Table 10). Specificity is the most common performance metric reported for PCR-based applications. In addition, the concentration of a host-associated genetic marker (gene copies/volume, mass, or cell count) in a non-target source is often reported for qPCR methodologies. Just like sensitivity testing (Section 3.2.1), it is important to consider the limit of detection definition, test quantity used, and any differences in methodology from one study to another when evaluating specificity findings. Table 10 summarizes available MST genetic marker occurrence data in non-target sources by methodology and geographic origin of reference waste samples. Even though there is a considerable amount of information available on the occurrence of MST genetic markers in non-target pollution sources, it is highly recommended that local reference pollution samples are tested in the area of interest prior to method implementation to confirm specificity performance. For more detailed information, please refer to Appendix A.4.0

4.1 Persistence of Faecal Indicator Bacteria (FIB)

Assessing the persistence of FIB in aquatic environments is complicated by the potential for waste inputs from multiple sources at any given time in a study, therefore persistence is generally measured in experiments where FIB are contained, as in laboratory glassware (Wanjugi and Harwood, 2014; Korajkic et al., 2013) or dialysis bags (Korajkic et al., 2013; Korajkic et al., 2014). Persistence studies can be very valuable for the selection of appropriate FIB for a particular application. For instance *Bifidobacterium* spp. have limited persistence in the environment and are very sensitive to chlorination, which could make them a poor choice for FIB monitoring in chlorinated waters (Resnick and Levin, 1981). Persistence experiments have been conducted under varying conditions, using many different models to assess changes in density over time, and therefore frequently provide discrepant results, which can lead to varying conclusions about the survival of FIB in surface waters. In general, predation (Wanjugi and Harwood, 2014; Korajkic et al., 2013, 2014), competition from other bacteria (Wanjugi and Harwood, 2013; Surbeck et al., 2010) and ultraviolet radiation exposure (Nguyen et al., 2015; Sassoubre et al., 2012) have a negative impact on FIB persistence, while the presence of sediments (Badgley et al., 2010) and high nutrient levels (Wanjugi et al., 2016) often increase FIB survival times. An overview of select key studies are summarized below. Please refer to the Section IV on Persistence and Transport for additional information.

Jeanneau and colleagues (2012) evaluated the persistence of FIB in sewage-spiked seawater, and reported the highest T_{90} value (\pm standard error) of 3.7 ± 0.1 days for a phylotype related to *Bifidobacterium adolescentis* (measured via qPCR), followed by 3.6 ± 0.8 days for culturable enterococci, 2.3 ± 0.2 days for the HF183 *Bacteroides* 16S rDNA marker; culturable *E. coli* had the

lowest T_{90} value of 1.7 ± 0.1 days in seawater (Jeanneau et al., 2012). In sewage-spiked freshwater, the same authors reported the highest T_{90} value (longest persistence) for culturable *E. coli* (5.8 ± 0.2 days), with lower values for enterococci (3.1 ± 0.5 days) and qPCR-quantified *B. adolescentis* (3.6 ± 0.2 days), and the lowest T_{90} value for the HF183 *Bacteroides* qPCR marker (1.7 ± 0.0 days) (Jeanneau et al., 2012). In freshwater mesocosms spiked with sewage and dog faeces, Anderson et al., (2005) reported faecal coliform decay rates of 0.27 to 0.37 \log_{10} (CFU/100mL) per day, respectively (Anderson et al., 2005). The reported faecal coliform decay rates in saltwater mesocosms spiked with sewage and dog faeces were 4.2 and 3.8 \log_{10} (CFU/100mL) per day, respectively. For enterococci relative to faecal coliforms, the same authors reported a greater decay rate in freshwater spiked with dog faeces, a similar decay rate in sewage-spiked freshwater, and a lower decay rate in sewage-spiked seawater. Decay rates in sediments were also reported to be lower than decay rates in the water column. These examples illustrate the difficulty of comparing studies that use different metrics to measure persistence, and that different bacterial species and DNA targets respond differently to environmental stressors, making generalizations about persistence very challenging.

In site studies of FIB persistence and transport in environmental habitats are possible when there is a clear connection between the infrastructure of interest and a waste stream. A systematic review of the FIB transport from pit latrines (infrastructure) to nearby groundwater sources has been reported; however, extrapolating transport distances to other locations can be challenging due poor characterization of flow rates, differences in soil types and groundwater conditions (Graham and Polizzotto, 2013). For example, the formation of a biologically active scum layer around the latrine pit can limit the movement of FIB from the pit area. Some studies have reported maximum transport distances of 10 meters (Banerjee et al., 2011), while others have reported transport up to 20 meters (Chidavaenzi et al., 2000). More information about the persistence of FIB in the environment and in sanitation technologies can be found in Chapters 15 and 16.

4.2 Overview of Persistence of Host-Associated Genetic Markers

A brief overview of the persistence literature available pertaining to host-associated bacterial MST genetic markers, as well as the discussion of some important methodological considerations for interpreting decay data are presented here. For more detailed information regarding persistence of human-associated MST markers (e.g. T_{90} times), please see chapters entitled "Using indicators to assess microbial treatment and disinfection efficacy" and "Evaluation of subsurface microbial transport using microbial indicators, surrogates and tracers." The majority of studies to date focus on investigating persistence of human-, ruminant-, and cow-associated indicators in aquatic habitats (Bae and Wuertz, 2009; Sokolova et al., 2012; Tambalo et al., 2012; Walters and Field, 2009). Some of the biotic and abiotic factors

commonly investigated include ambient sunlight (Korajkic et al., 2014; Green et al., 2011), water type (freshwater, estuarine, or marine) (Jeanneau et al., 2012; Green et al., 2011; Ahmed et al., 2014), temperature (Dick et al., 2010; Kreader, 1998; Okabe and Shimazu, 2007), influence of indigenous microbiota, and faecal pollution source (Bae and Wuertz, 2009; Sokolova et al., 2012; Tambalo et al., 2012; Walters and Field, 2009). Comparisons across studies and derivation of any overarching conclusions with respect to the effect of these stressors is challenging as many studies report conflicting results. For example, ambient sunlight has been reported to be detrimental by some researchers, but not others (Korajkic et al., 2014; Walters and Field, 2009; Green et al., 2011; Dick et al., 2010; Savichtcheva et al., 2007). It has been suggested that the effect of sunlight on host-associated indicators is linked to the physiological state of the organisms (Bae and Wuertz, 2009), as well as the stage of the decomposition process (Korajkic et al., 2014). A majority of studies tend to agree that persistence is typically longer at colder temperatures compared to warmer conditions (Kreader, 1998; Silkie and Nelson, 2007) and in marine waters compared to freshwater (Jeanneau et al., 2012; Green et al., 2011; Okabe and Shimazu, 2007; Schulz and Childers, 2011).

The apparent discord in literature is likely due to the wide variety of experimental designs employed, as well as lack of method protocol standardization, use of different units of measure, and varied data modeling practices. One of the important methodological factors likely to influence the outcome of a persistence study is whether the experiments were performed indoors or outdoors as the latter mimics ambient conditions more closely compared to bench-scale laboratory experiment with artificial lighting (Korajkic et al., 2014; Jeanneau et al., 2012; Bae and Wuertz, 2009; Sokolova et al., 2012; Tambalo et al., 2012; Green et al., 2011; Ahmed et al., 2014; Dick et al., 2010; Kreader, 1998; Okabe and Shimazu, 2007; Savichtcheva et al., 2007; Schulz and Childers, 2011). Observed persistence patterns can also depend on the type and amount of faecal source(s) inoculated as these factors vary widely. For example, the seeded faecal pollution source can range from a single *E. coli* laboratory strain to a composite mixture, such as sewage or septage waste. As a result, generalizations across studies seeded with different pollution sources can be misleading. Due to the potential

for bias and large discrepancies in faecal pollution decomposition from one locale to the next, it may be necessary to perform decay studies in the area of interest prior to water quality testing, if persistence data are needed to interpret host-associated indicator results.

5.0 Applications and Future Directions

There are many potential applications for FIB and host-associated genetic MST methods. FIB are commonly used around the world in regulatory settings for sewage effluent discharge control, recreational and aquaculture water quality monitoring, as well as drinking water safety assessments (see Tables 2 and 3) for over a century (Hacker and Blum-Oehler, 2007; Escherich, 1885). It is likely that FIB will continue to be employed in the regulatory arena with an expanded utility in greywater safety testing and monitoring irrigation waters used for agricultural food production.

There are currently no formal regulatory applications or standardized methods for any MST technology. However, the United States Environmental Protection Agency is working towards the development of standardized procedures for two human-associated qPCR methods including HF183/BacR287 and HumM2. Data acceptance metrics are available for these technologies (Shanks et al., 2016) and they have performed well in two separate multiple laboratory validation studies (Shanks et al., 2016; Layton et al., 2013). As these MST methods transition from research approaches to management tools, future studies will focus on potential regulatory and water quality management strategies.

Finally, it is important to recognize the role that emerging technologies will play in future applications of FIB and MST methods. Emerging technologies refer to new methodologies with the potential to improve FIB and MST indicator characterization. Emerging applications will doubtlessly harness the power of high throughput nucleic acid sequencing and other methodologies for the rapid and simultaneous measurements of multiple bacterial indicators. These novel technologies coupled with QMRA will likely provide future water quality managers, public health officials, and researchers with powerful tools to predict human health risk from exposure to faecal pollution.

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