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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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
E. coli	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
E. coli	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
E. coli	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 Entero1a gene via qPCR	Molecular	US EPA Methods 1609 and 1611	Ludwig and Schleifer 2000; Noble et al.; 2010
Bacteroides 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
Bifidobacterium 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
Bifidobacterium spp.	Identification of the Bifidobacterium gene via qPCR Identification of the BIF164 sequence via FISH	Molecular	NR	Gueimonde et al., 2004; Langendijk et al., 1995
Clostridium 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
Clostridium 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
Clostridium 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

[a]NR: 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	*E. coli*: <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	*E. coli*: <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; Repuclic 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 *E. coli*: 500 CFU/100mL Classification “Good” (95th percentile of $\log_{10}$ densities): Enterococci: 400 CFU/100mL *E. coli*: 1,000 CFU/100mL Classification “Sufficient” (90th percentile of $\log_{10}$ densities): Enterococci: 330 CFU/100mL *E. coli*: 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 *E. coli*: 250 CFU/100mL Classification “Good” (95th percentile of $\log_{10}$ densities): Enterococci: 200 CFU/100mL *E. coli*: 1,000 CFU/100mL (inland); 500 CFU/100mL Classification “Sufficient” (90th percentile of $\log_{10}$ densities): Enterococci: 185 CFU/100mL *E. coli*: 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) *E. coli* (freshwater only): 126 CFU/100mL (geometric mean); 410 CFU/100mL (10% statistical threshold value) 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) *E. coli* (freshwater only): 100 CFU/100mL (geometric mean); 320 CFU/100mL (10% statistical threshold value)	Recreational Water Quality Criteria (EPA 820-F-12-058)	USEPA, 2012

[a]Classification 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;

[b]requires filtration for drinking water and 4.0 $\log_{10}$ removal of *Cryptosporidium;*

[c]requires filtration for drinking water and 5.0 $\log_{10}$ removal of *Cryptosporidium;*
[d]requires filtration for drinking water and 5.5 $\log_{10}$ 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	*E. coli* (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	*E. coli*: <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	*E. coli*: <1 CFU/100mL or <5 MPN/100mL Total coliforms: <1 CFU/100mL or <5 MPN/100mL	Bolivian Norm NB 512 - Quality of potable water for human consumption (Norma Bolivia NB 512 – Calidad de agua potable para el consumo humano)	IBNORCA, 2016
Brazil	Drinking water	Entrance of Piped Distribution Network: Total coliforms: <1/100mL Faecal coliforms: <1/100mL Other Locations in Piped Distribution Network: 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) Faecal coliforms: <1/100mL Communal wells and springs (non-piped systems): Total coliforms: Absence in 100mL in 95% of samples and <10/100mL in 5% of samples Faecal coliforms: <1/100mL	Portaria No. 36/MS/GM: Norms and Standards for Potable Water Destined for Human Consumption	Brazilian Ministry of Health, 1990
Chile	Drinking water	Potable Water: Faecal coliforms: Nil/100mL Water in Piped Distribution Network: 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	Official Chilean Norm 409/1: Drinking Water	INN Chile, 1984
Colombia	Drinking water	*E. coli*: <1/100mL Total coliforms: <1 CFU/100mL or <2 MPN/100mL	Technical Norms for Potable Water Quality. Decree 475-1998.	Colombian Ministry of Health, 1998
Costa Rica	Drinking water	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)	Decree No. 25991-S: Regulations for the Quality of Potable Water	Costa Rican Ministry of Health, 1997

Ecuador	Drinking water	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) 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)	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	Total coliforms: <1 CFU/100mL or < 1.1 MPN/100mL Faecal coliforms: <1 CFU/100mL or < 1.1MPN/100mL *E. coli*: <1 CFU/100mL or < 1.1 MPN/100mL Heterotrophic plate count: <100 CFU/mL	Salvadoran Norm: Water, Potable Water (NSO 13.07.01:08)	CONACYT, 2009
Estonia	Drinking water	Distributed public water supply, containers and tanks: *E. coli*: <1 CFU/100mL Enterococci: <1 CFU/100mL Bottled into bottles or jerrycans: *E. coli*: <1 CFU/100mL Enterococci: <1 CFU/100mL *Pseudomonas aeruginosa:* <1 CFU/100mL Heterotrophic plate count at 22°C: 100 CFU/mL Heterotrophic plate count at 37°C: 20 CFU/mL	Quality and control requirements and analysis methods for drinking water	United Kingdom (Scotland), 2008
Honduras	Drinking water	Recommended Values: Total coliforms: <1/100mL Faecal coliforms: <1/100mL *E. coli*: 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	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	Regulations Concerning the Sanitary Quality of Drinking Water	Israeli Ministry of Health, 1991
Mexico	Drinking water	*E. coli*: <1/100mL 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 *E. coli*: <1/100mL	Chapter 2401-51. Public Water Supply System Regulations	Republic of Palau, 1996
Singapore	Piped drinking water	*E. coli* (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 *E. coli* (faecal coliforms): <1/100mL Satisfactory Classification: Total coliforms: 1 to 3/100mL *E. coli* (faecal coliforms): <1/100mL Suspicious Classification: Total coliforms: 4 to 10/100mL *E. coli* (faecal coliforms): <1/100mL Unsatisfactory Classification: Total coliforms: >10/100mL *E. coli* (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 *E. coli*: <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) *E. coli*: <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 Clostridium perfringens: <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	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) *E. coli*: <1/100mL (the situations below also represent non-compliance) Any positive *E. coli* repeat sample Repeat sample positive for *E. coli* following positive total coliform routine sample or vice versa Failure to take repeat samples following an *E. coli* positive routine sample or the failure to test for *E. coli* following a positive repeat sample for total coliform	National Primary Drinking Water Regulations: Revisions to the Total Coliform Rule	USEPA, 2006

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 (~ 44.5ºC). *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 10ºC and 45ºC, 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	*Bacteroidales*	HF183	16S rRNA *Bacteroides-Prevotella* group	End-point	Bernhard and Field, 2000
Human	*Bacteroidales*	HF183	16S rRNA *Bacteroides-Prevotella* group	SYBR	Seurinck et al., 2005
Human	*Bacteroidales*	HF183	16S rRNA *Bacteroides-Prevotella* group	TaqMan	Haugland et al., 2010
Human	*Bacteroidales*	BacH	16S rRNA *Bacteroidetes*	TaqMan	Reischer et al., 2007
Human	*Bacteroidales*	Bac-Hum UCD	16S rRNA *Bacteroidales*	Taqman	Kildare et al., 2007
Human	*Bacteroidales*	HumM2	Hypothetical protein	TaqMan	Shanks et al., 2009
Human	*Bacteroidales*	*B. thetaiotamicron*	1,6-alpha mannanase of *B. thetaiotamicron*	TaqMan	Yampara-Iquise et al., 2008
Human	Methanogens	*nifH*	*nifH* (nitrogenase) gene of *Methanobrevibacter smithii*	End-point	Ufnar et al., 2006
Human	Methanogens	*nifH*	*nifH* (nitrogenase) gene of *Methanobrevibacter smithii*	TaqMan	Johnston et al., 2010
Human	*Bifidobacteria*	*Bifidobacteria*	16S rRNA *B. adolescentis*	End-point	Bonjoch et al., 2004
Human	*Bifidobacteria*	*Bifidobacteria*	16S rRNA *B. adolescentis*	TaqMan	Gourmelon et al., 2010

Animal Group	Target Organism	Common Target Name	Specific Gene Target	Chemistry	Reference
Human	*Enterococcus*	*esp*	*esp* (enterococcus surface protein) from *E. faecium*	End-point	Scott et al., 2005
Human	*Enterococcus*	*esp*	*esp* (enterococcus surface protein) from *E. faecium*	SYBR	Ahmed et al., 2008
Ruminant	*Bacteroidales*	CF193	16S rRNA *Bacteroides-Prevotella* group	End-point	Bernhard and Field, 2000
Ruminant	*Bacteroidales*	Rum2Bac	16S rRNA *Bacteroidales*	TaqMan	Mieszkin et al., 2010
Ruminant	*Bacteroidales*	BacR	16S rRNA *Bacteroidetes*	TaqMan	Reischer et al., 2006
Ruminant	*Bacteroidales*	CowM2	Energy metabolism genes from *Bacteroidales*-like organisms	End-point	Shanks et al., 2006
Ruminant	*Bacteroidales*	CowM2	Secretory protein from *Bacteroidales*-like organisms	TaqMan	Shanks et al., 2008
Ruminant	*Bacteroidales*	CowM3	Energy metabolism genes from *Bacteroidales*-like organisms	End-point	Shanks et al., 2006
Ruminant	*Bacteroidales*	CowM3	Secretory protein from *Bacteroidales*-like organisms	TaqMan	Shanks et al., 2008
Porcine	*Bacteroidales*	PF163	16S rRNA *Prevotella* group	End-Point	Dick et al., 2005
Porcine	*Bacteroidales*	Pig2Bac	16S rRNA *Bacteroidales*	TaqMan	Mieszkin et al., 2009
Avian	*Helicobacter*	GFD	16S rRNA *Helicobacter* spp.	SYBR	Green et al., 2012
Avian	*Brevibacterium*	LA35	16S rRNA *Brevibacterium* spp.	SYBR	Weidhaas et al., 2010
Avian	*Catelicoccus*	Gull2	16S rRNA *Catelicoccus* 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 H_2S 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 H_2S 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
E. coli	Faeces (per wet g)	1.0 E+06 to 1.0 E+09
E. coli	Untreated Sewage (per 100mL)	1.0 E+07 to 1.0 E+08
E. coli	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
Bacteroides spp.	Faeces (per wet g)	1.0 E+08 to 1.0 E+10
Bacteroides spp.	Untreated Sewage (per 100mL)	1.0 E+09
Bifidobacterium spp.	Faeces (per wet g)	1.0 E+08 to 1.0 E+10
Bifidobacterium spp.	Untreated Sewage (per 100mL)	1.0 E+06 to 1.0 E+09
Clostridium spp.	Faeces (per wet g)	1.0 E+03
Clostridium spp.	Untreated Sewage (per 100mL)	1.0 E+04 to 1.0 E+06
Clostridium 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	*C. perfringens*	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	*C. perfringens*	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	*C. perfringens*	<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	*C. perfringens*	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	*C. perfringens*	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	*C. perfringens*	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	*C. perfringens*	2.51 E+08	1.04 E+11

Adapted from (Geldreich, 1978; Ashbolt et al., 2001); [a]CFU: Colony forming unit; [b]NR: 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

Area	Common Target Name	Number of samples	Sensitivity[a]	Gene Copy Concentration (Mean or Range) per 100mL	Reference
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 (± 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	*esp*	16	100%	9.8 E+03 to 3.8 E+04 gene copies/100mL	Ahmed et al., 2008
Australia	*esp*	10	100%	NR	Ahmed et al., 2009
			TaqMan		
India	HF183	5	100%	195 (± 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 (± 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 (± 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	*B. adolescentis*	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
USA	*nifH*	20	20 to 55%	NR	Layton et al., 2013
USA	*nifH*	16	100%	12 to 3.8 E+03 gene copies/100mL	Johnston et al., 2010
			End-point[c]		

Area	Common Target Name	Number of samples	Sensitivity[a]	Gene Copy Concentration (Mean or Range) per 100mL	Reference
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	*nifH*	8	100%	1.0 E+04 to 7.9 E+06 gene copies/100mL	Gourmelon et al., 2010
USA	*nifH*	39	100%	NR	McQuaig et al., 2009
USA	*nifH*	19	100%	NR	Harwood et al., 2009
USA	*nifH*	27	93%	NR	Ufnar et al., 2006
USA	*nifH*	20	20 to 55%	NR	Layton et al., 2013
Spain	*B. adolescentis*	45	95.6%	NR	Balleste et al,. 2010
Spain	*B. adolescentis*	12	100%	NR	Bonjoch et al., 2004
Spain, France, Sweden, UK, Cyprus, USA	*B. adolescentis*	114	92.7%	NR	Blanch et al., 2006
USA	*B. adolescentis*	3	66.6%	NR	Bachoon et al., 2010
Australia	*esp*	Not known	100%	NR	Neave et al., 2014
Spain	*esp*	13	77%	NR	Balleste et al,. 2010
USA	*esp*	26	92%	NR	Layton et al., 2009
USA	*esp*	55	100%	NR	Reischer et al., 2006
USA	*esp*	3	100%	NR	Korajkic et al., 2009
USA	*esp*	20	55%	NR	Masago et al., 2011

[a]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);

[b]NR: Not reported; [c]endpoint 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 (± 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	HF183	30	16.7%	204 (± 1.71 $\log_{10}$) gene copies/ng of total DNA	Odagiri et al., 2015
USA		20	100%	NR	Layton et al., 2013
USA	HF183	16	100%	1.47 E+03 (± 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 (± 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 (± 1.61 $\log_{10}$) gene copies/ng of total DNA	Odagiri et al., 2015
USA	BacHum-UCD	24	100%	NR	Layton et al., 2013
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

Area	Common Target Name	Number of samples	Sensitivity[a]	Gene Copy Concentration (Mean or Range)	Reference
India	HumM2	30	40%	37 (± 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 (± 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	*nifH*	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	*nifH*	70	29%	NR	Ufnar et al., 2006
USA	*esp*	12	83.3%	NR	Layton et al., 2009

[a]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);

[b]NR: Not reported; [c]Argentina, Austria, Ethiopia, Germany,Hungary, Hungary, Korea, Nepal, Netherlands, Romania, Spain, Sweden, Tanzania ,Uganda, UK; [d]Austria, Argentina, Australia, Ethiopia, Germany, Hungary, Korea, Nepal, Netherlands, Romania, Spain, Sweden, Tanzania, Uganda, UK, USA; [e]endpoint 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
nifH	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
nifH	16	93.7%	NR	McQuaig et al., 2009
nifH	25	100%	NR	Harwood et al., 2009
E. faecium esp	10	80%	NR	Scott et al., 2005
E. faecium esp	6	100%	NR	Masago et al., 2011

[a]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);

[b]NR: 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 (± 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 (± 0.50 $\log_{10}$) to 2.5 E+08 (± 0.13 $\log_{10}$) gene copies/g	Mieszkin et al., 2010
France	Rum2Bac	10	90%[a]	1.0 E+07 (± 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

[a]Represents any agricultural waste management practice such as lagoons, litter, etc.; [b]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); [c]Austria, Argentina, Australia, Ethiopia, Germany, Hungary, Korea, Nepal, Netherlands, Romania, Spain, Sweden, Tanzania, Uganda, UK; [d]NR: Not reported; [e]endpoint 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

[a]Represents any agricultural waste management practice such as lagoons, litter, etc.; [b]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); [c]NR: Not reported; [d]endpoint 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

[a]Represents any agricultural waste management practice such as lagoons, litter, etc.; [b]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).

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 (± 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.

Appendix A. Occurrence of Host-Associated Genetic Markers in Target and Non-

Target Sources

A.1.0 Human-Associated Methods

A.1.1 *Bacteroidales*

A.1.1.1 HF183/708R End-Point PCR.

For the HF183/708R end-point PCR method, initial specificity testing (10 non-human animal species; n=27 individual samples) indicated 100% specificity while sensitivity ranged from 84% in human faecal samples (n=13) to 100% in untreated sewage (n=3) (Bernhard and Field, 2000). Sensitivity and specificity of the HF183/708R assay in the end-point format has been tested in various regions of the United States (Bernhard and Field, 2000; Toledo-Hernandez et al., 2013; McQuaig et al., 2009; Shanks et al., 2010; Layton et al., 2013; Harwood et al., 2009), as well as Australia (Ahmed et al., 2008), Spain (Balleste et al., 2010), Canada (Edge et al., 2013; Fremaux et al, 2009) and France (Gourmelon et al., 2007). A wide range of sensitivity and specificity was reported, depending on the region and the reference pollution sources tested. In addition to the developing laboratory, five reports originating from the United States described performance of the end-point HF183/708R assay. Shanks *et al.* tested sensitivity against human faecal samples collected from 16 individuals, as well as 54 wastewater effluents collected across the country (Shanks et al., 2010). HF183/708R marker was detected in all 54 wastewater samples (100% sensitivity) and six (out of 16) individual human faecal samples (Shanks et al., 2010). Specificity was reported at 95%, as it cross-reacted with one (out of 10) dog samples, but not with other faecal samples collected from 21 non-target animal species (Shanks et al., 2010). A study conducted in Puerto Rico tested the end-point assay against 16 wastewater samples and 340 individual animal faecal samples collected from 12 non-target species eliciting a 75% sensitivity and 100% specificity as it was not detected in any of the non-target hosts (Toledo-Hernandez et al., 2013). Three hundred twenty-two non-target waste samples ranging from individual faecal samples to large-scale composites from Florida and Mississippi were tested to determine specificity of the HF183/708R end-point assay (Harwood et al., 2009). False-positives were detected in samples originating from dog, chicken and seagull for an overall reported specificity of 96% (Harwood et al., 2009). The same study also determined the assay to be 100% sensitive when tested against wastewater samples (n=48) and various on-site collection systems (e.g. lift stations, sewage lagoons and septic systems, n=80) (Harwood et al., 2009). Another Florida-based study performed specificity testing against individual faecal samples from 13 non-target animal sources (n=113) and 2 composite farm animal sources (McQuaig et al., 2009). The HF183/708R marker was found to cross-react with 13% of non-target samples, including one cat sample (out of five) and 14 (out of 55) dog samples (McQuaig et al., 2009). In the same study, sensitivity of the assay was determined to be 100% when tested against 39 wastewater samples and 16 on-site collection systems (McQuaig et al., 2009). As a part of the comprehensive method comparison study conducted in California and involving 27 laboratories worldwide, HF183/708R end-point assay was tested against human faecal samples, septage samples, wastewater samples, as well as composite faecal samples from nine non-target hosts (McQuaig et al., 2009). Sensitivity to human faecal sources ranged from 57% (sewage), to 71% (septage) to human faeces (96%), while the reported specificity was 96% (McQuaig et al., 2009). The performance of the HF183/708R end-point assay was also tested in Australia against 12 non-target animal species (total of 155 faecal samples), as well as 52 human samples, including primary wastewater effluent (n=15), secondary effluent (n=15), treated effluent (n=15) and 12 septic system samples (Ahmed et al., 2008). The HF183/708R marker was detected in all human samples and none of the non-target samples with a reported sensitivity and specificity of 100% (Ahmed et al., 2008). The sensitivity and specificity of HF183/708R end-point assay were considerably lower when tested in Spain (Balleste et al., 2010). Marker was challenged by testing it against wastewater, as well as effluents from poultry slaughterhouses, swine faeces, slurry from slaughterhouses and a farm, ruminant slaughterhouses and bovine farms. Reported sensitivity was 50% as it was detected in only 20 wastewater samples (out of 40), while specificity was higher (71%) with cross-reactions observed with cow (3 out of 19), poultry (15 out of 26) and swine (3 out of 28) (Balleste et al., 2010). In Canada, HF183/708R end-point assay was tested against 102 final wastewater effluents and it was detected in 74% of samples (Edge et al., 2013). Marker was also tested against faecal samples from eight non-target hosts and detected in one (out of 17) dog sample and one (out of 15) chicken sample, but it was not detected in cat, gull, Canada geese, mallard duck, cow and pig (total of 207) samples (Edge et al., 2013). Another Canada based study tested the HF183/708R marker against 62 individual human and wastewater samples, as well as 211 samples from 11 non-target animal groups (Fremaux et al., 2009). The HF183/708R marker was detected in 94% (51 out of 54) individual human samples, 100% (8 out of 8) wastewater samples and it exhibited 100% specificity (Fremaux et al., 2009). In France, end-point HF183/708R assay was challenged against individual human faecal samples (n=44), as well as wastewater (n=5) and sludge samples (n=6) (Gourmelon et al., 2010). Marker was detected in 43 individual human samples and all of the wastewater samples. Specificity was assessed by testing the marker against pig, cow, sheep, chicken and wildbirds, as well as pig manure. The HF183/708R marker was absent from pig (individual samples and manure), sheep and wildbird samples, but was detected in four (out of 32) cow samples and one (out of 10) chicken sample (Gourmelon et al., 2010).

A.1.1.2 HF183/708R SYBR Green Chemistry qPCR method.

Several years later, the end-point PCR HF183/708R method was adapted to a SYBR Green qPCR chemistry with a reported sensitivity ranging from 85.7% in human faecal samples (n=7) to 100% in sewage (n=4) (Seurinck et al., 2005). The same study reported that the SYBR Green HF183/708R assay cross-reacted with one chicken faecal sample, but tested negative against four other non-target animal groups. In addition to the developer's laboratory (located in Belgium), sensitivity and specificity of the HF813/708R assay using SYBR green chemistry has also been tested in Australia (Ahmed et al., 2009; Ahmed et al., 2015), Bangladesh (Ahmed et al., 2010), India (Odagiri et al., 2015) and the United States (Layton et al., 2013; Van De Werfhorst et al., 2011). In Australia, the performance was assessed by testing the marker against 32 wastewater influent samples and individual faeces and composite wastewater samples from five non-target groups including cattle, sheep, pigs, dogs and ducks (Ahmed et al., 2009). The HF183/708R SYBR green marker was found to be 100% sensitive and 98% specific as it was detected in all human samples and one dog faecal sample (Ahmed et al., 2009). A subsequent study conducted in three different climatic zones in Australia equivalent to subtropical, Mediterranean, and temperate conditions provided additional sensitivity data for the SYBR green HF183 assay (Ahmed et al., 2015). In this study, a total of 99 wastewater influent samples from three wastewater treatment plants located in the above described climatic zones was collected. The HF183/708R SYBR green marker was detected in all of the wastewater samples with a reported mean concentration of 8.0×10^5 gene copies/mL (Ahmed et al., 2015). A study conducted in Bangladesh tested the HF183/708R SYBR green assay against 45 faecal samples from humans, cattle, dogs, cats and chickens (Ahmed et al., 2010). Overall sensitivity was 87%, as it was detected in 13 (out of 15) human faecal samples (Ahmed et al., 2010). Specificity was 93% as it was detected in 1 dog and 1 cat sample (Ahmed et al., 2010). Reported levels in target sources ranged from 1.2×10^6 to 3.9×10^8/100 mg of faeces, while levels in dog and cat samples were 7.8×10^4 and 4.6×10^3, respectively (Ahmed et al., 2010). Another study from India tested the HF183/708R SYBR green marker on 60 composite samples comprised from cow (n=50), buffalo (n=50), sheep (n=50), goat (n=50), chicken (n=96), dog (n=50), as well as 30 individual human samples, 5 sewage samples and 20 samples from patients with diarrhea (Odagiri et al., 2015). The HF183/708R SYBR green assay indicated a sensitivity of 89% as it was detected in 26 healthy human faecal samples and all 5 wastewater samples (but not in any samples of patients with diarrhea) (Odagiri et al., 2015). When DNQ data was considered negative, sensitivity of the HF183/708R SYBR green assay was 63% (Odagiri et al., 2015). Cross-reactivity was observed with 100% to cow, buffalo, goat, sheep, and chicken, as well as 80% with dog faecal samples (Odagiri et al., 2015). Specificity was reported at 3% (DNQ considered positive) or 63% (DNQ considered negative) (Odagiri et al., 2015). Concentrations were reported as mean $\log_{10}$ gene copies per nanogram of total DNA in human faeces (0.96±1.64) and sewage (1.67±0.47), while reported levels in non-target samples were as follows: 1.63 (cow), 1.41±0.49 (buffalo), 2.23±1.0 (goat), 1.04±1.0 (dog), 3.09±1.11 chicken, and no sheep samples were within range of quantification (Odagiri et al., 2015). Two California based studies tested the performance of SYBR green HF183 assay (Layton et al., 2013; Van De Werfhorst et al., 2011). The first study tested sensitivity and specificity against individual human faecal samples, septage, sewage and faeces from five non-target animal hosts including cat, dog, gull, raccoon and rat (n=4) (Van De Werfhorst et al., 2011). The HF183/708R SYBR green marker was detected in 5 (out of 8) human faecal samples in concentrations ranging from 4.9×10^3 to 5.3×10^8 per gram (wet weight) (Van De Werfhorst et al., 2011). It was also present in 2 out of 3 septage samples in levels ranging from 9.8×10^7 to 4.9×10^8/L and in 10 out of 10 sewage samples ranging from 4×10^7 to 2.5×10^9/L (Van De Werfhorst et al., 2011). The HF183/708R SYBR green marker was also detected in 1 (out of 12) cat sample (2.6×10^3/gram, wet weight), but not in any other non-target samples (Van De Werfhorst et al., 2011). The same comprehensive method evaluation study described earlier for HF183/708R end-point assay also tested the performance of SYBR green and TaqMan chemistries (Layton et al., 2013). In this study, results from qPCR assays were reported under different conditions including classification of samples that were detectable but not quantifiable (DNQ) as positive or negative and by utilizing different reference units of measure (culturable indicator bacteria, wet mass, total DNA and qPCR for different general faecal indicators) (Layton et al., 2013). Sensitivity was determined for different types of human sources (faeces, septage or sewage), while specificity was tested against composite faecal samples from nine non-target hosts (Layton et al., 2013). When evaluated against 48 human faecal samples, sensitivity of the HF183/708R SYBR green assay was determined to be 100% or 92%, when considering DNQ as positive or negative, respectively (Layton et al., 2013). Testing against 104 non-target samples resulted in specificities of 78% and 89% with DNQ classified as positive or negative, respectively (Layton et al., 2013). When different human sources were considered separately, sensitivity to all three types was 100% with DNQ considered positive, but it varied when DNQ was considered negative (81% for sewage, 94% for septage and 100% for faeces) (Layton et al., 2013). Expressing the abundance in different units of measure (eg. Wet mass, total DNA, etc) resulted in a wide range of concentrations for both target and non-target sources. Median $\log_{10}$ marker concentrations in human fecal samples ranged from: 5.9±1.1 per mg of wet weight, 1.7±1.5 per nanogram of total DNA, 1.9±1.4 per culturable enterococci (USEPA, 2006), - 0.9±1.5 per enterococci via Entero1a qPCR (Haugland et al., 2005), 0.5±1.3 per culturable *E. coli* via membrane filtration, -0.2±1.2 per *E. coli* via EC23S857 qPCR (Chern et al., 2011), -2.2 ± 0.8 per *Bacteroidales* via GenBac3 qPCR (Siefring et al., 2008), -2.1 ± 0.3 per *Bacteroidales* via AllBac qPCR (Layton et al., 2006) and -1.5±0.5 per *Bacteroides* (Converse et al., 2009). Following the same order, median $\log_{10}$ concentrations in non-target hosts was as follows: 2.3±1, -0.2±0.8, -0.9±1.4, -3.7±1.3, -2.1±1.4, -3.2± 1.3, -5.4±1.9 and -5.1 (Layton et al., 2013).

A.1.1.3 HF183/BFDrev TaqMan qPCR.

A TaqMan qPCR HF183/BFDrev qPCR method soon followed with a reported sensitivity of 100% in human faecal samples (n=16; 3.17 ± 0.07 log_{10} gene copies/ng of total DNA) and sewage samples (n=14; median ~2.8 log_{10} gene copies/ng of total DNA) (Haugland et al., 2010). The product of the TaqMan qPCR HF183/BFDrev assay was not detected in composite preparations of cattle, pig and cat faecal samples (n=10 each animal source), but it did cross-react with composites of chicken faeces (n=10; 0.35± 0.07 log_{10} gene copies/ng of total DNA) and dog faeces (n=10; 0.36 ± 0.07 log_{10} gene copies/ng of total DNA) (Haugland et al., 2010). The widespread use of HF183/BFDrev qPCR technology and performance in multiple validation studies (Harwood et al, 2009; Boehm et al., 2013; Griffith et al., 2003) led a team of MST scientists to develop an improved TaqMan qPCR 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). The same California based method comparison study is the also evaluated sensitivity and specificity metrics for HF183/BFDrev TaqMan other than the developing laboratory (Layton et al., 2013). Sensitivity (n=60 samples) and specificity (n=130 samples) were determined to be 100% and 46% or 95% and 92% when DNQ samples were considered to be positive or negative, respectively (Layton et al., 2013). When considered by the animal source group, sensitivity was 100% across the board with DNQ deemed positive, but it ranged from 85% (sewage) to 100% (human faeces and septage) when DNQ was considered negative (Layton et al., 2013). Similar to HF183/708R SYBR green qPCR assay, abundance in target and non-target sources varied widely when expressed using different units of measure. The reported concentrations in target and non-target samples were: log_{10} 6.9±0.1/1.2±0.9 copies per milligram of wet weight, log_{10} 2.2±1.5/-0.5±0.8 copies per nanogram of total DNA, log_{10} 2.4±1.1/-1.1±1.5 copies per culturable enterococci (USEPA, 2006), log_{10} -0.3±1.7/-4±1.3 copies per enterococci qPCR (Haugland et al., 2005), log_{10} 1.3±0.6/-2.8±1.4 copies per culturable *E. coli* (via membrane filtration), log_{10} 0.5±0.3/-3.2±1.1 copies per *E.coli* qPCR (Chern et al., 2011), and log_{10} -1.7±0.6/-5.1±2.1 copies per GenBac3 qPCR (Siefring et al., 2008). A previously described study from India also evaluated sensitivity and specificity metrics of the HF183/BFDrev TaqMan assay (Odagiri et al., 2015). Regardless of whether DNQ samples were considered positive or negative, the assay had sensitivity of 29% as it was detected in 16.7% of healthy human faeces and 100% of sewage samples (assay was also detected in 40% of human samples with diarrhea) (Odagiri et al., 2015). Specificity was reported at 80%, irrespective of the DNQ classification as it cross-reacted with 40% of dog samples and 80% of chicken samples (Odagiri et al., 2015). Mean log_{10} gene copies per nanogram of DNA in human samples was as follows: 2.31 ± 1.71 (faeces from healthy humans), 2.29 ± 0.72 (sewage) and 1.70 ± 1.27 (faeces from humans with diarrhea) (Odagiri et al., 2015). Levels in dog and chicken faeces were 1.49 ± 1.12 and 3.52 ± 1.16, respectively (Odagiri et al., 2015).

A.1.1.4 BacH TaqMan qPCR.

BacH is also a TaqMan qPCR assay that targets the *B. doreii* 16S rRNA gene cluster (Reischer et al., 2007). In the original report, BacH was absent in faeces from 15 non-human animal groups (n=302 individual samples) and only cross-reacted with a single cat faecal sample (Reischer et al., 2007). BacH sensitivity ranged from 95% in human faecal samples (n=21; $6.6x10^9$-$9.1x10^{10}$ marker equivalents/g wet faeces) to 100% in wastewater and cesspit samples (n=21; $1.4x10^{10}$-$9.1x10^{10}$ marker equivalents/g) (Reischer et al., 2007). In addition to the developing laboratory located in Austria and a study conducted in India (Odagiri et al., 2015), performance metrics of BacH TaqMan assay have been evaluated in two other large method performance studies to date (Layton et al., 2013; Reischer et al., 2013), providing sensitivity and specificity data across sixteen countries including Argentina, Australia, Austria, Ethiopia, Germany, Hungary, India, Korea, Nepal, Netherlands, Romania, Spain, Sweden, Tanzania, Uganda, United Kingdom and United States. The United States based method evaluation study reported sensitivity (n=12 samples) and specificity (n=26 samples) as 100% and 77% and 75% and 85% when DNQ data were considered as positive or negative, respectively (Layton et al., 2013). Considering sensitivity in relation to the type of human source, the BacH qPCR assay was 100% sensitive when DNQ was considered positive, but it ranged from 50% (sewage) to 75% (septage) to 100% when DNQ was considered negative (Layton et al., 2013). The same study reported concentration of BacH qPCR marker in target and non-target samples using the above described different units of measure. Reported median log_{10} values for sensitivity and specificity were as follows: 7.5±0.2 and 2.8±0.9/mg of wet weight, 1.9±2 and 0.9±0.9/ng of total DNA, 2.1±1.8 and -1.5±1.8 per culturable enterococci (USEPA, 2006), 1.5±0.9 and -1.5±1.2 per culturable *E. coli*, 0.6±0.9 and -2.2±0.9 per *E. coli* qPCR (Chern et al., 2011). A global method comparison study encompassing six continents tested sensitivity and specificity on 280 individual faecal samples from humans and variety of animals including ruminants (cattle, sheep, deer, goat, chamois and lama), non-ruminant herbivores (horse, kangaroo, hare/rabbit, donkey, zebra, groundhog), omnivores (pig, wild boar), carnivores (dog, cat, coyote, opossum, otter) and birds (chicken, duck, geese, pigeons, starlings, turkey, gull and other wild birds) (Reischer et al., 2013). Overall, sensitivity and specificity of BacH qPCR was reported to be 77% and 53% as it was detected in 47 (out of 61) human faecal samples and 44 (out of 79) ruminant samples, 9 (out of 28) non-ruminant herbivore samples, 9 (out of 29) omnivore samples, 23 (out of 39) carnivore samples and 18 (out of 44) bird samples (Reischer et al., 2013). The concentration of BacH qPCR marker in human faeces ranged from not detectable to ~7 log_{10} marker copies per reaction, with reported median concentration of ~1.9 log_{10} (Reischer et al., 2013). Levels in non-target animal groups ranged from not detectable to ~4.5 log_{10} marker copies per reaction. Authors also expressed concentration data normalized to 1 nanogram of total DNA.

In that instance, BacH qPCR marker levels in target sources ranged from not detectable to ~5.5$\log_{10}$ (median ~1), while levels in non-target animal groups ranged from not detectable to ~3.8 $\log_{10}$ (Reischer et al., 2013). A previously described study from India reported sensitivity of the BacH TaqMan assay as 17%, irrespective of the DNQ classification as it was detected in 13.3% human faeces (from healthy individuals), 40% of wastewater samples and 30% of human faeces (from individuals with diarrhea) in the following concentrations expressed as mean $\log_{10}$ gene copies per nanogram of total DNA: 2.40±0.97 (healthy humans), 2.03±0.35 (sewage) and 2.24±1.03 (humans with diarrhea) (Odagiri et al., 2015). Reported specificity was 83%, regardless of DNQ classification, as the BacH TaqMan marker was detected in 30% of dog samples (1.09±0.87) and 70% of chicken samples (2.49±1.22) (Odagiri et al., 2015).

A.1.1.5 BacHum-UCD TaqMan qPCR.

BacHum-UCD is another human-associated marker developed in TaqMan qPCR format that targets 16S rRNA sequences of the *Bacteroidales* order (Kildare et al., 2007). Initial sensitivity testing indicated 66.7% and 100% sensitivity to human faecal samples (12/18) and wastewater samples (14/14), respectively (Kildare et al., 2007). The assay product was not detected in faecal samples from four non target animal groups (n=33), but it was detected in one of eight dog faecal samples (Kildare et al., 2007). BacHum-UCD qPCR was evaluated in different countries and in several method performance studies. Silkie and Nelson tested performance of the BacHum-UCD marker in California on raw sewage samples and pooled samples from four non-target groups (Silkie and Nelson, 2009). The BacHum-UCD marker was detected in all sewage samples (12 out of 12) with a mean concentration of 8.9 $\log_{10}$ gene copies/100mL of sewage. The BacHum-UCD marker cross-reacted with cow, horse and dog as it was detected in 1 (out of 11 cow pools resulting from 115 pooled samples) at 7.5 $\log_{10}$ gene copies/gram of dry weight faeces, 2 (out of 10 horse pools resulting from 85 individual samples) at 5.3 $\log_{10}$ gene copies/gram of dry weight of faeces, 9 (out of 10 dog pools resulting from 67 individual samples) at 7.6 $\log_{10}$ gene copies/gram of dry weight faeces (Silkie and Nelson, 2009). The BacHum-UCD marker was not detected in Canada geese samples (10 pools resulting from 94 individual samples). Another California based study tested performance of BacHum-UCD qPCR assay against a range of human sources (faeces, septage, sewage) and faeces from four non-target groups (Van De Werfhorst et al., 2011). The BacHum-UCD marker was detected in all human sources (8 faeces, 3 septage and 10 sewage samples) in concentrations (reported as gene copies) ranging from 6.4 x 10^4 to 5.1 x 10^8/gram wet weight (faeces), from 4.2 x 10^4 to 6.5 x 10^8/L (septage) from 6.0 x 10^7 to 8.5 x 10^9/L (sewage) (Van De Werfhorst et al., 2011). The BacHum-UCD marker was also found to cross-react with 10 cat samples (out of 12) with concentrations ranging from 2.0 x 10^3 to 3.9 x 10^5/gram of wet weight (faeces), 9 (out of 12 dog samples) ranging from 1.4 x 10^4 to 8.9 x 10^5, in one (out of 3) gull samples (4.4 x 10^2 per wet weight) and in two (out of five) raccoon samples ranging from 1.1 x 10^4 to 1.5 x 10^5/gram of wet weight (Van De Werfhorst et al., 2011). The BacHum-UCD marker was not detected in rat faecal samples. The California based method comparison study reported sensitivity (n=72) and specificity (n=156) as 97% and 37% when DNQ was considered positive or as 97% and 67% when DNQ was considered negative (Layton et al., 2013). When different types of human sources were considered separately, sensitivity was 100% for all three when DNQ samples were interpreted as positive and it ranged from 92% (sewage) to 100% (human faeces and septage) when DNQ samples were considered negative (Layton et al., 2013). The concentration of the BacHum-UCD marker in target and non-target sources varied by the unit of measure used and median values reported were 7.1±1/2.4±1.7/mg of wet weight, 2.7±1.7/0±1.7/ng of total DNA, 3±1.3/-0.6±1.3/culturable enterococci (USEPA, 2006), -0.4±1.5/-2.9±1.3/enterococci qPCR (Haugland et al., 2005), 1.9±1.1/-1.3±1.8/culturable *E. coli*, 1±0.9/-2.2±1.7/*E. coli* qPCR (Chern et al., 2011), -1.2±0.4/-3.9±1.9/GenBac3 qPCR (Siefring et al., 2008) and -0.4±0.2/-3.8±2.3/BacUni-UCD qPCR (Kildare et al., 2007). The previously described global method evaluation study reported overall sensitivity and specificity as 87% and 68%, respectively (Reischer et al., 2013). The BacHum-UCD marker was detected in 53 (out of 61) human faecal samples, 22 (out of 79) ruminant samples, 13 (out of 28) nonruminant herbivores, 6 (out 29) omnivores, 22 (out of 39) carnivores and 8 (out of 44) birds (30). Concentration in target and non-target sources expressed as $\log_{10}$ marker copies per reaction ranged from not detectable to ~6 (median ~2.5) for human sources and from not detectable to ~5 for non-target sources (Reischer et al., 2013). When data was expressed as $\log_{10}$ per nanogram of total DNA, levels in human faeces ranged from not detectable to ~5 (median ~1.8) and in non-targets from not-detectable to ~4 ((Reischer et al., 2013). A study conducted in India reported sensitivity as 49% or 29%, depending whether DNQ samples were considered positive or negative as it was detected in 40% of human faeces (from healthy individuals or individuals with diarrhea) and 100% of sewage samples (Odagiri et al., 2015). Concentrations were reported as mean $\log_{10}$ gene copies/ng of total DNA: 2.46±1.61 (healthy humans), 2.20 ± 0.75 (sewage) and 2.27 ± 1.06 (humans with diarrhea) (Odagiri et al., 2015). BacHum-UCD assay specificity was 78% (DNQ positive) or 80% (DNQ negative) since it was detected in 10% of buffalo samples, 10% of goat samples, 40% of dog samples, and 70% of chicken samples (Odagiri et al., 2015).

A.1.1.6 1,6-Alpha Mannanase *Bacteroides thetaiotamicron* TaqMan qPCR.

A TaqMan qPCR method targeting the 1,6-alpha mannanase gene from *Bacteroides thetaiotamicron* is reported by the developing laboratory, to exhibit a specificity of 100% (8 non-human animal species; n=283 individual samples) with a sensitivity of 100% for both human faecal samples (n=10; range 6.88 x 10^2-1.07 x 10^9 copies/g wet faeces) and sewage (n=20; 1.34 x 10^1-4.57 x 10^2 copies/ng of total DNA) (Yampara-Iquise et al., 2008). The performance of the TaqMan assay targeting 1,6-alpha mannanase gene from *Bacteroides thetaiotamicron* was

evaluated in the California based method performance study as well (Layton et al., 2013). Reported sensitivity (n=12) and specificity (n=26) were 100% and 54% when DNQ were considered positive and 92% and 96% when DNQ were considered negative. Considering sensitivity results by the animal group source, it was reported as 100% with DNQ results considered positive and it ranged from 75% (sewage) to 100% (human faeces, septage) when DNQ results were considered negative (Layton et al., 2013). Abundance in target and non-target samples was 5.3±0.1/0.9±0.7/mg of wet weight, 1±1.4/-0.9/0.4/ng of total DNA, 1.4±0.9/-1.1±1.2/culturable enterococci (USEPA, 2006), -0.2±1.9/-2.8±1.2/enterococci qPCR (20), 0.2±0.9/-3.7±1.6/culturable *E. coli* and -0.6±0.5/-4.3±1.2/*E. coli* qPCR (Chern et al., 2011). Another study evaluated concentrations of 1,6-alpha mannanase gene quantified by TaqMan qPCR and expressed as cell equivalents (CE) in raw sewage (RS), primary wastewater effluent (PE), secondary wastewater effluent (SE) and tertiary wastewater effluent (TE) as well as specificity tested against 226 non-human faecal samples originating from bird, cow, cat, dog, horse and pig faeces. Average $\log_{10}$ CE/100mL (± standard deviation) of 1,6-alpha mannanase gene were 6.63 ± 0.51 (RS), 6.75 ± 0.40 (PE), 4.13 ± 0.84 (SE) and 3.59 ± 1.12 (TE) (Srinivasan et al., 2011). Regrettably, results from the specificity testing were not reported (Srinivasan et al., 2011).

A.1.1.7 HumM2 TaqMan qPCR.

The HumM2 TaqMan qPCR method is another useful human-associated technology with a reported specificity of 97.2% (21 non-human animal species; n=249 individual samples) with 100% sensitivity in both human faecal samples (n=16) and untreated sewage (n=20; median ~2.8 $\log_{10}$ copies/ng of total DNA) (Shanks et al., 2009). The performance of the HumM2 qPCR assay was evaluated in the United States studies and in India (Odagiri et al., 2015). In the first report, the HumM2 marker was detected in 54 (out of 54) wastewater samples in the concentrations ranging from 1.8 $\log_{10}$ to ~3.5 $\log_{10}$ gene copy number per nanogram of total DNA, as well as 16 (out of 16) individual human faecal samples (3.42±0.05 $\log_{10}$ gene copies per nanogram of total DNA) (Shanks et al., 2010). Cross-reactions were observed with sheep and elk samples with reported mean concentrations of 2.25±0.05 and 1.82± 0.05 $\log_{10}$ gene copies/ng of total DNA, respectively (Shanks et al., 2010). The HumM2 marker was not detected in 20 other non-target animal hosts. Layton and collaborators reported HumM2 assay sensitivity (n=72) and specificity (n=156) values as 93% and 75% when DNQ were considered positive and as 67% and 94% when DNQ were considered negative. Considering sensitivity on the basis of three human sources, reported values range from 83% (sewage), to 96% (septage) to 100% when DNQ samples are considered positive and from 46% (sewage) to 54% (septage) to 100% when DNQ samples are considered negative (Layton et al., 2013). The concentration of HumM2 marker in target and non-target sources was 5.3±0.3 and 0.8±0.9/mg wet weight, 0.9±1.4 and -1.1±0.7/ng of total DNA, 1.1±1 and -0.9±0.8/culturable enterococci (USEPA, 2006), -1.6±1.7 and -3.7±1.3/enterococci qPCR (Haugland et al., 2005), 0.2±0.7 and -2.6 ±1.2/culturable *E. coli*, -0.8±0.5 and -3.2±0.7/*E. coli* qPCR (Chern et al., 2011), -2.9±0.7 and -6.2±1.9/GenBac qPCR (Siefring et al., 2008). A study from India reported sensitivity as 49% (DNQ positive) or 26% (DNQ negative) since the HumM2 TaqMan assay was detected in 40% of samples from healthy humans and 100% of sewage samples (as well as 10% of samples from humans with diarrhea) (Odagiri et al., 2015). Mean $\log_{10}$ concentrations/ng of total DNA in target sources were as follows: 1.57 ± 0.67 (healthy human), 1.95 (sewage) and 1.99 ± 0.97 (humans with diarrhea). Specificity varied from 70% to 92%, depending on whether DNQ samples were considered positive or negative (Odagiri et al., 2015). The HumM2 TaqMan marker was detected in 10% of cow samples (none within quantifiable range), 60% of goat samples (2.17), 30% of sheep samples (none within quantifiable range), 20% of dog samples (0.81), and 60% of chicken (0.88 ± 0.36) samples (Odagiri et al., 2015).

A.1.2 Methanogens

A.1.2.1 *nifH* End-Point PCR.

The *nifH* end-point PCR method is reported (by the developing laboratory) to have a specificity of 100% (10 non-human animal species; n=204 individual samples total) with a sensitivity ranging from 29% in human faecal samples (n=70) to 93% in sewage (n=27) (Ufnar et al., 2006). Sensitivity and specificity of the *nifH* end-point marker has been reported to date only in the United States. A Florida based study tested *nifH* end-point PCR specificity on individual samples from 13 non-target hosts, as well as two farm composite samples (McQuaig et al., 2009). Sensitivity was tested against 16 on-site collection samples, 39 wastewater influents and nine dechlorinated tertiary-treated wastewater effluents (McQuaig et al., 2009). The *nifH* end-point PCR marker was detected only in one (out of 24) individual cow samples and was detected in nearly all target samples, except one septic tank sample, but not in any final effluent samples (McQuaig et al., 2009). In another Florida based study, performance of *nifH* end-point PCR assay was tested against 343 individual and composite samples from 10 non-target groups and 44 target samples (19 wastewater samples and 25 on-site collection samples) (Harwood et al., 2009). The *nifH* end-point PCR marker was reported as 98% specific as it cross-reacted with two cow samples (out of 77), one dog sample (out of 100) and 2 seagull samples (out of 58), but was 100% sensitive as it was detected in all target samples (Harwood et al., 2009).

A.1.2.2 *nifH* TaqMan qPCR

A *nifH* TaqMan qPCR showed increased sensitivity (100%; n=16 ambient water samples with known sewage input; $1.2x10^{1}$-$3.8x10^{3}$ genome equivalents/100mL ambient water), but reduced specificity (50%; four ambient water samples spiked with same bird guano preparation) as reported by the developing laboratory (Johnston et al., 2010). Layton and collaborators tested sensitivity and specificity of the *nifH* TaqMan assay in a California based method comparison study (Layton et al., 2013). When results were interpreted with DNQ considered positive or

negative, sensitivity (n=60) was 78% and 60%, respectively while specificity (n=130) was 68% and 76%, respectively (Layton et al., 2013). Additionally, when different human sources were considered separately sensitivity ranged from 55% (sewage) to 85% (septage) to 95% (sewage) with DNQ samples interpreted as positive (Layton et al., 2013). When DNQ samples were considered negative sensitivity ranged from 20% (sewage) to 65% (septage) to 95% (human faecal samples) (Layton et al., 2013). Abundance of *nifH* qPCR (log_{10} transformed median value) in target samples ranged from 5.7±0.5 per milligram of wet weight, 1.3±1.6 per nanogram of total DNA, 2±1.2 per culturable enterococci (USEPA, 2006), -2.3±2.1 per enterococci qPCR (Haugland et al., 2005)(, 0.2±1.1 per culturable *E. coli* assayed via membrane filtration, -0.5±0.9 per *E. coli* qPCR (Chern et al., 2011) and -3.1±0.8 per GenBac3 qPCR (Siefring et al., 2008). Median log_{10} values in non-target samples were 3.4±1.2/mg of wet weight, 0.8±1.3/ng of total DNA, 1.6±2.2/culturable enterococci (USEPA, 2006), -1.9±2.2/enterococci qPCR (Haugland et al., 2005), -0.7±1.9/culturable *E. coli,* -1.3±1.7/*E. coli* qPCR (Chern et al., 2011) and -4.5±1.3/GenBac3 qPCR (Siefring et al., 2008).

A.1.3 Bifidobacterium

A.1.3.1 B. adolescentis End-Point PCR

A multiplex end-point PCR assay targeting 16S rRNA genes from *B. adolescentis* (ADO) and *B. dentium* (DEN) exhibited 100% specificity (3 non-human faecal samples; 8 individual samples total) (Bonjoch et al., 2004). The sensitivity of ADO (100%) was slightly better than that of DEN (91.7%) when tested against 12 sewage samples (Bonjoch et al., 2004). In addition to the developing laboratory located in Spain, the multiplex end-point PCR assay targeting *B. adolescentis* was also tested on samples collected from Spain, France, Sweden, United Kingdom, Cyprus and the United States. A Spain based study performed sensitivity and specificity testing on a total of 230 samples (Blanch et al., 2006). Sensitivity testing was carried out on 114 wastewater samples collected from municipal wastewater (n=77), hospital wastewater (n=21) and military camp wastewater (n=17) and the *B. adolescentis* end-point PCR marker was not detected in 6.3% of human derived samples (Blanch et al., 2006). Specificity was also lower, since the assay cross-reacted with 24.5% of animal samples comprised from slaughterhouse wastewater (n=57) and farm slurries (n=59) from different non-target groups (cattle, sheep, pigs, horses and poultry) (Blanch et al., 2006). A subsequent study also conducted in Spain, tested performance of the *B. adolescentis* end-point PCR assay on sewage samples from nine wastewater treatment plants, poultry wastewater effluents, swine faeces and slurry, ruminant slaughter houses and bovine farms (Balleste et al., 2010). The *B. adolescentis* end-point PCR marker was detected in 95.6% of wastewater effluents (43 out of 45), and was found to be 74.3% specific as it cross-reacted with 35.3% cow samples (6 out of 17), 18.2% poultry samples (4 out of 22), and 25.7% of swine samples (9 out of 35) (Balleste et al., 2010). Limited data exists in the performance of marker in the United States. When tested on raw sewage samples and against 22 samples from five non-target groups, the *B. adolescentis* end-point PCR marker was detected in two (out of three) sewage samples and three (out of 8) pig samples (Bachoon et al., 2010).

A.1.3.2 B. adolescentis TaqMan qPCR

A TaqMan qPCR assay that targets the 16S rRNA gene from *B. adolescentis* was subsequently developed with a reported specificity of 94.5% (6 non-human animal species; n=67 individual samples total) and sensitivity ranging from 90% in human faecal samples (n=10; $5x10^5$-$1x10^9$ log_{10} gene copies/g) to 100% in sewage (n=8; $1x10^4$-$7.9x10^6$ log_{10} gene copies/gram) (Gourmelon et al., 2010). No performance evaluations of *B. adolscentis* TaqMan qPCR assay were performed to date, aside from the initial developing laboratory report.

A.1.4 Enterococcus

A.1.4.1 esp Gene *E. faecium* End-Point PCR.

An end-point PCR assay targeting the Enterococcus surface protein (*esp*) from *Ent. faecium* exhibited 100% specificity (8 animal groups; n=102 individual samples) with sensitivity values ranging from 100% sensitivity in sewage samples (n=55) to 80% in septage samples (n=10) (Scott et al., 2005). An assay targeting the *esp* gene of *E. faecium* was tested in the United States, Spain and Australia. A Florida based study, tested sensitivity and specificity of the *esp* gene *E. faecium* end-point PCR assay on wastewater samples and individual samples (n=59) from two non-target groups (Korajkic et al., 2009). The *esp* gene *E. faecium* end-point PCR marker was detected in all sewage samples (n=3), but it cross-reacted with three seagull samples (out of 39) and one dog sample (out of 20) (Korajkic et al., 2009). In a California study, the *esp* gene *E. faecium* end-point PCR assay was tested against sewage samples and individual human faecal samples, as well as samples from five non-target hosts (Layton et al., 2009). Sensitivity of the *esp* gene *E. faecium* end-point PCR assay was reported as 92% as it was detected in 24 (out of 26) wastewater samples and it was also detected in ten (out of 12) individual human faecal samples (Layton et al., 2009). The *esp* gene *E. faecium* end-point PCR assay cross-reacted with 64% of non-target samples including all 16 dog samples, eight (out of 22) seagull samples, nine (out of 16) horse, 9 (out of 14) sea lion and all four seal samples (Layton et al., 2009). Increased performance was reported in a Michigan study where sensitivity was tested against untreated and treated wastewater, as well as sludge and on-site septic systems while specificity was tested against individual and composite samples from three non-target groups (Masago et al., 2011). The *esp* gene *E. faecium* end-point PCR marker was detected in all septic tank samples (n=6), 90% of untreated wastewater samples (9/10), 20% of treated wastewater samples (2/10), but not the wastewater sludge sample (n=1) nor any non-target samples (n=17) (Masago et al., 2011). The performance of the *esp* gene *E. faecium* end-point PCR assay was not as good in a Spanish study with reported sensitivity of 77% (10 out of 13) to

wastewater samples and specificity of 68% as it cross-reacted with ten (out of 13) pig samples and one (out of five) cow samples (Balleste et al., 2010). An Australian based study tested *esp* gene *E. faecium* end-point PCR assay performance with wastewater and faeces from 24 non-target species (Neave et al., 2014). The *esp* gene *E. faecium* end-point PCR marker was detected in all wastewater samples tested, as well as samples collected from species of wallaby, one species of wallaroo and a monkey (Neave et al., 2014). An end-point PCR assay targeting the Enterococcus surface protein (*esp*) from *E. faecium* exhibited 100% specificity (8 animal groups; n=102 individual samples) with sensitivity values ranging from 100% sensitivity in sewage samples (n=55) to 80% in septage samples (n=10) (60).

A.1.4.2 esp gene *E. faecium* SYBR Green qPCR

The method was later adapted to a SYBR Green qPCR chemistry with a reported sensitivity of 100% (n=16 wastewater samples; $9.8x10^3$-$3.8x10^4$ gene copies/100mL) (Ahmed et al., 2008). Performance of the SYBR Green *esp* qPCR assay was evaluated only in Australia to date (Ahmed et al., 2009). Authors tested 32 wastewater samples, as well as individual and composite samples from five non-target animal groups (n=50). The *esp* gene *E. faecium* SYBR green qPCR assay was reported to be 100% sensitive and specific (Ahmed et al., 2009).

A.2.0 Ruminant

A.2.1 Bacteroidales

A.2.1.1 CF193 End-point PCR

When originally developed, CF193 exhibited 100% specificity (6 non-ruminant animal species; n=28 individual samples total) with a sensitivity of 100% (6 ruminant or pseudo-ruminant animal species; n=31 individual samples total) (Bernhard and Field, 2000). In addition to the developing laboratory (located in the United States), the performance of the CF193 end-point ruminant MST assay has been tested by other laboratories in the United States (Raith et al., 2013; Shanks et al., 2010), as well as France (Gourmelon et al., 2007) and Spain (Balleste et al., 2010). Further testing in the US was carried out using 247 individual bovine faecal samples, as well as 175 faecal samples representing 24 different non-target animal species (Shanks et al., 2010). The CF193 end-point PCR assay was reported to be 99.9% specific as it cross-reacted with one horse sample (out of 7 tested) (Shanks et al., 2010). Overall prevalence of the CF193 end-point PCR marker was reported at 68% when tested against 11 different cattle herds, but ranged from none detected to 100% in individual samples within a population (Shanks et al., 2010). A multiple laboratory validation study conducted in the United States tested performance of the CF193 end-point PCR assay against pooled samples collected from over 100 individuals and representing 10 different species (human, horse, cow, deer, pig, goose, chicken, pigeon, gull and dog)(Raith et al., 2013). The CF193 end-point PCR assay was reported to be 67% sensitive and 94% specific (Raith et al., 2013). A French study tested sensitivity and specificity on individual faeces from humans (n=44), cows (n=32), sheep (n=12), chickens (n=10), wild birds (n=7) as well as 10 pig liquid manure samples, six sewage sludge (solids) and five sewage sludge liquid samples (Gourmelon et al., 2007). The ruminant CF193 end-point PCR marker was detected in all cow samples and 10 sheep samples, but was absent from all other non-target samples (Gourmelon et al., 2007). The CF193 end-point PCR marker was also not detected in any sludge or pig liquid manure samples (Gourmelon et al., 2007). A Spanish study reported considerably lower sensitivity values (0%) as the CF193 end-point PCR marker was not detected in any of the 19 cow faecal samples, but sensitivity was relatively high (99%) as it was absent from faecal samples of humans (n=39), swine (n=29) and present in one (out of 26) poultry samples (Balleste et al., 2010).

A.2.1.2 Rum2Bac TaqMan qPCR

As reported by the developing laboratory, the Rum2Bac method showed 97% sensitivity (2 ruminant animal species and bovine manure; n=30 individual samples; averages of 7.0±0.5-8.1±0.5 $\log_{10}$ copies/gram) with a specificity of 100% (4 non-ruminant animal species; n=40) (Mieszkin et al., 2010). The performance of the French ruminant Rum2Bac TaqMan marker was evaluated in the comprehensive United States method evaluation study described earlier (Raith et al., 2013). The reported sensitivity and specificity were both 100% with mean $\log_{10}$ gene copies in target sources ranging from 6.17 to 7.64 and <0.1 in non-target sources (Raith et al., 2013). Sensitivity and specificity data, as well as abundance in target and non-target samples were considered using different thresholds for a positive detection including raw data (all detections scored a positive), lower limits of quantification (LLOQ; 10 copies/reaction), 1 nanogram of total DNA, 5,000 copies per reaction of GenBac3 TaqMan qPCR marker (22), 104 MPN enterococci per reaction, or 0.1 mg wet weight of faecal material (Raith et al., 2013). Regardless of detection definition, sensitivity and specificity remained 100% with abundance expressed as mean $\log_{10}$ copies in target/non-target sources measuring 5.25/<0.1, 4.25/<0.1 and 4.31/<0.1 using LLOQ, 1ng total DNA and 0.1 mg wet weight as thresholds, respectively (Raith et al., 2013). However, specificity and abundance in target/non-target sources differed by detection definition: 97% specificity (false positive results with septage) and 5.25/0.80 with raw instrument data, 97% specificity and 3.35/<0.1 with 5,000 copies of GenBac3 TaqMan qPCR, 97% specificity (false positive results with septage) and 5.72/2.07 with 104 MPN enterococci (Raith et al., 2013).

A.2.1.3 BacR Taqman qPCR

Another TaqMan qPCR method targeting a different region of the 16S rRNA gene from ruminant-associated *Bacteroidales* (BacR) was found to be 100% sensitive (7 ruminant animal species; n=57 individual samples; average $4.1x10^9$ marker equivalents/g wet faeces) and did not cross-react with faecal samples collected from 11 non-ruminant animal species (n=131 individual or pooled samples)

(Reischer et al., 2006). In addition to the original developing laboratory study on BacR TaqMan qPCR assay reported by Austrian researchers, performance metrics were determined in France (Mieszkin et al., 2009), United States (Raith et al., 2013), Israel (Ohad et al., 2015), Canada (Ridley et al., 2014), and in a global method evaluation study with reference samples from Argentina, Austria, Australia, Ethiopia, Germany, Hungary, Korea, Nepal, Netherlands, Romania, Spain, Sweden, Tanzania, Uganda and United Kingdom (Reischer et al., 2007). A French study tested performance of the BacR TaqMan marker on pig samples (faeces, slurry, lagoon water and compost), as well as individual bovine (n=10), ovine (n=10), equine (n=10) and human (n=24) faecal samples (Mieszkin et al., 2009). Reported sensitivity was 100% as the BacR TaqMan marker was detected in all ruminant samples with an average estimated concentration of 10 ±0.3 $\log_{10}$ copies/gram of wet faeces (Mieszkin et al., 2009). Reported specificity was 89%, since BacR TaqMan was detected in 17%, 28% and 43% of pig slurry, lagoon water and compost samples, as well as 4% of human faecal samples (Mieszkin et al., 2009). A Canadian study evaluated performance of the BacR TaqMan assay by testing it against bovine (n=26), chicken (n=1), horse (n=2) and pig (n=3) faecal samples, as well as an unspecified number of wild animal samples, liquid dairy manure (n=2), liquid porcine manure (n=3), and 11 septic tank samples (Ridley et al., 2014). Reported sensitivity and specificity were 94.4% (one false negative result) and 93.9% (detection in septic tank and chicken faecal samples), respectively with an average concentration in ruminant faeces of 1.94 x 10^8 copies/gram (Ridley et al., 2014). An Israeli study reported sensitivity and specificity of BacR TaqMan assay as 100% and 99% when tested against an unspecified number of target and non-target animals (Ohad et al., 2015). The previously described United States method comparison study also tested performance metrics of BacR TaqMan assay (Raith et al., 2013). Reported sensitivity and specificity values were 100% and 58-100%, with abundance in target/non-target hosts of 6.17-7.64/< 0.1-1.87 mean $\log_{10}$ copies (Raith et al., 2013). Employing different detection definitions resulted in a wider range of values. When raw data were examined, sensitivity was 100%, while specificity was 85% (false positive results observed with chicken, dog, human and septage samples) and levels in target/non-target sources were 3.91/<0.1 mean $\log_{10}$ copies (Raith et al., 2013). Both sensitivity and specificity were 100% when a LLOQ definition was used with levels in targets/non-targets reported at 3.91 and < 0.1 mean $\log_{10}$ copies (Raith et al., 2013). Sensitivity remained the same when 1 ng of total DNA was used as a threshold, while specificity was 97% as chicken sample(s) provided false positives; levels in targets/non-targets were 3.10 and 0.88 mean $\log_{10}$ copies (Raith et al., 2013). Using 5,000 copies of GenBac3 TaqMan qPCR marker, 104 MPN enterococci, or 0.1 mg wet weight per reaction resulted in sensitivity of 100 %, but specificity ranged from 97% due to false positive(s) in chicken samples (enterococci and wet weight) to 100% (GenBac3 TaqMan qPCR) (Raith et al., 2013). Levels in target/non-target samples were as follows: 2.49/1.48 for GenBac3, 4.88/1.79 for enterococci and 3.47/2.42 for wet weight definitions (Raith et al., 2013). A global method evaluation study described earlier reported overall sensitivity and specificity values as 90% and 84%, respectively as the BacR TaqMan marker was detected in 71 (out of 79) ruminant samples, 4 (out of 28) non-ruminant herbivores, 2 (out of 29) omnivores, 12 (out of 39) carnivores, 9 (out of 44) birds, and 5 (out of 61) humans (Reischer et al., 2013)(30). Concentrations in target and non-target sources was expressed as $\log_{10}$ gene copies/reaction ranging from not detectable to ~7 (median ~3) for ruminant sources and from not detectable to ~5 for non-target sources (Reischer et al., 2013). When data was expressed as $\log_{10}$/nanogram of total DNA, levels in ruminant faeces ranged from not detectable to ~6 (median ~2.2) and in non-targets from not-detectable to ~4 (Reischer et al., 2013).

A.2.1.4 CowM2 End-point and TaqMan qPCR

As reported by the developing laboratory, CowM2 end-point PCR method exhibited 80% sensitivity to cattle faecal samples (n=148) and 100% specificity when tested against 26 animal species (n=279 individual samples) (Shanks et al., 2006). TaqMan qPCR version of the method demonstrated increased levels of specificity (100%) when tested against 15 non-cattle animal hosts (n=201) and similar sensitivity levels (100%) when tested against 60 individual cattle faecal samples (Shanks et al., 2008). The performance of CowM2 end-point PCR and TaqMan qPCR assays have been further evaluated in the United States (Raith et al., 2013; Shanks et al., 2010), Canada (Ridley et al., 2014), Israel (Ohad et al., 2015) and India (Odagiri et al., 2015). The first United States based study is the only study that performed evaluation on both CowM2 end-point PCR and qPCR assay chemistries (Shanks et al., 2010). Reported specificities for both end-point and TaqMan formats were 100% as neither marker was detected in any of the non-target groups tested (175 individual faecal samples from 24 different animal groups) (Shanks et al., 2010). Prevalence of the CowM2 end-point PCR assay in target species ranged from none detected to 100% when 11 different herds were examined (Shanks et al., 2010). Levels in target species for CowM2 TaqMan assay ranged from none detected to ~1 estimated $\log_{10}$ mean copy number/ng of total DNA (Shanks et al., 2010). A United States multiple laboratory method validation study reported sensitivity (100%) and specificity (97-100%), as well as mean $\log_{10}$ copy number in target (4.80-5.48) and non-target sources (<0.1 to 2.69) (Raith et al., 2013). Examining results by the different detection definitions netted the following results in the subsequent order (sensitivity/specificity/abundance in target and non-target sources expressed as mean $\log_{10}$ copies): 100%/100%, 3.14 and <0.1 when considering raw instrument data, 100%/100%, 3.14 and <0.1 when considering data above LLOQ, 75%/100%, 2.25 and <0.1/ng of total DNA, 50%/100%, 1.63 and <0.1/5,000 copies of GenBac3 TaqMan qPCR, 100%/100%, 3.78 and <0.1/104 MPN enterococci, 75%/100%, 2.32 and < 0.1/0.1mg wet weight (Raith et al., 2013). A Canada based study reported 100% specificity of CowM2 TaqMan qPCR assay when tested against chicken (n=1), horse (n=2), pig (n=3) faecal samples along with an indeterminate number of wild animal samples, liquid dairy manure (n=2), liquid porcine manure (n=3) and 11 septic tank samples (Ridley et al., 2014).

Reported sensitivity was 88.9% sensitivity (16 out of 18 target samples positive) (Ridley et al., 2014). The average concentration of the CowM2 TaqMan marker in target sources was 1.44 x 10^6 copies/gram (Ridley et al., 2014). An India based study tested performance metrics of the CowM2 TaqMan qPCR marker as well (Odagiri et al., 2015). When challenged by 30 individual human faecal samples, 5 sewage samples, and 60 pooled animal samples (from cow, buffalo, goat, sheep, dog and chicken) the assay exhibited 50% sensitivity and 100% specificity, regardless of whether DNQ samples were considered positive or not (Odagiri et al., 2015). The reported concentration in target sources ranged from ~1 to 2.2 $\log_{10}$ copies/ng of total DNA (Odagiri et al., 2015). An Israel based study reported sensitivity/specificity of CowM2 TaqMan assay as 50% and 89%, regrettably the number and type of target and non-target sources, as well as levels in target/non-target sources were not reported (Ohad et al., 2015).

A.2.1.5 CowM3 End-point and TaqMan qPCR

As reported by the developing laboratory, CowM3 end-point PCR method showed 91% sensitivity to cattle faecal samples (n=148) and 99% specificity when tested against 26 animal species (n=279 individual samples) (Shanks et al., 2006). Similar to CowM2, TaqMan version of CowM3 also showed increased specificity (100%) and similar sensitivity levels (98%) when tested against 60 individual cattle faecal samples (Shanks et al., 2008). Sensitivity and specificity of CowM3 marker has been evaluated in the United States (Raith et al., 2013; Shanks et al., 2010), Australia (Ahmed et al., 2013) and Israel (Ohad et al., 2015). Shanks *et al.* reported specificity of end-point and TaqMan chemistries as 98.9% (cross-reacted with two alpaca samples) and 100%, respectively when tested against 24 non-target animal groups (Shanks et al., 2010). Prevalence of CowM3 end-point PCR marker in 11 different cattle herds ranged from 0% to 100%, while levels of CowM3 TaqMan qPCR marker in the same samples ranged from non-detected to ~1 $\log_{10}$ estimated target copy/ng of total DNA (Shanks et al., 2010). A multiple laboratory validation study conducted in the United States reported sensitivity and specificity of CowM3 TaqMan qPCR assay as 100%, with levels in target/non-target of 4.52-5.89 and <0.1 mean $\log_{10}$ copies (Raith et al., 2013). When different detection definitions were applied, specificity remained 100%, but sensitivity and levels in target and non-target sources varied (Raith et al., 2013). Using raw instrument data, LLOQ, 1 nanogram of the total DNA 5.000 copies of GenBac3 TaqMan qPCR marker, 104 MPN of enterococci and 0.1 mg of wet weight as detection definitions, sensitivity/levels in target and non-target species were as follows: 100%/2.05 and <0.1, 75%/2.51 and <0.1, 50%/1.33 and <0.1, 0% none detected, 100%/2.69 and <0.1, 50%/1.65 and <0.1(Raith et al., 2013). An Australian based study evaluated performance of the CowM3 TaqMan qPCR assay with individual faecal samples from cattle (n=20), birds (n=10), chickens (n=10), dogs (n=10), ducks (n-10), kangaroos (n=10), pigs (n=10), possums (n=10), horses (n=10), as well as bovine (n=20) and human wastewaters (n=20) (Ahmed et al., 2013). Sensitivity of the CowM3 TaqMan qPCR assay was reported as 90% as it was detected in 16 cattle faecal samples and 20 bovine wastewater samples (Ahmed et al., 2013). Specificity was determined to be 90%, as it was detected in five dog samples, four duck samples, and two possum samples (Ahmed et al., 2013). A study conducted in Israel, reported sensitivity and specificity of CowM3 TaqMan assay as 93% and 99%, respectively (Ohad et al., 2015).

A.3.0 Porcine

A.3.1 Bacteroidales

A.3.1.1 PF163 End-point PCR

When originally developed, the end-point PCR method PF163 exhibited a specificity of 100% (pooled samples from 10 non-porcine animal species) with a sensitivity of 100% against two pooled porcine faecal samples (Dick et al., 2005). Performance of the PF163 end-point assay has been evaluated in the US (Toledo-Hernandez et al., 2013; Boehm et al., 2013; Lamendella et al., 2009), Canada (Fremaux et al., 2009) and France (Gourmelon et al., 2007). Lamendella *et al.* tested 215 faecal samples from pigs, cattle, humans, chicken, raccoons and horses as well as four manure pig pit and three waste lagoon samples (pig and/or cattle) (Lamendella et al., 2009).The assay was detected in all of the pig manure pits and lagoons and in 40 to 100% of pig faeces tested (Lamendella et al., 2009). PF163 end-point assay was also detected in 40% (9 out of 20) cattle, 30% (3 out of 10) human, 50% chicken (4 out of 8), 4% racoon (3/68) and 67% horse (8 out of 12) faecal samples (Lamendella et al., 2009). Puerto Rico study determined sensitivity and specificity of PF163 end-point assay by testing it against 340 faecal samples from cow (n=66), goat (n=32), horse (n=28), swine (n=30), monkey (n=9), fish (n=12), pigeon (n=11), chicken (n=97) and five wastewater samples (Toledo-Hernandez et al., 2013). Marker was detected in all of the pig samples, but it also cross-reacted with 100% of goat samples, 100% of horse samples and 80% of wastewater samples (Toledo-Hernandez et al., 2013). US based multi-laboratory validation study described earlier also performed sensitivity/specificity testing on the PF163 end-point assay and reported both to be greater than 80% (Boehm et al., 2013). A Canada based study tested PF163 marker against a total of 62 faecal samples from humans (individuals and sewage) and 50 samples from various animals including cow, pig, chicken, goose, moose, caribou, bison, goat and different species of deer. Assay exhibited 100% sensitivity and specificity (Fremaux et al., 2009). French study detected PF163 in all 25 pig faecal samples tested, as well as all of the pig liquid manure samples tested (n=10) with reported sensitivity of 100% (Gourmelon et al., 2007). Reported specificity was 98% as it was detected in two (out of 10) chicken samples but was absent from a44 human faecal samples, 32 cow faecal samples, 12 sheep faecal samples and seven wild bird samples (Gourmelon et al., 2007). PF163 was also not detected in any sewage sludge samples (n=6) or wastewater samples (n=5) (Gourmelon et al., 2007).

A.3.1.2 Pig2Bac TaqMan qPCR

The Pig2Bac TaqMan qPCR method demonstrated 100% sensitivity when tested against pig faecal samples (n=25; average 8.5±0.6 log_{10} copies/gm wet faeces), swine slurries (n=25; average 4.9±0.7 log_{10} gene copies/mL), lagoon waters (n=14; average 2.6±0.4 log_{10} gene copies/mL) and compost (n=14; average 5.3±0.6 log_{10} gene copies/gm) and 100% specificity (4 non-porcine animal species; n=54 individual samples as reported by the developing laboratory (Mieszkin et al., 2009). Performance of the Pig2Bac TaqMan qPCR assay was tested in a United States multiple laboratory validation study (Boehm et al., 2013) and in Israel (Ohad et al., 2015). The United States based study reported high sensitivity (100%, detected in 20 out of 20 pig samples), but low specificity (~40% to ~90%, depending on the laboratory) as it cross-reacted with samples from dog and human faeces, as well as septage (Boehm et al., 2013). Levels in target sources, reported as log_{10} median in units of copies per colony forming unit (CFU) of enterococci were 5.0 with all 20 samples within range of quantification (Boehm et al., 2013). For the non-target sources, 73% were not detected, 44% were detected but not quantified and 1% was within quantifiable range, but median was classified as not detected when expressed in log_{10} median units of copies per enterococci CFU (Boehm et al., 2013). A study conducted in Israel reported both sensitivity and specificity as 100%, but unfortunately non-target animals tested and levels in target samples were not specified (Ohad et al., 2015).

A.4.0 Avian

A.4.1 *Helicobacter* spp.

A.4.1.1 GFD SYBR Green qPCR

As originally reported, GFD exhibited 100% specificity when tested against 16 non-avian animal species (n=305 individual samples) and yielded 57% sensitivity when tested against 15 different avian species (n=768) (Green et al., 2012). In addition to the method developing laboratory, which evaluated method performance in the United States and New Zealand, sensitivity and specificity of the GFD SYBR Green assay was also measured with reference samples from 19 animal groups collected in the United States and Australia (Ahmed et al., 2016). In Australia and the United States, the prevalence of the GFD SYBR Green marker was reported as 58% and 30%, respectively with mean concentration of 5.2 x 10^3 gene copies/10 ng of total DNA (Ahmed et al., 2016). Specificity of the GFD SYBR Green marker was higher in the United States (100%) compared to Australia (94%) where it cross-reacted with dog, kangaroo, possum and sheep samples with a mean concentration in non-target samples of 56 gene copies/10 nanograms of total DNA (Ahmed et al., 2016).

A.4.2 Catelicoccus spp.

A.4.1.1 Gull4 TaqMan qPCR

Gull4 is a TaqMan qPCR method that targets the 16S rRNA gene of *C. marimammalium* (68). The method was tested for sensitivity against gull faeces (n=255), as well as various poultry and waterfowl species (n=249) and six non-avian species (n=180) (61). Gull4 is reported to be 86.7% sensitive to gull (average ~1x10^5 copies/ng of total DNA) and 15.3% for poultry and waterfowl (68). Specificity of the assay was nearly 100%, as it cross-reacted with only one pig faecal sample, but not 179 faecal samples from five other non-avian species (Ryu et al., 2012). No performance evaluations of *Catelicococcus spp.* Gull4 TaqMan qPCR assay were performed to date, aside from the initial developing laboratory report.

A.4.3 Brevibacterium spp.

A.4.3.1 LA35 SYBR Green and TaqMan qPCR

The LA35 SYBR Green chemistry qPCR method targets the 16S rRNA gene from *Brevibacterium* spp. (Weidhaas et al., 2010). Method sensitivity (76%) was determined using chicken litter (bedding) (n=17; 1.5x10^7-3.7x10^9 gene copies/gm) and individual chicken faecal samples (n=40; ≥ 2.8x10^4 gene copies/gm). In addition, LA35 exhibited 93% specificity when tested against 116 non-chicken individual faecal samples from five animal species and wastewater (Weidhaas et al., 2010). The method was recently adapted to TaqMan chemistry (Weidhaas et al., 2013), but there are no reports to date that further tested its performance. The performance of LA35 SYBR Green qPCR assay was tested in one United States based study to date. Sensitivity of the method was assessed by testing the assay against chicken litter (n=40) and poultry faecal samples (n=186) (Ryu et al., 2014). Overall, 97.5% of chicken litter samples and 22.6% of faecal samples were positive with mean values of ~7 (litter) and ~3.5 (poultry faeces) log_{10} copies/gram of sample (Ryu et al., 2014). The LA35 SYBR Green qPCR marker was detected in 8.9% of non-poultry avian species (5 out of 16 duck, 5 out of 25 Canada goose, 1 out of 11 guineafowl, 2 out of 64 gull, 1 out of 6 mallard and 3 out of 22 swan samples) (Ryu et al., 2014). Mean log_{10} copy number/gram of sample from non-poultry avian species was ~ 2.9 (Ryu et al., 2014). The LA35 SYBR Green qPCR marker was not detected in 8 non-target groups or any sewage samples (Ryu et al., 2014).

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