Chapter 9
Marine and Freshwater Fecal Indicators and Source Identification

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Glossary

Alternative fecal indicators	Fecal indicators that have not been fully validated for standard water quality methods, but show potential for increased sensitivity or specificity over current indicators.
Commensal	The general meaning of this word is sharing of food and originates from the Latin word *cum mensa*, meaning "sharing a table." In the context of bacteria and host interactions, the bacteria benefit from the host without causing harm.
Enterococci	The term "enterococci" is a general reference to members of the genus *Enterococcus*: however, in the context of water quality standards, enterococci often refers to *E. faecalis* and *E. faecium*, which can be enumerated using selective and differential media.

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Escherichia coli (*E. coli*)	Gram-negative bacteria found in the gastrointestinal tract of almost all warm-blooded animals. These bacteria are easily cultured and can be enumerated using selective and differential media.
Fecal indicator	A chemical or biological constituent that is found in fecal matter that can be used to demonstrate the presence of contamination.
Pathogen	A microbe or microorganism such as a bacteria, virus, fungi, prion, or protozoan that causes disease in its animal or plant host.
Polymerase chain reaction (PCR)	A scientific technique in molecular biology to amplify a single or few gene copies of a nucleic acid fragment across several orders of magnitude. Amplification results in the generation of thousands to millions of copies of a particular nucleic acid sequence.
Quantitative PCR	A technique based on PCR which simultaneously amplifies and quantifies a targeted nucleic acid molecule.

Fecal Indicator Definition

Fecal indicators are organisms or chemical constituents found in fecal material or wastewater that can be measured to demonstrate the presence of fecal pollution. Fecal waste from humans and other animals can contaminant surface waters and pose a serious threat to the environment and human health. Fecal pollution serves as a vehicle for disease transmission including pathogenic bacteria, viruses, or protozoa. Fecal waste also carries with it harmless *commensal* organisms that live in the gastrointestinal (GI) tract and are often used as fecal indicators since they are present in high numbers. The type and amount of pathogens found in fecal pollution is dependent on the host source (human, agricultural animal, wildlife) and the prevalence of illness in the host population. Therefore, employing fecal indicators that provide information about human and other animal contributions is critical for estimating the likelihood that pathogens are present and for directing remediation efforts.

Introduction

Fecal indicators when detected demonstrate the presence of fecal pollution. Fecal indicators play an important role in regulation. Governmental agencies charged with the protection of human health use indicators to assess recreational water quality. In much of the developed world, *Escherichia coli* and *enterococci* are the

organisms used for this purpose. Their quantitative link to human health risk in recreational epidemiology studies has led to development of water quality criteria to limit their concentrations in the USA and worldwide.

Conventional indicator methods focus on the cultivation of *E. coli* or enterococci cells isolated from an environmental sample. Culture-based methods are inexpensive and do not require extensive laboratory training to implement. However, these methods are time consuming, requiring 18–24 h to process samples. They also have other limitations such as the inability to discriminate between different animal sources and the potential of indicator microorganisms to persist and sometimes proliferate in the environment. As new scientific discoveries provide a broader view of the different microbes or chemicals associated with fecal pollution and specific sources, new indicators are being identified. These indicators are often referred to as alternative indicators since they have not been fully validated for use for standard methods in water quality testing, but show promise to address some of the limitations associated with conventional fecal indicator approaches.

Some alternative indicators are common to all sources of fecal pollution and can be used as general fecal pollution indicators. Others are associated with a particular host or group of animals. Host-associated indicators are useful for fecal source identification approaches, which are aimed at improving estimates of potential health risk due to pathogens, or identifying major pollution sources that should be remediated. Alternative indicators may take the place of conventional indicators as technology advances. Technologies such as real-time *quantitative PCR* (qPCR), flow cytometry, and advanced chemical analyses can detect previously uncultured microbes or chemicals associated with fecal pollution.

Impact of Fecal Pollution on Coastal Waters

Coastal waters are a valuable resource. Fecal pollution of beaches is not only a threat to human health [13, 52, 98, 195, 258], but can also result in economic losses to surrounding communities [113, 197]. Within the USA, the ocean and Great Lakes coasts encompass more than 15,000 miles of coastline and are the home of economic and recreational centers and unique and rich ecosystems. Many coastal areas are stressed because of dense development and subsequent anthropogenic impacts (Fig. 9.1). Studies have shown that with increasing urbanization, there is an increase of fecal pollution in waterways [91, 151, 261]. Agricultural land use in upper reaches of watersheds also contributes to fecal pollution in tributaries that ultimately discharge into the ocean or the Great Lakes [24, 261]. Fecal pollution is the major cause of biological water quality impairment in the USA and is the primary cause of recreational beach advisories and closing [252]. Currently, fecal pollution impacts are determined by measuring fecal indicator bacteria using conventional, culture-based approaches. In 2009, there were 18,682 advisories and closures at 2,876 beaches in the USA that are routinely monitored for fecal pollution.

Fig. 9.1 Plume of river water released into Lake Michigan from the Grand River (Photo provided by Dr. Philip Roberts, Georgia Institute of Technology)

The Link Between Waterborne Disease and Fecal Pollution

Fecal pollution may contain pathogens that can cause disease in humans. To date, there are more than 150 different agents of disease that can be considered waterborne pathogens. This list grows each year as additional emerging pathogens are identified. Table 9.1 lists common waterborne pathogens and their major host reservoirs. The primary reservoir of human viruses is humans themselves because viruses by nature are host specific; however, animal viruses may also be a concern if they are able to replicate in human hosts. Recent research has identified pigs as a reservoir of hepatitis E virus [99]. Sewage may contain high concentrations of human viruses and some studies have performed surveillance of the viral diseases in the community by monitoring sewage [213, 214]. Some pathogens are predominately found in nonhuman animal hosts, but if humans become infected, person-to-person or waterborne transmission may occur.

Exposure to contaminated water and potential waterborne pathogens most notably causes enteric illness, but skin, ear and eye, or respiratory illnesses may also

Table 9.1 Common pathogens responsible for enteric illnesses

Agent		Major sources	References
Viruses[a]			Reviewed by Fong and Lipp [76]
	Enteroviruses	Human	[32, 91, 122, 125, 167, 178]
	hepatitis A	Human	[86, 91, 122]
	Adenoviruses	Human	[51, 108, 122, 124, 125, 271, 273]
	Norovirus	Human	[17, 91, 263, 273]
	Rotavirus	Human	[85, 202]
	Astrovirus	Human	[47, 194]
Bacteria			
	Pathogenic *E. coli*	Humans and animals[b]	[5, 102, 109, 171, 260]
	Shigella	Humans and animals	[118]
	Vibrio cholerae	Humans and environment	[129, 130, 147]
	Campylobacter	Animals and humans	[8, 33, 112, 183, 254, 260, 270]
	Salmonella	Animals and humans	[21, 100, 144, 254]
	Yersinia enterocolitica	Animals	[104, 205]
	Aeromonas	Environment	[192, 215]
	Plesiomonas	Environment	[11, 166, 203]
	Vibrio parahemolyticus	Environment	[55, 127, 254]
Parasites			
	Cryptosporidium parvum	Animals and humans	[90, 112, 144, 149, 270]
	Giardia lamblia	Animals and humans	[90, 112]
	Entameba histolytica	Animals and humans	[152]

[a]Humans are the predominate source of human viruses, but in some cases, transmission from animals to human is possible. For example, this is suspected to be possible for hepatitis E [99], a calicivirus in the same virus family as norovirus

[b]Animals are the primary reservoir of *E. coli* O157:H7 [46], one strain of shiga-toxin-producing *E. coli*

Table 9.2 Common pathogens responsible for respiratory illness and skin infections

Agent		Primary source	References
viruses			
	Adenovirus	Humans	[51, 108, 122, 124, 125, 271, 273]
Bacteria			
	Vibrio parahemolyticus	Environment	[55, 127, 254]
	Vibrio vulnificus	Environment	[187, 264]
	Leptospira	Animals (wildlife)	[96, 236]
	Legionella spp.	Environment	[82, 186, 236]
	Staphylococcus aureus	Humans	[48, 49, 88, 254]

For an expanded list, please see [27, 168]

occur [27, 39, 65, 141, 195, 258]. Many waterborne disease agents are passed through the fecal-oral route, so any activities that involve ingesting contaminated water present a health risk. Ingesting contaminated seafood may also result in exposure to waterborne pathogens (Table 9.2). For respiratory diseases, inhalation of water droplets or direct contact with mucus membranes can expose a person to a disease-causing agent. Direct contact of contaminated water with wounds could result in an infection.

Recreational waters are of particular concern because swimmers can come into direct contact with contaminated water. Shellfish beds can also be impacted by fecal pollution and are regularly monitored to assure that harvested shellfish has not been subjected to contamination. In the Great Lakes, nearshore coastal waters are a drinking water source to nearly 40 million people. Stringent treatment requirements provide safe drinking water, but both source water and treated drinking water are closely monitored for evidence of fecal pollution to assure that treatment protocols are adequate. For a more complete discussion of this topic, see Chaps. 3–5 of this volume.

Important Attributes of Indicators. Fecal indicators can either be general indicators of fecal pollution or associated with a particular animal source. Many watersheds and coastal waters have mixed land use; therefore, both general fecal indicators and source-associated indicators have an important role in assessing water quality. Ideally, fecal indicators should be present in high levels in fecal pollution so that they can be used as a sensitive measure of the level of contamination when diluted to small concentrations in the environment. Fecal indicators should provide information about host source contribution when possible, whether it is from humans, or different agricultural animals or wildlife. Detection methods should be relatively simple and affordable considering that much of the hands-on monitoring is done by local health departments. Methods should lend themselves to rapid testing so that beach notification can happen in a timely manner.

Clearly, no single indicator can meet all of these goals. Therefore, it is critical to have multiple indicators that can be used in concert if needed. Different indicators will behave differently in various environments, e.g., marine waters versus freshwater [9, 92, 180, 227, 229]. Certain indicators may be appropriate for investigating sources of fecal pollution, or setting remediation goals, whereas others are better

suited for rapid detection for recreational water quality monitoring for any fecal pollution present. Water resource managers, public health officials, and researchers must work together to identify what information is needed and choose the most appropriate indicators. For example, *E. coli* is recommended for freshwater, but it has a very short half-life in the open waters of the Great Lakes [160]; therefore, highly persistent indicators such as *Clostridium perfringens* may be more useful for long-term monitoring [71, 146, 169]. Enterococci qPCR is being developed for rapid beach testing [93, 106], but is a general indicator and host-associated indicators within the order *Bacteroidales* may be more useful for identifying sources [31, 72, 236].

Important Attributes of Fecal Indicators
Routine monitoring: Specific for fecal pollution
Present when pathogens are present
Correlate well to illnesses
Easy to quantify
Simple and cost-effective methodology
Behave in the environment in the same manner as pathogens
No growth outside of the host environment
Amenable to rapid detection methodologies
Investigations: Specific for a source of fecal pollution
Sensitive, e.g., present in high numbers in most animals with a given source
Known or predictable ecology
Known or predictable relationship to pathogens

Detection of Conventional Indicators

Common fecal indicators that are used for water quality monitoring or recreational beaches are listed in Table 9.3. All of these indicators were originally identified as constituents of fecal pollution using selective and differential culture techniques. The earliest methods data back to late 1800s and early 1900s [14, 66] for coliform bacteria. There are two culture approaches for enumerating bacteria in water samples. The most probable number (MPN) methods involve culture-based detection in liquid broth using a series of dilutions. The dilutions in which organisms are detected can be used to calculate a statistical estimate of *enterococci* concentration for that sample. The second approach involves filtering samples through a membrane filter. The filter is transferred to solid selective media that is optimized for the growth of the target organisms and inhibitory for other organisms. Various chromogenic substrates or pH indicators can be incorporated to make the media differential for fecal indicator microorganisms. A review of conventional and novel indicators can be found in Edge and Boehm [69].

Culture-based methods continue to be widely used for detection of fecal indicator bacteria; however, the time required to obtain a result is a major limitation

Table 9.3 Historic and conventional fecal indicators

Organisms	Use	Limitations
Total coliforms	Early indicator used in surface waters, currently in use for drinking water[a] since detection of total coliforms provides information on general sanitation	Not specific for fecal pollution
Fecal coliforms	Used for recreational waters until late 1980s or 1990s in some US states, still in use as a standard for wastewater, recreational waters, and shellfish	Some fecal coliforms can grow in the environment
E. coli	Currently recommended by the USEPA for fresh recreational waters[b]. Replaced fecal coliforms as a more specific indicator of fecal pollution	More specific than fecal coliforms, but has been reported to persist and grow in the environment [7, 117, 134, 265]
Fecal streptococci	Early indicator for surface water quality	Not all are fecal specific
Enterococci	Currently recommended indicator for marine recreational waters. Replaced fecal streptococci	General indicator; some grow in the environment
Clostridium perfringens	Proposed in 1963 as an indicator of wastewater and receiving waters. Used in some European countries but not the USA	May survive for long periods in the environment.

[a]Total coliforms (TC) are used since they are a good measure of bacteriological contamination, regardless of fecal or environmental sources. New proposed rules would change the standard from TC to *E. coli*, e.g., more specific for fecal pollution

[b]Criteria are being revised; new criteria will be based on detection of enterococci by culture or qPCR (total *Bacteroides*)

of these methods for providing rapid (e.g., 4 h) results of beach water quality to assure timely public notification. Molecular methods such as qPCR can be used for detection of traditional fecal indicators [106, 182, 267].

Coliforms. Coliform bacteria are a group of bacteria that were the first indicators of fecal pollution. Coliforms are gram-negative, rod-shaped, facultatively anaerobic, non-spore-forming bacteria found in warm-blooded animals, as well as in soil, water, and vegetation. Coliforms are not a specific taxonomic group of bacteria, but are classified based on a number of characteristics. These organisms are identified by fermentation of lactose with the production of acid and gas at 35–37°C. Coliforms are also negative for cytochrome oxidase and positive for β-galactosidase. Coliforms are measured by using an MPN [16] or by enumeration of colony-forming units (CFU) using membrane filtration and selective and differential media such as MI [249]. These organisms generally are within the family *Enterobacteriaceae* and include the genera *Citrobacter*, *Escherichia*, *Enterobacter*, *Hafnia*, *Klebsiella*, and *Serratia*.

Coliform bacteria were one of the earliest indicators of water quality used in the USA, with individual states setting limits of 50–2,400 coliforms per 100 ml of water as a standard for recreation waters in the 1950s and 1960s [66].

Fecal coliforms. Fecal coliforms are a subgroup of coliforms and refer more specifically to coliforms derived from feces. Like coliforms, they are not a specific taxonomic group; they are based upon several morphological and physiological characteristics. These are defined by the same criteria as coliforms, but are thermotolerant and will grow at 44.5°C. *E. coli* is one of the major fecal coliforms found in feces, in addition to members of *Klebsiella*, *Enterobacter*, and *Citrobacter*. The designation of fecal coliforms was intended to improve specificity; however, some organisms included in this group can be found free living in the environment, most notably *Klebsiella* [42, 83, 177]. Beach water samples have also been found that have evidence of fecal coliforms that have replicated in the environment [158].

The first national water quality criterion for recreational waters was based upon fecal coliforms. In 1968, the National Technical Advisory Committee, commissioned by the Federal Water Pollution Control Administration (now referred to as the Environmental Protection Agency), determined that 400 fecal coliforms per 100 ml corresponded to an adverse GI health effect [66]. Subsequent recommendations stated that for recreational waters, within a 30-day period, the geometric mean should not exceed 200 fecal coliforms per 100 ml, and 10% of the samples should not exceed 400 fecal coliforms per 100 ml. Fecal coliforms are no longer used for recreation waters in most states, but the basis of the 1968 criteria is still used for regulating water quality of wastewater treatment plant effluents and for assessing river water quality. Fecal coliforms are also still used for shellfish testing (water overlying the reefs and oyster meats).

Escherichia coli (E. coli). *E. coli* is a fecal coliform that has been suggested to be more specific for fecal pollution than testing for the group of fecal coliforms and was recommended as an indicator for freshwater in 1986 by the United States Environmental Protection Agency (USEPA) [14, 247]. *E. coli* are present in the GI tract of most warm-blooded animals, and therefore a general indicator of fecal pollution. *E. coli* is a thermotolerant coliform that produces indole from tryptophan and it can be differentiated from other microorganisms based on β-glucuronidase activity. Selective and differential media tests for this activity using methods based on membrane filtration, modified mTEC [248], or MPN approaches such as the Colilert manufactured by IDEXX [68] are commonly used to identify *E. coli* in surface water samples. One testing methodology simultaneously detects coliforms and *E. coli* using β-galactosidase and β-glucuronidase activity, respectively, as discriminators [249]. Some epidemiology studies have shown a relationship between *E. coli* densities and GI illness [65, 195]. *E. coli* has some limitations as a fecal indicator at recreational beaches because it has been shown to persist and even grow in some aquatic environments, thereby potentially interfering with the relationship between *E. coli* and recent fecal pollution events [7, 26, 134, 265].

Enterococci. Enterococci are gram-positive cocci and are nearly universally present as commensal organisms in the intestine of human and nonhuman animal hosts. The most common species in human hosts are *E. faecalis* and *E. faecium* [58, 139].

The enterococci are a subgroup of the fecal streptococci. Fecal streptococci have also been referred to as Group D streptococci according to Lancefield serotyping. The fecal streptococci have historically been used as fecal indicators and include species from two genera: *Enterococcus* and *Streptococcus*. There are two *Streptococcus* species in the fecal streptococci group – *Streptococcus bovis* and *Streptococcus equinus* – that have been shown to survive poorly in water. Hence, in water, fecal streptococci and enterococci are thought to be equivalent [116].

In the USA and the EU, enterococci are used for monitoring marine bathing waters because epidemiology studies have linked their concentration to human health outcomes [256]. The standards are tied to approved culture-based methods for their quantification: multiple-tube fermentation, membrane filtration, and defined substrate assays. Clesceri et al. (1998) describe a multiple-tube method where azide dextrose broth is used followed by confirmation with Pfizer selective *Enterococcus* (PSE) media and brain-heart infusion broth with 6.5% NaCl. Both the EU and the USA have approved the use of defined substrate assays manufactured by IDEXX for the quantification of enterococci (Enterolert and Enterolert-E). The USEPA-approved method 1600 utilizes membrane filtration onto mEI media for quantification [250]. Studies that have compared these culture-based methods for quantifying enterococci often find the methods yield slightly different results [32].

The USEPA has developed a qPCR assay for the enumeration of enterococci which has been compared to membrane filtration results [106, 253]. Enterococci measured via qPCR often yield higher concentrations than culture-based measurements since it enumerates both live and dead bacteria [32]. Enterococci measured by qPCR have been linked to human health outcomes in epidemiology studies of marine and fresh water beaches [257–259]. Ongoing work is focused on better defining these links. As the USEPA formulated new recreational water quality criteria, qPCR for enterococci is expected to be included as a rapid method which allows beach managers and public health workers to post water quality advisories on the same day the sample is taken.

Clostridium perfringens. *C. perfringens* is member of the phylum *Firmicutes* and is a gram-positive, low GC content organism. *C. perfringens* was suggested as a potential indicator in 1963 [34], and gained acceptance in EU countries, but it was not chosen for use in the USA because it survives for long periods of time in the environment [14, 66]. Epidemiology studies report a relationship between *C. perfringens* and illness [268], while other studies found no relationship [39]. However, *C. perfringens* has been shown to be a useful fecal indicator in certain environments where other indicators are highly modulated by environmental factors. Studies in tropical waters suggested *C. perfringens* is a better indicator compared with fecal coliforms because it is a spore-forming organism and does not replicate in the environment [79, 80]. Because of its spore-forming ability, *C. perfringens* has been used as a tracer of long-term fecal pollution impacts in marine and freshwater systems [36, 54, 70, 110, 169]. *C. perfringens* has also been suggested as a good indicator in the open waters of the Great Lakes because it can serve as a conservative tracer of fecal pollution and may mimic protozoan cyst or oocyst survival [164, 169, 191].

Table 9.4 Examples of biological *alternative fecal indicators* that provide animal host information

	(Notes)	References
Organisms – 16S rRNA gene		
Bacteroidales	*Bacteroidales* associated with hosts have been identified	[25, 60, 75, 78, 133, 137, 138, 153, 165, 184]
Bifidobacterium		[35, 64, 148, 173]
Methanobrevibacterium	Member of Archaea and dominant in the GI	[128, 243–245]
Faecalibacterium		[161, 279]
Lachnospiraceae		[161, 175]
Ruminococcace		[161]
Functional genes		
esp gene in enterococci	Gene responsible for attachment on human epithelial cells	[93, 212]
Toxin genes in *E. coli*		[50, 131]
Beta-glucuronidase	Polymorphisms in this gene have been linked to different host types	[200]
Unknown genes/regions		
Cattle and human markers	Identified by genomic fragment enrichment	[220, 221]
Gull marker	Identified by subtractive hybridization	[101]
Phenotypes		
Antibiotic resistance of standard fecal indicators	Based on the theory that host exposed to antibiotics will have a higher percentage of antibiotic-resistant *E. coli* or enterococci	[97, 105, 188, 269]
Viruses		
F+ coliphage	Type I and IV associated with animals and II and III associated with humans	[114]
Bacteroides phage		[12, 126, 240]
Adenovirus, enterovirus, polyomaviruses	Viruses are host specific by nature, and therefore, detection of human viruses demonstrates human sources are present.	[6, 123, 162, 179, 193]

Alternative indicators. Ongoing research studies have identified a broad array of new potential indicators of fecal pollution. Molecular-based methods have made possible the characterization of organisms that previously were either not recognized as associated with fecal pollution, or were difficult to detect due to complex cultivation requirements. Alternative indicators may also employ unique chemical constituents. Alternative indicators are being developed as general detection of fecal pollution, such as total *Bacteroides* [53, 59], as well as source identifiers associated with a particular animal group (Table 9.4).

Different sources of fecal pollution can contribute different types and concentrations of pathogens (Table 9.1 and Table 9.2). For example, human fecal sources, particularly sewage, contain waste from a large number of people and are

considered a primary source of human enteric viruses. *Cryptosporidium* may be associated with cattle waste. Fecal indicators that provide information about the source will improve our ability to estimate the health risk due to pathogens as well as direct remediation efforts to major contributing sources of fecal pollution. The development of qPCR methodology has also advanced simple presence/absence detection to quantitative estimates of fecal pollution and provides a platform for the implementation of rapid methods.

16S rRNA gene targets. Many of the alternative indicators that have been described are based on detection of the organisms based on the 16S rRNA gene sequence. This gene is highly conserved among bacteria and has been used extensively to assign taxonomy.

Bacteroidales. Members of the order *Bacteroidales* are potentially useful indicators of fecal contamination because they generally are found in high numbers in fecal material of humans and other warm-blooded animals and are unlikely to survive in the beach environment [74, 136]. Early studies identified unique sequences in the *Bacteroides* 16S rRNA gene from human and ruminant *Bacteroides* species that are associated with respective fecal pollution sources [24, 136]. Sequencing of clone libraries demonstrated that sequences of members of the broader *Bacteroidales* group, rather than exclusively *Bacteroides* spp., are amplified with primers originally targeting total *Bacteroides* spp. [60]. Subsequent studies have used taxon-specific cloning to characterize *Bacteroidales* populations within humans and different animals and have identified a broad range of host-associated genetic markers [25, 60, 75, 78, 121, 133, 137, 138, 153, 165, 184]. Since culture techniques for isolation of these anaerobic bacteria are difficult to perform, molecular techniques have been developed to amplify, detect, and in some cases quantify the 16S rRNA genes of *Bacteroides* spp. from feces and ambient water [53, 59, 133, 153, 218, 262]. Many of these assays utilize the HF183 sequence first reported by Bernhard and Field [25]. The utility of the genetic markers has been tested extensively in fecal impacted environments, including beaches [1, 37, 84, 181, 207, 216]. In addition, numerous studies report information on the distribution of these host-associated genetic markers in target and non-target populations [3, 64, 133, 138, 143, 185, 224, 225, 228], relationship to pathogens [208, 209], and the decay of these genetic markers in marine and freshwaters [20, 61, 184, 210, 261].

Bifidobacterium. This genus represents another group of GI bacteria with particular species reported to be associated with human fecal pollution including *B. adolescentis*, *B. dentium*, and *B. longum* [35, 148, 155, 173]. Several technologies targeting *Bifidobacterium* genes are reported for multiplex PCR detection [35] and qPCR [150, 154]. *Bifidobacterium* typically occur at lower concentrations than *Bacteroidales* making them harder to detect in dilute ambient water samples [219] and exhibit a rapid decay based on bench-scale survival studies [201]. Thus, the detection of a *Bifidobacterium* host-associated genetic marker in a polluted water sample suggests a recent, high concentration contamination event.

Faecalibacterium. This genus of bacteria has been reported in humans and other animals and has been suggested as a potential target for development of host-associated genetic markers [81, 161, 246, 279]. Sewage and cattle have been

shown to have a high abundance of *Faecalibacterium* [161, 226]. Additional characterization of this group is needed to characterize phylotypes that are associated with specific animal sources.

Lachnospiraceae. *Lachnospiraceae* are found in high abundance in human fecal samples [57, 77, 242], sewage [161], and cattle [226]. *Lachnospiraceae* are included in the group *Clostridium coccoides* [107, 150]. The proportions of *Lachnospiraceae*, *Bacteroides*, and *Bifidobacterium* of the human microbiota vary among different animal species, and quantification of these proportions has been proposed as a method for fecal pollution source identification [81]. Additional characterizations of this group are needed to characterize phylotypes that are associated with specific animal sources [161].

Gene product targets. Molecular methods have also allowed for detection of genes that serve a functional role in the organism. In some cases, the function may be linked to specific host microbe interactions, making these genetic markers potentially good host-associated alternative indicators [221]. Genetic markers have been identified with a variety of molecular methods, including subtractive hybridization, genome fragment enrichment, and other metagenomic approaches.

Toxin genes *of* E. coli. Specific subpopulations of *E. coli* contain genes coding for toxins, including heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST). *E. coli* carrying toxins are generally clonal populations that are found within certain animal reservoirs and have been suggested as host-associated indicators. Specific sequences of the STII toxin gene were found to be associated with swine, but not present in sewage or dairy farm lagoons [132]. Cattle-associated LTIIa has also been reported [50, 131]. These toxin genes have a worldwide distribution [72]. The occurrence of *E. coli* positive for STII or LTIIa can be low in agricultural animal populations, potentially limiting the use of these genes for the identification of specific animal sources.

Esp *gene*. The enterococcal surface protein (*esp*) gene is a putative virulence factor in *Enterococcus faecium* that has been shown to be associated with enterococci from human origin [212]. Because this gene occurs at a low frequency, original detection methods involved an enrichment step where DNA is extracted from enterococci grown on selective media, followed by PCR. Comparison studies have shown the *esp* gene in enterococci to correlate with other human-associated genetic markers [4, 163] and this alternative indicator has been employed in numerous field studies [275]. Newer methods employ qPCR that can directly detect the *esp* gene [2].

gyrB. The genetic locus *gyr*B is a housekeeping gene (e.g., common to all bacteria because of a central function). Similar to 16S rRNA gene loci, housekeeping genes are generally highly conserved and therefore useful for identifying specific phylotypes. One study employed qPCR targeting *gyr*B in *Bacteroides fragilis* as an indicator of human specific fecal contamination [142].

Methanogens. *Methanobrevibacter smithii* is a dominant Archaea in the human gut [67]. The *nif*H gene of this organism has been used as a human-associated indicator [243]. Similar assays employing the same gene in *Methanobrevibacter ruminantium* have been developed [245]. Assays for quantification of the *nif*H

target have also been developed [22, 128]. An Archaea genetic marker may prove useful because it may have a different survival or ecology compared with bacterial indicators and pathogens.

Bacteroides thetaiotaomicron. *B. thetaiotaomicron* is found in high numbers in humans compared with other animals and is described as a niche organism in the human gut [274]. A genomic fragment that was generated with universal primers as a second unexpected amplicon was found to distinguish *B. thetaiotaomicron* from other animal species [241]. PCR primers specific for *B. thetaiotaomicron* were developed based on the sequence of this 547-bp genomic fragment and have been tested against a number of fecal samples from humans and nonhuman sources [45, 241]. A putative gene for complex polysaccharide degradation has also been used as a genetic marker for qPCR since the trait is hypothesized to be involved in host-associated metabolic pathway [277].

Metagenomics. The majority of host-associated genetic markers available to date target the 16S rRNA gene from a limited number of different microorganisms. Advancements in DNA sequencing and sorting technologies now allow researchers to survey the entire genome of all members of fecal microbial community. Different strategies include the use of competitive hybridization approaches [101], microarrays [145, 272], and 454 pyrosequencing [161, 226, 246]. Whole genome and community approaches vastly expand the number of candidate source-associated genetic markers and may allow for the development of even more refined source identification methods.

Viruses

F specific (F^+) coliphages. F^+ coliphage RNA coliphages have serologically distinct groups that predominate in humans (groups II and III) which are distinct from those commonly found in other animals (group I and IV) [114]. Comparison studies of different alternative indicators suggest F^+ coliphage types are reliable indicators of host sources, but the groups are not exclusive to either animal or human sources [28, 180]. Further, differential survival may influence source identification in natural waters and may need to be taken into account in interpreting source identification studies [38, 172]. However, viral indicators may correlate more closely to human viral pathogens as they may have a similar ecology in the environment.

Bacteroides *phages*. Phages infecting *B. fragilis* and *B. thetaiotaomicron* have been used as indicators of human fecal pollution [12, 126, 240]. Differential ability of host strains of *Bacteroides* to detect phages from different sources has been reported [196], as well as geographic variability. Culture methods have been developed to isolate diverse host *Bacteroides* strains [190]. In survival studies, two *B. fragilis* phages were shown to survive longer in seawater compared to MS2 coliphage [157].

Human polyomaviruses. Human polyomaviruses are widespread among human populations and have been suggested as indicators of human waste [6, 162].

Table 9.5 Chemical alternative fecal indicators

Chemical constituents
Fecal sterols
Optical brighteners
Caffeine
Personal care products and pharmaceuticals

This virus is excreted in the urine and therefore may be detected in the absence of human feces. Studies have compared detection of human polyomaviruses with detection of human *Bacteroides* HF183 genetic marker and enterococci carrying the *esp* gene and found a strong correlation [4, 163].

Chemicals. Chemical methods do not detect fecal bacteria. Instead, these methods are designed to detect chemical compounds associated with human activities or sanitary sewage. Chemical indicators may provide additional evidence as to source [87, 95]. These chemicals are often found in sewage treatment facility discharges and septic tank effluent. For example, optical brighteners are commonly found in laundry detergents and have been used to indicate the presence of human fecal pollution in environmental waters [41, 62]. Fecal sterols such as coprostanol are also reported to be associated with human fecal pollution [28, 115, 176, 239]. Other potential chemical fecal indicators include antibacterial compounds, pharmaceuticals, and caffeine [95, 278] (Table 9.5).

Quantification of Bacterial Indicators Using qPCR. Conventional or endpoint PCR allows for the selective amplification of a particular genetic marker at extremely low concentrations even in the presence of a mixture of heterologous DNA targets making it ideal for environmental applications. The final result of an endpoint PCR method is either the presence or absence of the DNA target. Even though the qualitative determination of fecal pollution in a water sample can be very useful information, researchers quickly recognized the added advantage of generating quantitative data. The ability to estimate the concentration of a DNA target in a known volume of water provides a means to investigate relationships between the concentration of a fecal indicator genetic marker and numerous factors such as illness rates in swimmers or efficiency of waste management practices.

qPCR relies on the continuous monitoring of PCR product accumulation as amplification occurs. Estimation of the concentration of a genetic marker is based on the theoretical premise that there is a log-linear relationship between the starting amount of DNA target in a reaction and the fractional thermal cycle where PCR product accumulation is first significantly detectable (Table 9.2); for review see [204]. qPCR applications designed to estimate fecal bacteria concentrations in recreational waters are gaining widespread attention due to the rapid nature of these methodologies (same day results), reports linking the occurrence of DNA targets to public health risk [106, 257, 258], and the development of host-associated fecal source identification assays [40, 133, 135, 138, 163, 185, 218, 222, 223]. However, there are many technical concerns that must be addressed before these qPCR applications are ready for implementation.

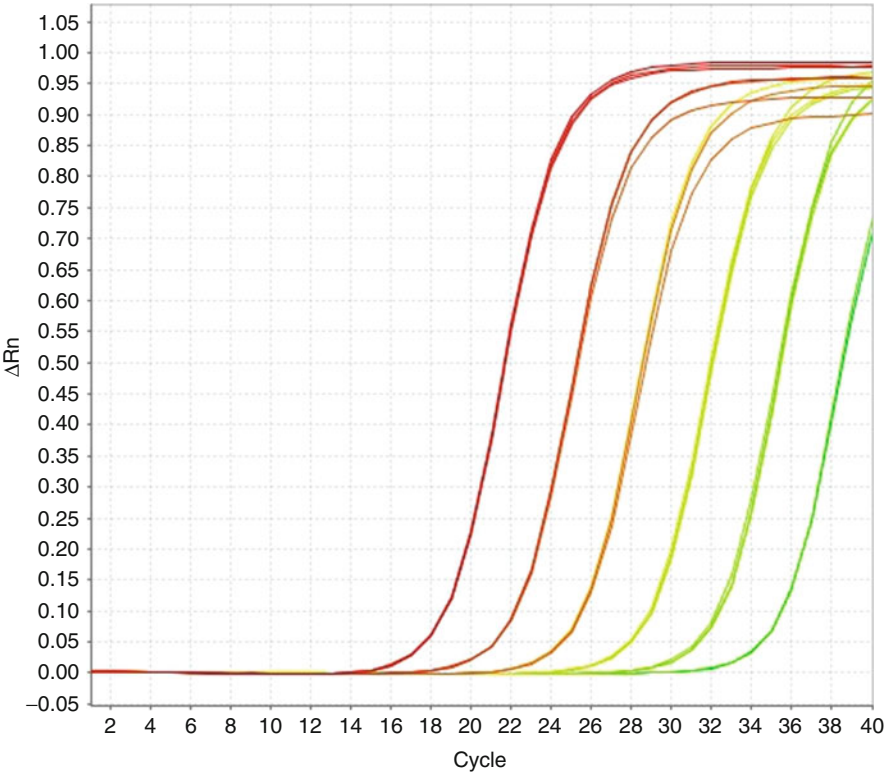

Fig. 9.2 Quantification of real-time polymerase chain reaction (qPCR) product can be achieved by observing an increase in fluorescence, indicating product formation, in relation to cycle number

It is important to recognize that a qPCR method consists of several protocols linked in succession including sample collection, sample preparation, nucleic acid purification, target amplification, and data interpretation. Each of these steps plays a critical role in the successful estimation of a DNA target concentration in an environmental sample. In addition, the extremely high level of sensitivity make qPCR methods highly susceptible to cross-contamination during field sampling, nucleic acid purification, and genetic marker amplification (Fig. 9.2). As a result, numerous studies have been conducted to address issues such as density and distribution of genetic markers in primary and secondary sources [60, 133, 199, 224, 225, 228], sample matrix interference during qPCR amplification [140, 198, 224, 255], estimating decay rates of DNA targets in ambient water [18, 23, 184, 261], loss of target DNA during nucleic acid recovery [106, 170, 238], and selection of a mathematical model to transform raw qPCR data into an estimation of concentration [230, 231].

Microbial Source Identification. Identification of the sources of fecal pollution is important for both developing remediation strategies and for estimating the

likelihood of pathogen occurrence. In most cases, the source of fecal pollution in a water body of interest is originally measured because of high amounts of conventional general fecal indicators (i.e., enterococci or *E. coli*). Methods and study designs for source identification, also referred to as "microbial source tracking" (MST) or "fecal source identification" (FSI), has been reviewed extensively [72, 206, 237].

Identifying fecal pollution sources involves understanding both the physical location of the inputs and the contributing host sources. Most source identification studies begin with spatial and temporal sampling since fecal pollution sources are rarely constant and the locations of inputs are not always obvious. Following release into the environment, the ecology of fecal indicators is greatly influenced by the residence time, type of water body (e.g., marine or freshwater, oligotrophic, or nutrient rich), predation, or even potential growth by some conventional indicators [31, 236]. Therefore, it is very difficult to take one or two samples and determine the major source contributing fecal pollution to an impacted body of water.

Spatial and temporal surveys are complemented by using alternative indicators that can provide information as to the host source of fecal pollution. Often, a first tier assessment will involve distinguishing human versus nonhuman fecal pollution [89, 181]. Cross reactivity needs to be considered, along with geographic relevance of a particular indicator. The possible fecal pollution sources within the watershed need to be considered when choosing the most appropriate alternative indicators. The use of alternative indicators for microbial source identification has been reviewed extensively [20, 72, 73, 206, 211, 235, 237].

Early approaches to microbial source identification focused on library-based methods, where either phenotypic traits or genotypes of indicator bacteria were characterized from a particular source and then compared to what was found in surface waters. Methods for characterizing *E. coli* or enterococci have included antibiotic resistance, ribotyping, and repetitive extragenic palindromic PCR [43, 44, 63, 103, 105, 159, 189, 217, 269]. There are multiple complications in using library-based methods that include applicability of the library across geographic locations, specificity of *E. coli* or enterococci indicators to a particular animal host, and complex genetic relationships among these indicators [10, 72, 159, 206, 236]. Further, creating a library is expensive and multiple water samples need to be analyzed because fecal pollution inputs are usually driven by storm events and can involve multiple animal sources. Most source identification methods have moved to marker-based, or non-library dependent, approaches. Marker-based approaches involve utilizing a chemical or biological constituent that is commonly found in the fecal pollution source of interest, in high abundance so that it can be detected easily and associated with a specific human or animal source (Figs. 9.3 and 9.4).

Fig. 9.3 Stormwater outfalls introduce fecal pollution from domestic pets and wildlife into rivers. Stormwater systems can also become contaminated with human sewage from leaking sanitary sewer systems (Photos provided by Dr. Sandra McLellan, University of Wisconsin-Milwaukee)

Fig. 9.4 Large gull populations are common non-point sources of fecal pollution on beaches (Photos provided by Dr. Sandra McLellan, University of Wisconsin-Milwaukee)

Ecology of Pathogens and Indicators in the Environment

The identification of a host-associated marker of fecal pollution goes beyond microbiology. Once the fecal indicator is discharged into the environment, it becomes necessary to understand the various fate and transport mechanisms that control the concentrations of indicators and pathogens at the point of sampling.

Fate processes include dark or photo-inactivation [32], growth [111], sorption and desorption to sediments [19, 94], and grazing by zooplankton [30]. Inactivation has received the most attention of these fate processes. Although a fair amount of work has examined the interaction of pathogens and indicators with sediments, the work has primarily been focused on porous media, and simplified conditions. More work on the interactions of microbial pollutants and sediments and particles in surface waters is needed, particularly given the widespread occurrence of some indicators and pathogens in sediments and beach sands [7, 26, 56, 117, 265, 275].

Transport processes that control indicator and pathogen transport in surface waters include advection and dispersion of waterborne organisms. These processes are fairly well understood [174] and once determined in a particular surface water, they can be used to model microbial pollution. The resuspension and deposition of sediment-bound organisms is more complicated. Some work has examined these processes for *E. coli* [119, 120] and fecal coliforms [234] in streams and lagoons. Yamahara et al. [275] present a conceptual model for how enterococci in beach sands are suspended into the water column. A better mechanistic understanding of how organisms in the sediment or sand are transported into the water column is warranted.

Of the fate and transport processes described above, perhaps the most important to consider when choosing an indicator for microbial source identification is the time scale of inactivation and its tendency to sorb to sediments. For example, if the goal is enterococci source identification for designing remediation strategies, then ideally, the persistence of the genetic marker will mirror that of enterococci. A health-protective goal may be to have no feces present in a water body. If this is the case, then a source identifier with very long-persistence may be needed. A source identifier that interacts strongly with sediments may be problematic as it may allow sediments to become a secondary, environmental source of the marker. Generally, sediments are believed to be a protective environment for microorganisms, particularly bacteria, where they may persist or even grow [276]. Future work on source identifiers will need to document the importance of sorption and interactions with sediments in general.

Estimating Risk of Pathogen Exposure Using Fecal Indicators

Using fecal indicators to link the presence of fecal pollution to waterborne disease risk is challenging. The types of pathogens that might be present will depend primarily on the source of fecal pollution. For example, sanitary sewer discharges (human sources) may contain high levels of human viruses, whereas wildlife is less likely to carry human

viruses, but may contain protozoan and bacteria that can infect humans. Comprehensive models that integrate data from several research fields such as occurrence of pathogens in fecal sources, dose–response relationships, source identifier decay behaviors, acceptable health risk, and route of transmission can be used to estimate risk and are termed quantitative microbial risk assessment (QMRA) [15, 232, 233]. The type of pathogen present will also depend on the prevalence of the disease-causing agent within the population at the time of contamination. Many human viruses are seasonal, and protozoans such as *Cryptosporidium* are prevalent during certain times of the year, such as spring when calves can shed high concentrations of this microorganism.

Factors that Diminish the Relationship Between Indicators and Pathogens
Seasonality of certain pathogens
Rate of infection in the host reservoir (herd, human population)
Differential decay
Differences in transport
Differences in sedimentation rates and partitioning to soil, sand, and sediments

Epidemiological Studies

Epidemiology studies have been conducted around the world to understand the correlative relationship between indicator concentrations and human health. The studies that have been conducted to date, and their methodologies, are summarized by Boehm and Soller (see Recreational Water Risk: Pathogens and Fecal Indicators. Most of the studies have focused on the health effects of recreational exposure to human fecal contamination from publicly owned treatment work discharges. These studies generally show a statistically significant correlation between enterococci and GI illness [256] in marine waters and *E. coli* and GI illness at freshwater beaches. Epidemiology studies are the cornerstone of the USA and EU water quality criteria and directives [31]. Acceptable illness rates are anchored to concentrations of indicator organisms in order to set acceptable contaminant levels. In the USA, 19 illnesses per 1,000 people is the acceptable illness level for marine water recreation, and in freshwater, the acceptable level is 8 illnesses per 1,000 people.

There are several important knowledge gaps in the understanding of how fecal contamination in recreational waters affects human health [31]. Few studies have documented the human health effects from exposure to nonhuman sources of fecal contamination including, but not limited to, bird and dog feces and urban and agricultural runoff [52, 98, 156]. A review of these studies suggests the relationship between indicator concentration and recreational waterborne illness risks is equivocal. Current studies with QMRA are trying to more fully understand the risks for exposure to animal feces in recreational waters [233].

Fecal Indicator Applications. There are numerous applications for fecal indicators and indicators need to be chosen that best serve a specific purpose or goal.

One primary purpose of an indicator is to evaluate the public health risk for recreational water. In this case, general indicators may be employed since beach managers will need to know if fecal pollution is present and at what level. Since the presence of pathogens is highly dependent on the source of fecal pollution, adequate protection of public health will depend on assuming that the indicators are derived from sources that carry the highest pathogen burden. Rapid detection of a fecal indicator is more important than the level of information provided by the indicator since water quality can change rapidly in the beach environment [29]. Ultimately, the source of fecal pollution needs to be identified and remediated to remove the health risk.

Fecal indicators also serve as important tools for sanitary survey practices and for prioritizing remediation strategies. While daily monitoring with a general indicator such as enterococci or *E. coli* will provide information on the extent of fecal pollution, the source needs to be identified in order to take corrective actions. Both extensive mapping of the physical location of fecal pollution inputs (where is it coming from?) and determination of the host sources (is it human or nonhuman sources?) are necessary. Host-associated alternative indicators are best suited for these applications.

Source identifiers can also be used to evaluate the success of best management practices and influence of many green infrastructure efforts in agriculture and urban run-off settings. For example, the installation of tile drainage systems or constructed wetlands is commonly used to control the flow of agricultural waste across the landscape during rain events. Host-associated methods provide an excellent metric for estimating the efficiency of these waste management practices.

Rapid Methods for Indicators

Recreational water quality monitoring has traditionally relied upon culture-based methods and therefore test results are not available to the public until, at the earliest, the following day. It is well established that water quality can change in a matter of hours [29, 266]. A high priority for beach managers is to utilize rapid testing methods, many of which are based on qPCR of fecal indicators. Studies have compared different rapid methods [93]. New water quality criteria that are being formulated by the USEPA are expected to include rapid methods for enterococci using qPCR.

BEACH Act Legislation. The Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000 is an amendment to the Federal Water Pollution Control Act (commonly known as the Clean Water Act). This legislation required states and tribes to adopt new or revised water quality standards by 2004. It also required the USEPA to publish new or revised criteria for pathogens and pathogen indicators. The BEACH Act authorized appropriations for states and tribes to develop and implement water quality monitoring and public notification programs at recreational beaches. The USEPA has identified scientific gaps that need to be filled in order to develop improved water quality criteria [251].

Future Directions

The identification of host-associated source identifiers represents the first step toward the successful implementation of a fecal indicator method. Several additional steps must be taken to complete the method development phase including method optimization, design of appropriate laboratory controls, and defining a data interpretation model. After method development, it is necessary to define the operational parameters of the method. In the case of qPCR, this might include factors such as generation of a calibration curve, defining the range of quantification, precision, and limit of detection. The next step is to characterize the robustness of the method by measuring specificity, host distribution of the source identifier, abundance of source identifier in target group, describing fate and transport mechanisms, establishing links to general fecal indicators, pathogens, and public health outcomes. Once the operational parameters and robustness of the method are adequately described, a multiple laboratory validation study should be conducted to address issues of reproducibility, variability between laboratories, normalization of results, standardization of controls, minimum requirements to establish laboratory efficiency, and requirements for laboratory training. It is important to note that this list is not comprehensive. There may be additional steps required depending on the intended use of the method.

Rapidly advancing technologies will provide new opportunities to expand the number and types of fecal indicators. Next-generation sequencing technologies have increased our capacity to analyze whole microbial communities, rather than single organisms. Advancing technologies will also allow for more detailed analyses of the dynamics of fecal indicators in the environment. Further, more sensitive, specific, and rapid detection strategies are needed to improve monitoring programs for devising pollution remediation strategies and for the protection of public health.

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