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Report on the 2nd Five-Year Review of EPA's Recreational Water Quality Criteria

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Acronyms

AFO	animal feeding operation
ALT	alanine aminotransferase
AMR	antimicrobial resistance
aOR	adjusted odds ratio
ARG	antimicrobial resistant genes
aRR	adjusted risk ratio
ATX	anatoxin-a
AWQC	ambient water quality criteria
B. theta	*Bacteroides thetaiotaomicron*
BASINS	Better Assessment Science Integrating Point and Nonpoint Sources
BAV	Beach Action Value
BEACH	Beaches Environmental Assessment and Coastal Health
BMAA	β-N-methylamino-L-alanine
BNR	biological nutrient removal
BTB	blood-testes barrier
BUN	blood urea nitrogen
bw	body weight
CAFO	combined animal feeding operation
CAT	*Catellicoccus*
CCE	calibrator cell equivalent
CDC	Centers for Disease Control and Prevention
CE	cell equivalents
CFR	Code of Federal Regulations
CFU	colony forming unit
CI	confidence interval
CSO	combined sewer overflow
CWA	Clean Water Act
DAL	double agar layer
dcNEOSTX	decarbamoyl neosaxitoxin
dhATX	dihydroanatoxin-a
D-HFUF	dead-end hollow-fiber ultrafiltration
DMF	direct membrane filtration
DNA	deoxyribonucleic acid
dPCR	digital polymerase chain reaction
E. coli	*Escherichia coli*
eDNA	environmental deoxyribonucleic acid
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
ESBL	extended spectrum beta-lactamase
EU	European Union
FDA	Food and Drug Administration

FIB	fecal indicator bacteria
FR	Federal Register
FSI	fecal source identification
g	grams
GC	gene copies
GI	gastrointestinal
GI.1	norovirus genogroup I, genotype 1
GII.4	norovirus genogroup II, genotype 4
GJs	gap junctions
GM	geometric mean
GTX	gonyautoxin
HAB	harmful algal bloom
HDA	helicase-dependent amplification
HESD	Health Effects Support Document
HSPF	Hydrological Simulation Program-Fortran
HPyVs	human polyomaviruses
HUC	Hydrologic Unit Codes
IC	inhibitory concentration
IEM	integrated environmental monitoring
IgG	Immunoglobulin G
Inv-IMS/ATP	inversely coupled immunomagnetic separation/adenosine triphosphate
i.p.	intraperitoneal
kg-d	kilograms per day
L	liter
LAMP	loop-mediated isothermal amplification
LD_{50}	lethal dose 50 percent (median lethal dose)
LDH	lactate dehydrogenase
LRV	log reduction value
LOAEL	lowest-observed-adverse-effect level
LWTX	lyngbyatoxins
M. smithii	*Methanobrevibacter smithii*
MC	microcystin
mL	milliliter
MPN	most probable number
MRA-IT	microbial risk assessment-interface tool
MSC	male-specific coliphage
MST	microbial source tracking
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
µg	microgram
NEEAR	National Epidemiological and Environmental Assessment of Recreational Water
NGI	NEEAR gastrointestinal
nmol/kg	nanomoles per kilogram

NIST	National Institute of Standards and Technology
NPDES	National Pollutant Discharge Elimination System
NOAEL	no-observed-adverse-effect level
NORS	National Outbreak Reporting System
ODH	Ohio Department of Health
OHEPA	Ohio Environmental Protection Agency
OR	odds ratio
ORD	Office of Research and Development (U.S. EPA)
PDV	phocine distemper virus
PFU	plaque forming unit
PI3K/AKT	phosphatidylinositol 3 kinase/protein kinase B
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PSP	Paralytic Shellfish Poisonings
QMRA	quantitative microbial risk assessment
qPCR	quantitative polymerase chain reaction
R-squared	Pearson's correlation coefficient squared
RBT	risk-based threshold
RfD	reference dose
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcriptase-polymerase chain reaction
RT-qPCR	reverse transcriptase-quantitative polymerase chain reaction
RVA	Group A Rotavirus
RWQC	Recreational Water Quality Criteria
SAL	single-agar layer
SCCWRP	Southern California Coastal Water Research Project
SDWA	Safe Drinking Water Act
spp.	species (plural)
SRM	Standard Reference Method
STEC	Shiga toxin-producing *E. coli*
STV	statistical threshold value
STX	saxitoxin
STX-Cou	Fluorescent coumarin-coupled STX
STXeq	saxitoxin equivalents
SWAT	soil and water assessment tool
SWCNT	single-walled carbon nanotubes
SWCNT-COOH	carboxylated single-walled carbon nanotubes
Ti/Abs	title and abstracts
TJs	tight junctions
TMDL	Total Maximum Daily Load
TSM	Technical Support Materials

TMRNEL	Tetramethylrhodamine nick end labeling
UK	United Kingdom
U.S.	United States
UV	ultraviolet
WHO	World Health Organization
WHOI	Woods Hole Oceanographic Institution
WOS	Web of Science
WQS	water quality standards
WRRF	water resource recovery facility
WWTP	wastewater treatment plant

Executive Summary

The Beaches Environmental Assessment and Coastal Health (BEACH) Act amendments to the Clean Water Act (CWA) Section 304(a)(9)(B) require the United States (U.S.) Environmental Protection Agency (EPA) to conduct a review of the current 304(a) National Recommended pathogen and pathogen indicator Recreational Water Quality Criteria (RWQC) every 5 years, and, as necessary, revise the RWQC. In conducting this review, EPA considered several factors, including the availability and evaluation of the latest scientific knowledge and additional implementation support needs. EPA conducted a systematic review and evaluation of scientific information and collection of updated information on recreational criteria implementation tools since the previous 2017 five-year review. Specifically, EPA evaluated health and epidemiological studies related to indicators of fecal pollution, studies related to children's health, new data on coliphages as indicators of enteric viruses, cyanotoxins health studies, antimicrobial resistance, fecal source identification, studies addressing advances in analytical methods, and implementation materials including advances in the use of models to predict water quality and assess risk. This report contains extensive information in these topic areas along with priorities for further work. EPA has completed a detailed review of the latest scientific knowledge and has determined that current science supports a revision of the 2012 RWQC and that there are several additional implementation tools that EPA can make available to manage recreational waters.

The available science demonstrates an increased health risk for children compared to adults when recreationally exposed to fecal contamination. Further, studies indicate that the use of culturable fecal indicator bacteria alone to evaluate recreational waters impacted by human sources can result in under-protection of human health. In these waters quantitative polymerase chain reaction (qPCR)-based criteria (i.e., based on a molecular testing method using qPCR) offer greater protection. Since 2017, EPA has published nationally validated protocols for qPCR methods and codeveloped (along with the National Institute of Standards and Technology [NIST]) a standard reference material for national use that supports the use of qPCR technologies. To address these issues, first EPA plans to develop additional criteria recommendations for qPCR-enumerated enterococci protective of children, which would also be protective of all recreators. Second, EPA plans to continue to develop recommendations for coliphages to help address potential risks from human enteric viruses in ambient waters. Third, EPA plans to explore how best to use human fecal source identifiers, such as HF183, for water quality management. Being able to demonstrate that a waterbody has been impacted by human fecal contamination will enable risk managers to use the appropriate tools to evaluate and manage risks for waters impacted by human sources.

I. Introduction

United States (U.S.) Environmental Protection Agency (EPA) has conducted a five-year review of its 2012 Recreational Water Quality Criteria (RWQC), as required by the Beaches Environmental Assessment and Coastal Health (BEACH) Act amendments to the Clean Water Act (CWA) Section 304(a)(9)(B). The last review was conducted in 2017. In conducting this review, EPA considered several factors, including the availability and evaluation of the latest scientific knowledge and additional implementation support needs. The Agency used the information in this "state-of-the science" report to assess whether new or revised RWQC are necessary at this time.

The development of the 2012 RWQC and five-year reviews are requirements of the BEACH Act of 2000. Through that Act, EPA has provided grants to states, territories, and tribes to implement water quality monitoring and notification programs for coastal recreation waters[1] (including the Great Lakes) since 2002. The 2012 RWQC included development of a beach advisory threshold for use in posting swimming advisories and the recommended ambient water quality criteria (AWQC). Swimming advisory decisions based on water quality monitoring are intended to reduce the risk to recreators and other users of these waters from illness associated with exposure to human fecal contamination and provide the public with information to make decisions about their actions. AWQC developed under CWA Section 304(a) are recommendations based on the latest science which states and authorized tribes can adopt as part of their water quality standards (WQS). In the case of the 2012 RWQC, EPA's recommendations were designed to protect primary contact recreation, in all coastal and non-coastal waters designated for recreational use. The criteria, once adopted by states and authorized tribes and approved by EPA under CWA section 303(c), become part of the regulatory structure of the state or authorized tribe for protection of primary contact uses for the applicable waters. The recreational criteria values that are part of a state or authorized tribe's approved WQS have a direct bearing on the issuance of National Pollutant Discharge Elimination System (NPDES) discharge permits, waterbody assessments, the decisions regarding attainment of WQS under CWA Sections 303(d) and 305(b), and the development of targets for total maximum daily loads (TMDLs) for restoring impaired waters.

The criteria values specified in the RWQC are for densities of culturable fecal indicator bacteria (FIB) in water. The FIB, enterococci, and *Escherichia coli (E. coli)* are not pathogenic under usual circumstances, but their presence in water above specified levels can indicate the presence of fecal contamination potentially containing viral, bacterial, or protozoan pathogens associated

[1] The BEACH Act of 2000 defines coastal recreation waters as follows:
The term "coastal recreation waters" means:
(i) The Great Lakes; and
(ii) Marine coastal waters (including coastal estuaries) that are designated under section 303(c) by a State for use for swimming, bathing, surfing, or similar water-contact activities.
The term "coastal recreation waters" does not include:
(i) Inland waters; or
(ii) Waters upstream of the mouth of a river or stream having an unimpaired natural connection with the open sea.

with an elevated risk of illness. Therefore, ensuring that the RWQC are consistent with the current state of the science and are protective of human health is key to protecting the health of users of all waters designated for primary contact recreation.

EPA identified the following objectives for this review of the 2012 RWQC:

- Identify the latest science and information available since the publication of the 2017 five-year review that may impact the 2012 criteria.
- Discuss the status of adoption of the RWQC.
- Include the latest science and information on health effects relevant to RWQC, coliphages, cyanotoxins, antimicrobial resistance (AMR), fecal source identification, and analytical methods that have the potential to impact recreational uses.
- Identify additional indicators and microbial methods including those that have become more refined or feasible and include this information.
- Provide information on the implementation materials for the 2012 criteria including site-specific tools, implementation materials for cyanotoxins, predictive and process modeling for both fresh and marine waters, outreach, and training.

An important goal of this review is to evaluate whether revisions to the 2012 RWQC are necessary based on the overall review of new information provided in the previous 5 years, described later in this report.

II. Background

A. Brief Description of the 2012 RWQC and Key Aspects of Implementation

The 2012 RWQC, which use enterococci and *E. coli* as predictors of gastrointestinal (GI) illnesses in recreational waters, are described below along with several other important aspects of how the criteria can be implemented.

1. Criteria Magnitude, Duration, and Frequency: Geometric Mean and Statistical Threshold Value

The 2012 RWQC consist of three primary components: magnitude, duration, and frequency.

Magnitude: The magnitudes of the bacterial indicators are the measured densities of the FIB from the water quality density distribution used for the criteria, expressed both as a geometric mean ([GM] 50th percentile value) and as a statistical threshold value ([STV] 90th percentile value).

Duration: The duration is the period over which excursions of the magnitude values are recorded and calculated. EPA recommended a duration of 30 days in the criteria for both the GM and the STV.

Frequency: The frequency is how often the GM or the STV are exceeded. EPA recommended no exceedances for the GM over the period of the duration.

Because the STV reflects the 90th percentile of the distribution of values used to determine the RWQC, the RWQC allowed for a 10 percent exceedance of the STV. EPA selected the estimated 90th percentile of the water quality distribution to account for the expected variability in water quality measurements, while limiting the amount of time allowed to exceed the STV as a threshold of water quality impairment.

EPA was clear that "both the GM and the STV would be part of the WQS, and therefore both targets would be used to determine whether a waterbody attains the WQS for primary contact recreation" (U.S. EPA, 2012a).

Table 1. 2012 RWQC Recommended GM and STV Values for 36 and 32 NGI Illnesses/1,000 Recreators for Marine and Fresh Waters

CRITERIA ELEMENTS	Recommendation 1 Estimated Illness Rate (NGI): 36/1,000		Recommendation 2 Estimated Illness Rate (NGI): 32/1,000	
Indicator	**GM** (CFU/100 mL)[a]	**STV** (CFU/100 mL)[a]	**GM** (CFU/100 mL)[a]	**STV** (CFU/100 mL)[a]
Enterococci (marine and fresh water)	35	130	30	110
E. coli (fresh water)	126	410	100	320

Duration and frequency: The waterbody GM should not be greater than the selected GM magnitude in any 30-day interval. There should not be greater than a 10 percent excursion frequency of the selected STV magnitude in the same 30-day interval. NEEAR = National Epidemiological and Environmental Assessment of Recreational Water; NGI = NEEAR gastrointestinal.

[a] EPA recommends using EPA Methods described at https://www.epa.gov/cwa-methods to measure culturable enterococci and *E. coli*. Units are colony forming units (CFU) per milliliter (mL).

Criteria values were provided for culture-enumerated FIB at two illness rates, 32 and 36 NGI illnesses per 1,000 swimmers. Based on EPA's analysis of the available information, either set of thresholds protects the designated use of primary contact recreation and, therefore, protects the public from the risk of exposure to harmful levels of pathogens from fecal contamination. The two sets of numeric concentration thresholds included in the 2012 RWQC provide states and authorized tribes flexibility to make their own risk-management decisions. The recommendations for criteria illness rate apply to both marine and fresh waters, regardless of the intensity of use of the beach.

In addition to recommending criteria values, EPA also provided states and authorized tribes with Beach Action Values (BAVs) for use in notification programs. The BAV was defined as the 75th percentile of the water quality distribution of values of *E. coli* and *Enterococcus* species (spp.) in the epidemiological studies used by EPA to establish a health link between gastrointestinal (GI) illness and levels of culturable FIB. EPA's intent was to provide the BAV for states and authorized tribes as a precautionary tool for beach management decisions. EPA recommended the BAVs for use by the states for issuing beach notifications/advisories in their public health programs, but not as part of the 2012 RWQC recommendations under CWA Section 304(a).

States, territories, and authorized tribes continue to make progress adopting RWQC. Thirty-one jurisdictions have adopted, and EPA has approved, revised RWQC for all primary contact waters. Three additional jurisdictions have adopted, and EPA has approved, revised RWQC for their coastal recreation (i.e., BEACH Act) waters only. One jurisdiction only includes fecal coliform as FIB in its WQS. Seven additional jurisdictions use fecal coliform as FIB for some but not all of their waters designated for primary contact recreation (e.g., they use enterococci as the FIB in their marine waters and fecal coliform in their fresh waters).

Although identified in 2017 as a possible barrier to adoption of the 2012 RWQC, criteria that are based on use intensity does not appear to be hindering continued adoption of the 2012 RWQC, as states and authorized tribes with coastal recreation waters have continued to adopt the 2012 RWQC without the inclusion of use intensities. More than half the states have adopted the 2012 RWQC or an equivalently protective criteria in all their waters designated for primary contact and an additional three states have adopted the 2012 RWQC for their coastal recreation waters; all authorized tribes with coastal recreation waters have adopted the 2012 RWQC.

While the culture-enumerated FIB at two illness rates were the recommended criteria in 2012, EPA also included quantitative polymerase chain reaction (qPCR)-based values as additional information. EPA developed and validated a molecular testing method using qPCR as a rapid analytical technique for the detection and quantitation of enterococci in recreational water (EPA Method 1611, EPA 2012b). EPA included qPCR-based values for the GM, STV, and BAV for both illness rates protective of the general population in the 2012 RWQC document. Because of potential matrix interference issues in water types other than those studied at the NEEAR effluent-affected beach sites, EPA's Office of Research and Development (ORD) continued research to characterize and refine the qPCR methodology (Haugland et al., 2016) resulting in the publication of Method 1609.1 (EPA Method 1609.1; U.S. EPA, 2015c).

Following publication of the 2012 recreational criteria, EPA provided additional information on tools for evaluating and managing recreational waters, such as predictive modeling and sanitary surveys, and stressed the need for a tiered approach to developing beach monitoring plans in the *2014 National Beach Guidance and Required Performance Criteria for Grants*. The Agency also provided Technical Support Materials for developing site-specific criteria and for the use of alternative indicators or methods at recreational beaches (see https://www.epa.gov/wqc/recreational-water-quality-criteria-and-methods).

B. How the 2nd Five-Year Review Was Conducted

1. Scope and Methods of the Review

This section describes the measures EPA has taken to assess advances in the state of the science supporting the 2012 RWQC since 2016, the date of the last five-year review's literature cut-off year (published in 2018). It also addresses advances in implementation. The measures include an inventory of the relevant scientific information published since 2016, a description of recreational criteria implementation tools applied at recreational settings, and information on sources of information and how information was accessed.

2. Inventory of Scientific Information Published Since 2016

A thorough inventory of scientific information published since 2016 for topics central to recreational waters monitoring and assessment is the core of this review (Figure 1). Several general categories of relevant information were identified:

i. Health studies, including epidemiological studies, outbreak studies, children's health studies, and the application of quantitative microbial risk assessment (QMRA) to water quality data and complex settings at recreational beaches.
ii. Summary of advancements in coliphage health studies and methods since 2016.
iii. Cyanotoxins health studies, methods, and criteria and implementation since 2016. Note that cyanotoxin recreational criteria are not subject to the BEACH Act because they are neither pathogens nor pathogen indicators. EPA included cyanotoxins in the literature reviews since EPA has recreational criteria for two cyanotoxins.
iv. Summary of scientific advancements in AMR since 2016.
v. Fecal source identification (also called microbial source tracking in the literature), including human and non-human fecal source markers and tracking.
vi. Performance, implementation, and updates of microbial methods for FIB and alternative indicators 2016 to present.

3. Summary of Recreational Criteria Implementation Tools Developed and EPA Outreach and Training Since 2016

A further category of activities and tools related to water quality monitoring and contextual assessment of beach settings was identified as highly relevant to the implementation of the BEACH Act and activities related to the 2012 RWQC. This category of implementation tools includes:

i. Site-specific implementation tools
ii. Sanitary surveys and watershed assessments
iii. Cyanotoxins implementation tools
iv. Predictive modeling
v. Process modeling

4. Sources of Information and How Information Was Accessed

The collection and analysis of information in each of these categories included accessing post-2016 information from two broad sources:

- EPA recreational water research and publications relating to that research.
- External (non-EPA) academic research conducted by researchers at academic institutions and government organizations that have focused on recreational water activities and science related to the BEACH Act.

5. A Systematic Review of Available Peer-Reviewed Literature

EPA performed systematic searches of the peer-reviewed literature for articles pertaining to health studies, including epidemiological studies of recreational water-contact activities, outbreak studies, advances in recreational exposure descriptions for children, characterization of children's illness susceptibility, observed illness rates in children upon exposure, and the application of QMRA to water quality data and complex settings at recreational beaches; advances in human and non-human fecal source identification (microbial source tracking); and advances in molecular methods used in recreational waters to measure indicators of fecal contamination. Multiple sets of search terms applicable to the topic were applied to references in Web of Science and PubMed (http://www.ncbi.nlm.nih.gov/pubmed) (see Appendix A for detailed search terms). Searches for gray literature were also performed. Abstracts were screened for relevance to the scope of the search. The literature search was limited to English-language, peer-reviewed citations published between January 2016 and November 2021, with the exception of searches pertaining to children's health studies. Searches pertaining to advances in recreational exposure descriptions for children, characterization of children's illness susceptibility, and observed illness rates in children upon exposure were limited to articles published between January 2018 and November 2021. Following the abstract screening, the full text of articles passing scope was reviewed for specific information related to each topic (see Appendix A for details). Diagrams of the process and results are provided in Figure 1, below. The steps of a systematic review as described in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol, which defines the minimum set of items for reporting in systematic reviews. The search strategy, search terms, screening criteria, and PRISMA diagrams are provided in Appendix A. Results of the systematic reviews and summaries of studies reviewed are included in Appendix B.

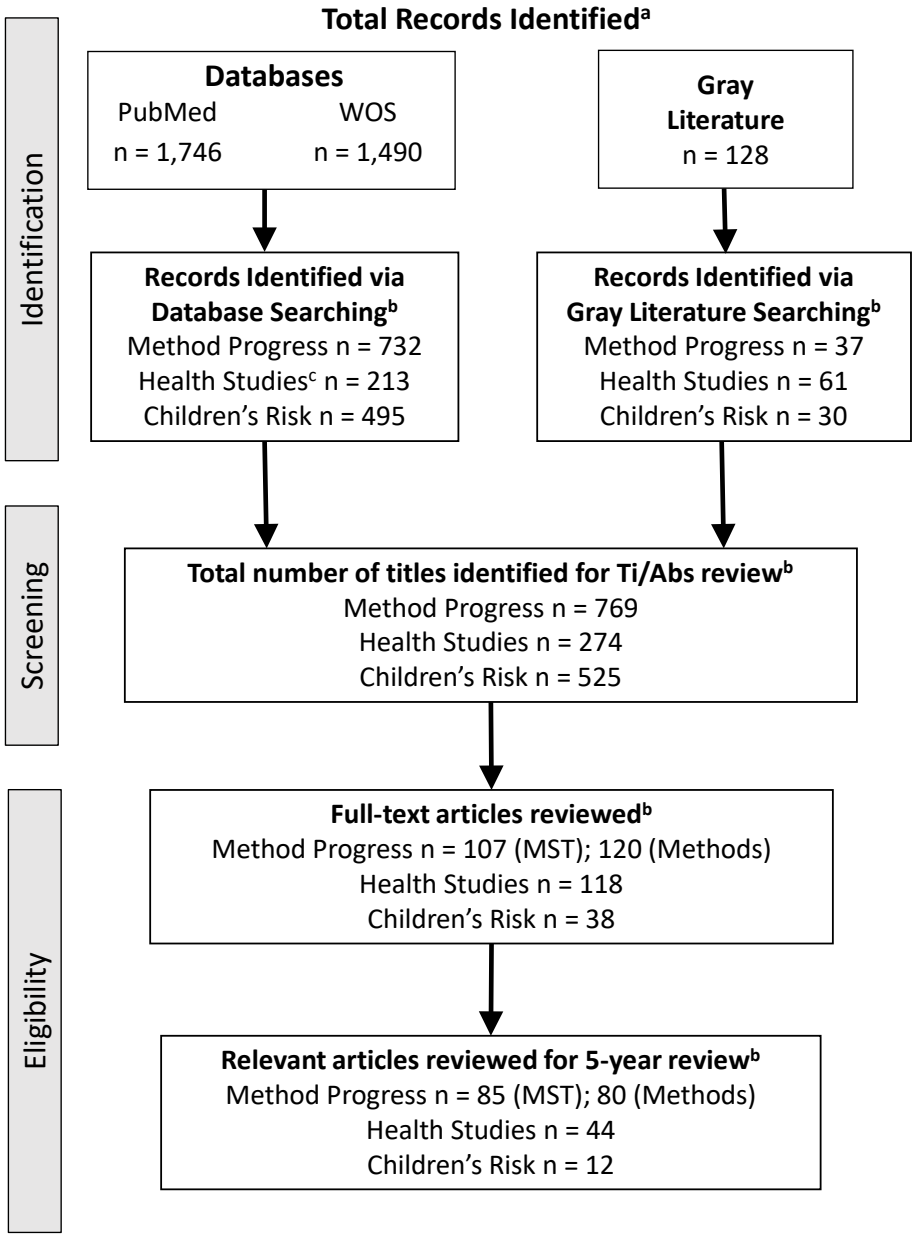

Figure 1. Process for Identifying Literature for Some of the Five-year Review Topics: 2016–2021

[a] Three literature searches (Methods Progress; Health Studies; Children's Risk) were conducted (Appendix A) to identify articles for four relevant topics: Microbial source tracking (MST); Molecular methods; Health studies; and Children's risk. The MST topic includes fecal source identification (FSI) and bacterial source tracking. Note that the literature on three additional topics, AMR, cyanotoxins, and coliphages, that is described in those sections was identified from additional literature searches conducted using different methods and is not shown here.

[b] Articles were cross-tagged to other document topics/sections when applicable, resulting in some articles being counted for more than one topic.

[c] Pre-screening prioritization of the Title/Abstracts (Ti/Abs) for Health Studies was conducted; 1,293 Ti/Abs were reduced to 162 based on machine learning supervised clustering using 37 seed title/abstracts (see Appendix A). Fifty-one cross-tagged articles were added after prioritization, resulting in 213 studies.

EPA also performed systematic searches of the peer-reviewed literature for articles pertaining to characterizing the human health effects of cyanotoxins from all routes of exposure. The cyanotoxins of interest for this topic included: anatoxins, cylindrospermopsin, microcystins, nodularins, and saxitoxins. The literature searches for anatoxins, microcystins, and cylindrospermopsin built upon work previously conducted by the Office of Water and covered the period from January 2014 to April 2021. The literature searches for all other cyanotoxins were conducted with no start date to April 2021. Multiple sets of search terms applicable to the topic were applied to references in Web of Science (WOS), PubMed, and Scopus (see Appendix A for search terms). Abstracts were screened for relevance to the scope of the search. Following the abstract screening, the full text of articles passing scope was reviewed for specific information related to each topic. Search strategy, search terms, screening criteria, and PRISMA diagrams are provided in Appendix A. Results of the systematic reviews are included in Appendix B.

III. Findings of the 2nd Five-Year Review

A. New Information on Health Effects after Exposure to Recreational Waters

The systematic literature review identified 44 health studies and 12 children's risk studies (Figure 1) that were evaluated for the Second Five-Year Review report (Appendix B). Additional relevant literature was identified via an ad hoc process (e.g., provided by subject matter experts). From this ad hoc process, twelve references relevant to health study information (Teunis et al., 2008, 2016, 2018, 2020; U.S. EPA. 2010, 2019; Boehm et al., 2015; Wade et al., 2019; Vanden Esschert et al., 2020; Egorov et al., 2021; Goh et al., 2021; California Water Boards, 2022) and two references relevant to children's risk (U.S. EPA, 2011 and Dufour et al., 2017) were identified.

Health studies of recreational water exposure broadly fall into three categories: epidemiological studies, QMRA-based studies, and outbreak reports or studies (also referred to as surveillance summaries).

- **Epidemiological studies** quantify associations between reported health symptoms (or biomarkers of infection) in participants and a measure of water quality, such as an indicator of fecal contamination, on the day of swimming exposure. These studies generally provide information on the level of illness experienced by recreators, and many have observed statistically significant associations between illness and measures of water quality (e.g., FIB). Epidemiological studies have informed the development of recreational water guidelines in the United States and elsewhere.

- **QMRA-based studies** characterize the probability of illness by focusing on the pathogens that can cause illness and, in this context, can provide risk-based thresholds (RBTs) for measures of water quality (including traditional and alternative surrogates of fecal contamination) that are based on the fecal sources affecting a waterbody.

- **Outbreak studies** are retrospective investigations that describe the conditions and consider possible etiologic agents resulting from clusters of reported illnesses. All three types of studies inform human health effects that recreators experience due to fecal contamination of recreational waters and may inform the understanding of the waterborne pathogens responsible for causing illness. This section presents studies identified in the published scientific literature in each of these three study categories, followed by a summary of major findings.

1. Recreational Water Epidemiological Studies

New Epidemiological Studies Conducted in the United States: 2016–2022

Children's Health Studies

The systematic literature search (see Figure 1 and Appendix A for method details) that identified new literature since the first five-year review (i.e., 2016–2021) found four recreational water epidemiology studies conducted in the United States that included enterococci data (Wade et al.,

2022; Arnold et al., 2016, 2017; Benjamin-Chung et al., 2017). Several studies evaluated the children's health risk from exposure to fecal contamination in the beach environment.

Wade et al. (2022) analyzed a pooled data set of over 80,000 beachgoing children, adolescents, and adults from 13 beach sites across the United States to compare illness risks and water quality by collecting measures of both culture- and qPCR-enumerated enterococci, exposure levels, beach sites, and health endpoints for different age groups. Sites previously studied by EPA included: West Beach, Huntington Beach, Washington Park Beach, Edgewater Beach, Fairhope Beach, Goddard State Park Beach, Surfside Beach, and Boquerón Beach. California sites studied by the University of California, Berkeley and the Southern California Coastal Water Research Project (SCCWRP) included: Mission Bay, Doheny Beach, Malibu Beach, and Avalon Beach. As part of the analysis, beach sites studied were categorized by the likelihood of human fecal contamination and whether the human source was a point source. Seventy percent of all participants had at least some contact with water and 27 percent stayed in the water 60 minutes or more. Children 12 years of age and under comprised approximately 25 percent of the total study population from all sites. The authors used odds ratios (ORs) among swimmers to characterize associations between levels of *Enterococcus* spp., measured by culture and qPCR, for different age groups across different exposures, beach category, and health endpoints (GI and respiratory illness). The authors found that GI symptoms were the most sensitive health endpoint associated with fecal contamination across all age groups, exposures, and site categories. Children were at higher risk of swimming-associated illness compared to older adolescents and adults with the highest mean ORs observed for GI illness among children under 10 years old at beaches affected by effluent. Statistically significant associations were consistently observed with *Enterococcus* qPCR cell equivalents (CE) across sites impacted by human fecal contamination. The highest odds ratios (OR = 2.32, 95% confidence interval [CI]: 1.33–4.06) were between *Enterococcus* qPCR and children 6 years old and under who spent at least 60 minutes in the water; ORs were similar for children in the "'10 and under" and "'12 and under" age groups. Respiratory illness was significantly associated with *Enterococcus* qPCR exposures among children 4 and under who spent 60 minutes or more in the water at sites affected by effluent. Cough was also associated with *Enterococcus* CFU at sites affected by effluent among children 4 and under, although small sample sizes resulted in imprecise estimates for these associations. Combining data across all sites, the association between GI illness and culturable enterococci, although positive, was not statistically significant for the general population (i.e., data from all participants combined). ORs for culturable enterococci and GI illness increased at "likely human-impacted" sites, particularly among those who swallowed water (1.37, 95% CI: 1.04–1.82) and there was evidence for increasing risk with younger aged children at sites affected by effluent. For example, among children under 6 years old at the sites affected by treated effluent the OR was 1.82 (95% CI: 1.14–2.91), which was significantly higher compared to adults 18 years old and over. ORs for severe GI illness[2] among swimmers, which accounted for approximately 20 percent of all reported GI illness, were statistically significant for *Enterococcus* qPCR (1.16, 95% CI: 1.03–1.31) and higher for children under 6 years old (1.50,

[2] Severe GI illness was defined as a GI illness episode that lasted three of more days or resulted in a visit to the hospital, doctor's office, or emergency room.

95% CI: 1.13–1.99), but not for culturable enterococci. The study conclusions were that age, source of fecal contamination, and the intensity of swimming exposure are important factors affecting the association between *Enterococcus* spp. Exposure from swimming-associated illnesses.

Arnold et al. (2016) conducted a meta-analysis of 13 beach sites and nearly 90,000 subjects. The study combined individual level-data from studies conducted by EPA and the University of California, Berkeley, which were coordinated to ensure similar designs and measurement methods. Data from 13 prospective cohorts were combined and integrated into a single data set. The beaches represented a range of recreational water conditions across the United States: four freshwater beaches were in the Great Lakes region, four marine beaches were in Southern California, and the remaining five marine beaches were spread along the Gulf Coast, Eastern Seaboard, and Puerto Rico. Nine of the beaches were located near a known source of treated sewage discharge and the remaining four beaches had more diffuse contamination from urban runoff. The primary outcome was incident diarrhea defined as three or more loose or watery stools in 24 hours. Swimmers were defined as those who immersed their body in water and were further classified on the basis of water quality, that is, whether the FIB, *Enterococcus*, exceeded the revised EPA guideline value of 35 CFU per 100 ml.

Compared to non-swimmers, diarrhea incidence increased with swimming exposure. *Enterococcus* levels were positively associated with diarrhea risk within age strata, with the largest absolute increases in risk among children 0 to 4 years old. Swimmers exposed to *Enterococcus* above EPA guidelines had higher diarrhea incidence compared to non-swimmers and compared to swimmers exposed to water below the guideline, but only at beaches with a known point source of human fecal pollution. Swimmers exposed to *Enterococcus* levels above the EPA guidelines had higher diarrhea incidence compared to non-swimmers as well as with swimmers exposed below the guideline in all age groups. The finding was more pronounced in children: 0 to 4 years old exposed to water above regulatory guidelines reported 103 episodes per 1,000 compared to 48 episodes per 1,000 among non-swimmers at beaches with a known point source. For swimmers that recreated in waters below the guideline level, the illness rate was 65 episodes per 1,000 compared, which is significantly higher than non-swimmers (48/1,000) but lower than the rate for swimmers who recreated in waters above the guideline level (103/1,000). The authors noted the uncertainties about the causes of diarrhea, which include non-infectious causes of diarrhea (e.g., swallowing excess saltwater), outcome reporting bias, or pathogens present in recreational waters that do not covary with *Enterococcus*. Children 0 to 4 and 5 to 10 years old had the most water exposure, stronger associations between levels of water quality and illness, and the largest attributable health burden. Overall, recreational water exposure was associated with increased risk of acute gastroenteritis resulting in missed daily activities with the highest risk and burden among young children.

Arnold et al. (2016) also performed a comparative analysis for association to health effects for FIB and qPCR. They found that the exposure-response relationship was more consistently monotonic for *Enterococcus* measured using qPCR methods versus culture methods across different analyses, even when restricted to beaches without a known point source of pollution.

Arnold et al. (2017) conducted a longitudinal cohort study of adult surfers (18 years old and older), a group of recreators with potentially high exposure profiles, in California waters to evaluate health impacts associated with water quality, measured by culturable FIB, under dry and wet weather conditions. The study collected illness information on GI illness, sinus infections, ear infections, fever, skin rashes, and infected wounds. Under dry conditions, recreational exposures were associated with an increased incidence of all health outcomes compared to the nonexposed group (adjusted incidence rate ratio = 1.86; 95% CI: 1.27, 2.71). With the exception of infected wounds, illness symptoms were not associated with increases in culturable enterococci levels. Culturable enterococci levels were strongly associated with multiple health outcomes after wet-weather exposure. In the wet weather flows discharging to the beaches, human fecal contamination from urbanized areas was identified. FIB (culturable enterococci, fecal coliforms, and total coliforms) were significantly associated with illness up to 3 days after rainfall. The median wet weather-associated excess risk at 35 CFU enterococci per 100 mL was 16 GI illnesses per 1,000 (95% CI: 5, 27) or less than half the rate of illness associated with the EPA's RWQC recommendations. The significance of this study is that it identifies wet weather as another factor that can affect loading of fecal contamination to recreational waters and can result in increased human health risk to recreators. The results support posting of beaches after rainstorms when human fecal sources are likely to affect recreational waters and management actions that would reduce fecal loading in urban runoff.

Benjamin-Chung et al. (2017) conducted a pooled analysis of six prospective cohort studies at coastal beaches in California, Alabama, and Rhode Island. Water quality was measured using culturable enterococci and coliphages. Knowledge of contamination inputs was used to interpret indicator monitoring. Human fecal pollution was unlikely on all study days at two of the beaches (Mission Bay and Malibu). At Fairhope and Goddard beaches, human fecal pollution was likely on all study days. The remaining two beaches (Doheny and Avalon) had evidence of variable human input such as groundwater influx conveying sewage through the sand or a berm that blocked direct discharge of contaminated water at the beach. Among all participants across the study sites and under all conditions combined, there was no association between GI illness and levels of culturable enterococci or coliphages. However, associations between culturable enterococci or coliphage levels and GI illness were observed when human fecal pollution was "likely present" and there was some evidence that the association with illness for male-specific coliphage (MSC; also known as F+ or F-specific) was stronger than for enterococci.

Studies Assessing Antibody Response

Four health effects studies were identified that characterized seroconversion, an immune response to infection, in recreators (Wade et al., 2018, Augustine, et al., 2020, Egorov et al., 2018, and Egorov et al., 2021).

Wade et al. (2018) collected saliva samples as part of the U.S. EPA Boquerón Beach epidemiological study to test for immunoglobulin G (IgG) responses to genogroup I.1 (GI.1) and genogroup II.4 (GII.4) noroviruses. Immunoconversions for noroviruses were observed in 34 subjects, or 2.6 percent of the study population (n = 1,298). Swimmers who reported submerging their heads while swimming had a significantly higher rate of immunoconversion compared to

non-swimmers. Immunoconversion was not statistically associated with GI symptoms. The results of this study demonstrate the potential for transmission of noroviruses among recreators at a marine beach. Another study by Wade et al. (2019) found similar results.

Augustine et al. (2020) demonstrated the use of a rapid salivary antibody assay to assess the prevalence of salivary antibodies against hepatitis A virus and immunoconversions in a population of beachgoers at Boquerón Beach, Puerto Rico. Results showed an immunoprevalence rate of approximately 16 percent among participants, which is less than half the overall immunoprevalence rate among U.S.-born persons 2 years old or older. Only 1.43 percent of the participants who provided all three samples were found to have hepatitis A virus immunoconversions. There was no statistically significant association between hepatitis A immunoconversions and any of the demographic or exposure risk factors tested. Previous health studies conducted at this location reported low rates of GI illness (e.g., <2 NGI/1,000 recreators), low levels of pathogens, and good water quality based on levels of enterococci below the EPA RWQC (U.S. EPA, 2010; Soller et al., 2016). This study demonstrated the potential of rapid assays for immunoconversions to a waterborne virus like hepatitis A virus to inform the protection of recreational water and public health (Augustine et al., 2020).

Egorov et al. (2018) conducted a prospective cohort study recruiting local families with children in Lawrence, Massachusetts (n = 1986) that overrepresented children (1,170 children <18 years; 58.8%). The results were that 12.5 percent of participants that immunoconverted to *Cryptosporidium,* tested through salivary immunoassay, were symptomatic. Immunoconversions by study participants to *Cryptosporidium* after recreational exposure to natural waterbodies or swimming pools was associated with adjusted odds ratios (aORs) of 2.3 (0.4;15.4) and 4.9 (1.6; 15.5), respectively. No association between immunoconversion with noroviruses and swimming in natural waterbodies (i.e., rivers, lakes, or oceans) was observed. However, only 3.6 percent of participant responses indicated swimming in pools and 2.5 percent in natural waterbodies, reducing the sample size included in analysis. For all oral exposure pathways analyzed, incidence rates of *Cryptosporidium* immunoconversions declined steadily with age: 8.5 per 100 person-years in children between 1 and 10 years of age, 5.6 per 100 person-years for age 11 to 20 years, 4.2 per 100 person-years for age 21 to 40, and 1.5 per 100 person-years in adults ages 41 to 85 years. Norovirus genogroups I and II also showed lower rates of immunoconversions in the group age 41 to 85 compared to ages 1 to 10. This community-level study demonstrated the utility of the salivary antibody immunoassay methodology for the detection of infections with *Cryptosporidium* and norovirus. In this study, the immunoconversions were related to potential exposure pathways, thus providing useful information for improving public health protection.

Egorov et al. (2021) conducted a prospective salivary antibody study at a Lake Michigan beach to study seroconversion to norovirus and *Cryptosporidium*. Among 872 study participants, there were seven cases of seroconversion, including six individuals with seroconversion to noroviruses and two to *Cryptosporidium*, with one study participant seroconverting to both pathogens. Those that seroconverted to norovirus were more likely to experience vomiting within 4 days of a beach visit ($p = 0.003$). This study provided further evidence that recreational water exposure can be associated with symptomatic illness and asymptomatic waterborne infections.

New Non-U.S. Epidemiological Studies: 2016–2022

Three recreational water epidemiology studies conducted outside the United States and one review of epidemiological data were identified (Joosten et al., 2017; Kuhn et al., 2018, and Verhougstraete et al., 2020; Young, 2016).

Joosten et al. (2017) conducted a prospective cohort study to assess risk factors for health complaints (GI illness, respiratory illness, and complaints associated with the skin) associated with recreational exposure to urban waters affected by wet weather-associated sewage overflows during canal swimming events in two Netherland cities in 2015. Prior to the events, *E. coli* and enterococci monitoring data from the canals were below the European Union (EU) thresholds. By the afternoon of the day of the event, *E. coli* levels greatly exceeded the EU threshold in one of the two cities while enterococci remained below the EU threshold. Thirty-one percent of swimmers (427 of 1,375 swimmers) reported GI illness symptoms following exposure to canal waters affected by sewage overflows resulting in an adjusted risk ratio (aRR) of 6.3 (95% CI: 4.1–9.5). Five out of seven stool samples provided by participants tested positive for various norovirus genotypes. One water sample from one of the canals tested positive for genogroup I norovirus and two water samples tested positive for genogroup II norovirus. The conclusion of this study is that the outbreak of acute GI illness in swimmers was related to the presence of norovirus, which was possibly linked to wet weather-associated sewage overflows affecting the canals.

Kuhn et al. (2018) conducted a prospective case-control study among Danish persons that included 446 children ages 1 to 5 years old (from a total of 3,119 study participants ages 1 to 30 years old) to identify risk factors for campylobacteriosis. Study participants provided information by completing an online questionnaire. Consumption of contaminated food, animal contact, bathing in fresh waters, contact with beach sand, and bathing in a paddling pool were identified as significant risk factors for increased risk of campylobacteriosis. The authors estimated that 4 percent of sporadic Danish campylobacteriosis cases may be caused by recreational water contact (Kuhn et al., 2018). The authors indicated that, based on their estimates, recreational water contact and contact with sand was likely linked to a large proportion of campylobacteriosis in Denmark's children and young adults.

Verhougstraete et al. (2020) developed adjusted risk difference models (excess gastrointestinal illness per swimming event) for children (<10 years of age) and "non-children" (≥10 years of age) across five Brazilian beaches using epidemiological data collected in the United Kingdom (UK) and Brazil. Water quality at the Brazilian beaches exceeded the maximum fecal streptococci levels measured in the UK studies 11.8 percent of the time. Risks associated with the elevated indicator levels equated to 53 and 96 NGI per 1,000 recreators for non-children and children, respectively. The study concluded that the World Health Organization (WHO) recreational water quality guidelines, based largely on the epidemiological study performed at UK beaches, were not appropriate as a basis for guideline development in tropical settings where there is minimal wastewater treatment. Pathogen profiles distinct to tropical waters and point

source discharges with minimal treatment were two factors identified for supporting the development of more regionally specific guidelines.

Young (2016) reviewed studies published before 2015 of infectious disease transmission in marine bathing waters, sources of pathogens in marine waters, and epidemiological evidence for the association between marine bathing and infectious disease. Numerous studies demonstrated an increased risk of gastrointestinal illness associated with marine swimming compared to non-swimming. However, an association between levels of FIB and illness among swimmers was not consistently found across the studies reviewed. The authors suggest that traditional FIB may not be predictive of human health impacts when human waste was not the predominant source of pathogens. In one study, 71 percent of gastrointestinal episodes in Southern California were estimated to occur when the water quality was considered safe for bathing, potentially implying that most cases of illness associated with marine bathing could arise from the lowest risk exposures, combined with high numbers of people exposed. The authors identified the need for research to identify additional markers for human health risk at non-point source beaches and the use of rapid methods to improve public health protection.

Summary of Findings Identified in New Epidemiological Studies

These studies vary in their focus, but conclusions highlight that:

- While FIB can be a useful indicator in waters impacted by raw and poorly treated sewage, the inconsistent performance of culture-enumerated FIB related to health outcomes was demonstrated in multiple studies, which suggests that other indicators (e.g., *Enterococcus* measured by qPCR) are more predictive of risk of illness.

- qPCR-enumerated enterococci performed better than FIB as a predictor of risk in waters dominated by human fecal sources, and there was some evidence that culture-enumerated enterococci were associated with increased GI illness when human fecal pollution was suspected based on knowledge of point sources.

- Some waters receiving human fecal contamination have measured viral pathogens at levels expected to lead to health outcomes and yet also be below EPA recommended water quality level for culturable FIB.

- Health risk in recreational waters is greatest in children 0 to 10 years old compared to both adolescents and adults.

- The health risk from recreating during or following wet weather compared to dry weather can be due to changes in fecal loading dynamics to a waterbody.

- Use of salivary assays to estimate seroconversion to waterborne pathogens can provide early information on infection by specific pathogens by recreators exposed to fecal contamination. Studies found evidence of seroconversion to norovirus and *Cryptosporidium* among swimmers and also found that those who seroconverted were

more likely to have gastrointestinal symptoms compared to those who did not seroconvert.

2. Use of QMRA to Understand Risks in Recreational Water Settings

QMRA studies published between 2016 and 2022 included studies that characterized human health risk from recreating in waters contaminated by human and non-human fecal sources; used QMRA to develop or evaluate risk-based water quality values for alternative indicators; and evaluated the importance or sensitivity of specific data as input to QMRA for recreational water purposes. The new studies described below report several important trends with respect to the use of QMRA to study recreational water risks. As with prior QMRA studies, the risks evaluated in these studies characterize risk of GI illness and do not include other health endpoints, such as respiratory illness.

Characterization of Risks from Human Fecal Sources

Soller et al. (2016) conducted a QMRA that included fecal indicator and pathogen monitoring concurrently with an epidemiological study to characterize the risk of GI illness at Boquerón Bay, Puerto Rico. Results of the QMRA were used to improve interpretation of a recreational water epidemiological study. Results of the water quality study component of the QMRA demonstrated low levels of pathogens and good water quality. The QMRA findings provide a plausible explanation for the lack of relationship between fecal indicator organism detection and swimming-related illness in the epidemiological study. The QMRA estimated a swimming-associated risk of less than 2 NGI per 1,000 recreation events, which was below the level of illness that the epidemiological study was designed to detect.

Vergara et al. (2016) conducted a QMRA to estimate the GI illness risk to a population from primary and secondary contact recreation in an urban catchment in Singapore using field measurements of norovirus, adenovirus, and *Cryptosporidium*. Land use in the catchment was mostly residential with some industrial and office areas and human contamination can be attributed to non-point sources of pollution (Vergara et al., 2016). Norovirus was detected more frequently and at higher levels compared to adenovirus. QMRA results showed a higher illness risk associated from exposure to norovirus compared to adenovirus. Risks for children (<18 years), based on children-specific recreational water ingestion data, were higher compared to adults and exceeded 36 NGI per 1,000 recreators approximately 5.6 percent of the time. The higher prevalence and illness risk of norovirus supported the use of norovirus as a reference pathogen for QMRA in recreational waters in Singapore.

Lapen et al. (2016) conducted a QMRA of freshwater recreation in rivers in Ontario, Canada. Using *Cryptosporidium* as the reference pathogen, two river basins in Ontario were monitored for *Cryptosporidium* oocysts levels and species/genotype data were collected. The QMRA included two approaches: one assumed all observed oocysts were infectious to humans, and for the second, risk was based on the fraction of oocysts that were *Cryptosporidium hominis* and/or *Cryptosporidium parvum*, which are the predominant human infectious forms of the parasite. Compared to assuming all oocysts are infective to humans, the estimated infection risk was one

order of magnitude lower when only human infectious forms were selected, and fluctuations in risk were also observed depending on seasonality. Results of this study demonstrate the variability in potential risk when different pollution sources can occur. Also, the availability of pathogen data at the species/genotype level can help inform more accurate estimates of potential human infectious risk in a QMRA.

Eregno et al. (2016) coupled discharge-based hydrodynamic modeling with QMRA to estimate the risk of infection from swimming in marine waters following a rainfall event with combined sewer overflow events (CSOs). Of the simulated bacteria, protozoa, and virus infection risks, the virus risk dominated, as represented by norovirus, and exceeded the infection health benchmark of 19 GI illnesses per 1,000 swimmers in the days after the rainfall event. This modeling result corroborates the findings of epidemiology studies (described above) that identified wet weather and sewage overflow as factors leading to increased pathogen levels and infection risks. The study demonstrates the potential utility of combining discharge-based hydrodynamic modeling with health modeling in QMRA to estimate risk under wet weather conditions to inform beach management conditions.

Soller et al. (2017) used QMRA to predict the GI illness risk to adult surfers associated with wet weather at marine beaches in Southern California impacted by urban stormwater. In the QMRA, sanitary survey information, monitoring data for fecal indicators, human microbial source markers, reference pathogens, site-specific dilution estimates, and literature-based data for incidental ingestion, pathogen dose-response, and morbidity were considered together to generate risk estimates associated with wet weather stream flows affecting coastal nearshore waters. As part of a broader "Surfer Health Study," data collection for the QMRA was conducted in parallel with a longitudinal recreational water epidemiological study focusing on surfer exposure (Arnold et al., 2017). Water quality monitoring showed the presence of human fecal contamination and fecal-associated pathogens in the stormwater discharging to the coastal water where surfers were exposed. The results of the health modeling demonstrated that enteric viruses, as indexed by norovirus, were an important etiologic agent of GI illness among surfers. The QMRA was bolstered by a sensitivity analysis characterizing the uncertainty associated with using published norovirus dose-response information. QMRA risk estimates from this study corroborated the reported epidemiological results (Arnold et al., 2017). Predicted average illness levels were lower at higher levels of FIB when compared to the epidemiological data that informed EPA's national recommendations. For both the QMRA (Soller et al., 2017) and epidemiological (Arnold et al., 2017) components of the "Surfer Health Study," the predicted and reported GI illness levels represent a specific short-term high-risk scenario associated with exposure to human fecal contamination present in coastal stream discharges containing urban stormwater. The QMRA methodology used in this study and the reported results can be useful in developing alternative health-based water quality criteria specific to risks associated with wet weather impacts in an urbanized setting.

Bortagaray et al. (2020) assessed the risk of infection and illness for Group A Rotavirus (RVA) in the Santa Lucia and Uruguay watersheds in Uruguay that are affected by raw sewage from two cities. The authors performed qPCR on surface water samples and developed a QMRA

framework for people who use surface waters from the rivers. RVA was detected at all sampling locations in both watersheds with an approximate detection frequency of 40 percent of samples. Detection frequency increased during the coldest month of the year. The mean level of RVA was 1.3×10^5 genomic copies per liter (L). Due to the frequency and occurrence of RVA, QMRA results demonstrated that populations using both rivers had comparable risks of infection and illness.

Shoults et al. (2021) conducted a reverse QMRA for a natural swimming pool relying on biological treatment processes, including a biofilm filter, a zooplankton filter, a hydro-botanic filter, and a submerse filter in constructed wetlands coupled with ultraviolet (UV) irradiation prior to the treated water being returned to the pool. The authors found that of the four reference pathogens included (norovirus, *Campylobacter*, *Cryptosporidium*, and *Giardia*), only norovirus exceeded the median recreational water risk benchmark. Log reduction values (LRVs) for the reference pathogens were developed. The authors indicated that more than 1 day of treatment would be needed to achieve the risk benchmarks after heavy bather use. Finally, the authors concluded that ultraviolet disinfection had little effect on reducing the treatment time required.

Abia et al. (2016) conducted a QMRA to evaluate the public health risk associated with exposure to pathogenic bacteria in polluted river water under undisturbed conditions and conditions of sediment resuspension in the Apies River, Gauteng, South Africa. River water was monitored for *E. coli* levels and the presence or absence of three feces-associated enteric pathogens: *Salmonella* spp., *Shigella* spp., and *Vibrio cholerae*. The authors assumed that 8 percent of generic *E. coli* counts were pathogenic. Ingestion rates of 1 mL, 50 mL, and 100 mL were used to represent different levels of ingestion exposure. The results indicated that risks of infections increased during the wet season. A 2-log increase in water *E. coli* count following sediment disturbance led to approximately 10 times higher probability of infection than when sediments were undisturbed.

Liao et al. (2016) linked a watershed-scale microbial fate and transport model with a stochastic dose-response model in a QMRA to predict human health risks from different fecal sources in an urban watershed for comparison with regulatory benchmarks in order to prioritize remediation efforts. Results indicated that human illness risks were consistently higher than 36 illnesses per 1,000 people for the study watershed, even when the predicted FIB levels were in compliance with the *E. coli* GM standard of 126 CFU per 100 mL. Sanitary sewer overflows were associated with the greatest risk of illness, which is of particular concern, given increasing indications that sewer leakage is ubiquitous in urban areas. Uncertainty analysis suggested the accuracy of risk estimates would be improved by additional site-specific pathogen data. The authors recommend integrating this QMRA with water quality management planning to provide greater clarity to stakeholders and decision makers (Liao et al., 2016).

Characterization of Risks from Non-Human Fecal Sources and Mixed Sources

Gitter et al. (2020) used QMRA to estimate the probability of GI illness from exposure to a freshwater creek impacted by a mixture of sources. Pathogen concentration was estimated based on FIB MST source apportionment of human, wildlife, cattle, and domestic animals, assuming all detected FIB were from fresh contamination. The results indicate that the risk of illness from

norovirus, representing human fecal sources, contributed the greatest risk to human health. The most frequent sources of *E. coli* at the sites monitored were from non-human sources (Gitter et al., 2020).

Lim et al. (2017) conducted a source-apportionment QMRA to assess risk of GI illness at a Southern California beach (Baby Beach, City of Dana Point) that did not have a known source of human fecal contamination. Dry weather inputs of enterococci included bather shedding and birds. Wet weather inputs of enterococci included the potential presence of sewage in stormwater outflows (up to 20% of enterococci loading), animal feces from wildlife and dogs, and nonpathogenic sources, such as plant and soil associated sources. Historical enterococci measurement data were used to evaluate risks in dry and wet weather scenarios. During dry weather, the median recreational waterborne illness risk at this beach is below the EPA's RWQC target illness rate of 36 illness cases per 1,000 bathers regardless of the fecal source contributing enterococci. During wet weather, the median recreational waterborne illness risk predicted by the QMRA depends on the assumed level of human waste associated with stormwater; the risk is below the EPA RWQC illness risk benchmark 100 percent of the time, provided that <2 percent of the FIB in stormwater are of human origin.

Evaluating Alternative Water Quality Surrogate Measures and Derivation of Risk-Based Thresholds

Boehm et al. (2018) investigated the risk of gastrointestinal illness associated with swimming in surface waters with aged sewage contamination. A systematic literature search and meta-analysis compiled decay rate constants for reference pathogens and human feces-associated source markers, which were incorporated into the QMRA. The QMRA evaluated HF183, a human-associated fecal source marker, as an index for sewage present and thereby provided insight into how risk relates to HF183 concentrations in surface water. Risks were modeled for aged sewage and sewage of unknown age. The risks from recreational exposure to fresh and aging sewage were primarily attributed to the reference pathogen norovirus. Based on the range of decay values (n = 52) from the literature, HF 183 was shown to decay (~0.4 $\log_{10}$ d^{-1}) faster than norovirus as sewage ages. Therefore, the risk associated with a fixed number of gene copies of HF183 increases with the age of contamination. A HF183 risk-based water quality threshold for fresh sewage was 9,700 copies per 100 mL and 900 copies per 100 mL after sewage aged for 2.5 days.

Ahmed et al. (2018a) estimated concentrations (genome copies [GC]) of sewage-associated markers in 100 mL of beach water sample contaminated with either fresh untreated or secondary treated sewage that exceeded the median illness rate of 36 per 1,000 people at a single event for reference pathogens norovirus and human adenovirus 40/41. The concentration of 3.22×10^3 GC of the HF183 in 100 mL of water sample corresponded to a risk above the GI illness benchmark value when beach water was contaminated with fresh untreated sewage and roughly the same for waters contaminated by secondary treated effluent.

Wu et al. (2020) identified RBTs (corresponding with 36 illnesses per 1,000 swimmers) within a QMRA context in a hypothetical waterbody contaminated by a continuous loading of both

human and non-human fecal contamination. Their results indicated that a larger difference in decay rates between pathogen and indicator leads to an increased miscalculation of gastroenteritis risk compared to calculations that do not account for differential decay. Given the continuous loading scenario, the difference in RBT between fresh and aged contamination was less for human marker HF183 in the human contamination scenarios than for animal markers in the animal contamination scenarios. The median RBT for human contamination was approximately 3.5 to 3.7 $\log^{10}$ gene copies per 100 HF183.

Goh et al. (2021) estimated RBT of human polyomaviruses (hPyVs), *Bacteroides thetaiotaomicron* (*B. theta*), and *Methanobrevibacter smithii* (*M. smithii*) that correspond to the acceptable GI illness risks associated with recreational activities in Singapore using a QMRA approach. Among the three MST markers, hPyVs showed the highest specificity (100%) to sewage samples, followed by *M. smithii* (97%) and *B. theta* (90%). All MST markers showed 100 percent sensitivity toward sewage contamination, with *B. theta* present in highest abundance in sewage, followed by hPyVs and *M. smithii*. Field data showed that the MST markers at threshold concentrations were able to classify the safe level in more than 83 percent of the samples. This study presents the range of threshold concentrations in MST markers in freshwater and seawater matrices by considering two scenarios (i.e., decay and dilution) under different exposures (i.e., minimum, average, and maximum ingestion rates for swimming activity).

Brown et al. (2017) interpreted measured concentrations of *Catellicoccus* (CAT) genes (from gull contamination) in recreational waters. The authors took 37 gull fecal samples to measure the CAT gene concentrations and integrated the measurements in a QMRA framework using dose-response functions from reference pathogens. They estimated that when the level of CAT surpasses 4×10^6 copies per 100 mL of water, the median predicted illness exceeds three illnesses per 100 swimmers.

Boehm and Soller (2020) incorporated updated data for microbial decay rates and aging of mixtures of human sewage contamination to derive a risk-based water quality threshold for the human marker HF183 in ambient waters using QMRA. The authors identified a threshold of 1–525 HF183 copies per 100 mL that corresponded with the health benchmark of 32 illnesses per 1,000 for different contamination scenarios. This threshold was estimated for a scenario in which human contamination in sewage of different ages co-occurs with contamination from gull feces.

Schoen et al. (2020) used QMRA to explore the RBT of various markers for different recreational water quality scenarios based on a series of assumptions and uncertainties. For example, they found that fresh sewage RBT estimates were not always protective when aged sewage was present, and aged sewage RBT estimates often fell below the marker lower limit of quantification. Conservative RBT estimates of 9.3×10^2 and 9.1×10^3 (copies/100 mL) for HF183/BacR287 and CPQ_056, respectively, were predicted when fresh sewage was greater (by volume) than aged at the time of measurement. Conversely, genetic markers may not be effective indicators when aged sewage contributes the majority of pathogens but minimal marker levels, relative to fresh contamination. This study describes the importance of taking into account the

impact of a potentially slower decay rate and higher abundance when modeling public health risk. The results highlight the utility of QMRA that incorporates pollutant age and mixture scenarios, and the potential influence of site-specific factors on estimating RBT values. The approach discussed in this study has been refined in multiple previous studies (Boehm et al., 2015, 2018; Boehm and Soller, 2020) and supports establishing health protective RBTs for alternative indicators.

Van Abel et al. (2017) conducted a QMRA to assess risk in surface waters used for drinking, domestic, and recreational purposes in South Africa. Water monitoring results for three rivers demonstrated a frequency of occurrence of norovirus genogroup I (50%) and genogroup II (74%), with genogroup I occurring at lower levels than genogroup II. Risk estimates were based on the occurrence of norovirus, exposure route and the dose-response model selected. Risk of norovirus infection was sensitive to the choice of dose-response model chosen. Recreational risk varied with the level of exposure (i.e., swimming, playing on the shore adjacent to the river, and boating) with swimming yielding the highest risk. Depending on the dose-response model, river waters frequently exceeded an annual recreational water illness benchmark of 0.03 (genogroup I: 46%–96%, genogroup II: 74%–96%). The risk burden was greater for individuals from vulnerable subpopulations who may utilize untreated surface waters for multiple uses.

Sunger et al. (2019) used QMRA incorporating literature values of pathogen densities in secondary treated wastewater effluents to simulate the risk of illness from swimming in freshwater receiving wastewater effluent. Data from both disinfected and non-disinfected secondary effluent were used. Enteric viruses, as indexed by norovirus, dominated the estimate risk of GI illness.

Dose-response modeling has advanced for several waterborne pathogens that are commonly used as reference pathogens in recreational water QMRAs. Adenovirus (Teunis et al., 2016), norovirus (Teunis et al., 2020), and *Campylobacter* (Teunis et al., 2018) dose-response modeling has been further developed by combining data sets and conducting Bayesian analyses to better characterize infection and illness rates. Teunis et al. (2020) reported a meta-analysis of clinical challenge studies for norovirus and norovirus outbreaks from consumption of oysters. Data included were from 14 challenge studies using six different norovirus inocula and nine outbreaks. The study showed how challenge studies and "natural experiments" (outbreaks) can be combined in a multilevel, hierarchical dose-response framework. Overall, the results of the study confirm the high infectivity of norovirus reported by earlier studies (Teunis et al., 2008). Teunis et al. (2020) indicate that an updated dose-response model may be applied in QMRA, with the potential benefit of differentiating between norovirus genogroups and host secretor status.

Evaluating Parameter Selection and Sensitivity Analyses

Ballesté et al. (2018) examined the impact of decay rates of fecal source identification (FSI) markers and FIB from different sources. The authors concluded that it is important to take into consideration the rate of inactivation of bacteria and FSI markers in QMRA or other models used to model pollution sources in freshwaters. Decay rates were found to be affected by changes in

temperature (seasons), sunlight, predation rates, presence of organic matter, fecal source, and alkalinity. Intrinsic characteristics of indicators and markers for specific sources influenced seasonal changes in decay rates.

Federigi et al. (2019) used PRISMA guidelines to review QMRAs of recreational waters published between 2003 and 2018. PRISMA guidelines define the minimum set of items for reporting in systematic reviews. They found research gaps included: 1) a lack of epidemiological data on the main pathogens of concern in specific geographic areas; 2) a lack of site-specific water quality data; 3) little consideration of recovery efficiency or infectivity of pathogens; 4) lack of consideration of host-specific factors (like previous immunity or immunodeficient); and 5) lack of QMRA model validation. The authors conclude that the use of QMRA is "very promising for management of recreational waters and as a mean[s] to improve the regulations," but additional research in the areas identified could improve the reliability of results.

Poma et al. (2019) evaluated type of methodology used for finding the best fit for pathogen data and reported that this is a critical consideration in QMRA, especially in data sets with numerous non-detects, because different approaches can result in very different estimations of risk. Five different alternatives to represent censored data were evaluated. They found that the decision on how to treat censored data, especially when they are abundant in the data set, is crucial. These findings led the authors to suggest that, if in doubt about which approach to use, one should adopt a more protective approach, such as using half the detection limit.

Summary of Findings from New QMRA Studies

- Additional evidence shows that waters affected by human fecal inputs can pose higher health risks compared to non-human fecal sources.
- Several studies utilized norovirus as a primary reference pathogen to characterize risk from human enteric viruses in waters affected by human fecal pollution.
- QMRA combined with epidemiological data can be a robust approach for characterizing elevated health risks in acute exposure recreational scenarios.
- Children-specific exposure data can be used in a QMRA to estimate potential illness risk to recreating children.
- Across analyses, the age of fecal contamination was identified as an important factor affecting risk estimates and calculation of RBT values for alternative indicators.

3. Outbreak Studies of Illnesses in Ambient Recreational Waters Associated with Enteric Pathogens

Outbreaks of illness in recreational waters are defined as the occurrence of similar illnesses in two or more persons, epidemiologically linked by location and time of exposure to recreational water or to pathogens, toxins, or chemicals aerosolized or volatilized from recreational water into the surrounding air (Graciaa et al., 2018). The National Outbreak Reporting System (NORS) is a

passive reporting system through which state and local health officials voluntarily report outbreaks to the Centers for Disease Control and Prevention (CDC). CDC reported NORS information on leading etiologies causing outbreaks nationally in ambient recreational waters between 2000–2014 (Graciaa et al., 2018). Vanden Esschert et al. (2020) discussed three outbreaks in detail including reported FIB monitoring data for two of the outbreaks. Additionally, four recreational water-related outbreaks of illness occurring outside the United States were identified in the peer-reviewed scientific literature (Mosnier et al., 2018; Polkowska et al., 2018; Schets et al., 2018; Sips et al., 2020). During 2000–2014, CDC reported 140 untreated recreational water-associated outbreaks resulting in at least 4,958 cases and two deaths of which 95 outbreaks were confirmed to have a pathogen etiology, including five outbreaks with multiple etiologies (Graciaa et al., 2018). The 95 outbreaks represented at least 3,125 cases; enteric pathogens caused 80 (84%) resulting in 2,704 cases, with norovirus linked to 21 outbreaks (22%), pathogenic *E. coli* linked to 19 (20%), *Shigella* linked to 14 (15%), and *Cryptosporidium* linked to 12 (13%). Of the eight outbreaks related to toxins or chemicals, seven (88%) were caused by algal toxins associated with harmful algal blooms (HABs). Most outbreaks were associated with freshwater venues (84%) and occurred during the June–August timeframe (81%). Statistical analyses indicated the outbreak number was not significantly different over the 15-year reporting period (Graciaa et al., 2018).

CDC recorded 119 outbreaks associated with ambient recreational water for the period 2009–2019. In 69 of the outbreaks (58%), enteric pathogens were identified as the cause: norovirus (19 [22%]; 1,858 cases); Shiga toxin-producing *E. coli* (STEC) (19 [22%]; 240 cases); *Cryptosporidium* (27 [19%]; 237 cases); and *Shigella* (14 [16%]; 713 cases) (Vanden Esschert et al., 2020). In July 2018, 97 people reported becoming ill with GI symptoms after swimming at Woods Pond Beach in Maine. Recreators reporting head submersion or swallowing water were approximately three times more likely to be ill. Two stool specimens collected from four ill people tested positive for norovirus genogroup I although the source of contamination was not identified (Vanden Esschert et al., 2020). In August 2019, 69 cases of STEC infection were identified in persons who reported swimming at a public lake in Minnesota. No *E. coli* monitoring results from this outbreak exceeded state recreational water criteria during April–October. No evidence of a point source of fecal contamination was identified, but investigators did report multiple swimmers and lifeguards had contact with the lake while ill (Vanden Esschert et al., 2020). In July 2019, 24 cases of shigellosis were identified in California following contact with Santa Ana River water. *E. coli* concentrations in Santa Ana River water exceeded acceptable levels and ranged from 350 to 1,600 most probable number (MPN) per 100 mL. Flow in the Santa Ana River consists of discharges from multiple wastewater treatment plants (WWTPs) and non-point sources of pathogens affect the quality of the river (California Water Boards, 2022).

Four international outbreak investigations that demonstrated an increased risk burden among children recreating in contaminated recreational water and/or provided specific etiological information on the cause of the outbreak were found in the literature search. Norovirus was identified as a cause of outbreaks at public beaches (Polkowska et al., 2018; Schets et al., 2018)

or other untreated recreational water venues (Sips et al., 2020). One study characterized a *Cryptosporidium*-related outbreak among children in an isolated village with poor sanitation (Mosnier et al., 2018).

Outbreaks of norovirus affected at least 1,093 people swimming at beaches in Finland in 2014 reported via a web-based survey (Polkowska et al., 2018) and 100 people swimming in a lake in the Netherlands in 2012 (Schets et al., 2018); norovirus was detected in 19 of 23 (Polkowska et al., 2018) and 4 of 5 (Schets et al., 2018) stool samples, respectively. No point sources of human fecal contamination were identified at the lakes involved in either outbreak.

Polkowska et al. (2018) conducted a retrospective cohort study on an outbreak of gastroenteritis in Tampere, Finland, which included children from 0 to 17 years old. Beach water monitoring demonstrated acceptable levels of FIB (range from 0 to 5 CFU enterococci per 100 mL and 1 to 27 CFU *E. coli*/100 mL). The authors developed a web-based survey to identify infection sources and risk factors. Cases in the general population included children who visited beaches at four different lakes. Identified risk factors included exposure by getting water into the mouth while swimming, swallowing water while swimming, submersing the head under water, and playing at the beach. Generally, children 0 to 17 years old were more frequently affected, which the authors related to higher exposure associated with recreating activities. Risks associated with submerging the head under water were higher for children 0 to 4 years compared to the general population (risk ratio 3.55 vs. 3.24). For other risk factors, the risk ratio was either lower for children compared to the general population or not significant. The potential sources of enteric pathogens detected (norovirus, *Campylobacter jejuni*, and rotavirus) were infected beachgoers. Environmental health officers reported poor hygienic conditions at public beaches, including children urinating or defecating on the beaches, overflowing trash receptacles, and used diapers observed on the beach. (Polkowska et al., 2018). In both outbreaks, infected individuals may have contaminated lake waters and contributed to secondary transmission of norovirus between recreators. The authors suggested a need for new indicators of water quality for norovirus and development of evidence-based recommendations regarding timing of safe reopening of recreational water venues associated with outbreaks.

Schets et al. (2018) reported an outbreak of norovirus infection in Gelderland, the Netherlands, among swimmers at a freshwater lake. Most cases were children with gastroenteritis symptoms, and 82 percent of the cases had been in the water for 30–60 minutes. An epidemiological investigation, combined with detection of norovirus genogroup I in stool samples of patients and sand samples collected at the lake, suggested that exposure to the recreational lake resulted in the outbreak. The most likely source of contamination was determined to be an infected human and the authors concluded that active communication about human shedding of viruses during and after diarrhea is needed along with guidance to refrain from swimming when contamination is suspected.

Outbreaks of recreational water-associated GI illness reported since 2017 show that children can be disproportionately susceptible to health effects from exposure to pathogens in recreational waters compared to adults (Mosnier et al., 2018; Schets et al., 2018; Sips et al., 2020).

Mosnier et al. (2018) describe an increase in cryptosporidiosis reported over a 6-month period among children living in a remote area along the Maroni River in French Guiana. Questionnaires were distributed to collect data on demographics, food consumption, river interaction (play, washing), symptoms, and outcome. Stool samples were collected and analyzed 3 months after the onset of symptoms. Mosnier et al. (2018) report on a specific outbreak cluster of 14 cases, all children, aged between 4.5 and 38 months, and all linked to *Cryptosporidium hominis*. The outbreak was notable because it represented a peak in occurrence compared to previous sporadic cases and occurred during a dry period. Human behavior and activities in the river area were suspected to be the driver for the outbreak among the children. Numerous playgrounds on the riverside included clothes washing, bathing, cooking/eating, playing, and defecation. Fecal contamination and high turbidity were noted at water supply locations. Although this outbreak likely resulted via multiple factors occurring at this location, it does provide evidence that children, who have immature immune systems, can be at a disproportionate risk of contracting severe infectious diseases when pathogen prevalence increases in a population.

Sips et al. (2020) described a norovirus outbreak in a natural playground in the Netherlands where untreated water flows from a nearby river into a recreational lake. The Public Health Service received 21 case notifications via the national online reporting system and an additional 100 cases (mostly children) were identified following an online query of the local health-related Facebook platform. Water and fecal samples from humans and birds were tested and human introduction of norovirus was identified as the most likely cause of the outbreak.

Summary of Findings from New Outbreak Reports

- Outbreak data document increased risk to children in recreational waters
- Outbreak surveillance in the United States and elsewhere continues to indicate the importance of human enteric viruses as a leading etiologic agent of illness in ambient recreational waters.
- Some outbreaks documented high levels of FIB in waters linked to the outbreak, but some outbreaks occurred despite low densities of FIB.

4. New Information on Children's Exposure in Recreational Waters

In 2019, EPA published *Recommended Human Health Recreational Ambient Water Quality Criteria or Swimming Advisories for Microcystins and Cylindrospermopsin*, which used data from Dufour et al. (2017) to characterize incidental ingestion for children ages 6 to 10 years old and 7 to 11 years old compared to adults (Cyanotoxin AWQC, U.S. EPA, 2019). Section 4.2.3.1 of the Cyanotoxin AWQC includes graphical visualizations of ingestion volumes for different age groups, information on the length of time an individual spends in the water (duration of exposure), and a calculation of the daily incidental ingestion rate. Children ages 6 to 10 years old have a daily incidental ingestion rate higher than any other age group. Section 7.3.2 of the Cyanotoxin AWQC discusses exposure factors for children younger than 6 years old. Data for ingestion by children <6 years old is more qualitative in nature and there are large uncertainties

given the lack of measured incidental ingestion data specifically for this age group, thus it is challenging to compare this group directly to other age groups. However, using assumptions and estimates for exposure for younger children from EPA's 2011 *Exposures Factor Handbook* and the data reported from Schets et al. (2011), the daily ingestion rate for children <6 years old was estimated to be lower than the 6- to 10-year-old age group. Section 7.2.1 of the Cyanotoxin AWQC presents data on duration of recreational exposures from both DeFlorio-Barker et al. (2017) and EPA's *Exposure Factors Handbook* (U.S. EPA, 2011).

DeFlorio-Barker et al. (2018) compiled self-reported swimming durations from epidemiological study surveys from 12 beaches in which participants were asked to estimate, in minutes, the total time they spent in the water. The study combined the data for time spent in the water with incidental ingestion volumes reported in Dufour et al. (2018) to estimate the incidental ingestion of water swallowed per swimming event by age group. Parents or guardians were responsible for answering survey questions assessing exposures such as getting water in the mouth or swallowing water, on behalf of their minor children. The study results represent 7,534 children ages 8 to 12 years old and 5,998 children ages 4 to 7 years old recreating in fresh and marine water. Marine recreators spent more time in the water compared to freshwater recreators. The authors suggest that behaviors may have been influenced by the warmer water at most of the marine sites (California and Gulf Coast) compared to the freshwater sites in the Great Lakes. Children ages 4 to 7 and 8 to 12 years old had the highest exposures. Children 6 to 12 years old were estimated to have the highest ingestion amounts and male children ingested more water compared to females. This study provides additional evidence that children have higher exposure in recreational water compared to adults and supports the results of other studies that show children have higher risks of GI illness from recreational exposures.

The Influence of Children's Behavior on Exposure to Recreational Waters

Recent studies of children's behavior support other conclusions that they spend more time in water and engage in more vigorous activity. Ferguson et al. (2019) conducted an observational study focused on children ages 1 to 6 years old and their behavior patterns at recreational marine beaches in Florida and Texas. Analyzing data from more than 400 parental surveys in both locations, the authors found that over 46 percent of the study participants stay for 3 hours or longer at the beach when going with their children, increasing their potential time of exposure compared to adults who stay for less than 3 hours. Survey respondents also indicated that children ages 1 to 6 years old spent 31–43 percent of the time in the water, 27–35 percent in the intertidal zone, and 34–45 percent in dry sand. The authors found that age was the most relevant factor in how and where children play (gender being less influential), with dry sand being the most common zone for children 1 to 6 years old, although 6-year-old children spent the most time in water. Hygiene practices like washing hands before eating at the beach significantly varied by region ($p < 0.001$), indicating that only 37 percent of children washed their hands before eating in Florida compared to 63 percent in Texas. Lastly, the authors observed that the time spent digging in the sand was longer than previously published estimates. Although the data were self-reported, they provide insights about children's behaviors in marine coastal beaches.

Ferguson et al. (2021), used a virtual timing device to quantify real-time, sequential micro-activity pattern data collected from videos of 120 children at four different beaches. This study is the first to use this approach extensively for collecting data on children's behavior at beaches. Across all sexes, age groups, and beaches, wading was the most common activity and seawater was the most common location where children played. For all age groups (0 to 6 years old), the majority of time was time spent wading (activity) for both male (40.7%) and female (41.5%), which corresponds to the seawater location where the most time was spent by both female (45.8%) and male (47.5%) children. The fraction of time spent wading was 38.9 percent for children 0 to 24 months, 37.6 percent for children 25 to 48 months old, and 45.1 percent for children greater than 48 months. Although the study did not address ingestion directly, the authors reported mouth contacts and for the majority of time, the mouth was not in contact with any surface. When the mouth did contact a surface, the most common item was food (2%), the next common was drinks (1.2%). The authors concluded that, based on activity patterns, exposures for young females and males may be similar, and for dermal exposure and ingestion exposure, the right hand is influential.

Summary of Findings from New Studies on Children's Exposure

New studies on children's exposure indicate that risk differences can be influenced by one or more of the following:

- Children's recreational exposure is greater than any other age group. In fact, studies showed that ages 6 to 12 years old had greatest total volume of ingestion of recreational water, independent of body size.

- Children's greater exposure is due to behaviors such as increased time spent in water, length of time spent playing in the sand, and more vigorous activity, which are associated with greater health risk compared to adolescents and adults.

5. Summary of Major Findings from New Information on Health Effects and Children's Exposure

- Recent publications have advanced understanding and further confirm that epidemiological and outbreak data demonstrate that children 0 to 10 years have a higher risk of illness compared to adults when exposed to fecal contamination in recreational waters.

- Children's behavior, such as increased time spent in water, length of time spent playing in the sand, and more vigorous activity, can increase their potential exposure to fecal contamination and is associated with greater health risk compared to other life stages, especially those 18 and over. Children's recreational exposure is greater than adolescents and adults because they ingest more water in proportion to their body weight than the other age groups.

- Studies demonstrate that human fecal contamination can pose higher risks of illness in recreators relative to some non-human fecal sources.

- While FIB can be a useful indicator where raw and poorly treated sewage dominates water quality, multiple studies discuss the inconsistent performance of culture-enumerated FIB related to health and point to other indicators to better represent the potential risk of illness (e.g., *Enterococcus* measured by qPCR).

- Waters receiving human fecal contamination can have viral pathogens present at health-relevant levels and yet also be below recommended water quality levels for culturable FIB.

- The health risk of recreating during or following wet weather compared to dry weather can be different due to changes in fecal loading dynamics to a waterbody.

- Recent QMRA studies corroborate past findings that waters affected by human fecal inputs can pose higher health risks compared to non-human fecal sources.

- Several QMRAs utilized norovirus as a primary reference pathogen to characterize risk from human enteric viruses in waters affected by human fecal pollution.

- Across QMRA studies, the age of fecal contamination was identified as an important factor affecting risk estimates and calculation of RBT values for alternative indicators.

- Outbreak surveillance in the United States and elsewhere continues to indicate the importance of human enteric viruses as a leading etiologic agent of illness in ambient recreational waters, even where low densities of FIB occur.

- Multiple outbreak investigations demonstrate the increased health burden children experience when exposed to fecal pollution in ambient waters.

B. New Information on Coliphages

Since the finalization of the first five-year review, new peer-reviewed scientific literature on coliphages has been published. In particular, EPA has focused on literature assessing coliphages as indicators of fecal contamination and their ability to act as a surrogate for enteric viruses in human fecal contamination that impacts recreational waters. The sources of the new literature for this section on coliphages include studies cited in the literature review performed by Nappier et al. (2019a) and thorough discussions with experts (e.g., U.S. EPA, 2017a) and colleagues.

In 2019, a systematic literature review and meta-analysis characterizing the occurrence of coliphages in raw wastewater and ambient waters was published (Nappier et al., 2019a). The review identified studies published up to January 2017 of coliphages occurrence in raw wastewater and ambient waters of sufficient quality and study design to support estimating coliphage distributions accounting for geographic region and season. Study results reviewed

consistently found that somatic coliphages occur in significantly higher numbers compared to MSC in both raw wastewater and ambient waters, which is consistent with other published results. In ambient waters, somatic coliphages were more prevalent compared to MSC. Analysis of the available quantitative data and distribution estimates showed higher densities of somatic coliphages compared to MSC in raw wastewater, but this difference is not significant when accounting for geographic region or season. Seasonal trends for both somatic and MSC were noted with a winter peak for both coliphage types, which is similar to the seasonal variation reported in the literature for noroviruses. The distributions for somatic and MSC reported in Nappier et al. (2019a) could be used to support various risk assessment applications, such as water reuse, shellfish, and understanding recreational water risk.

Additional studies found in the published scientific literature since 2017 have emphasized the need for a viral surrogate to evaluate risk of human fecal contamination affecting ambient waters. Culturable FIB are more effectively reduced by conventional WWTP processes than are enteric viruses, such as adenovirus and norovirus, and protozoa (Dias et al., 2018; Korajkic et al., 2022). Because FIB are disproportionately inactivated by most water treatment and environmental degradation processes compared to viruses, Farkas et al. (2020) concluded that FIB are "poor indicators of viral infection risk" and should not be used as the sole indicator of human fecal contamination in water quality monitoring programs. Furthermore, Farkas et al. (2020) pointed out that somatic coliphages and male-specific ribonucleic acid (RNA) bacteriophages are used to assess wastewater contamination but are not always specific to human feces. While some studies corroborated the finding that FIB are useful as a fecal contamination indicator, the level of FIB may not be a good indicator of waterborne viral pathogens (Dias et al., 2018 Korajkic et al., 2018).

1. Research on Coliphages

Since the first five-year review, a series of studies were published measuring coliphage occurrence trends in recreational waters and wastewater (Wanjugi et al., 2018, Korajkic et al., 2020a; Jones et al., 2022), environmental decay and fate and transport characteristics of coliphages (Korajkic et al., 2019a; McMinn et al., 2019; McMinn et al., 2020; Boehm et al., 2019), wastewater disinfection effectiveness on coliphages (Korajkic et al., 2022; Jones et al., 2022), co-occurrence of coliphages and common host-associated fecal source markers (Li et al., 2021), and wastewater treatment process efficacy on the removal of pathogens and indicators (Ryu et al., 2021).

Wanjugi et al. (2018) characterized the incidence of culture-enumerated MSC and somatic coliphages, *E. coli* and enterococci in recreational waters in the Great Lakes basin. FIB concentrations were consistently higher than the concentration of MSC and somatic coliphages. Somatic coliphage levels were consistently higher compared to MSC. Ultraviolet light absorption and water temperature, but not FIB levels, were closely associated with coliphage concentrations suggesting different persistence trends between FIB and coliphages in Great Lake waters. In general, physico-chemical properties and recreational area parameters (e.g., meteorological)

were better predictors of FIB compared to coliphages at Great Lake recreational sites tested in this study (Wanjugi et al., 2018).

Korajkic et al. (2020a) reported geospatial trends of culturable bacteriophages (MSC, somatic, total coliphages, and GB-124 phage), and multiple fecal pollution-related genetic markers in 49 primary influent wastewater samples collected from rural and urban WWTPs across the United States. Total coliphages were enumerated using the CB390 host. Like the pattern reported for the Great Lakes region by Wanjugi et al. (2018), coliphages consistently occurred at levels lower than FIB at a national scale. On average, there was a trend of higher somatic coliphage levels compared to MSC levels, however, $\log_{10}$ plaque forming units (PFU) per 10 mL concentrations of somatic, MSC, and total coliphages were not significantly different from each other ($p \geq 0.469$). Notably, the indicators tested in this study exhibited a geographic stability in their occurrence, regardless of urban or rural location designation. However, the data collected represent a three-month monitoring effort during the winter season. This study provides a large, paired data set consisting of multiple viral and bacterial indicators that can support new or updated QMRA models (Korajkic et al., 2020a).

Other studies reported on decay characteristics of both coliphage types and adenoviruses in lake water (McMinn et al., 2020), the effect of various biotic and abiotic environmental parameters on die-off of coliphages and other fecal microbiota in ambient waters (Korajkic et al., 2019a), as well as fate and transport of coliphages and other indicators of fecal pollution in a constructed wetland designed to treat an impaired creek (McMinn et al., 2019).

Boehm et al. (2019) conducted a systematic review and meta-analysis of decay rates of mammalian viruses and coliphages in surface waters. The decay rate constants of MSC and somatic coliphages were similar to those of enterovirus, human astrovirus, and rotavirus A. Norovirus (specifically Norwalk virus), hepatovirus A, and mastadenovirus had smaller decay rate constants than coliphages. Decay rate constants were higher with increasing water temperatures. The decay rate constants for coliphages were smaller in fresh water versus estuarine and marine waters. However, this pattern was not observed for mammalian viruses, most of which had insufficient data to make a comparison (Boehm et al., 2019).

McMinn et al. (2020) conducted an in situ mesocosm study in a freshwater lake to compare decay characteristics of MSC and somatic coliphages to human adenovirus 2. Effects of sunlight and indigenous protozoans on viral decay characteristics were also evaluated. The results demonstrated that decay of coliphages and adenovirus were similar in the mesocosms and that the greatest log reductions were observed when viruses were exposed to both sunlight and predation. Somatic coliphages were the most affected by sunlight (McMinn et al., 2020).

Korajkic et al. (2019a) summarized the most recent published literature on the decay of fecal microorganisms in the aquatic environment. Studies included in the review demonstrated longer survival of coliphages in waters < 20°C, and faster decay of coliphages, FIB, and viral pathogens (measured by culture-based techniques) in marine waters relative to freshwaters (Korajkic et al., 2019a).

McMinn et al. (2019) monitored levels of culture-enumerated FIB, MSC, somatic coliphages, and clostridia as well as genetic markers for *Campylobacter*, *E. coli* and enterococci, human-associated (HF183) and bird-associated (GFD) MST markers through a constructed wetland to evaluate its effectiveness at reducing microbial contamination from sewer overflows in stream water fed to the wetland. Increases in fecal indicator concentrations in the wetland system were noted. The increase could be attributed to indicator regrowth within the wetland and authors noted elevated bird activity in areas corresponding to increases in fecal indicators. Lack of reduction in fecal indicators due to fecal inputs by wildlife has been noted in other studies. General fecal indicators, such as enterococci and coliphages, widely occur in warm-blooded animals so bird deposition in the wetland can lead to false assumptions on the effectiveness of the constructed wetland. The concentration of MSC and somatic coliphages did not significantly change through the wetland treatment system. Somatic coliphages were correlated to culture-enumerated *E. coli* levels ($p = <0.0001$, $R^2 = 0.486$). Some treatment efficacy in the wetland was noted based on decreases of HF183 through the system, whereas increases in the GFD marker were observed due to the elevated bird activity.

Korajkic et al., (2022) evaluated the effectiveness of two wastewater disinfection strategies (chlorination and UV light exposure) on removal of coliphages, FIB, and viral pathogens. Somatic, MSC, and total (CB-390 host strain) coliphages were measured from influent, and 20–40 L final effluent samples concentrated using dead-end hollow-fiber ultrafiltration (D-HFUF). Regardless of the effluent disinfection strategy, FIB were generally more sensitive to chlorine and UV disinfection compared to coliphages. Both MSC and somatic coliphages were equally susceptible to chlorination, but MSC were more resistant to UV light. Comparisons of coliphage removal to that of viral pathogens was rarely feasible due to many samples with no detectable pathogens, but removal of MSC correlated to that of adenovirus removal when chlorination was the treatment strategy. MSC, GB-124 bacteriophage, and crAssphages were the most resistant to UV disinfection and performed similarly to infectious enteric viruses and adenoviruses.

Li et al. (2021) evaluated paired measurements of coliphages (MSC and somatic), FIB, and FSI genetic markers for human, ruminant, canine, and avian feces at beach and river sites in the Great Lakes region to study the co-occurrence of fecal indicators and host-associated genetic markers. Study findings demonstrated that general fecal indicators used for monitoring water quality can influence the interpretation of paired FSI measurements and potentially lead to prioritization of different pollutant sources for remediation. Routine monitoring with somatic coliphages or MSC often led to contradictory fecal pollution source trends compared to bacterial indicators. Both FIB and coliphages can originate from multiple animal sources. Knowledge of the sources of contamination contributing to a waterbody would improve interpretation of the general fecal indicator monitoring results.

Ryu et al. (2021) characterized the occurrence of MSC and somatic coliphages in primary influent and their associated LRVs after secondary treatment and chlorine or UV disinfection. Coliphages were detected in all influent samples (range 20 to 11,700 PFU/100 mL) but had low detection rates in effluent samples (7% of effluent samples had detectable MSC and 14% of effluent samples had detectable somatic coliphages). For both coliphage types, LRVs were

similar (approximately 5-$\log_{10}$ reduction) for both chlorine and UV disinfection. LRVs for coliphages were greater than the LRV for adenovirus, suggesting less stability than adenovirus.

Fitzmorris et al. (2022) conducted a narrative review using the One Water framework to assess the strengths and weaknesses of using bacteriophages as viral indicators in wastewater, biosolids, reclaimed water, recreational water, and shellfish for public health purposes. This review indicated that scientists are reaching a consensus that bacterial indicators do not account or represent the risk that viral pathogens pose to public health. Fitzmorris et al. (2022) suggested that the fate and transport phenomena of bacterial pathogens is different for viral pathogens present in surface water, groundwater, and wastewater (affected by, for example, treatment processes, size differences, and lack of metabolic overhead for biological survival). Therefore, monitoring for fecal indicator viruses, specifically coliphages, could support bacterial fecal indicator monitoring as part of site-specific health-protection criteria.

2. New Epidemiology Studies Including Coliphages

Epidemiological studies evaluating the association between coliphages measured in waterbodies and GI illness have been conducted since the first five-year review (Griffith et al., 2016; Benjamin-Chung et al., 2017). The interest in understanding this association is because enteric viral pathogens have been recognized as the leading causative agents of recreational water illnesses in the United States (Graciaa et al., 2018; Vanden Esschert et al., 2020). As a result, coliphages have also been considered in the context of QMRA to integrate monitoring efforts with risk assessment modeling to estimate potential health impacts (Boehm, 2019).

Griffith et al. (2016) combined results across three epidemiological prospective cohort studies at three California beaches to compare culturable MSC with culturable enterococci in relationships with illness among swimmers. Culturable MSC measured by EPA Method 1602 had a stronger association with GI illness than culturable enterococci enumerated by EPA Method 1600, at Avalon and Doheny, where human fecal pollution was known to be affecting the beaches. Human source markers performed as well or better compared to culturable enterococci at the site with known human sewage inputs from faulty infrastructure. Overall, site-specific conditions at each beach determined which indicator best predicted GI illness.

Benjamin-Chung et al. (2017) conducted a pooled analysis of six prospective cohort studies at coastal beaches in California, Alabama, and Rhode Island. Water quality was measured using enterococci and coliphages. Under all conditions combined, there was no association between GI illness and levels of enterococci or coliphages. Associations between coliphage levels and GI illness were observed only when human fecal pollution was likely present. Additionally, when human fecal contamination was present, culturable enterococci only performed well when coliphages were also detected. When enterococci levels were <35 CFU per 100 mL, coliphages were detected in 72 percent (somatic) and 79 percent (male-specific) of samples taken, indicating that viral pathogens may be present in waters receiving human fecal contamination even when water quality is below the EPA criteria value. The study findings included similar associations between somatic coliphages or enterococci with gastrointestinal illness. Additionally, in human

fecal-contaminated marine waters, there was some indication of MSC having a stronger association with illness than enterococci.

3. QMRA

Boehm (2019) used a QMRA framework to estimate RBTs associated with the illness benchmark of 32 NGI per 1,000 recreators for MSC and somatic coliphages in ambient waters receiving untreated wastewater inputs. The framework considers recreational exposure to various ages of wastewater and the associated coliphages and enteric pathogens to account for the microbial decay over time. The effect of temperature difference on decay rates were also considered. Exposure to fresh, unaged wastewater contamination resulted in estimated RBTs for somatic (60 PFU/100 mL) and MSC (30 PFU/100 mL) coliphages. The estimated RBT generally decreased as the wastewater ages because coliphages decay more quickly compared to norovirus, which was used as a reference pathogen in the QMRA, effectively indicating the estimated risk increases per indicator over time. Estimated RBTs decrease more quickly at higher temperatures (25°C vs. 15°C). When the age of contamination is unknown, estimated RBTs for both coliphages ranged between 1 and 10 PFU per 100 mL.

4. Studies of Wastewater Treatment Efficacy

The fate and persistence of norovirus, MSC, and somatic coliphages were evaluated throughout the treatment process at nine water resource recovery facilities (WRRFs) that utilized various types of secondary and tertiary treatment and disinfection (Worley-Morse et al., 2019). Reduction of norovirus genogroups I and II was more similar to reduction of coliphages than FIB (Worley-Morse et al., 2019). Reduction of MSC and somatic coliphages was variable and dependent on the specific unit processes employed by a given WRRF (Worley-Morse et al., 2019). Facilities with non-biological nutrient removal (BNR) activated sludge and chlorine (de facto chloramine) disinfection had less reduction of coliphages (1–3 $\log_{10}$) compared to FIB (5 $\log_{10}$) (Worley-Morse et al., 2019). Greater than a 4 $\log_{10}$ reduction was reported for both FIB and coliphages at facilities that employed BNR processes and UV or ozone disinfection (Worley-Morse et al., 2019). Although a 4 $\log_{10}$ reduction was reported for FIB at a facility using peracetic acid for disinfection, only a 2–3 $\log_{10}$ reduction was reported for coliphages (Worley-Morse et al., 2019). Worley-Morse et al. (2019) conclude that many WRRFs in the United States, in particular those with disinfection steps using peracetic acid and chlorine in the presence of ammonia (or chloramines), have greater reduction of FIB than coliphages. Thus, viral indicators may serve as better predictors of the fate of enteric viruses, including norovirus (Mann et al., 2019; Worley-Morse et al., 2019).

Jones et al. (2022) conducted a systematic review and meta-analysis of occurrence of coliphages in effluent. Jones et al. found somatic coliphages and MSC density data from WWTP effluent that had undergone primary, secondary, or tertiary treatment, or disinfection. The densities of MSC and somatic coliphages in WWTP effluent were significantly lower than the densities in the influent after receiving secondary biological treatment (with and without nutrient removal) or tertiary treatment (e.g., treatment ponds or phosphate removal), or disinfection through chlorination, UV irradiation, or both processes. This systematic review suggested that the

combination of tertiary treatment processes may result in greater viral inactivation in WWTP effluent compared to a single effluent disinfection step. Jones et al. (2022) also concluded that considering somatic coliphages and MSC as indicators of fecal contamination of sewage and treated effluent continues to be useful to assess its potential impact on public health and wastewater utilities.

5. Summary of New Information on Coliphages

- Both somatic and MSC consistently occur in raw wastewater across the United States. Somatic coliphages are generally more numerous than MSC, and thus have potential to be used as indicators of fecal contamination in waters affected by sewage and wastewater treatment (particularly disinfected effluent).

- Viral indicators, such as coliphages, may perform better as surrogates of the fate of human enteric viruses compared to culture-enumerated FIB during wastewater treatment.

- Coliphages can exhibit a higher decay rate relative to viral pathogens in ambient waters.

- Because coliphages have been shown to have a stronger relationship to illness than FIB where human fecal sources were present, knowledge of the fecal sources affecting a particular waterbody can improve interpretation of coliphage monitoring data.

C. New Information on Cyanotoxins

While cyanotoxins are not subject to the five-year review since they do not meet the criteria of pathogens and pathogen indicators, EPA assessed the new information on health effects after cyanotoxin exposure from literature searches conducted in 2021 by EPA (ORD and Office of Science and Technology [OST]; see Appendix A for more information on methods). The 2021 literature search focused on six cyanotoxins: anatoxins, β-methylamino-L-alanine (BMAA), cylindrospermopsin, microcystins, nodularins, and saxitoxins. From the relevant articles identified in the 2021 literature search, new (defined as published since 2016) studies of health effects after exposure to cyanotoxins are described here.

Cyanobacteria, also called blue-green algae, are naturally occurring photosynthetic bacteria found in freshwater and brackish waters. Under certain environmental conditions, such as elevated levels of nutrients, warmer temperatures, still water, and plentiful sunlight, cyanobacteria can rapidly multiply to form HABs. Some species of cyanobacteria are able to produce toxic compounds, known as cyanotoxins, which can be harmful to human and animal health (U.S. EPA, 2019). EPA developed a public HAB web portal that contains information on cyanobacteria and managing cyanotoxins in drinking and recreational waters (https://www.epa.gov/cyanohabs).

U.S. exposure information indicates that cyanobacterial HABs have been reported in ambient waters in all states and occasionally in marine waters (U.S. EPA, 2019; Woods Hole Oceanographic Institute [WHOI], 2016). During a cyanobacterial HAB, the toxin concentration can rapidly increase and may become elevated before a visible bloom is observed (Chorus et al., 2000; WHO, 2021b; U.S. EPA, 2019) and persist after the bloom fades. Therefore, human exposures can occur before and after the visible signs of a bloom. The most common cyanotoxins found in ambient waters in the United States are microcystins, cylindrospermopsin, anatoxin-a, and saxitoxins (U.S. EPA, 2009a; U.S. EPA, 2019). Nodularins are typically produced by HABs in brackish waters and share similarities to microcystins in both structure and adverse human health effects resulting from exposure. Exposure to elevated levels of cyanobacteria and the toxins they produce during recreational activities could lead to adverse health effects ranging from a skin rash, fever-like symptoms, body aches, respiratory irritation, and gastrointestinal symptoms to more serious adverse health effects associated with organ damage or neurological effects (U.S. EPA, 2019).

1. RWQC Guidance Values and Health Advisories for Cyanotoxins

In 2019, EPA published recreational water quality criteria and/or swimming advisories for two cyanotoxins, microcystins and cylindrospermopsin, based on the best available science (U.S. EPA, 2019). EPA recommended criteria for these two cyanotoxins include a magnitude (8 µg/L microcystins or 15 µg/L cylindrospermopsin) and duration (not to be exceeded in more than three 10-day assessment periods over the course of a recreational season) for freshwaters with a recreational designated use (Table 2). The recommended magnitude represents the concentration of microcystins or cylindrospermopsin at or below that which is not expected to result in adverse human health effects from short-term recreational exposure to the toxins via incidental ingestion by children while swimming in freshwaters. The recommended values are based on critical studies identified from the available toxicity information described in the Health Effects Support Document for the Cyanobacterial Toxin Microcystins (U.S. EPA, 2015a) and Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin (U.S. EPA, 2015b). These recommendations are intended as guidance to states, territories, and authorized tribes to consider when developing WQS. Alternatively, these recommendations can be used as the basis of swimming advisories for notification purposes in recreational waters to protect the public.

Table 2. Recreational Criteria or Swimming Advisory Recommendations for Microcystins and Cylindrospermopsin[a]

Application of Recommended Values	Microcystins			Cylindrospermopsin		
	Magnitude (µg/L)	Duration	Frequency	Magnitude (µg/L)	Duration	Frequency
Recreational Water Quality Criteria	8	One in 10-day assessment period across a recreational season	More than three excursions in a recreational season, not to be exceeded in more than 1 year[b]	15	One in 10-day assessment period across a recreational season	More than three excursions in a recreational season, not to be exceeded in more than 1 year[b]
Swimming Advisory		1 day	Not to be exceeded		1 day	Not to be exceeded

[a] These recommendations can apply independently within an advisory program or in water quality standards. States can choose to apply either or both toxin recommendations when evaluating excursions within and across recreational seasons.

[b] An excursion is defined as a 10-day assessment period with any toxin concentration higher than the criteria magnitude. When more than three excursions occur within a recreational season, and that pattern reoccurs in more than 1 year, it is an indication the water quality has been or is becoming degraded and is not supporting its recreational use. Units are micrograms (µg) per L.

The WHO *Guidelines on Recreational Water Quality - Volume 1: Coastal and Fresh Waters* (WHO, 2021a) derived guideline values for four cyanotoxins: anatoxin-a and analogs (60 µg/L), cylindrospermopsins (6 µg/L), microcystins (24 µg/L), and saxitoxins (30 µg/L). Differences between EPA's and WHO's values for microcystins and cylindrospermopsin are due to differences in input values such as children's body weights and incidental ingestion. For microcystins, WHO selected a different critical study compared to EPA for the basis of developing recreational values. Additional information for these four cyanotoxins can be found in WHO's *Toxic Cyanobacteria in Water, 2nd Ed* (Chorus and Welker, 2021).

2. Health Studies

Since the last five-year review, new studies of health effects after exposure to cyanotoxins have been published. Two single-dose, acute toxicity studies (Chernoff et al., 2020; Chernoff et al., 2021) and three repeat-dose, chronic toxicity studies (Li et al., 2016a; Wang et al., 2016b; Zhou et al., 2020) of health effects following oral exposure to microcystin were identified. One repeat-dose, chronic toxicity study of health effects following oral exposure to cylindrospermopsin (Chernoff et al., 2018) was published. Review of new data from new studies on cylindrospermopsin and microcystins does not suggest changes to the reference doses (RfDs). Further details of the studies for microcystins and cylindrospermopsin are described below.

Microcystins

Acute Studies

When EPA developed the Health Advisories in 2015 and the RWQC for Microcystins in 2019, EPA derived an RfD for all microcystins using microcystin-LR (MC-LR) as a surrogate based on MC-LR having a robust toxicological data set and relatively high potency among the microcystin congeners (U.S. EPA, 2015a). Lethal dose 50 percent (LD_{50}) studies of the most common

microcystin congeners identified a relationship between the congeners with hydrophobic L-amino acids (-LA, -LR, and -YR) and the highest toxicity, and between hydrophilic amino acids, such as microcystin-RR, and lower toxicity (Stoner et al., 1989; Gupta et al., 2003).

New studies on microcystin have been published since 2016. Chernoff et al. (2020, 2021) characterized the acute toxicity of multiple congeners of microcystin. Chernoff et al. (2020) reported acute toxicity for oral exposure of BALB/c mice to 10 different microcystin congeners (MC-LR, MC-LA, MC-LF, MC-LW, MC-LY, MC-RR, [Asp3]MC-RR, [Asp3,Dhb7]MC-RR, MC-WR, and MC-YR) by gavage as single 7 mg/kg doses. Significant differences in the single-dose acute toxicity of microcystin congeners were observed, with MC-LR, MC-LA, MC-LY, and MC-YR having effects on ≥ seven toxicity indicators e.g., moribundity, increases in relative liver weight, and changes in clinical chemistry parameters. Noticeably, MC-LA and MC-LR showed the greatest toxicity, including significant increases in moribundity. Consistent with previously published studies, hydrophobic congeners of microcystin (e.g., MC-LR) were more toxic than the hydrophilic congeners (e.g., MC-RR) (U.S. EPA, 2015a). Of the hydrophobic congeners tested, MC-LR was more toxic than MC-LW, MC-LF, and MC-WR.

In a follow up study, Chernoff et al. (2021) administered single doses of several microcystin congeners (LA, LR, LY, and RR) by gavage to BALB/c mice at levels ranging from 0.5 to 11 mg/kg, depending on the congener (Chernoff et al., 2021). All animals were euthanized 24 hours post-dosing. In this study, MC-LA was the most toxic congener among the four with toxicity. MC-LA induced significant toxic effects in the liver that led to increases in total serum bilirubin at 3 mg/kg, which was identified as the lowest-observed-adverse-effect level (LOAEL). In a 28-day exposure of MC-LR, Heinze (1999) identified a LOAEL of 50 µg per kilograms per day (µg/kg-d), 60 times lower than the acute LOAEL identified by Chernoff et al. (2021) for MC-LA. The Heinze (1999) LOAEL for MC-LR was used for the derivation of the EPA's recreational criteria. Therefore, microcystin recreational criteria based on MC-LR is protective of short-term, 1- to 28-day exposures to all four microcystin congeners. This newly identified study (Chernoff et al., 2021) does not change the microcystin recreational criteria as the LOAEL from an acute exposure study of MC-LR is protective of primary contact exposures to other hydrophobic microcystins.

Chronic Studies

Three new studies of health effects after chronic exposure to microcystin were identified. Wang et al. (2016b) administered 1, 10, or 40 µg/L of MC-LR in drinking water for 6 months to female BALB/C mice to determine the impact of chronic, low-dose exposure to MC-LR on the lungs (Wang et al., 2016b). The authors observed alveolar collapse and lung cell apoptosis with altered cell junction integrity at all doses. The authors were not able to detect the presence of MC-LR in lungs by immunoblot analysis and quantitative dose-response data were not provided.

Li et al. (2016a) characterized the chronic toxicity of MC-LR per L in adult male C57BL/6 mice treated with 1, 5, 10, 20, and 40 µg MC-LR per L in drinking water for 12 months. Statistically significant differences in lung/body weight ratios, caused by an increase in the level of lung inflammatory cytokines that resulted in thickening of the alveolar septa, were observed (Li et al.,

2016a). The authors did not provide quantitative data, however, so a dose-response curve could not be developed.

Zhou et al. (2020) evaluated the effects of chronic exposure to MC-LR on mice testes. Male mice (age and strain not specified) were exposed to 0, 1, 10, or 100 µg/L MC-LR (purity >96%; dissolved in 0.1% methanol) in drinking water for 90 or 180 consecutive days. Mice were noted to be 15 to 25 grams (g), but body weights in Figure 1 presented in the study appear to be more than 25 g on day 0. Therefore, it is not clear if the mice used were adults. Average intake was estimated to be 0.15, 1.5, and 15 µg/kg-d by the study authors based on an assumed, rather than measured, water intake of 1.5 mL per 10 g body weight and presumably the average biweekly body weights. Body weight was significantly decreased in 10 and 100 µg/L groups beginning at 154 and 126 days, respectively. There are methodological issues with the lack of measured water intake, the lack of reporting about the age of the animals, and the uncertainties with body weight measurements at the start of the study. No significant change or trend in relative testes weight at either 90 or 180 days was observed. However, absolute testes weights were not reported. Based on decreases in body weights, it would be expected that there would be a change in absolute testes weights. The lack of these data is a limitation in assessing effects on the testes. Histological evaluation indicated an increase in abnormal seminiferous tubules in the 100 µg/L group at both 90 and 180 days and in the 10 µg/L group at 180 days. Zhou et al. (2020) cite methods used by Chen et al. (2011). As noted in section 7.4.2 of EPA (2015a), peer reviewers identified concerns with the histological procedure used by Chen et al. (2011) and the issues do not appear to have addressed in Zhou et al. (2020). Of 4,950 proteins quantified by isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomics, 20 proteins (8 upregulated and 12 downregulated) were significantly different between the 10 µg/L and control group. The altered protein expression corresponded to 15 pathways, including renin-angiotensin system, extracellular matrix-receptor interaction, phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) signaling pathway, focal adhesion, tight junction (TJs), and gap junction (GJs). The histological analysis found that the blood-testes barrier (BTB) was significantly more permeable in the two higher dose groups compared to the control group. The results suggest that MC-LR causes dysfunction of the BTB through affecting TJs and GJs. Adverse effects in the Zhou et al. (2020) study occurred at the two higher dose groups, with an estimated intake of 1.5 and 15 µg/kg-d, which are lower than the LOAEL of 50 µg/kg-d identified in the Heinze (1999) study used to develop the EPA's recreational criteria recommendation for microcystins. However, the multiple methodological concerns, described above, of the Zhou et al. (2020) study precludes EPA from considering the data to develop revised criteria.

The three chronic effects studies identified as part of EPA's literature review assessed the potential adverse respiratory (Li et al., 2016a; Wang et al., 2016b) and male reproductive (Zhou et al., 2020) effects after long-term exposure to MC-LR. However, limitations associated with the study designs and reporting prevented the quantitative use of the information and would not support revising the RfD used to derive the 2019 recreational criteria recommendation for MC.

Cylindrospermopsin

One new oral toxicity study of cylindrospermopsin was identified. The study exposed adult male and female mice to cylindrospermopsin (75 to 300 µg/kg-d) by gavage for 90 days (Chernoff et al., 2018). Health effects observed were elevated organ to body weight ratios of the liver and kidney at all dose levels, increase in serum alanine aminotransferase (ALT) activity, decreases of blood urea nitrogen (BUN) and serum cholesterol concentrations in males, plus high monocyte counts in both genders compared to the negative control. The study identified a LOAEL of 75 µg/kg-d based on significant effects in liver and kidney/body weight ratios, reduced BUN, increased serum monocytes, and multiple signs of histopathology. The health effects as well as the histopathological findings are consistent with the toxicological findings found in the earlier studies of Humpage and Falconer (2002, 2003) that were used for the derivation of EPA's RfD for cylindrospermopsin. Although a comparison of no-observed-adverse-effect levels (NOAELs) between the Chernoff et al. (2018) and Humpage and Falconer (2002, 2003) studies is not possible, the higher LOAEL (75 µg/kg-d) reported in the Chernoff study is higher than the 60 µg/kg-d dose reported in the Humpage and Falconer study, so the new information would not support revising the RfD used to derive EPA's 2019 recreational criteria recommendation for cylindrospermopsin.

Other Cyanotoxins

EPA identified new toxicity studies for anatoxin-a and saxitoxins but not for nodularins. There is not enough information to support the derivation of RfDs for either anatoxin-a or nodularins. The EPA literature search did identify a number of new studies, including dose-response studies, for saxitoxins.

Anatoxin-a

One new study was identified on the lethal dose of anatoxin-a (ATX) and its congener dihydroanatoxin-a (dhATX) (Puddick, et al., 2021). Purified anatoxin-a and dhATX from the benthic cyanobacterium *Microcoleus autumnalis* were administered to female Swiss albino mice by intraperitoneal (i.p.) injection and by oral ingestion (gavage and feeding) for 14 days to determine the LD_{50}. The researchers observed a difference in lethal doses for each exposure route, with i.p. injection dhATX was less toxic than anatoxin-a (0.73 mg/kg for dhATX and 0.23 mg/kg for anatoxin-a), but dhATX was more toxic than anatoxin-a with gavage (2.5 mg/kg for dhATX and 10.6 mg/kg for anatoxin-a) and feeding (8 mg/kg for dhATX and 25 mg/kg for anatoxin-a). The study design focused on identifying lethality only and did not analyze for nonlethal health effects such as neurotoxicity, histopathology, hematology, and serum chemistry, and therefore, this study could not be used to derive a noncancer RfD for ATX or dhATX.

Zhong et al. (2020) is an in vitro study that evaluated the immunotoxicity of anatoxin-a using *Carassius auratus* lymphocytes. Non-adherent lymphocytes isolated from the kidneys of *C. auratus* fish (6–10 months old) were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium with 5 percent fetal bovine serum, free from antibiotics, and exposed to 0, 0.01, 0.1, 1, and 10 mg/L for 12 hours. In vitro immunotoxicity from exposure to anatoxin-a was evidenced by increases in apoptosis of lymphocytes, which increased in a dose-dependent manner, based on

both deoxyribonucleic acid (DNA) fragmentation and flow cytometry. Flow cytometry results indicated that the percentage of apoptotic lymphocytes exposed to 0.01, 0.1, 1, and 10 mg/L of anatoxin-a reached 18.89, 22.89, 39.23, and 35.58 percent, respectively, with less than 15 percent observed in controls. Ultrastructural changes associated with anatoxin-a exposure identified using transmission electron microscopy-included cytoplasmic condensation, vacuolation, and swollen mitochondria in *C. auratus* lymphocytes. Oxidative stress also increased in a dose-dependent manner as measured by increases in reactive oxygen species (ROS) and malonaldehyde, and decreases in superoxide dismutase, glutathione, catalase, glutathione reductase, glutathione peroxidase, and glutathione-s-transferase. In vivo studies are needed to confirm the immunotoxicity findings of Zhong et al. (2020).

No human epidemiological data were identified that assessed health effects after exposure to anatoxin-a. One new human case report of food poisoning following exposure to sea figs contaminated with anatoxin-a was identified but the study did not measure or report dose information (Biré et al., 2020).

Studies assessing adverse effects from either the inhalation or dermal route of exposure were not identified for anatoxin-a. Anatoxin-a was detected on glass fiber filters using in field-deployed air sampler during an active harmful algal bloom in Massachusetts in 2019 (Sutherland et al., 2021), but the study was not designed to quantify or characterize the human health risk from inhalation of anatoxin-a in water droplets.

For anatoxin-a, the current available information on health effects falls short for determining an RfD. In the absence of EPA recreational water recommendations for anatoxin-a, some states have adapted the WHO health-based reference values for acute anatoxin-a exposure (WHO, 2021b). EPA will continue to evaluate new scientific data on anatoxin-a and its effects as they become available.

Saxitoxins

Saxitoxins are a group of 57 analogs grouped by structural similarity, including the parent compound (STX), neosaxitoxin (neoSTX), gonyautoxins (GTXs), C-toxins, decarbamoylsaxitoxins, and lyngbyatoxins (LWTXs). STX equivalents (STXeq) are often reported in studies as the total concentration of STX variants, or they may represent concentrations adjusted for toxicity (WHO, 2020). New studies on health effects after exposure to saxitoxin have been published since 2016. EPA has initiated the development of a Health Effects Support Document (HESD) for STXs. Health effects studies, both human studies and toxicological studies, were identified and reviewed through EPA's literature search. The available human studies include case reports of morbidity and mortality resulting from paralytic shellfish poisonings (PSPs) in adults and children, as well as epidemiologic and case-series studies that assessed human health effects after exposure to saxitoxins. Development of this HESD will continue in 2023.

Human Case Reports

A case of PSP poisoning in the United States was reported in 2016 when an elderly female who ate clams harvested in Roslyn Beach in Kodiak, Alaska, reported symptoms of nausea with dry heaving, weakness, respiratory depression, and shock (Coleman et al., 2018). PSP testing of the clams from the suspected meal determined a saxitoxin concentration of 277 µg per 100 g, 309 µg per 100 g of neosaxitoxin, 576 to 2,490 µg per 100 g of multiple GTX, 7.52 to 11.3 µg per 100 g of decarbamoyl, and 10.8 to 221 µg per 100 g of C-toxins. Saxitoxins, neosaxitoxins, and GTX1-4 were also found in the urine of the patient (64.0 µg/g-creatinine, 60 µg/g-creatinine, and 492-4,780 µg/g-creatinine, respectively).

Knaack et al. (2016) evaluated 11 patients with suspected PSP. Of the 11 patients, four were confirmed to have STX-PSP by urine testing (24–364 ng STX/g-creatinine) and five patients had clinical manifestations of PSP. Results revealed that dysphagia and dysarthria appeared to be stronger indicators of PSP than paresthesia and nausea, which are commonly used to clinically diagnose patients with PSP. Meal remnants obtained from six presumptive PSP cases were analyzed and all six samples tested positive for PSP toxins. The results of this report are limited because only 4 of 11 patients were confirmed to have PSP.

Animal Studies

Diehl et al. (2016) conducted a toxicological study in rats exposed to two doses of saxitoxin equivalents orally via drinking water for 30 days and then assessed for behavioral effects. The results of the behavioral test battery indicate that the STX-exposed rats had decreases in learning and memory processes compared to negative controls (Diehl et al., 2016).

Selwood et al. (2017) conducted a series of studies to determine the acute toxicity of STX and multiple STX analogs administered by i.p. injection, as compared to oral gavage or consumption. Exposure data were used to identify the LD_{50} and NOAEL. Mice were first dosed a step below the best preliminary estimates of the LD_{50}, and subsequent animals receive a lower dose (if the previous animal dies) or a higher dose (if the previous animal survives). In general, the LD_{50} values for each STX analog were lower following i.p. injection than they were with oral administration. This was attributed to slower absorption of the toxins via the oral route. Nonlethal health effects of exploratory behavior, grip strength, changes in abdominal breathing, and lethargy were assessed and used as the basis for the NOAEL values for oral gavage (544 nanomoles per kilogram [nmol/kg] for STX; 276 nmol/kg [neoSTX] to 25,500 nmol/kg [C3&4] for STX analogs) and dietary exposures (4,360 nmol/kg [decarbamoyl neosaxitoxin (dcNEOSTX)] to 17,400 nmol/kg [C1&2] for STX analogs; not determined for STX). The symptoms of toxicity manifested by the STX analogs following the oral gavage and feeding routes of exposure were proportional to those for i.p. injection except that the onset of toxicity, recovery from sublethal doses, and time to death were delayed for the oral exposure routes. The recovery and time to death results for the different exposure routes (i.p. injection < gavage < feeding) indicate the toxin has to be delivered to systemic circulation before the effects are manifested (Selwood et al., 2017).

In Vitro Studies

Peripheral blood mononuclear cells obtained from wild-captured harbor seals were used to study the effects of STX on immune cell modulation and phocine distemper virus (PDV) replication (Bogomolni et al., 2016). Exposure to 10 ppb STX led to a 78 percent increase in lymphocyte proliferation compared to the control. In STX-exposed cultures, there was an 8-fold increase in lymphocyte fraction PDV loads on average at day 5 post-infection, and a 2.5-fold increase in the supernatant fraction PDV load at day 9 post-infection. Given these findings, the authors suggest that exposure to STX could increase systemic virus dissemination upon in vivo infection in marine mammals.

D'Mello et al. (2017) investigated the cytotoxicity of STX and other cyanotoxins on primary human astrocytes using the lactate dehydrogenase (LDH) release assay. LDH activity was significantly increased with 0.1 nmol STX, and inhibitory concentration (IC) IC_{20} and IC_{50} (concentration giving 20% or 50% of the maximum inhibitory response) values were determined to be 0.65 and 0.95 nmol, respectively. Cell proliferation was significantly decreased with STX exposure at both the IC_{20} and IC_{50} doses, with proliferation at the IC_{50} dose at 46.4 ± 2.7 percent of the control.

Two mammalian neuronal cell lines were used to study effects of extended, low-dose exposure to STX on neuronal development (O'Neill et al., 2017). STX exposure prevented normal morphological changes in neuronal morphology even with the lowest concentration tested. The effects observed appeared to be dose dependent. The results support a conclusion that STX exposure can cause a change in neuronal cell morphologies in both the central and autonomic nervous system.

The interactions between pristine single-walled carbon nanotubes (SWCNTs) or carboxylated single-walled carbon nanotubes (SWCNT-COOH) and STX were evaluated to understand the potential effects of those interactions on cell toxicity (Ramos et al., 2017). No effects on cell viability or cell proliferation were reported with any treatment at 30 minutes. At 24 hours, exposure to STX with SWCNT led to a significant decrease in cell viability compared to controls. No other statistically significant effects at 24 hours were found. These data suggest a weak interaction between STXs and SWCNTs that could alter the effect of STX on mammalian neuronal cells.

Abi-Khalil et al. (2017) investigated the immunotoxicity and localization of STX in hemocytes from a species of oyster. Fluorescent coumarin-coupled STX (STX-Cou) was detected in the cytoplasm of oyster hemocytes but did not co-localize with the mitochondria. The negative control, Gua-Cou, showed no specific localization of fluorescent coumarin labeling, suggesting a STX-specific accumulation in the cytoplasm of oyster hemocytes. Upon evaluation of phosphatidylserine translocation and membrane permeability, a strong green-fluorescent signal indicated the presence of phosphatidylserine on the cell surface in both Etoposide and STX-treated hemocytes. Additionally, the signal depended on dose; it was detected after treatment with 3.3 µM STX but not with 0.8 µM STX (Abi-Khalil et al., 2017).

The apoptotic potential of STX was determined as the imaging of cells exposed to 0.8 µM STX showed marked chromatin condensation at the nucleus periphery, a hallmark of apoptosis. A similar effect was noted in the positive control (50 µM of Etoposide) but not the sterile sea water negative control. DNA fragmentation and apoptosis were determined in cells exposed to 0.8 µM STX for 3.5 hours. Tetramethylrhodamine nick end labeling (TMRNEL) revealed that hemocytes exposed to 0.8 µM STX exhibited a greater proportion of cells with fragmented nuclear DNA compared to controls. 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay results showed that exposure to 3.3 µM STX resulted in a decrease (34% ± 8%) in living cells similar to that of 50 µM Etoposide (49% ± 8%), however, no effect was observed after exposure to 0.8 µM STX. Over a 2-hour period, exposure to STX did not exhibit a detectable effect on ROS concentrations, suggesting that cell death observed in other assays is not due to ROS (Abi-Khalil et al., 2017).

Some state and international agencies have developed health-based values for saxitoxins in drinking and recreational waters. The Ohio Environmental Protection Agency (OHEPA) issues Recreational Public Health Advisories at 0.8 µg/L (OHEPA, 2022). The Ohio Department of Health (ODH) also issues "Do Not Drink" advisories for saxitoxins in drinking water: 0.3 µg/L for bottle-fed infants and children younger than school age, pregnant women, nursing mothers, individuals with pre-existing liver conditions or who are immunocompromised, and individuals receiving dialysis treatment; and 1.6 µg/L for all people of all ages as well as for pets and livestock (ODH, 2020). WHO (WHO, 2020) estimated an acute oral RfD for saxitoxins of 0.5 µg/kg-body weight (bw) based on mild shellfish poisoning symptoms from 500 case reports exposed to PSP, as reported by the European Food Safety Authority (EFSA, 2009). EFSA estimated a NOAEL of 0.5 µg STXeq kg-bw based on human PSP symptoms and supported by animal studies: based on the point of departure of the lowest acute NOAEL for neoSTX of 87 µg/kg-bw (Munday et al., 2013) from a gavage toxicology study, an acute oral RfD for neoSTX of 0.87 µg/kg-bw (applying an uncertainty factor of 100) was derived. This value is of the same order of magnitude as the reference values obtained with human data (WHO, 2020).

Nodularins

No new oral toxicity studies of nodularins, published since 2016, were identified in EPA's 2021 literature search. EPA has previously reviewed the available toxicity data for nodularins and found that the previously published (before 2016) data are inadequate to derive an RfD to use to support developing RWQC and/or Swimming Advisories.

3. Summary of New Information on Cyanotoxins

- EPA has identified several repeat-dose, acute, and chronic toxicity studies of health effects of cyanotoxins published in the last 5 years.
- For cylindrospermopsin and microcystins, these new studies either supported the existing reference doses identified in EPA's HESDs or did not provide new information to support derivation of a revised RfDs.
- EPA evaluated several toxicity studies characterizing effects from exposure to anatoxin-a, saxitoxins, and nodularins.

- Work is underway at EPA to evaluate data to support development of an HESD for saxitoxins.
- The limited available toxicity information available for anatoxin-a and nodularins does not support development of an HESD at this time.

D. New Information on Antimicrobial Resistance

To identify new literature on AMR and antimicrobial resistant genes (ARGs), EPA reviewed three relevant review articles that were published since the last five-year review (Nappier et al., 2020; Korajkic et al., 2020b; Franklin et al., 2021).

1. EPA AMR Research

The review article, Korajkic et al. (2020b), on AMR in *Enterococcus* species in marine and estuarine environments indicates that *E. faecium* and *E. faecalis* exhibit resistance to the highest number of antibiotics, followed by *E. casseliflavus*, *E. hirae*, and *E. durans*. Further, studies found that all *Enterococcus* species were resistant to erythromycin. The highest proportion of resistant enterococci were measured in the water column (>18%), with other important reservoirs of resistant enterococci being feces and tissues of animals such as seabirds, whales, and clams, and to a lesser degree sediments and sands. Regarding methodology, the review identified a lack of standardized procedures to study, detect, and classify antibiotic resistant *Enterococcus* in the environment and recommended the establishment a standardized framework for studying antibiotic resistance in the environment.

The review article by Franklin et al. (2021) on the available molecular methods to analyze AMR in surface waters was conducted to support One Health Assessments (https://www.epa.gov/healthresearch/one-health). This article described three state-of-the-art methodologies that can provide information about the presence, diversity, and dynamics of ARG targets in the environment including surface waters (Franklin et al., 2021). The three techniques, high-throughput qPCR, metagenomics, and whole-genome sequencing, are all culture-independent and can target a high diversity of genes simultaneously. The article noted that there is value in combining molecular information with culture-based approaches because molecular methods can identify most, if not all, genetic elements that may confer AMR in a single environmental isolate and/or a microbial community, while culture-based methods can provide phenotypic confirmation of AMR in environmental isolates. Used together, the methods can more fully characterize AMR in surface waters.

The state-of-the-science review by Nappier et al. (2020) of AMR bacteria and ARGs in recreational waters reported that new studies found that ARGs can be acquired through spontaneous mutations and can persist in microbial populations via selective pressure. For example, AMR and heavy metal resistance genes often co-occur on the same mobile genetic elements (e.g., plasmids), resulting in a co-selection and propagation of mobile genetic elements carrying ARGs, even in the absence of antibiotics. Since resistant genes can move between pathogenic and nonpathogenic microorganisms, a better mechanistic understanding of horizontal gene transfer and AMR selection is warranted to improve AMR monitoring. Nappier et al. (2020) also reported that studies indicate that untreated wastewater, treated WWTP effluent,

medical waste, pharmaceutical production, biosolids, agriculture, aquaculture, plant, and animal agriculture (e.g., concentrated animal feeding operations [CAFOs]), and the feces of birds and other wildlife can be sources of AMR bacteria and ARGs. Such observations are consistent with prior studies that show differential effects of different treatment processes on AMR bacteria and ARGs.

2. Summary of New Information on AMR

- Treatment processes commonly used at WWTPs are not typically calibrated to target removal of AMR bacteria or ARGs.

- An increased understanding of horizontal gene transfer mechanisms and AMR selection can be used to improve monitoring for AMR in ambient waters.

- A combination of techniques (including both molecular- and culture-based) are necessary to fully understand AMR in surface waters.

E. New Information on Human and Non-Human FSI

FSI techniques are used to characterize fecal sources potentially present in polluted waters. These methods rely on the detection and/or quantification of host-identifiers such as chemical or microbial targets closely associated with a particular pollution source (Hagedorn et al., 2011). Accurate and reliable FSI technologies have the potential to improve future water quality management in the United States. The 2012 RWQC (U.S. EPA, 2012a) has provisions that recommend these FSI methods for use as a sanitary characterization tool and has published information to support alternative criteria eligibility of FSI (U.S. EPA, 2014a).

EPA has invested considerable resources to develop, validate, standardize, and implement FSI technologies. High-priority research areas include completion and publication of standardized EPA methods for human-associated qPCR methods, development and public release of a companion standard reference material, further investigation of data interpretation approaches, and science to advance virus-based human FSI method development.

Since 2017, EPA researchers have published 15 peer-reviewed studies, released two nationally validated human-associated FSI qPCR methods, and codeveloped a standard reference material in collaboration with the National Institute of Standards and Technology (NIST). In addition to EPA activities, there are numerous significant contributions by other researchers that have advanced FSI recreational water science.

The systematic literature review identified 85 relevant articles (Figure 1) discussing advances in MST or fecal source identification techniques (Appendix B). Additionally, 22 relevant references were identified via an ad hoc process (Blatchley et al., 2007; Shanks et al., 2009, 2010, 2011, 2014; Sinclair et al., 2009; Okoh et al., 2010; Soller et al., 2010a,b; Hagedorn et al., 2011; Boehm et al., 2013; Ervin et al., 2013; Dutilh et al., 2014; U.S. EPA, 2014a; Qiu et al, 2015;

Stachler et al., 2017; Korajkic et al., 2020a; McKee et al, 2020; Shrestha et al., 2020; Ahmed et al., 2021; Kralj et al., 2021; Sivaganesan et al., 2022).

1. Human FSI Research

Advances in Bacterial-Based FSI

Researchers have advanced methods for bacterial-based identification of human fecal sources in recreational water. In particular, the *Bacteroides* HF183 genetic marker is well characterized (Ahmed et al., 2016b; Holcomb and Stewart, 2020; Jiang et al., 2018; Somnark et al., 2018; Staley and Edge, 2016; Vadde et al., 2019) and routinely used for qPCR studies due to its strong human-host association (Devane et al., 2019; Staley and Edge, 2016; Symonds et al., 2017). HF183 was found to be a more specific marker for combined sewage overflows than human polyomavirus, pepper mild mottle virus, and six other human pathogens in Cobbs and Tacony Creeks in Philadelphia (McGinnis et al., 2018). HF183 was also readily detected at Lake Pontchartrain beach and was used to determine that rainfall events introduce large amounts of human fecal bacteria into lake waters (Xue et al., 2018). Green et al. (2019) assessed HF183 and ruminant-associated markers during dry weather in Onondaga Creek, New York. They found that the upstream ruminant inputs decayed and/or were diluted out and that the high levels of urban bacterial contamination were human associated and most likely due to failing infrastructure and/or illicit discharges independent of rain events. In addition to HF183, other human-associated genetic markers have been used for identifying human sources in recreational waters, such as HumM2, HumM3, N*if*H, and BifHM (Devane et al., 2019; Zeki et al., 2021; Zhang et al., 2020; Ballesté et al., 2018; Shanks et al., 2009).

Napier at al. (2017) assessed the presence of four human-associated *Bacteroides* markers (HF183, BsteriF1, BuniF2, and HumM2) with self-reported gastrointestinal illness, diarrhea, and respiratory illnesses. The analysis used data from 12,060 adult visitors who enrolled in the NEEAR studies at six beaches from 2003 to 2007. The HF183/HumM2 detection rate at NEEAR marine and freshwater beaches were 26 percent and 28 percent, respectively. Overall, findings suggest inconsistent associations between illness and the detection of human-associated *Bacteroides* markers. The authors concluded that quantitative measures of human fecal pollution, rather than presence-absence, may be necessary to improve assessment of potential links between public health risk and the occurrence of human fecal pollution.

Mattioli et al. (2020) used HF183 as a microbial tracer to evaluate potential sources of contamination of a drinking water well linked to multiple outbreaks of norovirus documented over a month-long period. This is a new application for human-associated FSI genetic markers, used to mitigate exposure.

Advances in Virus-Based FSI

Enteric viruses, such as noroviruses, are reported to be the dominant etiological agents of recreational waterborne disease (Sinclair et al., 2009; Soller et al., 2010b). These enteric viruses

can react to waste treatment and environment stressors in different ways compared to bacterial fecal indicators (Blatchley et al., 2007; Okoh et al., 2010; Qiu et al., 2015). Viral FSI technologies offer an alternative to current bacterial-based methods; however, many are either not sensitive enough or lack sufficient host specificity (Boehm et al., 2013). An ideal viral FSI method would target a virus that is both closely associated with a particular animal host and is highly abundant. There is a growing body of evidence suggesting that some virus-based FSI genetic targets are highly abundant in sewage, are closely associated with human waste, and persist for longer periods of time in wastewater and environmental settings compared to many bacterial fecal indicators.

Since 2017, there have been significant advances in the performance characterization of recently reported crAssphage-like human-associated genetic markers (Stachler et al., 2017) identified from a metagenome cross-assembly (Dutilh et al., 2014). EPA scientists and external partners conducted studies demonstrating a uniform distribution of crAssphage-like sequences in untreated wastewater across the contiguous United States (Korajkic et al., 2020a). Notable efforts include evidence that crAssphage-like sequences are highly abundant in sewage occurring at similar concentrations to HF183 genetic markers (Ahmed et al., 2018b), correlate with some enteric viral pathogens (Crank et al., 2020), more closely mimic enteric virus decay rates compared to bacterial-based FSI targets (Ahmed et al., 2021; Crank et al., 2019), and can be recovered in multiple ambient surface water types (Petcharat et al., 2020). However, research also suggests, like any other methodology, that standardized protocols are important for generating consistent and reliable measurements within and between laboratories (Ahmed et al., 2020).

Advances in Anthropogenic Chemical-Based FSI

Napier et al. (2018) used data from seven NEEAR study beaches impacted by WWTP discharges for *Enterococcus* spp. measured by qPCR and 62 potential anthropogenic chemical markers. The markers were assessed for potential associations with self-reported GI illness, diarrhea, and respiratory illnesses. Across all beaches, no individual chemical marker or chemical category showed strong or consistent association between *Enterococcus* spp. measured by qPCR and health outcomes.

New Tools for Human-Associated FSI

EPA developed and published two methods (Method 1696.1 and 1697.1; U.S. EPA, 2022a,b) and collaborated with NIST to develop a standard reference material (SRM 2917) that functions with 11 qPCR FSI protocols, including human-associated HF183/BacR287 and HumM2 protocols (Kralj et al. 2021). These accomplishments represent two key advances for FSI science since 2017 and are discussed in more detail in the methods section (Section III.F).

A series of studies were conducted to support the implementation of EPA Methods 1696.1 and 1697.1. Notable contributions included the demonstration of genetic target occurrence uniformity in untreated wastewater across the contiguous United States (Korajkic et al., 2020a), a large-scale field demonstration at 29 freshwater sites (Li et al., 2019), and use of QMRA modeling to

investigate links to public health risk in raw sewage (Schoen et al., 2020), as well as the use of a new censored data analysis approach to rank recreational water sites based on human fecal pollution levels (Cao et al., 2018a) to investigate potential links with rainfall (Shrestha et al., 2020) and describe human fecal pollution source trends in waters routinely monitored for bacterial and viral fecal indicators (Li et al., 2021).

2. Non-Human FSI Advances

Since 2017, there has been a growing interest in the use of non-human FSI methods to characterize wildlife, pet, and agricultural fecal sources potentially present in recreational waters, especially using qPCR-based methods. Non-human fecal source information can help prioritize impaired sites, focus mitigation resources, and manage public health risk. Most research activities focused on avian-, ruminant-, and swine-associated genetic markers.

Ruminant (i.e., cattle and deer), avian (i.e., gull and Canada goose), and domestic pets (i.e., dogs) can contribute to elevated FIB levels in ambient surface waters (Ervin et al., 2013). In response, EPA conducted a series of studies to explore the implementation of non-human FSI methods. Notable contributions include the large-scale implementation of ruminant-, cattle-, avian-, swine- and dog-associated qPCR methods at over 30 freshwater sites (Li et al., 2019, McKee et al., 2020), development of SRM 2917 in collaboration with NIST, which functions with avian, ruminant, dog, and swine FSI qPCR methods (Sivaganesan et al., 2022; Willis et al., 2022), and characterization of decay trends in fresh and marine waters (Korajkic et al., 2019b).

A variety of host-associated avian genetic markers have been investigated, including the general avian marker GFD targeting *Helicobacter* spp. (Ahmed et al., 2016a; Symonds et al., 2017; Vadde et al., 2019; Zhang et al., 2020), the poultry-associated markers LA35 and CL targeting *Brevibacterium* sp. (Gibson et al., 2017), and the poultry-associated BifPL targeting *Bifidobacterium* (Ballesté et al., 2018). Multiple gull-associated markers targeting *Catellicoccus marimammalium* were also evaluated including CAT (Wu et al., 2017), Gull2 (Cloutier and McLellan, 2017; Symonds et al., 2017), Gu112 (Thulsiraj et al., 2017), and qGull4 (Staley et al., 2018a,b). Overall, findings suggest that these methods can be highly specific for avian targets, however, most methods exhibit less than ideal sensitivity.

Multiple swine-associated markers were investigated, including Pig2Bac, targeting *Bacteroidales* (Ballesté et al., 2018; Derx et al., 2021; He et al., 2016; Kongprajug et al., 2019; Liang et al., 2021; Somnark et al., 2018; Vadde et al., 2019; Zhang et al., 2020), L.amylovorus, targeting *Lactobacillus amylovorus* (He et al., 2016; Zhang et al., 2020), and PF, targeting *Bacteroidales* (Symonds et al., 2017). In general, Pig2Bac exhibited a high specificity and sensitivity suggesting that this methodology may be a reliable method for future recreational water quality testing.

Ruminant FSI methods are generally grouped into cattle-associated and ruminant-associated methods. Several studies explored the use of cattle-associated qPCR methods including Bac3qPCR (Kongprajug et al., 2020) and CowM2/CowM3 (Xue et al., 2018; Xue et al., 2019). Ruminant-associated methods can identify cattle as well as other animals that share the same

digestive physiology such as deer and elk, among others. Numerous ruminant-associated methods were investigated in field studies targeting BacCow (Seidel et al., 2017; Symonds et al., 2017); BifCW, targeting *Bifidobacterium* (Ballesté et al., 2018); and *Bacteroidetes* 16S ribosomal RNA (rRNA) genes, including BoBac (Bushon et al., 2017) and BacR (Derx et al., 2021; Kolm et al., 2019). Some ruminant sources such as cattle may under some circumstances represent a similar health risk to humans (Soller et al., 2010a). In addition, cattle-associated genetic marker shedding patterns are linked to animal diet (Shanks et al., 2011, Shanks et al., 2010) and age (Shanks et al., 2014) resulting in potentially different occurrence expectations from one monitoring site to another.

Feng and McLellan (2019) analyzed *Bacteroides* populations in sewage and non-human animal hosts by targeting the V4–V5 and V6 16S rRNA gene hypervariable genome regions. Findings suggest that the most abundant *Bacteroides* in untreated sewage were not human fecal associated but sewage infrastructure derived. A qPCR assay was developed targeting an abundant sewage infrastructure-associated *Bacteroides*. The authors assert that a qPCR assay that targets organisms closely associated with the sewer pipe habitat can potentially provide source information that is independent of the human fecal microbiome and could be useful for identifying sewage pollution in water. Beyond microbial populations, Staley et al. (2018a) evaluated environmental DNA (eDNA) utilizing the mitochondrial cytochrome oxidase I genetic marker for DNA barcoding, which identifies DNA from various animal species. eDNA from cow and chicken were only detected in creek and beach surface waters in Lake Ontario following an extreme rain event. The authors note that caution should be used when interpreting eDNA results because these sequences may not be of fecal origin. Advances in community-based FSI science suggest that these approaches can complement FIB and quantitative FSI methods such as qPCR, as well as identify new infrastructure-associated genetic targets. Additional research is warranted to further characterize the utility of these methodologies.

3. Community-based FSI

Microbial community-based FSI methods are an active area of research for characterizing recreational waters. Characterizing the occurrence of microbial groups utilizing sequencing for identification can provide information about fecal sources and other microbial inputs. In addition to genomics (nucleic acid sequencing), metabolomics (characterization of metabolites) has been explored for characterizing recreational waters. For example, Beale et al. (2017) utilized metagenomics sequencing (hypervariable V5 and V6 regions of the 16S rRNA gene) to report the most abundant metabolically active bacteria in water samples from five sites along the Brisbane River, Queensland, Australia. They also conducted gas chromatography mass spectrophotometry analysis of water samples to characterize metabolites, such as sugars, fatty acids, among others. Additional research is warranted to investigate the impacts of agricultural practices, sewage treatment, and environmental endpoints using community-based approaches (Beale et al., 2017). Another study utilized amplicon sequencing of the V3–V4 region of the bacterial 16S rRNA gene to determine human sewage, avian, and horse fecal sources in the Navesink River in New Jersey (Phelan et al., 2019).

4. Summary of New Information on FSI

- EPA Methods 1696.1 and 1697.1 are now available and facilitate the use of human-associated qPCR technologies in recreational settings (see more details in Section III.F, Analytical Methods).

- Field demonstrations have been published that use EPA Methods 1696.1 and 1697.1 and establish their performance in wastewater and surface waters.

- Release of SRM 2917, a high-quality standard control material that functions with 11 FSI qPCR methods indicative of human (HF183/BacR287, HumM2, CPQ_056, CPQ_064), cattle (CowM2, CowM3), ruminant (Rum2Bac), canine (DG3, DG37), swine (Pig2Bac), and avian (GFD) fecal sources, facilitates implementation of FSI qPCR methods.

- A new censored data analysis approach has been published that allows for quantitative analyses of FSI qPCR data sets consisting of high proportions of non-detections and detections below the limit of quantification.

- As the use of FSI tools for human fecal pollution characterization becomes a more common practice, practitioners are recognizing that the contribution of non-human fecal pollution sources plays an integral role in water quality management.

- Recent studies on microbial community-based FSI offer new strategies and tools to characterize fecal pollution sources; however, additional studies are needed to evaluate performance and demonstrate utility for recreational water quality monitoring.

- Understanding the decay of microorganisms associated with specific fecal pollution sources is important for many FSI applications. Unlike culture-based FIB methods, FSI genetic methods target nucleic acids instead of cells capable of growing and reproducing on selective and differential media. This fundamental difference can contribute to variable persistence behaviors in ambient surface water settings.

- Potential surrogates for enteric viral pathogens, such as enterococci qPCR, culturable MSC and somatic coliphages, and human-associated genetic markers (HF183 and Hum M2), have been included in health study evaluations and provide additional information for assessing potential risk of illness (Griffith et al., 2016; Benjamin-Chung et al., 2017; Ahmed et al., 2018a; Boehm et al., 2018; Boehm and Soller, 2020; Schoen et al., 2020; Wade et al., 2022).

- Advances have been made in establishing potential links between public health risk and human-associated FSI marker measurements using QMRA (see Section III.A, QMRA, for details).

F. New Information on Analytical Methods for Recreational Waters

As discussed above, EPA has identified five types of indicators of risk in recreational waters: FIB, coliphages, cyanotoxins, antimicrobial resistant bacteria and genes, and human and non-human FSI genetic markers. Following is a discussion of the new research and scientific information on the analytical methods for identifying and quantifying these potential risk indicators.

The systematic literature search identified 80 articles (Figure 1) relevant to analytical methods (Appendix B). Additional relevant articles were identified via an ad hoc process (U.S. EPA, 2012b, 2015c, 2020; Sivaganesan et al., 2018, 2022; Steele et al., 2018; Nshimyimana et al., 2019; Lane et al., 2020a; Kralj et. al., 2021; Bone et al., 2022).

1. FIB

The 2012 RWQC protect human health from exposure to fecal contamination by detecting and enumerating FIB. There are many analytical methods to enumerate *E. coli* and enterococci in recreational waters. These methods either enumerate by growing the bacteria on selective and differential media (culture methods) or by quantifying specific sequences of DNA from the FIB (molecular methods). Currently, there are more than a dozen microbiological methods approved for use under the CWA to monitor ambient water (see Code of Federal Regulations (CFR) 40 CFR 136.3.IH) and each method is a culture-based method.

Culture methods have been used to monitor water quality for decades. In May of 2021, EPA approved a culture method for *E. coli* in ambient water that requires only 10 hours to provide a result (May 19, 2021, Federal Register (FR) 86 FR 27235 and 40 CFR136.3.IH.3) and https://www.epa.gov/cwa-methods). This incubation time is the shortest of the culture methods. The length of time culture methods take to provide results means that people may have already been exposed to fecally contaminated water, and water quality may have changed by the time the result is available. Lag time between sampling and results may lead to unnecessary or delayed action.

Since 2016, significant work has been conducted to update existing microbial methods, or add methods with a faster turnaround time, applicable to the FIB *E. coli* and enterococci including updated molecular-based qPCR methods and draft digital PCR (dPCR) methods. Molecular methods can provide results faster than culture methods. EPA Method 1609.1 produces enterococci results in about 4 hours, allowing monitoring in the morning and the ability to take action to close a beach by noon, if necessary, and thereby reducing the number of swimmers exposed to fecal contamination as indicated by elevated levels of FIB (EPA Method 1609.1). This method is based on detecting specific sequences of enterococci DNA using qPCR technology. Though faster than culture methods, molecular methods are generally more expensive and technically more difficult to perform than culture methods. The target sequence can be detected in viable but non-culturable, and metabolically inactive, cells and it is possible to

detect the target sequence associated with free DNA from cells that have lysed. Research in molecular water monitoring methods is advancing rapidly and there are additional molecular technologies under development or that are being deployed in the field in addition to qPCR.

PCR-Based Methods for FIB

EPA has made scientific advancements in qPCR methods, including the development and publication of new methods, the development of standard control materials, the completion of multiple laboratory performance studies, and studies characterizing linkages between FIB qPCR and culture-based measurements in recreational waters.

A qPCR approach offers the advantage over culture methods of providing rapid results (2–6 hours versus 10–48 hours), allowing managers to make same-day decisions to protect beachgoers. Since the initial public release of the *Enterococcus* qPCR method (EPA Method 1611; U.S. EPA, 2012b), EPA has made significant advances, including addressing potential matrix interference issues in water types other than those studied at the NEEAR effluent-affected beach sites culminating in the publication of an improved qPCR-based method for enterococci enumeration (EPA Method 1609.1; U.S. EPA, 2015c) and the development of a draft *E. coli* qPCR protocol (Haugland, et al., 2021). Despite the advantages of a qPCR water quality monitoring approach, the use of these methods by state water quality programs has been slow (Shrestha and Dorevitch, 2020).

Since 2017, EPA has published 18 manuscripts, developed standard control materials, and released updated automated data analysis tools to help address research and implementation priorities. Recent studies are summarized below.

Implementation of qPCR methods requires the generation of a standard curve to interpret results. Quality and consistency of standard curves strongly influences the precision and reproducibility of qPCR measurements. To address this need, EPA researchers developed a reference material (IDTSMART-KAN_Std1_Xho), using droplet digital PCR (ddPCR) and advanced statistics, for both the *Enterococcus* and *E. coli* qPCR methods in the Great Lakes region (Sivaganesan et al., 2018). To help facilitate qPCR method implementation on a national scale, EPA researchers recently collaborated with NIST to codevelop SRM 2917, which is expected to replace the initial reference material in future recreational water analyses (Kralj et al., 2021; Sivaganesan et al., 2022; Willis et al., 2022). EPA also reviewed qPCR methods for microbial water quality monitoring (Nappier et al., 2019b).

To evaluate the performance of the *E. coli* qPCR method and establish custom data acceptance metrics for future practitioners, EPA helped organize two ad hoc multi-laboratory studies. The first effort consisted of 21 participating laboratories, primarily from the Great Lakes region using a standardized protocol with the same set of reagents and consumables (Sivaganesan et al., 2019). Findings suggested that a qPCR-based methodology can be successfully implemented across a large network of laboratories and led to the development of custom data acceptance metrics. In the second study, the feasibility of multiple laboratories to meet newly developed data acceptance metrics was evaluated with most participants, both new and experienced,

demonstrating acceptable qPCR measurements (Aw et al., 2019). Given these findings, a companion data analysis workbook was customized to incorporate new data acceptance metrics and streamline calculations (Lane et al., 2020a).

Several studies were conducted to characterize linkages between FIB qPCR and culture-based measurements in recreational waters. One effort investigated the temporal stability of enterococci culture and qPCR paired measurements at freshwater recreational beaches over a 24-hour period (Wymer et al., 2021). Findings reinforced the concept that same-day qPCR results are more indicative of current water quality conditions compared to culture results obtained 24 hours after sample collection. Another study generated nearly 7,000 *E. coli* culture and qPCR paired measurements from 101 Lake Michigan recreational beach sites (Haugland et al., 2021). This data set provided the State of Michigan with the scientific basis for the adoption of a statewide Beach Notification Value for the *E. coli* qPCR method (Lane et al., 2020b) and could serve as a blueprint for other states, territories, and authorities.

In 2020, EPA developed and evaluated a dPCR method using 38 recreational fresh and marine water samples collected from 24 states across the country. The results were used to develop draft quantitative quality control criteria that could be used to assess laboratory proficiency prior to conducting a multi-laboratory validation study. The method incorporates elements from ORD's qPCR methods as well as a dPCR method being piloted in San Diego, California, recreational waters (U.S. EPA, 2020). The results from the study indicate that the dPCR procedure is suitable for multi-laboratory validation in both fresh and marine waters for *E. coli* and/or enterococci (Bone et al., 2022).

2. Coliphage Methods

As discussed in Section III.B, coliphages are better indicators of some enteric viruses in recreational waters compared to traditional methods measuring FIB. In 2018, EPA published updated culture-based single-agar layer (SAL) methods to enumerate MSC and somatic coliphages in recreational waters and wastewater (Method 1642) and in secondary wastewater with no disinfection (Method 1643) (U.S. EPA, 2018ab). EPA Method 1642 includes ultrafiltration to concentrate larger sample volumes (2 L), needed for recreational water monitoring to address potential low coliphage densities in ambient waters downstream of human fecal inputs. EPA conducted a multi-laboratory validation study (U.S. EPA, 2018c) of Method 1642 for MSC and somatic coliphages in fresh and marine recreational waters and secondary wastewater effluents with disinfection. EPA Method 1643 reflects the results of a multi-laboratory validation study of EPA Method 1602 for 100 mL secondary (no disinfection) wastewater samples for MSC and somatic coliphages and is used for monitoring secondary (no disinfection) wastewater matrices under the CWA. For both methods, the multi-laboratory validation results allowed for the development of initial precision and recovery and ongoing precision and recovery and matrix spike quality control acceptance criteria. Results from both methods can be obtained in 16 to 24 hours.

EPA researchers published a study comparing performance of EPA Method 1642 to that of two other alternative methods (modified SAL and direct membrane filtration [DMF]) capable of

processing large volumes of surface waters (1 L per coliphage type) (McMinn et al., 2018). All methods were evaluated for the following metrics: percentage of samples with no detectable coliphage, concentrations of coliphage, cost, and time required to process a single sample. Method 1642 outperformed both alternative methods as it generated the least percentage of non-detects (≤35%), the highest coliphage concentrations (0.30–1.65 $\log_{10}$ PFU/L) and required the least amount of time, while cost per sample was comparable to that of DMF.

An additional study evaluated performance of Method 1642 on freshwater and marine ambient water samples collected nationwide and assessed the effect of spike titer protocols (i.e., SAL versus double agar layer [DAL]), which are required for initial and ongoing precision recovery tests (Korajkic et al., 2021). Study findings indicated that average percent recovery (53%–72%) was comparable to previous reports, confirming a consistent performance of Method 1642 across a wide variety of water types. Somatic coliphage recovery was not affected by water type, but MSC results were significantly lower in marine waters compared to freshwater. Similarly, somatic coliphage were not affected by spike titer protocol, but utilization of DAL significantly underestimated MSC recovery compared to SAL.

Currently, there are *E. coli* host strains under evaluation that detect both MSC and somatic coliphages. These host strains include CB-390 and C-3000 (Rose et al., 2004; Guzmán et al., 2008; Agulló-Barceló et al., 2016; Korajkic et al., 2020a). A single host strain could simplify Methods 1642 and 1643 by allowing the enumeration of both MSC and somatic coliphages in a SAL assay.

Other New Studies for FIB Using PCR

Some notable studies compared EPA methods to other methods. For example, Byappanahalli et al. (2018) compared culture methods (EPA Method 1603 for *E. coli* and Method 1600 for enterococci) to qPCR-based methods (modified EPA Method 1611 qPCR for *Enterococcus*) at coastal beaches and rivers. Overall, the culture-based and qPCR-based results for both indicators were correlated in this study. While the qPCR methods would have resulted in fewer water quality advisories or notifications, the authors concluded that the increased benefit of same-day results provided by qPCR provides better protection overall.

Dorevitch et al., (2017) analyzed nine Chicago beaches by *E. coli* culture (Colilert method) and *Enterococcus* spp. qPCR (EPA Methods 1609 and 1609.1). This study looked at the two pieces of information available to beach managers on a given day: *Enterococcus* qPCR results of samples collected that morning and *E. coli* culture results of samples collected the previous day. The study found that if the same-day qPCR results had not been available, culture-based results from water samples collected the day before would have triggered unnecessary, belated beach advisories 24 percent of the time. And, on 4.7 percent of the beach-days, culture results would have resulted in a "failure to act" (meaning, failure to trigger advisories) when advisories were needed (based on exceedance of the same-day qPCR results). Furthermore, the 71.3 percent concordance of beach actions between same-day qPCR and culture results from water samples collected the day before can be explained entirely by chance. The authors concluded that there is

little scientific rationale for continued *E. coli* culture testing in Chicago beaches waters for the purposes of public notification.

Digital PCR

Detection of enterococci in water samples was compared using ddPCR, qPCR (EPA Method 1609.1), and culture-based Enterolert (Crain et al., 2021). A comparison of enterococci qPCR and ddPCR monitoring data demonstrated an Index of Agreement of 0.89 and a significant Pearson's correlation coefficient of $r = 0.87$ ($p < 0.001$). A subset of data used for the ddPCR and qPCR comparison also included Enterolert data, which was evaluated using linear regression to develop an intrinsic copy number equation that was then used to adjust the direct comparison between ddPCR and Enterolert monitoring data. A ddPCR advisory threshold of 1,413 gene copies per 100 mL was derived from this analysis and determined to be equivalent to the BAV of 104 MPN per 100 mL used in California (Crain et al., 2021). The ddPCR threshold value was implemented as a BAV to monitor coastal beach water quality at San Diego County beaches in 2022 (County of San Diego Department of Environmental Health, http://www.sdbeachinfo.com).

Other FIB Method Technologies

Multiple methods for the real-time measurement of *E. coli* and enterococci have been evaluated, including culture-based methods (chromogenic and fluorogenic substrates), cultivation-independent detection methods (i.e., flow cytometry, biosensors), and microbial identification by mass spectrometry (matrix-assisted laser desorption ionization time-of-flight, as reviewed by Bonadonna et al., 2019). A critical review suggests that none of these technologies are currently able to achieve real-time analysis. Additional research is warranted to develop real-time approaches for monitoring FIB.

Kolm et al. (2017, 2019) developed helicase-dependent amplification (HDA) assays to detect different fecal targets in surface water including fecal indicators. Only a heating block is required for use of the assay, and the sample is applied directly to a test strip that detects and displays the amplification products by marker-specific hybridization probes using a colorimetric reaction in 2 hours. The HDA assay yielded comparable results in sensitivity, specificity, and limits of detection to qPCR. The authors suggest that this low-cost simple assay that requires minimal training could be used instead of qPCR to yield comparable results (Kolm et al., 2019).

Loop-mediated isothermal amplification (LAMP) is a nucleic acid diagnostic method that is potentially useful for testing of water samples in low-resource settings lacking sophisticated laboratory equipment and highly trained personnel (Martzy et al., 2017; Nieuwkerk et al., 2020). Oliveira et al. (2020) used LAMP to detect *E. coli* (*uid*A) in polluted lake and river samples. Quantification of *Enterococcus* spp. and *E. coli* using an MPN-LAMP approach and qPCR protocol were correlated, demonstrating that the MPN-LAMP method could be used for water quality testing in areas that have limited resources (Fu et al., 2021).

3. Cyanotoxin Methods

As discussed in Section III.C, under certain environmental conditions cyanobacteria can rapidly multiply and form HABs and some species may produce cyanotoxins. EPA has developed

several methods for the analysis of various cyanotoxins in ambient water, including *Method 546: Determination of Total Microcystins and Nodularins in Drinking and Ambient Waters* (U.S. EPA, 2016); *Single Laboratory Validated Method for Determination of Cylindrospermopsin and Anatoxin-a in Ambient Freshwaters* (U.S. EPA, 2017b); and *Single Laboratory Validated Method for Determination of Microcystins and Nodularin in Ambient Freshwaters* (U.S. EPA, 2017c).

Since 2017, EPA has published seven molecular technology-related peer-reviewed studies on employing molecular tools for the detection of toxigenic cyanobacteria and cyanotoxins (Chen et al., 2017; Zhu et al., 2019; Lu et al., 2019, 2020; Li et al., 2020; Wang et al., 2021; Tanvir et al., 2021; Duan et al., 2022). EPA's research found that there are high correlations between microcystin and the *mcy* gene specific qPCR/reverse transcriptase (RT)-qPCR signals, while *mcy* specific RT-qPCR can signal activities of toxin-producing genes and toxigenic species and indicate the initiation of bloom-formation and toxin production and the subsequent subsidence of toxin production. An early warning signal of one-week prior to microcystin production using qPCR/RT-qPCR methods was identified (Lu et al., 2020). An additional study developed early warning threshold values of *mcy* gene copy or transcript numbers corresponding to the recommended swimming advisories for microcystins (Duan et al., 2022). The qPCR and RT-qPCR threshold values provided here are specific for Harsha Lake, but they have been supported by the data from lab culture experiments and current research collaborations with regions/states (Duan et al., 2022).

In 2020, EPA developed qPCR detection methods for the detection of microcystin producers (Lu et al., 2020), and filed a patent (U.S. Serial Number 16/142,319) on CyanoHAB qPCR assays. This patent is for detecting genus-specific toxigenic cyanobacteria, providing a standardized qPCR protocol, and the early warning process for cyanotoxin production. EPA is presently developing qPCR detection methods for anatoxin, saxitoxin, and cylindrospermopsin producers in lakes and rivers and qPCR methods for the detection of toxic/nontoxic benthic cyanobacteria.

4. AMR Bacteria

As discussed above in Section III.D, AMR bacteria can be produced by untreated wastewater and many other sources and pose a risk to human health. No standard methods exist for measuring AMR in recreational waters. Researchers are using a variety of different methods to study environmental AMR with variations in the bacteria or genes targeted, the antibiotics tested, and type of method (culture or molecular methods). This variation hinders cross comparison of data sets. WHO has selected *E. coli*, specifically extended spectrum beta-lactamase (ESBL)-*E. coli*, for global surveillance spanning human, animal, and environmental samples (Matheu et al., 2017; WHO, 2018, 2019).

Human and Non-Human FSI

As described in Section III.C, the source of fecal contamination provides important information needed to fully estimate the health risk and remediation options for any fecally contaminated waters. Since 2017, EPA researchers have published 15 peer-reviewed studies on FSI, released

two nationally validated human-associated microbial source tracking qPCR methods, and codeveloped a standard control material in collaboration with NIST.

5. Human FSI

EPA and numerous external partners collaborated to publish nationally validated EPA Methods 1696.1 and 1697.1 for human-associated qPCR assays HF183/BacR287 and HumM2, respectively (U.S. EPA, 2022a,b). These methods include standardized protocols, custom data acceptance metrics, a self-administered method proficiency testing protocol, and an automated data analysis tool (https://www.epa.gov/cwa-methods/other-clean-water-act-test-methods-microbiological).

EPA Method 1696.1 and 1697.1 implementation requires the use of a standard control material to interpret results. In response, EPA designed a control material that functions with 11 qPCR FSI protocols, including HF183/BacR287 and HumM2, and partnered with NIST to develop a large-scale preparation for mass distribution on a national scale. The result is SRM 2617. This material was subject to a rigorous set of certification experiments by NIST using dPCR (Kralj et al., 2021); performance was confirmed by EPA using qPCR (Sivaganesan et al., 2022; Willis et al., 2022) and it was made available to the public in April 2022.

Adaptation of dPCR for FSI

Since 2017, research focusing on the development and implementation of dPCR protocols for FSI applications has increased (Pendergraph et al., 2021; Staley et al., 2018b; Steele et al., 2018; Zimmer-Faust et al., 2021). dPCR can offer greater precision, superior trace level detection, and is less prone to amplification inhibition compared to qPCR (Cao et al., 2016) . However, the conversion of well-characterized qPCR protocols to a dPCR platform is not always straightforward. For example, some researchers have observed a decrease in specificity with dPCR protocols (Nshimyimana et al., 2019; Staley et al., 2018b), while others report the opposite compared to a respective qPCR method (Nshimyimana et al., 2019). In addition, dPCR is ideal for multiplex applications when the goal is to simultaneously amplify two or more targets in the same reaction. Multiplexing can streamline laboratory testing workflow, reduce costs, and offer increased certainty in fecal pollution characterization. Duplex dPCR-based FSI protocols have been developed by combining the widely used HF183 16S rRNA human-associated genetic marker with a crAssphage-like method (CPQ_056) (Ahmed et al., 2019) and an enterococci assay (Cao et al., 2018b).

Emerging Technologies for FSI

Emerging methods are also being tested for utility in FSI. Three methods, a test strip (Kolm et al., 2017, 2019), inversely coupled immunomagnetic separation/adenosine triphosphate (Inv-IMS/ATP) (Zimmer-Faust et al., 2018), and LAMP (Jiang et al., 2018), are discussed in more detail above. Briefly, a test strip was used to differentiate between spring/groundwater, surface water, and wastewater exhibiting a high degree of agreement with paired qPCR measurements (Kolm et al., 2017). A ruminant-associated version of the test strip correctly detected six ruminant sources but did not react with human and 15 other non-ruminant sources (Kolm et al.,

2019). LAMP was used to identify human fecal contamination in water by detecting as few as 17 copies per mL of human-associated *Bacteroides* HF183 sequence with no false positives from dog or cat feces (Jiang et al., 2018).

6. Summary of New Findings on Analytical Methods

- Analytical methods continued to advance over the previous 5 years, providing more precise and rapid tools to assess the quality of recreational waters.
- Development of dPCR methods (e.g., Crain et al., 2021) offers the promise of rapid results as compared to bacterial culture methods.
- Publication of EPA Methods 1642 and 1643 for culture-based coliphage methods allows enumeration of MSC and somatic coliphage types in recreational waters.
- Development of FSI methods (1696.1 and 1697.1) and standard control material (SRM 2617) advances useful tools to mitigate and further inform the human health risk once fecal contamination has been identified.

G. New Implementation Tools

This section discusses the development of implementation tools over the past 5 years, including those for the 2012 RWQC and 2019 Cyanotoxins AWQC; advances in both predictive and process modeling tools; and an update on the status of outreach and training efforts, as well as information on BEACH Act grant funding.

1. Implementation of the 2012 RWQC

Site-Specific Alternative Recreational Criteria

As scientific advancements lead to new technologies for quantifying indicators of fecal contamination in terms of improvements in rapidity, sensitivity, specificity, and method performance for site-specific applications, states and authorized tribes could consider using these methods to develop site-specific water quality criteria. Information demonstrating that these new site-specific alternative criteria developed by states and authorized tribes are scientifically defensible and protective of the recreational use is necessary to support new or revised water quality standards.

To help states and authorized tribes in this effort, EPA recently developed and made public an Excel spreadsheet tool (AltCalc Tool; U.S. EPA, 2021a,b,c) that complements EPA's *Site-Specific Alternative Recreational Criteria Technical Support Materials for Alternative Indicators and Methods* (Alternative Indicators TSM; U.S. EPA, 2014a; available online at: https://www.epa.gov/wqc/recreational-water-quality-criteria-and-methods). The Alternative Indicators TSM outlines the scientific information needed before an alternative indicator/method can replace the use of a recommended or approved method on a site-specific basis and describes how to compare indicators after users have identified a candidate site and an alternative indicator/method that has desirable attributes. The AltCalc Tool facilitates this comparison by

providing a user-friendly way to compute the index of agreement and Pearson's correlation coefficient squared (R-squared) statistics for microbial water quality data sets from two different microbial water quality methods. The index of agreement and R-squared values are used to assess whether the alternative water quality method is appropriate for replacing an existing water quality method using the steps in EPA's Alternative Methods TSM (pages 16–19).

Several states have taken advantage of the information in the Alternative Indicators TSM (available online at: https://www.epa.gov/wqc/recreational-water-quality-criteria-and-methods). In Michigan, the state and EPA collaborated on a large-scale project to evaluate EPA Draft Method C for enumerating *E. coli* with qPCR compared to previously approved EPA culture-based methods (Best et al., 2018; Lane et al., 2020b; Haugland et al., 2021). Results from this study were used to facilitate efforts to implement qPCR-based *E. coli* detection for rapid recreational water quality monitoring in the State of Michigan. See the Outreach, Training, and Grants section below for more information. In California, EPA Region 9 approved a pilot program for the rapid ddPCR test to assess beach water quality to provide same-day notices in San Diego County. (Wang et al., 2016a; California Water Boards Water Quality Coordinating Committee, 2017; U.S. EPA, 2020; Crain et al., 2021). Other examples include Illinois (Shrestha and Dorovitch, 2019), Hawaii (Fujioka et al., 2015; Esgaib Vaz Guimarães, 2017), and Wisconsin (Hernandez, 2018).

Sanitary Surveys

In July 2020, EPA released its updated Sanitary Survey App for Marine and Fresh Waters with enhanced features to help states, territories, and tribes gather sanitary survey data to identify sources of fecal contamination and potential HAB events affecting coastal recreation waters. In 2021, EPA was able to allow increased access to the Sanitary Survey App for use by local governments, participatory scientists, non-governmental organizations, and the public. The surveys allow users to collect and share data on potential sources of fecal pollution and information on potential HAB events in local surface waters, including designated recreational waters. The data from the Sanitary Survey App can be exported for use in predictive models and for sharing within or between groups.

The Sanitary Survey App is now a mobile web application and can be used on any device (i.e., phone, tablet, computer). Special features include photo storage, real-time geolocation, links to websites such as the National Weather Service to access data, and free data storage. Additional information is located on the Sanitary Surveys for Recreational Waters web page.

The EPA Sanitary Survey App is available for use by states, territories, tribes, local governments, citizen science and environmental groups, and the public. EPA conducted extensive outreach since the release of the app in July 2020. Stakeholders were notified of the availability of the app by email, listserv announcements, social media posts, press releases, and presentations at four national conferences, two state beach conferences, and several state and tribal meetings. EPA also provided more than 15 virtual training webinars that included demonstrations of the freshwater and marine routine surveys. EPA plans to continue its outreach efforts to raise awareness and encourage use of the app by all stakeholders.

2. Cyanotoxins

EPA published materials to support implementation of the 2019 recommended recreational water quality criteria and/or swimming advisories for microcystins and cylindrospermopsin. These materials include the *Final Technical Support Document: Implementing the 2019 Recommended Human Health Recreational Ambient Water Quality Criteria or Swimming Advisories for Microcystins and Cylindrospermopsin* (U.S. EPA, 2021d), and guidance on EPA's Monitoring and Responding to Cyanobacteria and Cyanotoxins in Recreational Waters web page.[3] The technical support document contains several questions and answers relating to the recommended recreational water quality criteria and/or swimming advisories for microcystins and cylindrospermopsin. The information in this document is primarily intended to support states, authorized tribes, and territories interested in adopting the recommended criteria into their state or tribal WQS or using the recommended values as the basis for swimming advisories and related public notification purposes. The document provides recommendations on how to monitor for cyanobacteria and two of their known cyanotoxins (microcystins and cylindrospermopsin) in waterbodies and information on how to complete assessments, list impaired or threatened waters, and develop TMDLs. EPA's Monitoring and Responding to Cyanobacteria and Cyanotoxins in Recreational Waters web page and materials found there provide detailed information to support states and authorized tribes in their efforts to monitor and respond to cyanobacterial blooms and cyanotoxins in recreational waters. EPA also developed HABs infographics that can be used by federal agency, state, territorial, tribal, and local government partners to help them educate recreators about the potential dangers of HABs in both marine and freshwaters.

To aid in tracking the occurrence of HABs and their toxins in the nation's freshwaters, EPA developed the Tracking CyanoHABs Storymap. This user-friendly, interactive resource compiles state-issued recreational waterbody and drinking water health advisories for HABs from across the country. Each month, EPA collects available information on HAB advisories in drinking and recreational freshwater systems that state health and/or environmental agencies publicly report online. This information is displayed in two interactive maps that illustrate current advisories and advisories since 2015.

In addition, EPA facilitated a series of workshops across the country to build relationships and identify shared HABs-related goals, needs, and barriers among federal, state, and tribal CWA and Safe Drinking Water Act (SDWA) programs. The workshops, held from 2015 to 2018 across the country, provided information on the health outcomes associated with exposure to HABs, strategies for the prevention and management of HABs in surface waters, and effective treatment techniques for HABs-related cyanotoxins in drinking water. In addition, the workshops provided a forum for state and tribal health and environmental agencies to exchange information on their HABs programs, experiences, and needs. EPA also held a series of webinars specific to tribal waters in 2021. Information on these and other webinars can be found on EPA Newsletter and Collaboration and Outreach on HABs website.

[3] https://www.epa.gov/cyanohabs/monitoring-and-responding-cyanobacteria-and-cyanotoxins-recreational-waters

3. Predictive Modeling Research

The 2012 RWQC encourage the use of predictive models to supplement water quality monitoring to enable more timely beach notification decisions. Predictive modeling uses past water quality data and current observed hydrometeorological measurements to estimate water quality at a given time. Accurate and reliable predictive models can improve water quality management.

EPA maintains an active research program focusing on priority research areas identified in the *2017 Five-Year Review of the 2012 Recreational Water Quality Criteria* (U.S. EPA, 2018d) including development of predictive models and technology transfer to stakeholders. EPA has organized and participated in multiple workshops and training sessions on the use of Virtual Beach across the United States, including in-person workshops in Ohio, South Carolina, and New England. Additionally, EPA has held over a dozen online training sessions and webinars in the last 5 years. Together, these online and in-person training opportunities reached approximately four hundred participants.

4. Process Modeling Research

Process or deterministic models are valuable tools for the simulation of contaminant sources, concentrations, transport, and exploration of mitigation scenarios in recreational waters. Single models and integrated environmental modeling (IEM) are two types of approaches that can be applied when simulating the fate and transport of microbial contaminants at the watershed scale. IEM consists of a construct of modules and/or models that simulate individual processes where data is exchanged seamlessly to produce an output that targets the problem of interest (Kim et al., 2018; Whelan et al., 2018). Over the past 5 years, EPA has published multiple QMRA software modules and tutorials. The workflow was developed to estimate risk associated with inputs of microbial contaminants from a variety of land uses at the watershed scale (Whelan et al., 2017a,b). Data collection has been automated for watershed analysis, allowing Hydrologic Unit Code (HUC)-8 or Pour Point analyses (Whelan et al., 2015). The IEM automatically delineates watersheds and sub-watersheds and the system accounts for minimum stream lengths, minimum subwatershed sizes, and minimum land use type sizes. Local data files contain a Microbial Properties Database that provides physico-microbial properties relevant to release, fate, transport, exposure, and effects modeling (Whelan et al., 2017c). Precipitation data can be entered from either monitored gauges or radar/remote sensing precipitation sources. The system automatically estimates microbial loads on all sub-watersheds based on the number of animals reported per land use patterns by calculating manure-based source terms and processes point and non-point sources of fecal microbial contaminants. The information is used to prepopulate a watershed model, the Hydrological Simulation Program-Fortran (HSPF), with microbial decay and transport constants (Kim et al., 2017). The system is compatible with both graphical and tabular viewers in HSPF and Better Assessment Science Integrating Point and Nonpoint Sources (BASINS) and allows users to investigate water flows and microbial concentration time series at multiple locations throughout the watershed. Finally, the system is designed to be linked to a pathogen risk model, the microbial risk assessment-interface tool (MRA-IT). Supporting

information for the use, training, and application of the QMRA software can be found in the Quantitative Microbial Risk Assessment Tutorial – Primer.

Modeling the fate and transport of MSC and somatic coliphages was another example of an IEM application where information produced in laboratory studies contributed with critical data inputs for the development of a hydrodynamic coliphage model in the Great Lakes. EPA evaluated the light-induced inactivation of MSC and somatic coliphages using both laboratory and wastewater treatment plant-isolated strains (Zepp et al., 2018; Nelson et al., 2018). Solar simulators were used to develop biological weighing functions and ultraviolet inactivation constants that, in turn, were used to model the inactivation of coliphages over a range of conditions in aquatic environments. A three-dimensional model of coliphage inactivation and transport was developed for Washington Park Beach in Lake Michigan using the organism-specific action spectra developed in the laboratory studies (Safaie et al., 2020). Results indicated that coliphage models outperformed a previously tested *E. coli* model suggesting that coliphages may serve as conservative indicators of microbiological contamination.

EPA published a study that evaluated the importance of sediment resuspension in FIB loading calculations in watersheds of various sizes (Bradshaw et al., 2021). While suspended transport was the dominant mechanism for FIB movement regardless of stream size, FIB loads from overland and bedload transport should be considered when performing modeling and microbial assessments at the watershed scale.

EPA also modeled FIB using a high-frequency, multi-year microbial data set with the Soil and Water Assessment Tool (SWAT) (Sowah et al., 2020). SWAT was applied to understand the sources and parameters impacting microbial water quality in a mixed-used watershed in southeastern United States. A key scientific advancement of this work was the development of a criterion to evaluate the performance of bacterial models in SWAT.

5. Outreach and Grants

EPA Stakeholder Technology Transfer

An essential step in the successful implementation of recreational water quality, laboratory-based methods is technology transfer. Molecular and newly developed coliphage culture methods can be technically demanding, requiring specialized equipment, detailed procedures, and specialized training making it challenging to implement on a national scale. To help address this need, EPA ensures public access to standardized protocols and companion implementation tools, as well as provides technical support and hands-on training opportunities. Since 2017, EPA has provided support to 56 different federal, state, territory, or local laboratories, as well as to more than 20 academic research groups. These activities help facilitate the proper use of newly developed recreational water quality laboratory methods.

6. BEACH Act Grant Funding

Under the BEACH Act, EPA awards grants to eligible state, territorial and tribal applicants to help them and their local government partners monitor water quality at coastal and Great Lakes beaches. When bacteria levels are too high for safe swimming, these agencies notify the public

by posting beach warnings or closing the beach. Since 2001, state, and local governments, territories and tribes have been awarded nearly $206 million in EPA BEACH Act grants to monitor beaches for FIB, maintain and operate public notification systems, and report results of monitoring and notification activities to EPA. While beach grant funding totals allocated by Congress were $9.2 million in 2019 and 2020, $10.1 million has been awarded in 2022. See https://www.epa.gov/beach-tech/beach-grants for detailed information on BEACH Act grant awards.

IV. Summary of Major Findings and Priorities for Further Work

A. Summary of Major Findings

1. Summary of Findings Identified in New Epidemiological Studies

- Recent publications have advanced understanding and further confirm that epidemiological and outbreak data demonstrate that young children (i.e., 10 years old and under) have a higher risk of illness compared to adults when exposed to fecal contamination in recreational waters.

- Young children's (i.e., 10 years old and under) behavior, such as increased time spent in water, length of time spent playing in the sand, and more vigorous activity, can increase their potential exposure to fecal contamination and is associated with greater health risk compared to other age groups, especially those 18 and over.

- Children's recreational exposure is greater than adolescents and adults because they ingest more total volume of water, independent of body weight, than do the other age groups.

- Studies demonstrate that human fecal contamination can pose higher risks of illness in recreators relative to some non-human fecal sources.

- While FIB can be a useful indicator where raw and poorly treated sewage dominates water quality, multiple studies discuss the inconsistent performance of culture-enumerated FIB related to health and point to other indicators to better represent the potential risk of illness (e.g., *Enterococcus* measured by qPCR).

- Waters receiving human fecal contamination can have viral pathogens present at health-relevant levels and yet also be below recommended water quality levels for culturable FIB.

- The health risk of recreating during or following wet weather compared to dry weather can be different due to changes in fecal loading dynamics to a waterbody.

- Recent QMRA studies corroborate past findings that waters affected by human fecal inputs can pose higher health risks compared to non-human fecal sources.

- Several QMRAs utilized norovirus as a primary reference pathogen to characterize risk from human enteric viruses in waters affected by human fecal pollution.

- Across QMRA studies, the age of fecal contamination was identified as an important factor affecting risk estimates and calculation of RBT values for alternative indicators.

- Outbreak surveillance in the United States and elsewhere continues to indicate the importance of human enteric viruses as a leading etiologic agent of illness in ambient recreational waters, even where low densities of FIB occur.

- Multiple outbreak investigations demonstrate the increased health burden children experience when exposed to fecal pollution in ambient waters.

2. Summary of New Information on Coliphages

- Both somatic and MSC consistently occur in raw wastewater across the United States, with somatic coliphages more numerous than MSC, and thus have potential to be used as indicators of fecal contamination in waters affected by sewage.

- Viral surrogates, such as coliphages, may perform as better surrogates of the fate of human enteric viruses compared to culture-enumerated FIB during wastewater treatment.

- Coliphages can exhibit a higher decay rate relative to viral pathogens in ambient waters.

- When human fecal sources are suspected based on location but not water quality measures, MSC had a stronger relationship to illness. Knowledge of the fecal sources affecting a particular waterbody, especially human contamination sources, can improve interpretation of monitoring data.

3. Summary of New Information on Cyanotoxins

- New data were identified for saxitoxins and work is underway at EPA to evaluate data to support development of an HESD.

- For cylindrospermopsin and microcystins, new studies reviewed did not provide new information to support derivation of a revised RfDs.

- Limited new toxicity information is available for anatoxin-a and available literature reviewed does not support development of an HESD at this time.

- No new toxicity information was identified on nodularins and available literature reviewed does not support development of an HESD at this time.

4. Summary of New Information on AMR

- An increased understanding of horizontal gene transfer mechanisms and AMR selection can be used to improve monitoring for AMR in ambient waters.

- A combination of techniques (including both molecular- and culture-based) are necessary to fully understand AMR in surface waters.

5. Summary of New Information on FSI

- Release of SRM 2917, a high-quality standard control material that functions with 11 FSI qPCR methods indicative of human (HF183/BacR287, HumM2, CPQ_056, CPQ_064), cattle (CowM2, CowM3), ruminant (Rum2Bac), canine (DG3, DG37), swine (Pig2Bac), and avian (GFD) fecal sources, facilitates implementation of FSI qPCR methods.

- Large-scale field demonstrations were conducted using new human-associated EPA Methods 1696.1 and 1697.1 to establish performance in wastewater and surface waters.

- As the use of FSI tools for human fecal pollution characterization becomes a more common practice, practitioners are recognizing that the contribution of non-human fecal pollution sources plays an integral role in water quality management.

- Understanding the decay of microorganisms associated with specific fecal pollution sources is important for many FSI applications.

6. Summary of New Findings on Analytical Methods

- Analytical methods continued to advance over the previous 5 years, providing more precise and rapid tools to assess the quality of recreational waters.

- Development of dPCR methods (e.g., Crain et a., 2021) offers the promise of rapid results as compared to bacterial culture methods and also easier implementation as compared to qPCR methods.

- Publication of EPA Methods 1642 and 1643 for culture-based coliphage methods allows identification of human health risk from viral pathogens.

- Development of FSI methods (1696.1 and 1697.1) and standard control materials (SRM 2617) advances useful tools to mitigate and further inform the human health risk once fecal contamination has been identified.

- The exposure-response relationship was more consistently monotonic for *Enterococcus* measured using qPCR methods versus culture methods across different analyses, even when restricted to beaches without a known point source of pollution.

B. Assessment of the Need to Revise the 2012 RWQC

Based on a review of the latest scientific knowledge EPA has determined that the 2012 RWQC need to be revised and that there are several additional implementation tools that EPA can make available to manage recreational waters.

The available science demonstrates an increased health risk for children compared to adults when recreationally exposed to fecal contamination. Since 2017, EPA has published nationally

validated protocols for qPCR methods and codeveloped a standard reference material for national use that supports the use of qPCR technologies in recreational settings.

Based on the findings in this review:

1. EPA plans to develop additional criteria recommendations for qPCR-enumerated enterococci protective of children, which would be protective of all recreators.
2. EPA plans to continue to develop recommendations for coliphages to help address potential risks from human enteric viruses in ambient waters.
3. Finally, EPA plans to explore how best to use human fecal source identifiers, such as HF183, for water quality management. Being able to demonstrate that a waterbody has been impacted by human fecal contamination will enable risk managers to use the appropriate tools to evaluate and manage risks for waters impacted by human sources.

C. Priorities for Further Work

Health Studies

The analysis of risks to children during recreation is a significant driver for future research. Further analysis of *Enterococcus* spp. qPCR data for children is needed for consideration in criteria development. An evaluation of how QMRA can be used to address risk to children from swimming exposure is needed as is guidance on QMRA. Additional health studies would be valuable to assess the risks to children during recreation. While studies have shown that children have greater exposure to recreational water, which could explain greater illness rates, it is important to conduct studies to understand whether the children's developing immune system is a biological factor that increases their susceptibility to illness.

Coliphages

Based on the findings of studies conducted to date, future research efforts should focus on improving and refining virus detection methodologies in estuarine and marine waters, as well as on determining coliphage occurrence trends in these water types and across seasons through measurements. It is also important to further understand the treatment efficacies of different combinations of traditional and advanced treatment processes by comparing levels of multiple enteric viruses and coliphages. Studies to investigate the relationship between viral surrogates and human fecal pollution may be useful to support the development of risk-based standards to protect public health from pathogenic viruses in ambient waters. Finally, research is needed to understand the decay patterns of coliphages and viral pathogen in recreational waters and wastewater.

Cyanotoxins

EPA has reviewed the available toxicity data for saxitoxin and is preparing an HESD to determine whether there is adequate toxicity data to derive an oral RfD.

There is a need to synthesize the information on human health risk from exposure to cyanotoxins and to conduct additional studies to support health assessments for individual and groups of

cyanotoxins. EPA's ORD is working with other EPA offices and regions to develop a systematic literature review and evidence map to characterize the human health effects of exposure cyanotoxins from any route of exposure. The scope of the review includes available epidemiological and toxicological literature for anatoxins, BMAA, cylindrospermopsin, microcystins, nodularins, and saxitoxins.

Future research needs include research on the effects of cyanotoxins on both aquatic life and human health. There is a need for research on the toxicity of HABs on aquatic life and aquatic-dependent wildlife and the development and validation of analytical methods for cyanotoxins in diverse sample matrices in order to evaluate cyanotoxins accumulation in aquatic species and the food web. Research needs on the toxicity of HABs to humans include short-term, sub-chronic, and chronic toxicity and epidemiology studies of anatoxin-a and derivatives and nodularins, research to evaluate risk associated with dermal exposure to algal toxins, and research to evaluate risk associated with inhalation of algal toxins, including long-term exposure. In addition, a systematic literature review of the impact of climate change on HAB formation is needed.

Antimicrobial Resistance

Future research needs include evaluation of best management practices for AMR attenuation, characterization of AMR occurrence in human stool, characterization of environmental reservoirs of AMR, characterization of AMR targets at subtropical beaches, and application of QMRA to investigate the risk of recreational exposure to AMR in surface-waters. Specific research needs include data on exposure pathways and dose-response relationships for various health outcomes. More data are needed on the variability and load contributions of potential sources (i.e., WWTPs, animal feeding operations [AFOs], CAFOs, medical/health care facilities, wildlife, slaughterhouse waste streams, landfills, septic tanks, cesspools, and cemeteries) of AMR bacteria and ARGs in surface waters to better understand their behavior in the environment and across wastewater treatment processes. Further, active surveillance in surface waters of clinically relevant AMR bacteria strains is needed to provide exposure information for protection of human health. Standardized methods for culturable and gene targets are needed to study, detect, and classify AMR bacteria in surface waters.

Human and Non-Human Fecal Source Identification

Further advances in avian FSI applications are needed, especially the development of methods with improved host distribution. Further research is needed to develop a toolbox approach for ruminant fecal source characterization in recreational waters. Additional research is warranted to further characterize the utility of these methodologies.

Considering findings from EPA and others, monitoring of crAssphage-like human-associated genetic markers could offer substantial advantages for future water quality management and warrant further research.

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The EPA publication of nationally validated protocols and the public release of SRM 2917 combined with key scientific studies provides necessary tools and information for the use of human-associated qPCR technologies in recreational settings. Future research needs to support national implementation of EPA Methods 1696.1 and 1697.1 include interlaboratory performance assessment of SRM 2917, method performance assessment in subtropical marine waters, further science to support data interpretation, and adaptation to a digital PCR platform. Finally, additional efforts are needed toward the development of human-associated virus-based microbial source tracking methods to complement bacterial HF183/BacR287 (U.S. EPA, 2022a) and HumM2 (U.S. EPA, 2022b) protocols.

Non-Human Fecal Sources Technical Support Materials

EPA is developing a TSM to support derivation of site-specific alternative water quality criteria for ambient recreational waters where the predominant contamination is from non-human fecal sources. The TSM describes the use of QMRA to derive site-specific alternative water quality criteria for ambient recreational waters. Specifically, this TSM provides information on how to use a risk-based approach to develop water quality criteria that are equally health protective as EPA's 2012 RWQC for waterbodies predominantly affected by non-human fecal contamination. The QMRA framework discussed in the TSM is based on the current science, EPA's FIB recommendations for enterococci or *E. coli*, and associated enumeration methods (EPA Methods 1600, 1603; U.S. EPA, 2009b, 2014b). Additional information includes characterization of the exposure scenario, selection the QMRA parameters, information on dose-response, information on risk characterization, descriptions of the reference pathogens, and examples of the types of supporting evidence that are useful.

Analytical Methods

Several scientific advances in methods have been made in the past 5 years. The use of dPCR platform should continue to be explored in the future. A large-scale performance assessment of FIB qPCR methods in marine waters is needed.

Implementation Tools

Predictive Modeling

Future research needs for predictive modeling approaches to improve recreational water quality management include the development of a web-based Virtual Beach solution providing an easy-to-use interface with more powerful analytics and the completion of current Great Lakes and Boston Harbor predictive modeling efforts. There is also a need to assess predictive model performance in subtropical recreational marine waters.

Process Modeling

Multiple research activities are needed to augment the development of site-specific criteria using process models; these include improving modeling of human versus non-human sources at the watershed scale. It will also be important to enhance the understanding of watershed connectivity as well as potential impacts on marine recreational coastal environments from mixed-used watersheds. Process modeling can also be improved by implementing novel tools for calibration of bacterial models such as global sensitivity analysis. IEM future research should also address developing a link between process modeling approaches and the new EPA Sanitary Survey App. Information gathered by the app could be automatically imported into watershed microbial models, thus speeding up the process of parameterization and model calibration.

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Appendix A. Literature Search and Review Strategies

1. Background

Context

Under the Beaches Environmental Assessment and Coastal Health (BEACH) Act amendments to the Clean Water Act (CWA) Section 304(a)(9)(B) EPA is required to complete a review of its recommended recreational water quality criteria every 5 years. In 2018, EPA published a review of the 2012 304(a) recommended recreational water quality criteria (RWQC). These 2012 criteria were based on the latest research and science at the time including epidemiology studies conducted in the 2000s. The criteria are designed to protect the public from exposure to harmful levels of pathogens while participating in water-contact activities in recreational waters. An important goal of the five-year review is to document the assessment of whether revisions to the 2012 RWQC are necessary. A key component of that assessment is a consideration of the state of the science and advances made since the previous five-year review that support the recommended RWQC and enhance its implementation. The EPA's RWQC Review will be the second five-year review. Systematic literature search and review will be conducted on five topics relevant to the 2nd five-year RWQC Review:

1. Advances in molecular microbial source tracking (MST) for recreational water applications (supports Section III.E of this report).
2. Advances in molecular methods (supports Section III.F of this report).
3. Advances in quantitative microbial risk assessment (QMRA) and epidemiological studies (supports Sections III.A.1, III.A.2, III.A.3 of this report).
4. Characterization of children's risk from exposure to recreational water contaminated by fecal contamination (supports Sections III.A.4 of this report).
5. Cyanotoxins: microcystins, cylindrospermopsin, and anatoxin-a (supports Section III.C of this report).

This document includes the literature search strategies for the above topics. Each of the topic areas have associated focus areas and research questions. The literature search for Topics 1 and 2 were conducted in a single effort as they are closely related by focusing on *methods*. In addition, articles that were relevant for more than one topic area, were cross-tagged between topics, except for cyanotoxins which was conducted under a separate effort.

Systematic Approach

The basic steps of this systemic review include:

1) Planning: scoping, research questions, keywords, title/abstract (Ti/Abs) eligibility criteria
2) Literature search
3) Prioritization, clustering (optional depending on how many Ti/Abs obtained)
4) Ti/Abs screening (optional machine learning)
5) Literature retrieval
6) Study quality metrics evaluation

7) Data extraction
8) Data analysis and visualization

This document includes the literature search and the eligibility (inclusion/exclusion) criteria for Ti/Abs screening and study quality metrics.

2. Topics 1 and 2: Methodological Progress

This search strategy specifically focuses on 1) advances in MST (or fecal source identification and bacterial source tracking) for recreational water applications, and 2) advances on molecular methods. The first topic includes advances in method standardization, standard control or reference materials, water sample inhibition or matrix interference, genetic markers, field studies to identify and/or remediate fecal sources. The second topic focuses on advances in the development of quantitative polymerase chain reaction (qPCR), digital polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), next generation sequencing, metagenomic deoxyribonucleic acid (DNA) sequencing, and hypervariable 16S ribosomal ribonucleic acid (RNA) gene sequencing used in recreational waters to measure indicators of fecal contamination.

Focus Areas:

- Advances in method standardization.
- Development of standard control or reference materials.
- Occurrence of amplification inhibition and/or matrix interference.
- Statistical analysis or other approaches to address non-detects or results below the level of quantification.
- Field studies to identify and/or remediate fecal sources.
- Advances in the development of qPCR, digital PCR, RT-PCR, next generation sequencing, metagenomic DNA sequencing, and hypervariable 16S ribosomal RNA gene sequencing used in recreational waters to measure indicators of fecal contamination, pathogen indicators or waterborne pathogens.
- Advances in non-molecular yet novel approaches to culture methods and combinations of FIB.
- Data interpretation statistical/modeling approaches (fecal score, QMRA, censored data).

Research Question: What methodological (tools, markers, approaches) advances have been made since 2016 in the area of recreational surface water-based monitoring (e.g., fecal indicator organisms, pathogens, or MST) including method standardization (standards or reference material) and controls (inhibition or quality control)?

Literature Search Strategy for Topics 1 and 2: Methodological Progress

The searches are limited to the English language only.

Databases for searches include PubMed (terms searched in All Fields) and Web of Science (terms searched in Topic).

Dates: January 2016 to November 9, 2021 (described below)

Keywords were tested to develop the strategies.

Topic 1 and 2: Methodological Progress Keyword Test Summary

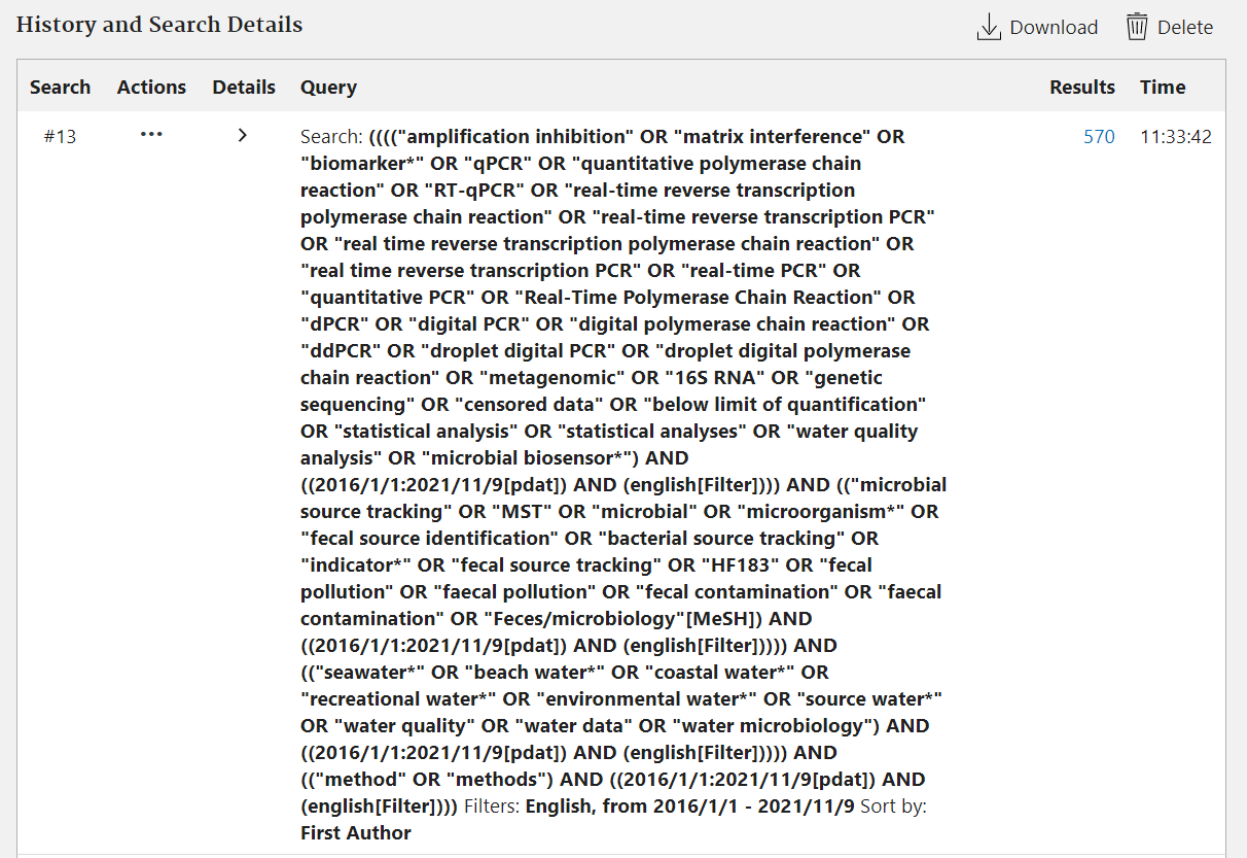
Search Date	Date Limit	Search Strategy Sets	Number of Title/Abstracts		
			PubMed	WoS	Unique Total
November 9, 2021	January 2016–search date	Organism AND Water AND Detail AND Methodology	570	326	717

Topic 1 and 2: Database Search: PubMed

Date of Search: November 9, 2021 (range January 2016 through November 9, 2021)
Fields Searched: All Fields
Limits: English Only; January 1, 2016, November 9, 2021

Set Name	Search Strategy for PubMed	Results (Number of Titles)
Organism	("microbial source tracking" OR "MST" OR "microbial" OR "microorganism*" OR "fecal source identification" OR "bacterial source tracking" OR "indicator*" OR "fecal source tracking" OR "HF183" OR "fecal pollution" OR "faecal pollution" OR "fecal contamination" OR "faecal contamination" OR "Feces/microbiology"[MeSH])	378,129
Water	("seawater*" OR "beach water*" OR "coastal water*" OR "recreational water*" OR "environmental water*" OR "source water*" OR "water quality" OR "water data" OR "water microbiology")	36,185
Detail	("amplification inhibition" OR "matrix interference" OR "biomarker*" OR "qPCR" OR "quantitative polymerase chain reaction" OR "RT-qPCR" OR "real-time reverse transcription polymerase chain reaction" OR "real-time reverse transcription PCR" OR "real time reverse transcription polymerase chain reaction" OR "real time reverse transcription PCR" OR "real-time PCR" OR "quantitative PCR" OR "Real-Time Polymerase Chain Reaction" OR "dPCR" OR "digital PCR" OR "digital polymerase chain reaction" OR "ddPCR" OR "droplet digital PCR" OR "droplet digital polymerase chain reaction" OR "metagenomic" OR "16S RNA" OR "genetic sequencing" OR "censored data" OR "below limit of quantification" OR "statistical analysis" OR "statistical analyses" OR "water quality analysis" OR "microbial biosensor*")	481,175
Methodology	("method" OR "methods")	3,088,876
Total	**(Organism AND Water AND Detail AND Methodology)**	**570**

Screenshot of Topic 1 and Topic 2 PubMed Search



History and Search Details

Download Delete

Search	Actions	Details	Query	Results	Time
#13	•••	›	Search: ((((("amplification inhibition" OR "matrix interference" OR "biomarker*" OR "qPCR" OR "quantitative polymerase chain reaction" OR "RT-qPCR" OR "real-time reverse transcription polymerase chain reaction" OR "real-time reverse transcription PCR" OR "real time reverse transcription polymerase chain reaction" OR "real time reverse transcription PCR" OR "real-time PCR" OR "quantitative PCR" OR "Real-Time Polymerase Chain Reaction" OR "dPCR" OR "digital PCR" OR "digital polymerase chain reaction" OR "ddPCR" OR "droplet digital PCR" OR "droplet digital polymerase chain reaction" OR "metagenomic" OR "16S RNA" OR "genetic sequencing" OR "censored data" OR "below limit of quantification" OR "statistical analysis" OR "statistical analyses" OR "water quality analysis" OR "microbial biosensor*") AND ((2016/1/1:2021/11/9[pdat]) AND (english[Filter]))) AND (("microbial source tracking" OR "MST" OR "microbial" OR "microorganism*" OR "fecal source identification" OR "bacterial source tracking" OR "indicator*" OR "fecal source tracking" OR "HF183" OR "fecal pollution" OR "faecal pollution" OR "fecal contamination" OR "faecal contamination" OR "Feces/microbiology"[MeSH]) AND ((2016/1/1:2021/11/9[pdat]) AND (english[Filter])))) AND (("seawater*" OR "beach water*" OR "coastal water*" OR "recreational water*" OR "environmental water*" OR "source water*" OR "water quality" OR "water data" OR "water microbiology") AND ((2016/1/1:2021/11/9[pdat]) AND (english[Filter])))) AND (("method" OR "methods") AND ((2016/1/1:2021/11/9[pdat]) AND (english[Filter]))) Filters: **English, from 2016/1/1 - 2021/11/9** Sort by: **First Author**	570	11:33:42

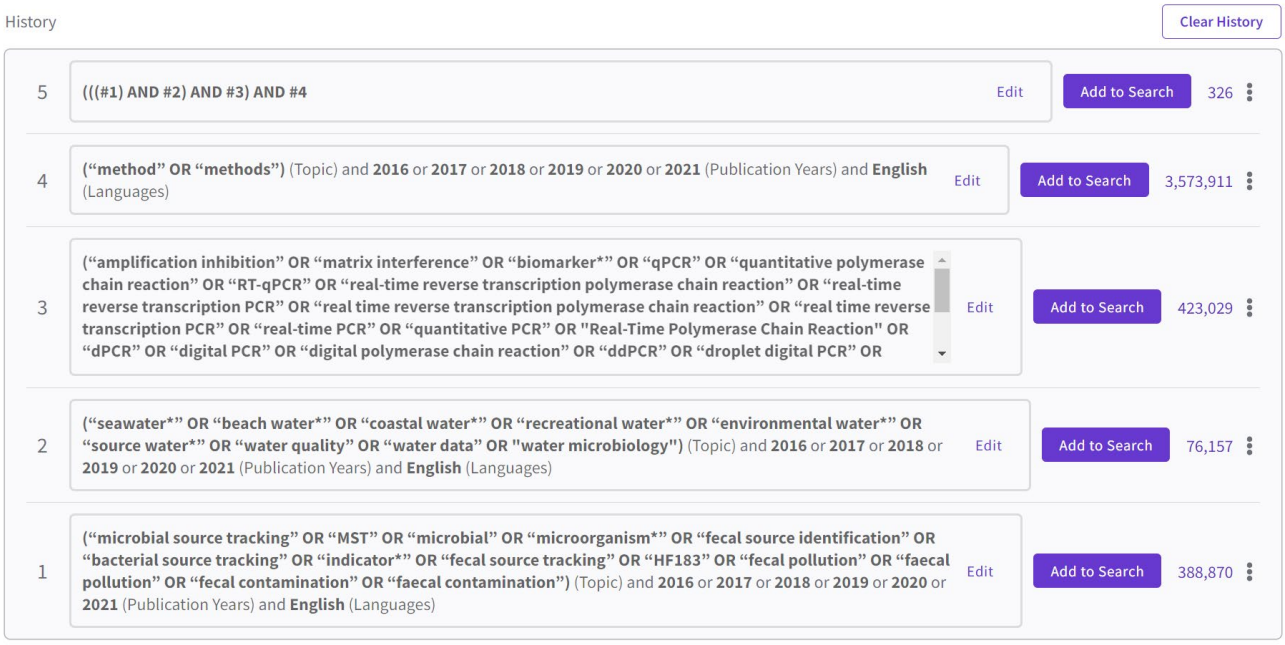
Topic 1 and 2: Database Search: Web of Science

Date of Search: November 9, 2021 (range 2016 through 2021)
Fields Searched: Topic
Limits: English Only; 2016 to 2021

Set Name	Search Strategy for Web of Science	Results (Number of Titles)
Organism	("microbial source tracking" OR "MST" OR "microbial" OR "microorganism*" OR "fecal source identification" OR "bacterial source tracking" OR "indicator*" OR "fecal source tracking" OR "HF183" OR "fecal pollution" OR "faecal pollution" OR "fecal contamination" OR "faecal contamination")	388,870
Water	("seawater*" OR "beach water*" OR "coastal water*" OR "recreational water*" OR "environmental water*" OR "source water*" OR "water quality" OR "water data" OR "water microbiology")	76,157

Set Name	Search Strategy for Web of Science	Results (Number of Titles)
Detail	(“amplification inhibition” OR “matrix interference” OR “biomarker*” OR “qPCR” OR “quantitative polymerase chain reaction” OR “RT-qPCR” OR “real-time reverse transcription polymerase chain reaction” OR “real-time reverse transcription PCR” OR “real time reverse transcription polymerase chain reaction” OR “real time reverse transcription PCR” OR “real-time PCR” OR “quantitative PCR” OR “Real-Time Polymerase Chain Reaction” OR “dPCR” OR “digital PCR” OR “digital polymerase chain reaction” OR “ddPCR” OR “droplet digital PCR” OR “droplet digital polymerase chain reaction” OR “metagenomic” OR “16S RNA” OR “genetic sequencing” OR “censored data” OR “below limit of quantification” OR “statistical analysis” OR “statistical analyses” OR “water quality analysis” OR “microbial biosensor*”)	423,029
Methodology	(“method” OR “methods”)	3,573,911
Total	**(Organism AND Water AND Detail AND Methodology)**	**326**

Screenshot of Web of Science Topic 1 and Topic 2 Search



Topic 1 and 2: Gray Literature Search

Date of Search: November 9, 2021

NOTE: Google uses strict character limits. Strings containing more than 32 words are not allowed.

Organization	URL	Keywords /Search string	Results (Duplicates Removed)
FDA	FDA.gov	site:fda.gov AND ("microbial source" OR "MST" OR "fecal source" OR "bacterial source" OR "HF183" OR "fecal pollution") AND ("standard" OR "reference" OR "inhibition" OR "quality" OR "PCR" OR "indicator*") AND (water OR "beach") AND ("PCR" OR "polymerase chain reaction" OR metagenomic OR "censored data" OR "genetic sequencing" OR "statistical analysis")	14
NPS	NPS.gov	site:nps.gov AND ("microbial source" OR "MST" OR "fecal source" OR "bacterial source" OR "HF183" OR "fecal pollution") AND ("standard" OR "reference" OR "inhibition" OR "quality" OR "PCR" OR "indicator*") AND (water OR "beach") AND ("PCR" OR "polymerase chain reaction" OR metagenomic OR "censored data" OR "genetic sequencing" OR "statistical analysis")	3
FWS	FWS.gov	site:fws.gov AND ("microbial source" OR "MST" OR "fecal source" OR "bacterial source" OR "HF183" OR "fecal pollution") AND ("standard" OR "reference" OR "inhibition" OR "quality" OR "PCR" OR "indicator*") AND (water OR "beach") AND ("PCR" OR "polymerase chain reaction" OR metagenomic OR "censored data" OR "genetic sequencing" OR "statistical analysis")	2
NAS	Nap.edu	site:nap.edu AND ("microbial source" OR "MST" OR "fecal source" OR "bacterial source" OR "HF183" OR "fecal pollution") AND ("standard" OR "reference" OR "inhibition" OR "quality" OR "PCR" OR "indicator*") AND (water OR "beach") AND ("PCR" OR "polymerase chain reaction" OR metagenomic OR "censored data" OR "genetic sequencing" OR "statistical analysis")	4
WHO	Who.int	site:who.int AND ("microbial source" OR "MST" OR "fecal source" OR "bacterial source" OR "HF183" OR "fecal pollution") AND ("standard" OR "reference" OR "inhibition" OR "quality" OR "PCR" OR	17

Organization	URL	Keywords /Search string	Results (Duplicates Removed)
		"indicator*") AND (water OR "beach") AND ("PCR" OR "polymerase chain reaction" OR metagenomic OR "censored data" OR "genetic sequencing" OR "statistical analysis")	
TOTAL			40

Date Limited: 2016 to November 9, 2021 [Present]

Topic 1 and 2: Title/Abstract Screening Criteria and Tagging

Two separate individuals screened and applied the following tags to each title/abstract. In cases where screener 1 and 2 disagreed a subject matter expert provided conflict resolution and determined the final tagging result. Each title/abstract was tagged as follows:

Screening Tag	Description of Tag
Top Level Tags – choose one [include, unclear, or exclude]:	
Include Topic 1	Obtain full text, advance to full text screening (see topic description below).
Include Topic 2	Obtain full text, advance to full text screening (see topic description below).
Unclear	Requires full text to determine whether within scope. Unclear if applicable to recreational waters, for example, drinking water, groundwater, wastewater studies. OK to include "other possible tags."
Exclude	Do not obtain full text. Not within scope. OK to include notes. OK to include "other possible tags."
If Include – choose all that apply [these four tags only apply if 'include']:	
MST (Topic 1)	• Advances in method standardization related to MST. • Development of standard control or reference materials for MST. • Occurrence of amplification inhibition and/or matrix interference for methods used for MST. • Field studies to identify and/or remediate fecal sources. • Risk assessment with MST genetic markers (epidemiological studies, QMRA). • **In Scope:** ▪ MST in natural ambient surface water samples. ▪ HF183 (human fecal marker), markers for livestock, wildlife, other sources of fecal contamination. ▪ Review Articles about MST data or methods. • **NOT in Scope:** ▪ MST for other water types: drinking water, ground water, wastewater.

Screening Tag	Description of Tag
	▪ MST that does not use to microbial markers (e.g., caffeine, detergent brighteners, plant viruses, other indicators of water quality such as dissolved oxygen, turbidity).
Molecular Methods (Topic 2)	• Advances in the development of qPCR, digital PCR, rt-PCR, next generation sequencing, metagenomic DNA sequencing, and hypervariable 16S RNA gene sequencing used in recreational waters to measure indicators of fecal contamination, pathogen indicators or waterborne pathogens. • Advances in statistics/modeling for data interpretation (fecal score, QMRA, censored data, etc.). • **In Scope:** ▪ Methods applied to natural ambient surface water samples. ▪ Methods that detect (presence/absence) or enumerate (quantify) pathogens or indicators (including but not limited to fecal coliform, enterococci, *E. coli*, coliphage, norovirus, *Cryptosporidium*, *Giardia*). ▪ Methods that use spiked samples if the method is for enumerating microbes in ambient waters. ▪ Review articles for methods. • **NOT in Scope:** ▪ Methods for drinking water, ground water, wastewater. ▪ Methods that do not pertain to microbes (e.g., caffeine, detergent brighteners, plant viruses, other indicators of water quality such as dissolved oxygen, turbidity).
Priority or Review (for Topic 1)	• Articles that provide a review of one of the major topics will be prioritized. Other papers that seem particularly of interest and should be prioritized can also have this tag. Use judgment with this tag. Not all review articles need to have this tag—just the review articles that are particularly on point.
Priority or Review (for Topic 2)	• Articles that provide a review of one of the major topics will be prioritized. Other papers that seem particularly of interest and should be prioritized can also have this tag. Use judgment with this tag. Not all review articles need to have this tag—just the review articles that are particularly on point.
Other Possible Tags – choose all that apply [Ti/Abs indicates the paper may contain information on]:	
QMRA or Epi (Topic 3)	• Advances in QMRA and epidemiological studies in analyzing indicators of fecal contamination (specifically: coliform bacteria, fecal streptococci, anaerobic bacteria) in recreational waters. • Do not include if only bacteriophages.
Children's risk (Topic 4)	• Health effects that children experience following exposure to microbially contaminated surface waters, including illness rates/health outcomes from outbreak reports, epidemiological or other health studies.
Explanation – Free text notes field	• *Optional additional keywords including but not limited to: antimicrobial resistance (AMR), combined sewer overflows (CSO), sanitary sewer overflow (SSO), somatic coliphage, male-specific coliphage (MSC), F-specific coliphage (MSC). • Additional notes about the paper.

Topic 1: Study Quality Evaluation

Articles that were 'include' or 'unclear' moved to full text screening. The full text was reviewed for scope to confirm the article is within the scope. Articles that pass scope were screened for study quality and articles that pass study quality were qualified for data extraction.

Scope

Is the study within the Topic 1 scope? Does it include information on microbial source tracking in recreational waters?

- Yes – continue with study quality.
- No – stop evaluation.

Study Quality Metrics – If Any of the Metrics Are Unacceptable, the Study Was Not Included for Data Extraction.

Sampling Methodology:

- Acceptable – Sampling is described or referenced via a citation. Information may include equipment, sampling depth, time of day, process/reasons for selection of sampling sites. Sampling location map is best, but not required. Site is described, including at a minimum the water type (fresh or marine).
- Unacceptable – If any of the following is missing, rate as unacceptable: date of sampling, water type (matrix), sample storage before assay. Not enough information is included to evaluate the sampling or the sampling procedures have critical problems.

Analytical Methodology:

- Acceptable – Methodology is well described. Information may include filtration/concentration and extraction methods, analytical equipment, reagents, instrument calibration, level of detection (LOD), level of quantification (LOQ), controls, standards, recovery, matrix adjustments, cycling protocol, primers.
- Unacceptable – Controls are not described and it is unclear whether controls were conducted. Or not enough information is included to evaluate the method.

Spatial and Temporal Variability:

- Acceptable – sample size is more than one. Replicates and multiple timepoints may be included.
- Unacceptable – Sample size is not reported or only one sample was taken or sample size is unclear or indecipherable for the summary statistics.

Reporting of Results:

- Acceptable – Raw data are included (check supplemental materials) or summary results are clearly described, such as GM, arithmetic mean, SD, SE, median, and other percentiles. Frequency of detection is reported. Variability and uncertainty are discussed/captured in summary statistics.
- Unacceptable – Samples described in the methods are not reported in the results or vice versa. Unclear whether summary data include non-detects.

Quality Assurance:

- Acceptable – Quality assurance is discussed or can be implied given discussion of controls and recoveries.
- Unacceptable – Not enough information is included to evaluate whether quality assurance (QA) was an issue, or QA issues have been identified that interfere with the reliability of the study, or the study is a non-peer-reviewed publication.

Topic 2: Study Quality Evaluation

Articles that were 'include' or 'unclear' moved to full text screening. The full text was reviewed for scope to confirm the article is within the scope. Articles that pass scope were screened for study quality and articles that pass study quality were qualified for data extraction.

Scope

Is the study within the Topic 2 scope? Does it include information on methods for measuring indicators (e.g., *E. coli*, enterococci, coliphages) or pathogens in recreational waters?

- Yes – continue with study quality.
- No – stop evaluation.

Study Quality Metrics – If Any of the Metrics Are Unacceptable, the Study Was Not Included for Data Extraction.

Sampling Methodology:

- Acceptable – Sampling is described or referenced via a citation. Information may include equipment, sampling depth, time of day, process/reasons for selection of sampling sites. Sampling location map is best, but not required. Site is described, including at a minimum the water type (fresh or marine).
- Unacceptable – If any of the following is missing, rate the study as unacceptable: date of sampling (including the range of sampling dates), water type, sample storage before assay. Not enough information is included to evaluate the sampling, or the sampling procedures have critical problems.

Analytical Methodology:

- Acceptable – Methodology is well described. Information provided may include filtration/concentration and extraction methods, analytical equipment, reagents, instrument calibration, LOD, LOQ, controls, standards, recovery, matrix adjustments, cycling protocol, primers.
- Unacceptable – Controls are not described, it is unclear whether controls were conducted, or not enough information is included to evaluate the methodology.

Spatial and Temporal Variability:

- Acceptable – Sample size is more than one. Replicates and multiple timepoints may be included.
- Unacceptable – Sample size is not reported, or only one sample was taken, or sample size is unclear or indecipherable for the summary statistics.

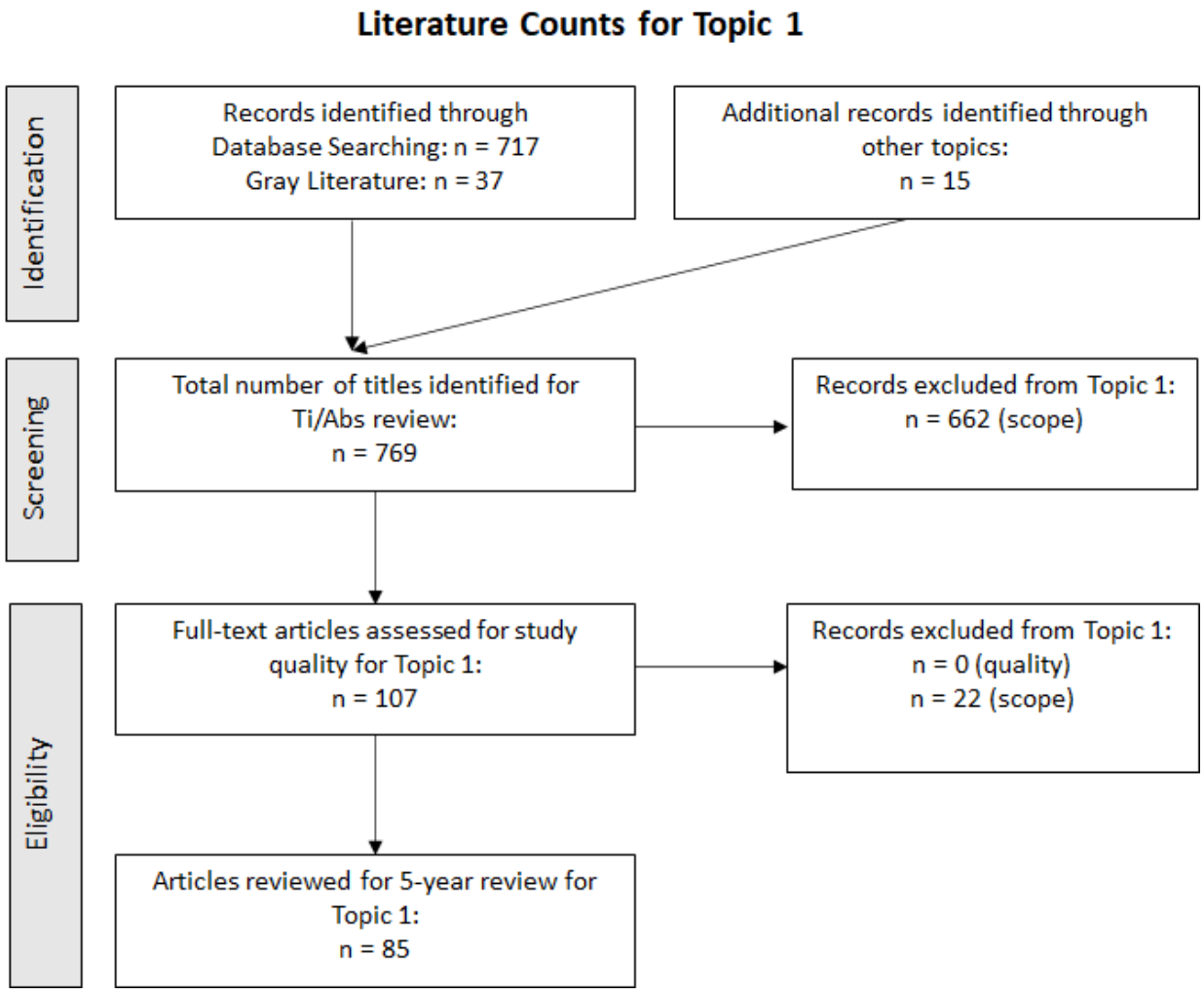
Reporting of Results:

- Acceptable – Raw data are included (check supplemental materials) or summary results are clearly described, such as geometric mean (GM), arithmetic mean, standard deviation (SD), standard error (SE), median, and other percentiles. Frequency of detection is reported. Variability and uncertainty are discussed/captured in summary statistics.
- Unacceptable – Samples described in the methods are not reported in the results or vice versa. Unclear whether summary data include non-detects (i.e., an analytical sample where the concentration is deemed to be lower than could be detected using the method employed by the laboratory).

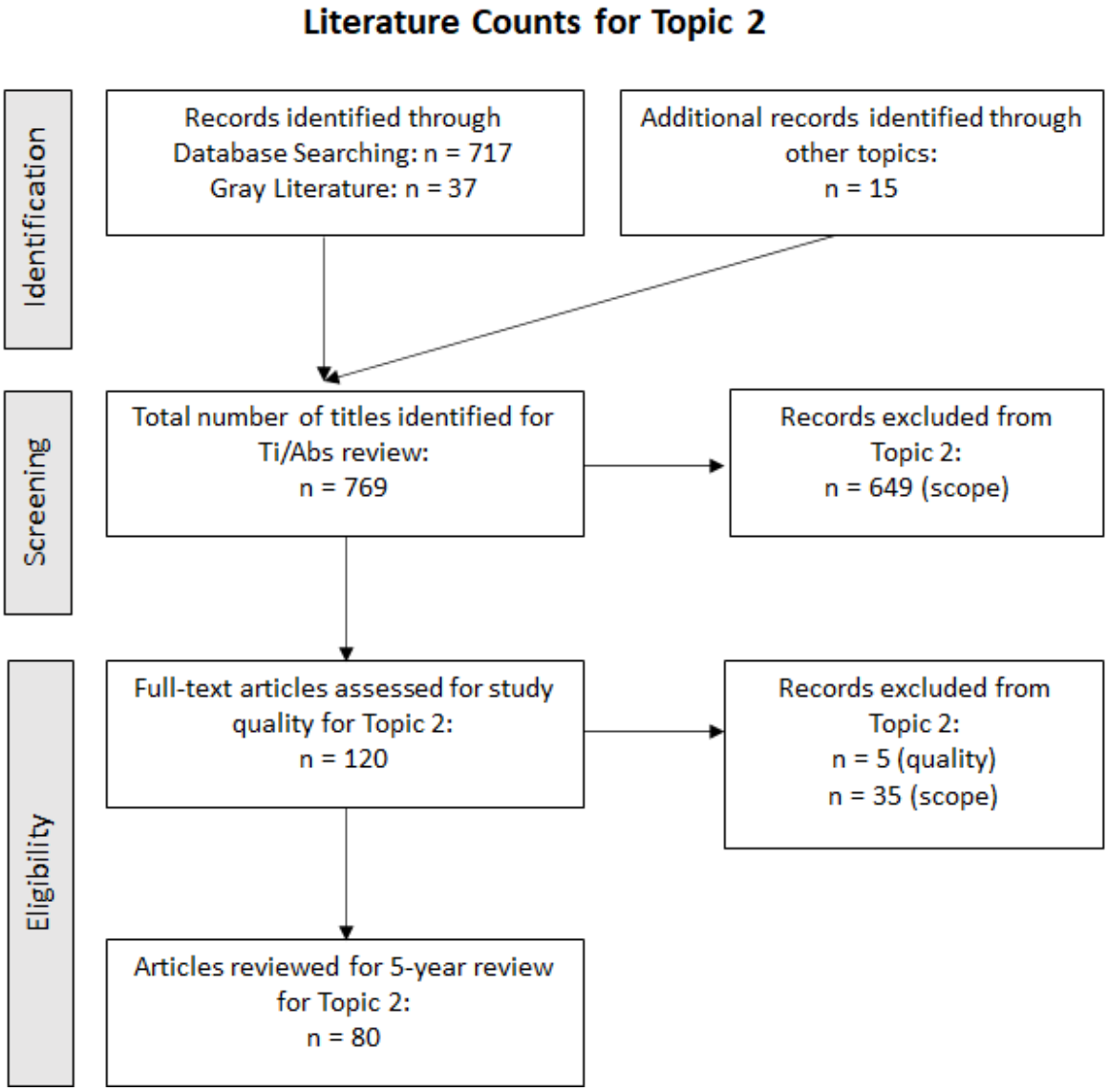
Quality Assurance:

- Acceptable – QA is discussed or can be implied given discussion of controls and recoveries.
- Unacceptable – Not enough information is included to evaluate whether QA was an issue, or QA issues have been identified that interfere with the reliability of the study, or the study is a non-peer-reviewed publication.

Topic 1: PRISMA Diagram



Topic 2: PRISMA Diagram



3. Topic 3: Quantitative Microbial Risk Assessment (QMRA) and Epidemiological Studies

This topic focuses on advances estimating human health risks from exposure to fecal contamination using QMRA and findings from epidemiological studies in measuring indicators of fecal contamination except bacteriophages (e.g., coliform bacteria, fecal streptococci, anaerobic bacteria, pathogens) in recreational waters. The search will also determine if epidemiological studies and QMRA measured for exposure and health effects endpoints, as well as for indicators of fecal contamination, MST, or pathogens. This systematic literature review will be conducted from January 2016 to November 22, 2021.

Focus Areas:

- Advances in risk characterization
- Parameter selection for exposure and health outcomes assessments
- Approaches used to describe microbial distributions
- Approaches used to characterize the source(s) of pathogens or indicators e.g., MST

Research Question: What advances have been made in estimating human health risks from exposure to fecal contamination in recreational waters using QMRA and epidemiological studies?

Literature Search Strategy for Topic 3: Quantitative Microbial Risk Assessment (QMRA) and Epidemiological Studies

The searches are limited the language to English only.

Databases for searches include: PubMed (terms searched in All Fields) and Web of Science (terms searched in Topic)

Dates: January 2016 to November 22, 2021 [present]

Keywords were tested to develop the strategies.

Topic 3: Quantitative Microbial Risk Assessment (QMRA) and Epidemiological Studies Keyword Test Summary

Search Date	Date Limit	Search Strategy Sets	Number of Title/Abstracts		
			PubMed	WoS	Unique Total
November 22, 2021	January 2016–search date	Organism AND Source AND Water AND Detail	777	976	1293

Topic 3 Database Search: PubMed

Date of Search: November 22, 2021 (range January 2016 through November 22, 2021)
Fields Searched: All Fields
Limits: English Only; January 1, 2016, to November 22, 2021

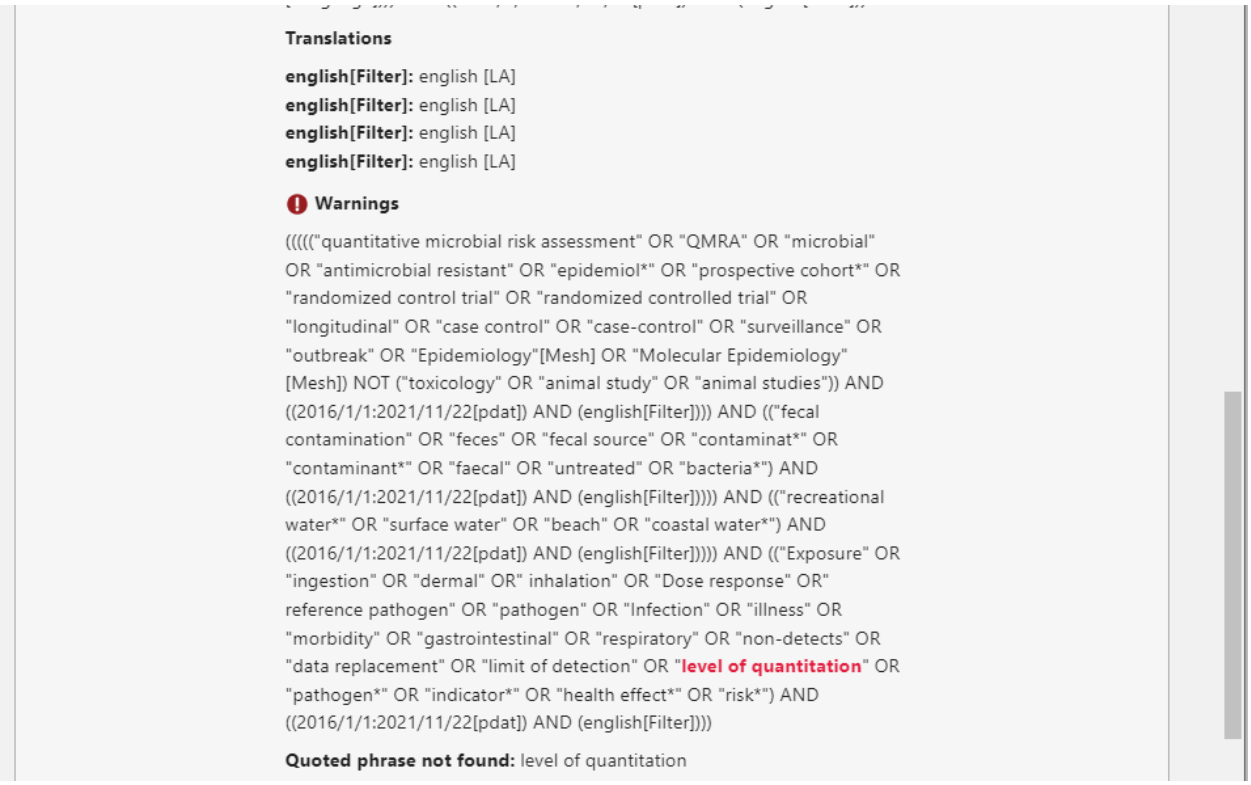
Set Name	Search Strategy for PubMed	Results (Number of Titles)
Organism	((“quantitative microbial risk assessment” OR “QMRA” OR “microbial” OR “antimicrobial resistant” OR “epidemiol*” OR “prospective cohort*” OR “randomized control trial” OR “randomized controlled trial” OR “longitudinal” OR “case control” OR “case-control” OR “surveillance” OR “outbreak” OR “Epidemiology”[Mesh] OR “Molecular Epidemiology”[Mesh]) NOT (“toxicology” OR “animal study” OR “animal studies”))	1,296,600
Source	(“fecal contamination” OR “feces” OR “fecal source” OR “contaminat*” OR “contaminant*” OR “faecal” OR “untreated” OR “bacteria*”)	492,062
Water	(“recreational water*” OR “surface water” OR “beach” OR “coastal water*”)	20,643
Detail	(“Exposure” OR “ingestion” OR “dermal” OR” inhalation” OR “Dose response” OR” reference pathogen” OR “pathogen” OR “Infection” OR “illness” OR “morbidity” OR “gastrointestinal” OR “respiratory” OR “non-detects” OR “data replacement” OR “limit of detection” OR “level of quantitation” OR “pathogen*” OR “indicator*” OR “health effect*” OR “risk*”) *Quoted phrase not found: “level of quantitation”*	2,435,377
Total	**(Organism AND Source AND Water AND Detail)**	**777**

Screenshot of Topic 3 PubMed Search

#12 ••• Search: **(((((("quantitative microbial risk assessment" OR "QMRA" OR "microbial" OR "antimicrobial resistant" OR "epidemiol*" OR "prospective cohort*" OR "randomized control trial" OR "randomized controlled trial" OR "longitudinal" OR "case control" OR "case-control" OR "surveillance" OR "outbreak" OR "Epidemiology"[Mesh] OR "Molecular Epidemiology"[Mesh]) NOT ("toxicology" OR "animal study" OR "animal studies")) AND ((2016/1/1:2021/11/22[pdat]) AND (english[Filter]))) AND (("fecal contamination" OR "feces" OR "fecal source" OR "contaminat*" OR "contaminant*" OR "faecal" OR "untreated" OR "bacteria*") AND ((2016/1/1:2021/11/22[pdat]) AND (english[Filter])))) AND (("recreational water*" OR "surface water" OR "beach" OR "coastal water*") AND ((2016/1/1:2021/11/22[pdat]) AND (english[Filter])))) AND ((("Exposure" OR "ingestion" OR "dermal" OR" inhalation" OR "Dose response" OR" reference pathogen" OR "pathogen" OR "Infection" OR "illness" OR "morbidity" OR "gastrointestinal" OR "respiratory" OR "non-detects" OR "data replacement" OR "limit of detection" OR "level of quantitation" OR "pathogen*" OR "indicator*" OR "health effect*" OR "risk*") AND ((2016/1/1:2021/11/22[pdat]) AND (english[Filter]))) Filters: **English, from 2016/1/1 - 2021/11/22** Sort by: **Publication Date** 777 10:32:16

((("quantitative microbial risk assessment"[All Fields] OR "QMRA"[All Fields] OR "microbial"[All Fields] OR "antimicrobial resistant"[All Fields] OR "epidemiol*"[All Fields] OR "prospective cohort*"[All Fields] OR "randomized control trial"[All Fields] OR "randomized controlled trial"[All Fields] OR "longitudinal"[All Fields] OR "case-control"[All Fields] OR "case-control"[All Fields] OR "surveillance"[All Fields] OR "outbreak"[All Fields] OR "Epidemiology"[MeSH Terms] OR "Molecular Epidemiology"[MeSH Terms]) NOT ("toxicology"[All Fields] OR "animal study"[All Fields] OR "animal studies"[All Fields])) AND (2016/01/01:2021/11/22[Date - Publication] AND "english"[Language]) AND (("fecal contamination"[All Fields] OR "feces"[All Fields] OR "fecal source"[All Fields] OR "contaminat*"[All Fields] OR "contaminant*"[All Fields] OR "faecal"[All Fields] OR "untreated"[All Fields] OR "bacteria*"[All Fields]) AND (2016/01/01:2021/11/22[Date - Publication] AND "english"[Language])) AND (("recreational water*"[All Fields] OR "surface water"[All Fields] OR "beach"[All Fields] OR "coastal water*"[All Fields]) AND (2016/01/01:2021/11/22[Date - Publication] AND "english"[Language])) AND ((("Exposure"[All Fields] OR "ingestion"[All Fields] OR "dermal"[All Fields] OR "inhalation"[All Fields] OR "Dose response"[All Fields] OR "reference pathogen"[All Fields] OR "pathogen"[All Fields] OR "Infection"[All Fields] OR "illness"[All Fields] OR "morbidity"[All Fields] OR "gastrointestinal"[All Fields] OR "respiratory"[All Fields] OR "non-detects"[All Fields] OR "data replacement"[All Fields] OR "limit of detection"[All Fields] OR (("level"[All Fields] OR "levels"[All Fields]) AND ("quantitate"[All Fields] OR "quantitated"[All Fields] OR "quantitates"[All Fields] OR "quantitating"[All Fields] OR "quantitation"[All Fields] OR "quantitations"[All Fields] OR "quantitative"[All Fields] OR "quantitatively"[All Fields] OR "quantitativeness"[All Fields] OR "quantitatives"[All Fields] OR "quantitive"[All Fields] OR "quantitively"[All Fields])) OR "pathogen*"[All Fields] OR "indicator*"[All Fields] OR "health effect*"[All Fields] OR "risk*"[All Fields]) AND (2016/01/01:2021/11/22[Date - Publication] AND "english"[Language]))) AND ((2016/1/1:2021/11/22[pdat]) AND (english[Filter]))

Translations



Translations

english[Filter]: english [LA]
english[Filter]: english [LA]
english[Filter]: english [LA]
english[Filter]: english [LA]

Warnings

(((((("quantitative microbial risk assessment" OR "QMRA" OR "microbial" OR "antimicrobial resistant" OR "epidemiol*" OR "prospective cohort*" OR "randomized control trial" OR "randomized controlled trial" OR "longitudinal" OR "case control" OR "case-control" OR "surveillance" OR "outbreak" OR "Epidemiology"[Mesh] OR "Molecular Epidemiology" [Mesh]) NOT ("toxicology" OR "animal study" OR "animal studies")) AND ((2016/1/1:2021/11/22[pdat]) AND (english[Filter]))) AND (("fecal contamination" OR "feces" OR "fecal source" OR "contaminat*" OR "contaminant*" OR "faecal" OR "untreated" OR "bacteria*") AND ((2016/1/1:2021/11/22[pdat]) AND (english[Filter])))) AND (("recreational water*" OR "surface water" OR "beach" OR "coastal water*") AND ((2016/1/1:2021/11/22[pdat]) AND (english[Filter])))) AND (("Exposure" OR "ingestion" OR "dermal" OR" inhalation" OR "Dose response" OR" reference pathogen" OR "pathogen" OR "Infection" OR "illness" OR "morbidity" OR "gastrointestinal" OR "respiratory" OR "non-detects" OR "data replacement" OR "limit of detection" OR "**level of quantitation**" OR "pathogen*" OR "indicator*" OR "health effect*" OR "risk*") AND ((2016/1/1:2021/11/22[pdat]) AND (english[Filter])))

Quoted phrase not found: level of quantitation

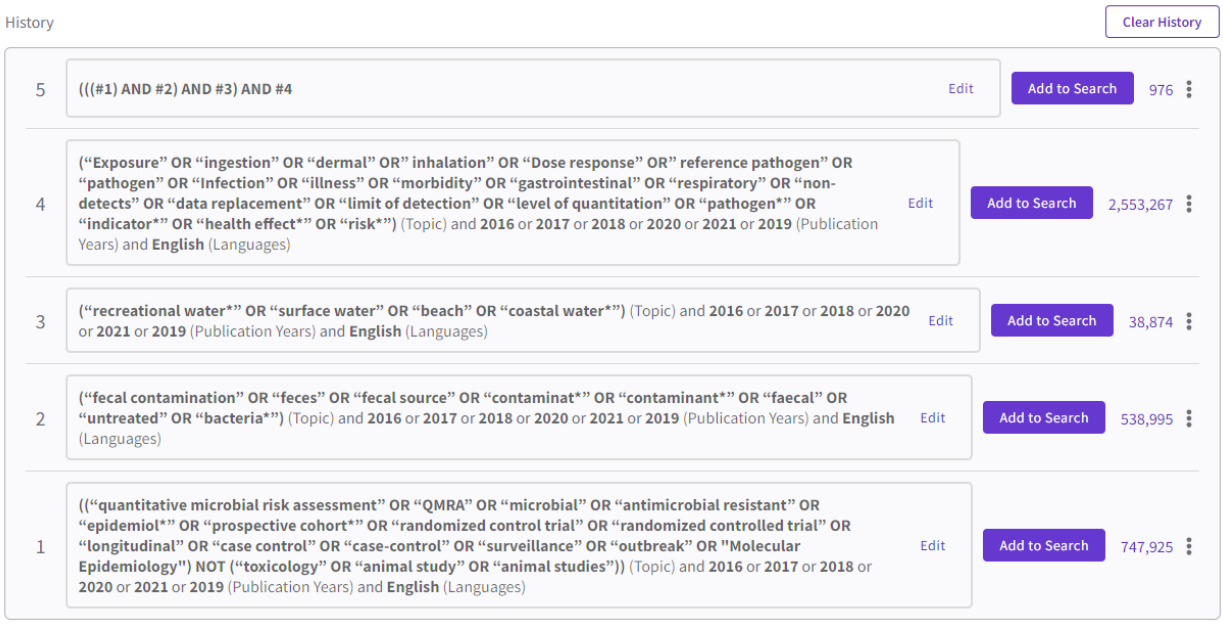
Topic 3 Database Search: Web of Science

Date of Search: November 22, 2021 (range 2016 through 2021)
Fields Searched: Topic
Limits: English Only; 2016 to 2021
Search run via Google Scraper Tool

Set Name	Search Strategy for Web of Science	Results (Number of Titles)
Organism	((“quantitative microbial risk assessment” OR “QMRA” OR “microbial” OR “antimicrobial resistant” OR “epidemiol*” OR “prospective cohort*” OR “randomized control trial” OR “randomized controlled trial” OR “longitudinal” OR “case control” OR “case-control” OR “surveillance” OR “outbreak” OR “Molecular Epidemiology”) NOT (“toxicology” OR “animal study” OR “animal studies”))	747,925
Source	(“fecal contamination” OR “feces” OR “fecal source” OR “contaminat*” OR “contaminant*” OR “faecal” OR “untreated” OR “bacteria*”)	538,995
Water	(“recreational water*” OR “surface water” OR “beach” OR “coastal water*”)	38,874

Set Name	Search Strategy for Web of Science	Results (Number of Titles)
Detail	(“Exposure” OR “ingestion” OR “dermal” OR” inhalation” OR “Dose response” OR” reference pathogen” OR “pathogen” OR “Infection” OR “illness” OR “morbidity” OR “gastrointestinal” OR “respiratory” OR “non-detects” OR “data replacement” OR “limit of detection” OR “level of quantitation” OR “pathogen*” OR “indicator*” OR “health effect*” OR “risk*”)	2,553,267
Total	**(Organism AND Source AND Water AND Detail)**	**976**

Screenshot of Web of Science Topic 3 Search



Topic 3 Gray Literature Search

Date of Search: November 19, 2021

Limits: 2016 to 2021; PDFs only

NOTE: Google uses strict character limits. Strings containing more than 32 words are not allowed.

Organization	URL	Keywords /Search string	Results (Duplicates Removed)
FDA	FDA.gov	site:fda.gov AND (“risk assessment” OR “QMRA” OR “epidemiol*” OR “cohort” “longitudinal” OR “outbreak”) AND (Feces OR fecal) AND (“recreational water”) AND (“Exposure” OR “ingestion” OR “dermal” OR ”inhalation” OR “Dose response” OR “Infection” OR “illness” OR “morbidity” OR “gastrointestinal” OR “respiratory” OR “detection” OR “pathogen*” OR “indicator*” OR “health effect”) NOT (“toxicology”)	52
NPS	NPS.gov	site:nps.gov AND (“risk assessment” OR “QMRA” OR “epidemiol*” OR “cohort” “longitudinal” OR “outbreak”) AND (Feces OR fecal) AND (“recreational water”) AND (“Exposure” OR “ingestion” OR “dermal” OR ”inhalation” OR “Dose response” OR “Infection” OR “illness” OR “morbidity” OR “gastrointestinal” OR “respiratory” OR “detection” OR “pathogen*” OR “indicator*” OR “health effect”) NOT (“toxicology”)	2
FWS	Fws.gov	site:fws.gov AND (“risk assessment” OR “QMRA” OR “epidemiol*” OR “cohort” “longitudinal” OR “outbreak”) AND (Feces OR fecal) AND (“recreational water”) AND (“Exposure” OR “ingestion” OR “dermal” OR ”inhalation” OR “Dose response” OR “Infection” OR “illness” OR “morbidity” OR “gastrointestinal” OR “respiratory” OR “detection” OR “pathogen*” OR “indicator*” OR “health effect”) NOT (“toxicology”)	1
NAS	Nap.edu	Site:nap.edu AND (“risk assessment” OR “QMRA” OR “epidemiol*” OR “cohort” “longitudinal” OR “outbreak”) AND (Feces OR fecal) AND (“recreational water”) AND (“Exposure” OR “ingestion” OR “dermal” OR ”inhalation” OR “Dose response” OR “Infection” OR “illness” OR “morbidity” OR “gastrointestinal” OR “respiratory” OR “detection” OR “pathogen*” OR “indicator*” OR “health effect”) NOT (“toxicology”)	0
WHO	Who.int	Site:who.int AND (“risk assessment” OR “QMRA” OR “epidemiol*” OR “cohort” “longitudinal” OR “outbreak”) AND (Feces OR fecal) AND (“recreational water”) AND	6

Organization	URL	Keywords /Search string	Results (Duplicates Removed)
		(“Exposure” OR “ingestion” OR “dermal” OR ”inhalation” OR “Dose response” OR “Infection” OR “illness” OR “morbidity” OR “gastrointestinal” OR “respiratory” OR “detection” OR “pathogen*” OR “indicator*” OR “health effect”) NOT (“toxicology”)	
Total	**All domains**	**Same search in each domain**	**61**

Topic 3 Prioritization of Ti/Abs for Screening

Using prioritization tools built into litstream were used to evaluate the Ti/Abs that were returned from the above data searches.

Supervised clustering litstream tools were used to predict whether the Ti/Abs are of interest based on six different algorithms. Seed papers feed into the prediction algorithm.

Seed papers for Topic 3 included:

1. Abdelzaher, A.M., Wright, M.E., Ortega, C., Rasem Hasan, A., Shibata, T., Solo-Gabrieie, H.-M., Kish, J., Withum, K., He, G., Elmir, S.M., Bonilla, J.A., Bonilla, T.D., Palmer, C.J., Scott, T.M., Lukasik, J., Harwood, V.J., McQuaig, S., Sinigalliano, C.D., Gidley, M.L., Wanless, D., Plano, L.R., Garza, A.C., Zhu, X., Stewart, J.R., Dickerson, J.W., Yampara-Iquise, H., Carson, C., Fleisher, J.M., Fleming, L.E. 2011. Daily measures of microbes and human health at a non-point source marine beach. Journal of Water and Health, 9(3): 443-457.
2. Ashbolt, N.J., Bruno, M. 2003. Application and refinement of the WHO risk framework for recreational waters in Sydney Australia. Journal of Water and Health, 1(3): 125-131.
3. Ashbolt, N.J., Schoen, M.E., Soller, J.A., Roser, D.J. 2010. Predicting pathogen risks to aid beach management: The real value of quantitative microbial risk assessment (QMRA). Water Research, 44(16): 4692-4703.
4. Boehm, A.B., Soller, J.A., Shanks, O.C. 2015. Human-associated fecal quantitative polymerase chain reaction measurements and simulated risk of gastrointestinal illness in recreational waters contaminated with raw sewage. Environmental Science & Technology Letters, 2(10): 270-275.
5. Boehm, A.B., Graham, K.E., Jennings, W.C. 2018. Can We Swim Yet? Systematic Review, Meta-Analysis, and Risk Assessment of Aging Sewage in Surface Waters. Environ Sci Technol. 52(17):9634-9645.
6. Colford, J., Wade, T., Sandhu, S., Wright, C., Lee, S., Shaw, S., Fox, K., Burns, S., Benker, A., Brookhart, M., van der Laan, M., Levy, D. 2005. A randomized control trial of in-home drinking water intervention to reduce gastrointestinal illness. American Journal of Epidemiology 161(5): 472-82.

7. Colford, J.M., Jr., Wade, T.J., Schiff, K.C., Wright, C.C., Griffith, J.F., Sandhu, S.K., Burns, S., Sobsey, M., Lovelace, G., Weisberg, S.B., 2007. Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination. Epidemiology 18(1): 27-35.
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The ‘ensemble score’ is the number of algorithms that predicted the Ti/Abs would be relevant. The ‘count of ensemble score’ is the number of Ti/Abs that got each ensemble score. The ‘# of seeds’ is the number of the seed articles that got each ensemble score. Ti/Abs that received a score of 1 to 6 were included in the screening. Ti/Abs that received a score of 0 were not screened.

Ensemble Score	Count of Ensemble_Score	# of Seeds
0	1131	
1	77	1
2	39	3
3	33	5
4	30	14
5	15	11
6	5	3
Grand Total	**1330**	**37**

Topic 3 Title/Abstract Screening Criteria and Tagging

Two separate individuals screened and applied the following tags to each title/abstract. In cases where screener 1 and 2 disagreed a subject matter expert provided conflict resolution and determined the final tagging result. Each title/abstract was tagged as follows:

Screening Tag	Description of Tag
Top Level Tags – choose one [include, unclear, or exclude]:	
Include (Topic 3 QMRA/epi)	Obtain full text, advance to full text screening • Advances in QMRA and epidemiological studies in analyzing indicators of fecal contamination (specifically: coliform bacteria, fecal streptococci, anaerobic bacteria) in recreational waters. • Do not include if only bacteriophages. • **In Scope:** ▪ QMRA (or MRA) modeling infectious disease risk in ambient surface water samples. Or QMRAs that link risk to fecal indicators. Epidemiological studies conducted on recreational exposures to surface waters. Can be primary (e.g., swimming) or secondary (e.g., boating) contact exposure. ▪ Papers that advance specific parameters for QMRA might also be of interest. Use your judgment. The authors should talk about how the paper fits into QMRA. ▪ Review Articles about QMRA or epi in rec waters are also OK. • **NOT in Scope:** ▪ Studies for other water types: drinking water, ground water, wastewater. ▪ Studies prior to 2016 (2016 and earlier) are not in scope.
Unclear	Requires full text to determine whether within scope. Unclear if applicable to recreational waters, for example, drinking water, groundwater, wastewater studies. OK to include "other possible tags."
Exclude	Do not obtain full text. Not within scope. OK to include notes. OK to include "other possible tags."
If "Include" this tag might also apply [this tag only applies if 'include']:	
Priority or Review (for Topic 3)	• Articles that provide a review of one of the major topics will be prioritized. Other papers that seem particularly of interest and should be prioritized can also have this tag. Use judgment with this tag. Not all review articles need to have this tag – just the review articles that are particularly on point

Screening Tag	Description of Tag
Other Possible Tags – choose all that apply [Ti/Abs indicates the paper may contain information on]:	
MST (Topic 1)	• Advances in method standardization related to MST. • Development of standard control or reference materials for MST. • Occurrence of amplification inhibition and/or matrix interference for methods used for MST. • Field studies to identify and/or remediate fecal sources. • Risk assessment with MST genetic markers (epidemiological studies, QMRA).
Molecular Methods (Topic 2)	• Advances in the development of qPCR, digital PCR, rt-PCR, next generation sequencing, metagenomic DNA sequencing, and hypervariable 16S RNA gene sequencing used in recreational waters to measure indicators of fecal contamination, pathogen indicators or waterborne pathogens. • Advances in statistics/modeling for data interpretation (fecal score, QMRA, censored data, etc.).
Children's Risk (Topic 4)	• Health effects that children experience following exposure to microbially contaminated surface waters, including illness rates/health outcomes from outbreak reports, epidemiological or other health studies.
Explanation – Free Text Notes Field	• *Optional additional keywords including but not limited to: antimicrobial resistance (AMR), combined sewer overflows (CSO), sanitary sewer overflow (SSO), somatic coliphage, male-specific coliphage (MSC), F-specific coliphage (MSC). • Additional notes about the paper.

Topic 3 Study Quality Evaluation

Articles that were 'include' or 'unclear' moved to full text screening. The full text was reviewed for scope to confirm the article is within the scope. Articles that pass scope were screened for study quality and articles that pass study quality were qualified for data extraction.

Scope

Is the study within the Topic 3 scope? Does it include information on QMRA characterizing risk in fecally contaminated recreational waters or recreational water epidemiological studies including indicators of fecal contamination (e.g., coliform bacteria, fecal streptococci, anaerobic bacteria)?

- Yes – continue with study quality.
- No – stop evaluation. (Choose 'no' if the study is only about bacteriophages/coliphages, does not include a recreational water exposure route [e.g., incidental ingestion, inhalation, dermal].)

Study Quality Metrics – If Any of the Metrics Are Unacceptable, the Study Was Not Included for Data Extraction.

QMRA Documentation:

- Acceptable – The narrative defines the concern driving the risk assessment, the purpose, and objectives of the risk assessment, the scope of analysis, some sort of conceptual model, and analytical approaches are described. Media or matrix is clear. Health endpoint is clear.
- Unacceptable – Not enough information is included to evaluate the QMRA methods or critical problems are identified. Matrix/media is not identified. Health endpoint is not clear.
- NA – Not a QMRA.

QMRA Parameters:

- Acceptable – Parameters are well described. Quantitative parameters include some justification/logic/basis for selection.
- Unacceptable – Numeric parameters are not provided, not adequately referenced or are unclear. Or not enough information is included to evaluate the parameters.
- NA – Not a QMRA.

Epi Documentation:

- Acceptable – Site and conditions at the site are described. Population is described. Study design is described. Odd ratios or other risk metrics are included and clearly presented. Confounding variables, if included for some analyses, are articulated and presented.
- Unacceptable – Unclear or indecipherable methods. Type of epidemiological study design is not included or cannot be evaluated with the information provided.
- NA – Not an epidemiological study.

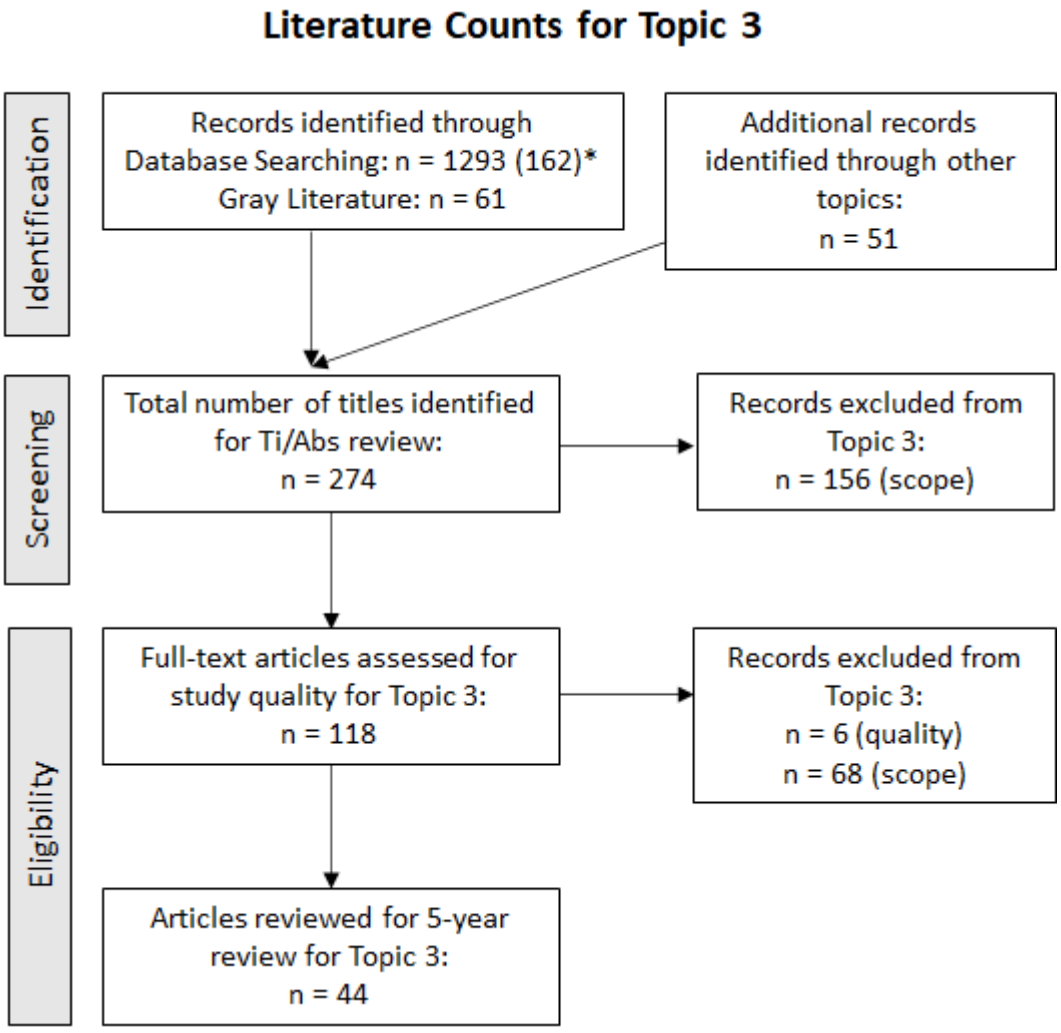
Reporting of Results:

- Acceptable – Raw data are included (check supplemental materials) or summary results are clearly described. Variability and uncertainty are discussed or captured in summary statistics.
- Unacceptable – Reporting of results is unclear, does not match methods (results with no method description or methods with no results reported), or is insufficient to evaluate the study.

Quality Assurance:

- Acceptable – Quality assurance is discussed or can be implied in the discussion. Peer-reviewed or reputable government or institutional gray literature are acceptable.
- Unacceptable – Not enough information is included to evaluate whether QA was an issue, or QA issues have been identified that interfere with the reliability of the study, or the study is a non-peer-reviewed publication.

Topic 3: PRISMA Diagram



*Automated prioritization tools were used to bin Ti/Abs. The most relevant Ti/Abs group was screened.

4. Topic 4: Characterization of Children's Risk from Exposure to Recreational Water Contaminated by Fecal Contamination

This topic specifically focuses on the health effects that children experience following exposure to fecal-contaminated surface waters, including infection or illness rates/health outcomes from outbreak reports, epidemiological or other health studies, exposure behavior studies conducted in swimming pools that may have been performed for children or a comparison between adults and children. The systematic literature review will be conducted from January 2018 to November 8, 2021.

Focus Areas:

- Advances in recreational exposure description for children
- Characterization of children's illness susceptibility
- Observed illness rates in children upon exposure

Research Question: What advances have been made in characterizing the differential susceptibility between children and adults and how exposure behaviors (contact, inhalation, and ingestion patterns) to waterborne fecal pathogens for children differ from adults?

Literature Search Strategy for Topic 4: Characterization of Children's Risk from Exposure to Recreational Water Contaminated by Fecal Contamination

The searches are limited the language to English only.

Databases for searches include: PubMed (terms searched in All Fields) and Web of Science (terms searched in Topic)

Dates: January 2018 to November 8, 2021 [present]

Keywords were tested to develop the strategies.

Topic 4: Characterization of Children's Risk from Exposure to Recreational Water Contaminated by Fecal Contamination Keyword Test Summary

Search Date	Date Limit	Search Strategy Sets	Number of Title/Abstracts		
			PubMed	WoS	Unique Total
November 8, 2021	January 2018–search date	Organism AND Source AND Water AND Detail AND Population	399	188	484

Topic 4: Database Search: PubMed

Date of Search: November 8, 2021 (range January 2018 through November 8, 2021)
Fields Searched: All Fields
Limits: English Only; January 1, 2018, to November 8, 2021

Set Name	Search Strategy for PubMed	Results (Number of Titles)
Organism	((“quantitative microbial risk assessment” OR “QMRA” OR “epidemiol*” OR “prospective cohort*” OR “randomized control trial” OR “randomized controlled trial” OR “survey*” OR “longitudinal” OR “case control” OR “case-control” OR “surveillance” OR “outbreak” OR “Epidemiology”[Mesh]) NOT (“toxicology” OR “animal study” OR “animal studies”))	975,149
Source	(“fecal contamination” OR “feces” OR “fecal source” OR “contaminated” OR “contaminant*”)	57,458
Water	(“recreational water*” OR “surface water” OR “ambient water” OR “water” OR “beach*”)	253,558
Detail	(“ingestion” OR “inhalation” OR “dermal” OR “ear” OR “eye” OR “behavior” OR “exposure*” OR “recreational exposure*” OR “duration” OR “pathogen*” OR “dose” OR “infection” OR “illness” OR “gastrointestinal illness” OR “respiratory illness” OR “waterborne gastrointestinal illness*” OR “waterborne illness” OR “health burden” OR “outbreak” OR “surveillance” OR “severity” OR “susceptibility” OR “risk” OR “lifestage” OR “life stage” OR “negative health outcome*” OR “adverse health outcome*” OR “health outcome*”)	1,929,700
Population	(“child” OR “Child”[Mesh] OR “children*”)	494,592
Total	**(Organism AND Source AND Water AND Detail AND Population)**	**399**

Screenshot of Topic 4 PubMed Search

History and Search Details

Download Delete

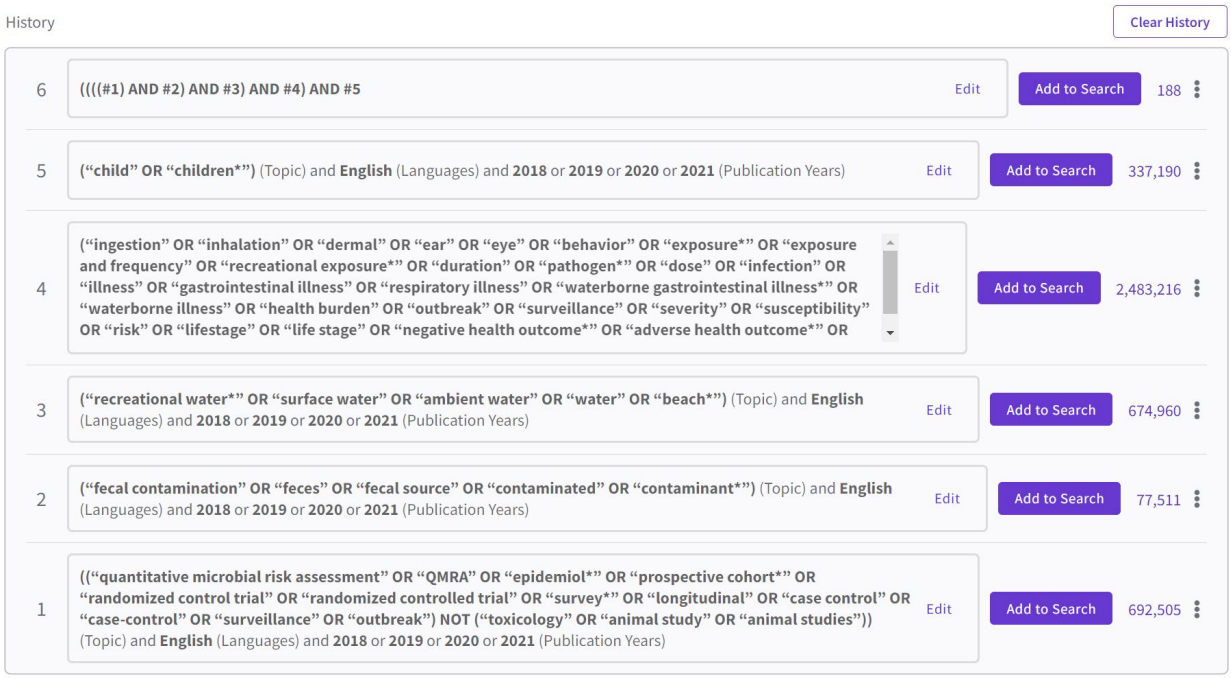
Search	Actions	Details	Query	Results	Time
#95	•••	›	Search: **((((((("quantitative microbial risk assessment" OR "QMRA" OR "epidemiol*" OR "prospective cohort*" OR "randomized control trial" OR "randomized controlled trial" OR "survey*" OR "longitudinal" OR "case control" OR "case-control" OR "surveillance" OR "outbreak" OR "Epidemiology"[Mesh]) NOT ("toxicology" OR "animal study" OR "animal studies")) AND ((2018/1/1:2021/11/8[pdat]) AND (english[Filter]))) AND (("fecal contamination" OR "feces" OR "fecal source" OR "contaminated" OR "contaminant*") AND ((2018/1/1:2021/11/8[pdat]) AND (english[Filter])))) AND (("recreational water*" OR "surface water" OR "ambient water" OR "water" OR "beach*") AND ((2018/1/1:2021/11/8[pdat]) AND (english[Filter])))) AND (("ingestion" OR "inhalation" OR "dermal" OR "ear" OR "eye" OR "behavior" OR "exposure*" OR "recreational exposure*" OR "duration" OR "pathogen*" OR "dose" OR "infection" OR "illness" OR "gastrointestinal illness" OR "respiratory illness" OR "waterborne gastrointestinal illness*" OR "waterborne illness" OR "health burden" OR "outbreak" OR "surveillance" OR "severity" OR "susceptibility" OR "risk" OR "lifestage" OR "life stage" OR "negative health outcome*" OR "adverse health outcome*" OR "health outcome*") AND ((2018/1/1:2021/11/8[pdat]) AND (english[Filter])))) AND (("child" OR "Child"[Mesh] OR "children*") AND ((2018/1/1:2021/11/8[pdat]) AND (english[Filter]))) Filters: English, from 2018/1/1 - 2021/11/8**	399	21:43:57

Topic 4 Database Search: Web of Science

Date of Search: November 8, 2021 (range 2018 through 2021)
Fields Searched: Topic
Limits: English Only; 2018 to 2021

Set Name	Search Strategy for Web of Science	Results (Number of Titles)
Organism	((“quantitative microbial risk assessment” OR “QMRA” OR “epidemiol*” OR “prospective cohort*” OR “randomized control trial” OR “randomized controlled trial” OR “survey*” OR “longitudinal” OR “case control” OR “case-control” OR “surveillance” OR “outbreak”) NOT (“toxicology” OR “animal study” OR “animal studies”))	692,505
Source	(“fecal contamination” OR “feces” OR “fecal source” OR “contaminated” OR “contaminant*”)	77,511
Water	(“recreational water*” OR “surface water” OR “ambient water” OR “water” OR “beach*”)	674,960
Detail	(“ingestion” OR “inhalation” OR “dermal” OR “ear” OR “eye” OR “behavior” OR “exposure*” OR “exposure and frequency” OR “recreational exposure*” OR “duration” OR “pathogen*” OR “dose” OR “infection” OR “illness” OR “gastrointestinal illness” OR “respiratory illness” OR “waterborne gastrointestinal illness*” OR “waterborne illness” OR “health burden” OR “outbreak” OR “surveillance” OR “severity” OR “susceptibility” OR “risk” OR “lifestage” OR “life stage” OR “negative health outcome*” OR “adverse health outcome*” OR “health outcome*”)	2,483,216
Population	(“child” OR “children*”)	337,190
Total	**(Organism AND Source AND Water AND Detail AND Population)**	**188**

Screenshot of Web of Science Topic 4 Search



Topic 4: Gray Literature Search

Date of Search: November 18, 2021
Limits: English Only; 2018 to 2021
Search run via Google Scraper Tool

NOTE: Google uses strict character limits. Strings containing more than 32 words are not allowed.

Organization	URL	Keywords /Search string	Results
FDA	FDA.gov	site:fda.gov AND ("risk assessment" OR "QMRA" OR "epidemiol*" OR "cohort" OR "randomized" OR "longitudinal" OR "control" OR "survey") AND (Feces OR "contaminant") AND ("Water" OR "beach") AND (child*) AND ("ingestion" OR "inhalation" OR "dermal" OR "ear" OR "eye" OR "behavior" OR "exposure*" OR "pathogen*" OR "infection" OR "illness" OR "outbreak" OR "surveillance" OR "susceptibility" OR "risk" OR "health outcome")	7
NPS	Nps.gov	site:nps.gov AND ("risk assessment" OR "QMRA" OR "epidemiol*" OR "cohort" OR "randomized" OR "longitudinal" OR "control" OR "survey") AND (Feces OR "contaminant") AND ("Water" OR "beach") AND (child*) AND ("ingestion" OR "inhalation" OR "dermal" OR "ear" OR "eye" OR "behavior" OR "exposure*" OR "pathogen*" OR "infection" OR "illness" OR "outbreak" OR "surveillance" OR "susceptibility" OR "risk" OR "health outcome")	4
FWS	Fws.gov	site:fws.gov AND ("risk assessment" OR "QMRA" OR "epidemiol*" OR "cohort" OR "randomized" OR "longitudinal" OR "control" OR "survey") AND (Feces OR "contaminant") AND ("Water" OR "beach") AND (child*) AND ("ingestion" OR "inhalation" OR "dermal" OR "ear" OR "eye" OR "behavior" OR "exposure*" OR "pathogen*" OR "infection" OR "illness" OR "outbreak" OR "surveillance" OR "susceptibility" OR "risk" OR "health outcome")	9
NAS	Nap.edu	site:nap.edu AND ("risk assessment" OR "QMRA" OR "epidemiol*" OR "cohort" OR "randomized" OR "longitudinal" OR "control" OR "survey") AND (Feces OR "contaminant") AND ("Water" OR "beach")	0

Organization	URL	Keywords /Search string	Results
		AND (child*) AND ("ingestion" OR "inhalation" OR "dermal" OR "ear" OR "eye" OR "behavior" OR "exposure*" OR "pathogen*" OR "infection" OR "illness" OR "outbreak" OR "surveillance" OR "susceptibility" OR "risk" OR "health outcome")	
WHO	Who.int	site:who.int AND ("risk assessment" OR "QMRA" OR "epidemiol*" OR "cohort" OR "randomized" OR "longitudinal" OR "control" OR "survey") AND (Feces OR "contaminant") AND ("Water" OR "beach") AND (child*) AND ("ingestion" OR "inhalation" OR "dermal" OR "ear" OR "eye" OR "behavior" OR "exposure*" OR "pathogen*" OR "infection" OR "illness" OR "outbreak" OR "surveillance" OR "susceptibility" OR "risk" OR "health outcome")	10
Total	**All domains**	**Same search in each domain**	**30**

Topic 4: Title/Abstract Screening Criteria and Tagging

Two separate individuals screened and applied the following tags to each title/abstract. In cases where screener 1 and 2 disagreed a subject matter expert provided conflict resolution and determined the final tagging result. Each title/abstract was tagged as follows:

Screening Tag	Description of Tag
Top Level Tags – choose one [include, unclear, or exclude]:	
Include (Topic 4 Children's Risk)	Obtain full text, advance to full text screening. • Health effects that children experience following exposure to microbially contaminated surface waters, including illness rates/health outcomes from outbreak reports, epidemiological or other health studies. • **In Scope:** ▪ Papers on children's risks from exposure to microbes in surface waters ▪ Review Articles about children's risk in rec waters are also OK ▪ Case studies, surveys • **NOT in Scope:** ▪ Studies for other water types: drinking water, ground water, wastewater. ▪ Non-microbial risks (drowning, animal related injuries). ▪ Drinking surface water. ▪ Schistosomes/Schistosomiasis (and other waterborne diseases that are not typically found in U.S.).
Unclear	Requires full text to determine whether within scope. Unclear if applicable to recreational waters, for example, drinking water, groundwater, wastewater studies. OK to include "other possible tags."

Screening Tag	Description of Tag
Exclude	Do not obtain full text. Not within scope. OK to include notes. OK to include "other possible tags."
If "Include" this tag might also apply [this tag only applies if 'include']:	
Priority or Review (for Topic 4)	Articles that provide a review of one of the major topics will be prioritized. Other papers that seem particularly of interest and should be prioritized can also have this tag. Use judgment with this tag. Not all review articles need to have this tag – just the review articles that are particularly on point.
Other Possible Tags – choose all that apply [Ti/Abs indicates the paper may contain information on]:	
MST (Topic 1)	• Advances in method standardization related to MST. • Development of standard control or reference materials for MST. • Occurrence of amplification inhibition and/or matrix interference for methods used for MST. • Field studies to identify and/or remediate fecal sources. • Risk assessment with MST genetic markers (epidemiological studies, QMRA).
Molecular Methods (Topic 2)	• Advances in the development of qPCR, digital PCR, rt-PCR, next generation sequencing, metagenomic DNA sequencing, and hypervariable 16S RNA gene sequencing used in recreational waters to measure indicators of fecal contamination, pathogen indicators or waterborne pathogens. • Advances in statistics/modeling for data interpretation (fecal score, QMRA, censored data, etc.).
QMRA or Epi (Topic 3)	• Advances in QMRA and epidemiological studies in analyzing indicators of fecal contamination (specifically: coliform bacteria, fecal streptococci, anaerobic bacteria) in recreational waters. • Do not include if only bacteriophages.
Explanation – Free text notes field	• *Optional additional keywords including but not limited to: antimicrobial resistance (AMR), combined sewer overflows (CSO), sanitary sewer overflow (SSO), somatic coliphage, male-specific coliphage (MSC), F-specific coliphage (MSC). • Additional notes about the paper.

Topic 4: Study Quality Evaluation

Articles that were 'include' or 'unclear' moved to full text screening. The full text was reviewed for scope to confirm the article is within the scope. Articles that pass scope were screened for study quality and articles that pass study quality were qualified for data extraction.

Scope

Is the study within the Topic 5 scope? Does it include information on health effects that children experience following exposure to microbially contaminated surface waters, including illness rates/health outcomes from outbreak reports, epidemiological or other health studies, published risk assessments (e.g., quantitative microbial risk assessments) that may have been performed for children or a comparison between adult and children's risk? Note that studies that have been conducted in ambient waters using ingestion estimates are relevant to this topic.

- Yes – continue with study quality.
- No – stop evaluation.

Study Quality Metrics – If Any of the Metrics Are Unacceptable, the Study Was Not Included for Data Extraction.

Study Documentation:

- Acceptable – The narrative defines the concern driving the study, the purpose, and objectives of the study, and the scope of analysis. Some sort of conceptual model is discussed and analytical approaches are described.
- Unacceptable – Not enough information is included to evaluate the study or critical problems are identified.

Study Parameters:

- Acceptable – Parameters are well described. Quantitative parameters include some justification/logic/basis for selection.
- Unacceptable – Numeric parameters are not provided or are unclear, or not enough information is included to evaluate the parameters.
- NA – not a QMRA.

Epidemiological Documentation:

- Acceptable – Site, conditions at the site, study population, and study design are described. Odd ratios or other risk metrics are included and clearly presented. Confounding variables, if included for some analyses, are articulated and presented.
- Unacceptable – Unclear or indecipherable methods. Type of epidemiological study design is not included or cannot be evaluated with the information provided.
- NA – not an epi study.

Reporting of Results:

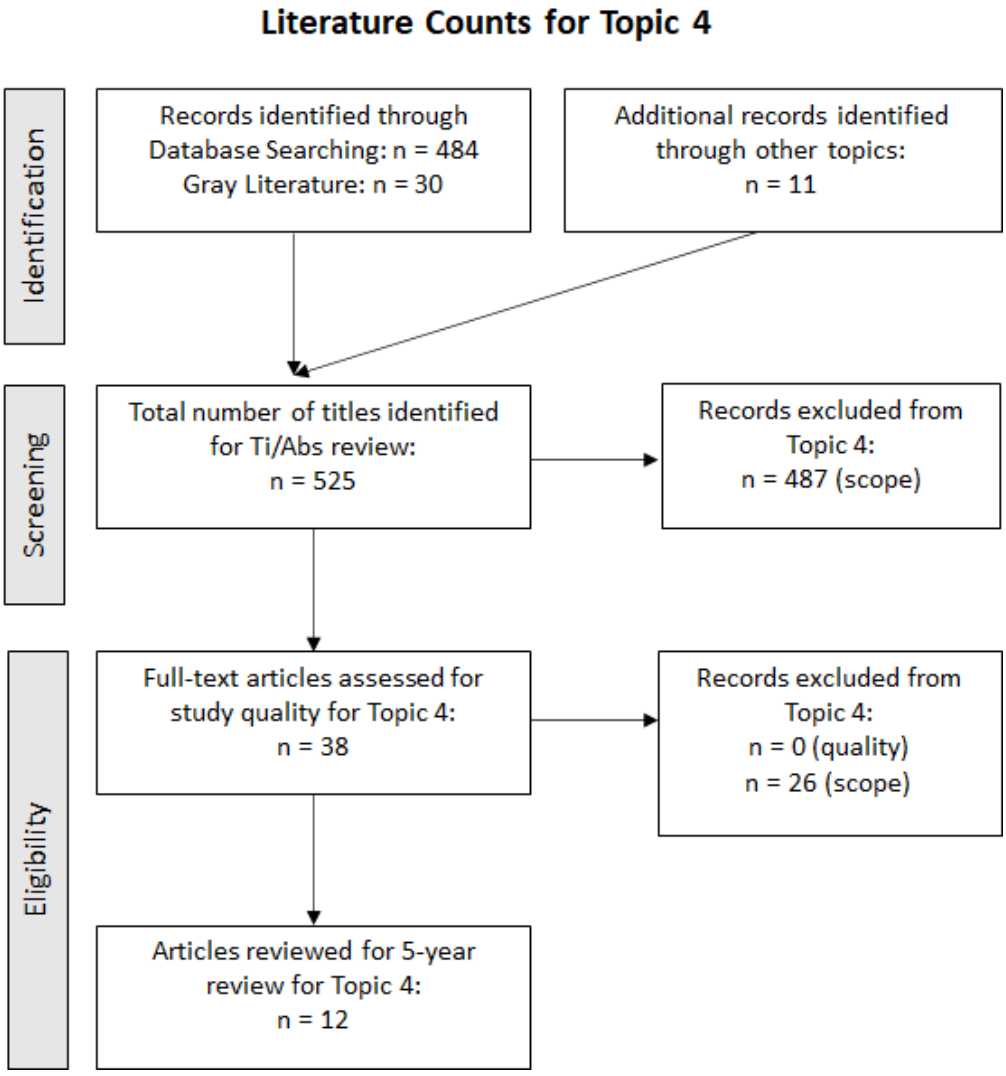
- Acceptable – Raw data are included (check supplemental materials) or summary results are clearly described. Variability and uncertainty are discussed or captured in summary statistics.
- Unacceptable – Reporting of results is unclear, does not match methods (results with no method description or methods with no results reported), or is insufficient to evaluate the study.

Quality Assurance:

- Acceptable – Quality assurance (QA) is discussed or can be implied in the discussion. Peer-reviewed or reputable government or institutional gray literature are acceptable.

- Unacceptable – Not enough information is included to evaluate whether QA was an issue, or QA issues have been identified that interfere with the reliability of the study, or the study is a non-peer-reviewed publication.

Topic 4: PRISMA Diagram



5. Topic 5: Cyanotoxins

This topic is focused on characterizing the human health effects of cyanotoxins from all routes of exposure. Specifically, the cyanotoxins of interest for this topic are anatoxins, BMAA, cylindrospermopsin, microcystins, nodularins, and saxitoxins. The literature searches for anatoxins, microcystins, and cylindrospermopsin built upon work previously conducted by the Office of Water and was conducted from 2014 to April 2021. The literature searches for all other cyanotoxins were conducted with no start date limit to April 2021. Topic 5 literature search was conducted separately from the literatures searches on Topics 1 through 4.

Focus Areas: Human Health, Mechanistic, Toxicokinetic, and Pharmacokinetic (PK)/Physiologically based Pharmacokinetic (PBPK) Models.

Research Question: What epidemiological and toxicological literature is available for anatoxins, BMAA, cylindrospermopsin, microcystins, nodularins, and saxitoxins, that could be informative for derivation of a human health toxicity value? In addition, what supplemental material is available that could help support a human health risk assessment for these cyanotoxins?

Literature Search Strategy for Topic 5: Cyanotoxins

Please note that the original literature search (April 2021) did not separate the searches by cyanotoxin; nor did it date limit searches for microcystin and cylindrospermopsin. Therefore, a second literature search was conducted in April 2022 to replicate the April 2021 literature search but separate the results by cyanotoxin and date limit the microcystin and cylindrospermopsin results. Additionally, a third literature search was conducted to specifically capture anatoxin studies from 2014, as those were outside the date limit set in the initial April 2021 search. Results from all searches are shown below.

Databases for searches include: PubMed (terms searched in All Fields) and Web of Science (terms searched in Topic) and Scopus (terms searched in title, abstract, and keyword fields). After 2021, use of Scopus is no longer supported by HERO.

Dates: Listed for each search

Topic 5 Database Search: PubMed (April 2021)

Date of Search: April 13, 2021

- Anatoxins: January 2015 to present
- BMAA: no limit
- Cylindrospermopsin: no limit
- Microcystins: no limit
- Nodularins: no limit
- Saxitoxins: no limit

Fields Searched: All Fields

Search Strategy for PubMed (April 2021)	Results (Number of Titles)
((“microcystins”[tw] OR “microcystin”[tw]) OR (“microcystin-LA”[tw] OR “96180-79-9”[rn]) OR (“microcystin-LF” OR “154037-70-4”[rn]) OR (“microcystin-LR”[tw] OR “101043-37-2”[rn]) OR (“microcystin-LY” [tw] OR “123304-10-9” [rn]) OR (“microcystin-RR”[tw] OR “111755-37-4”[rn]) OR (“microcystin-YR”[tw] OR “101064-48-6”[rn]) OR “microcystin-LW”[tw] OR “[Asp3]MCRR”[tw] OR “Asp3,Dhb7]MCRR”[tw] OR “MCWR”[tw]) OR ((“nodularins”[tw] OR “nodularin”[tw]) OR (“nodularin-R”[tw] OR “118399-22-7”[rn]) OR “Nodularin-Har”[tw] OR “Nodularin-V (motuporin)”[tw] OR “[D-Asp1]NOD”[tw] OR “[DMAdda3]NOD”[tw] OR “[dhb5]NOD”[tw] OR “[Glu4(Ome)]NOD”[tw] OR “[MeAdda3]NOD”[tw] OR “[(6Z)-Adda3]NOD”[tw]) OR (((“anatoxins”[tw] OR “anatoxin”[tw]) OR (“anatoxin-a”[tw] OR “64285-06-9”[rn]) OR “homoanatoxin-a”[tw] OR “dihydroanatoxin-a”[tw] OR “2,3-epoxy-anatoxin-a”[tw] OR “4-hydroxy-anatoxin-a”[tw] OR “4-oxo-anatoxin-a”[tw] OR “dihydrohomoanatoxin-a”[tw] OR “Anatoxin-a(s)”[tw] OR “(guanitoxin (GNT))”[tw]) AND (2015/01/01 : 2021/04/01[dp])) OR (“Cylindrospermopsin”[tw] OR (“7-epicylindro-spermopsin”[tw] OR “143545-90-8”[rn]) OR “7-deoxycylindrospermopsin”[tw] OR “7-deoxy-desulfo-cylindrospermopsin”[tw] OR “7-deoxy-desulfo-12-acetylcylindrospermopsin”[tw]) OR (“BMAA”[tw] OR “beta-Methylamino-L-alanine)”[tw]) OR ((“saxitoxins”[tw] OR “saxitoxins”[tw]) OR (“stx-dihydrochloride”[tw] OR “35554-08-6”[rn]) OR “Gonyaulax-toxin”[tw] OR “Saxitoxin”[mh]) OR (“Carbamate”[tw] OR “NeoSTX”[tw] OR “GTX1”[tw] OR “GTX2”[tw] OR “GTX3”[tw] OR “GTX4”[tw] OR “GTX5 (B1)”[tw] OR “GTX6 (B2)”[tw]) OR (“Decarbamoyl”[tw] OR “dcSTX”[tw] OR “dcNEOSTX”[tw] OR “dcGTX1”[tw] OR “dcGTX2”[tw] OR “dcGTX3”[tw] OR “dcGTX4”[tw]) OR (“Deoxydecarbamoyl”[tw] OR “doSTX”[tw] OR “doGTX2”[tw] OR “doGTX3”[tw])	15,263

Topic 5 Database Search: PubMed (April 2022)

Date of Search: April 14, 2022

- Anatoxins: January 2015 to April 2021
- BMAA: no limit to April 2021
- Cylindrospermopsin: January 2014 to April 2021
- Microcystins: January 2014 to April 2021
- Nodularins: no limit to April 2021
- Saxitoxins: no limit to April 2021

Fields Searched: All Fields

Cyanotoxin	Search Strategy for PubMed	Results (Number of Titles)
Anatoxins	((“anatoxins”[tw] OR “anatoxin”[tw]) OR (“anatoxin-a”[tw] OR “64285-06-9”[rn]) OR “homoanatoxin-a”[tw] OR “dihydroanatoxin-a”[tw] OR “2,3-epoxy-anatoxin-a”[tw] OR “4-hydroxy-anatoxin-a”[tw] OR “4-oxo-anatoxin-a”[tw] OR “dihydrohomoanatoxin-a”[tw] OR “Anatoxin-a(s)”[tw] OR “(guanitoxin (GNT))”) AND (2015/01/01 : 2021/04/13[dp])	117

Cyanotoxin	Search Strategy for PubMed	Results (Number of Titles)
BMAA	("BMAA"[tw] OR "beta-Methylamino-L-alanine)"[tw]) AND (1800/01/01:2021/04/13[dp])	459
Cylindrospermopsin (Date Limited)	("Cylindrospermopsin"[tw] OR ("7-epicylindro-spermopsin"[tw] OR "143545-90-8"[rn]) OR "7-deoxycylindrospermopsin"[tw] OR "7-deoxy-desulfo-cylindrospermopsin"[tw] OR "7-deoxy-desulfo-12-acetylcylindrospermopsin"[tw]) AND (2014/01/01:2021/04/13[dp])	278
Microcystins (Date Limited)	("microcystins"[tw] OR "microcystin"[tw]) OR ("microcystin-LA"[tw] OR "96180-79-9"[rn]) OR ("microcystin-LF" OR "154037-70-4"[rn]) OR ("microcystin-LR"[tw] OR "101043-37-2"[rn]) OR ("microcystin-LY" [tw] OR "123304-10-9" [rn]) OR ("microcystin-RR"[tw] OR "111755-37-4"[rn]) OR ("microcystin-YR"[tw] OR "101064-48-6"[rn]) OR "microcystin-LW"[tw] OR "[Asp3]MCRR"[tw] OR "Asp3,Dhb7]MCRR"[tw] OR "MCWR"[tw]) AND (2014/01/01:2021/04/13[dp])	2,076
Nodularins	(("nodularin-R"[tw] OR "118399-22-7"[rn]) OR "Nodularin-Har"[tw] OR "Nodularin-V (motuporin)"[tw] OR "[D-Asp1]NOD"[tw] OR "[DMAdda3]NOD"[tw] OR "[dhb5]NOD"[tw] OR "[Glu4(Ome)]NOD"[tw] OR "[MeAdda3]NOD"[tw] OR "[(6Z)-Adda3]NOD"[tw]) AND (1800/01/01:2021/04/13[dp])	259
Saxitoxins	(("saxitoxins"[tw] OR "saxitoxins"[tw]) OR ("stx-dihydrochloride"[tw] OR "35554-08-6"[rn]) OR "Gonyaulax-toxin"[tw] OR "Saxitoxin"[mh]) OR ("Carbamate"[tw] OR "NeoSTX"[tw] OR "GTX1"[tw] OR "GTX2"[tw] OR "GTX3"[tw] OR "GTX4"[tw] OR "GTX5 (B1)"[tw] OR "GTX6 (B2)"[tw]) OR ("Decarbamoyl"[tw] OR "dcSTX"[tw] OR "dcNEOSTX"[tw] OR "dcGTX1"[tw] OR "dcGTX2"[tw] OR "dcGTX3"[tw] OR "dcGTX4"[tw]) OR ("Deoxydecarbamoyl"[tw] OR "doSTX"[tw] OR "doGTX2"[tw] OR "doGTX3"[tw]) AND (1800/01/01:2021/04/13[dp])	9,887
Total Unique References		**12,692**

Topic 5 Database Search: Web of Science (April 2021)

Date of Search: April 13, 2021

- Anatoxins: January 2015 to present
- BMAA: no limit
- Cylindrospermopsin: no limit
- Microcystins: no limit
- Nodularins: no limit
- Saxitoxins: no limit

Fields Searched: Topic

Search Strategy for Web of Science	Results (Number of Titles)
((TS=”microcystins”OR TS=”microcystin”) OR (TS=”microcystin-LA” OR TS=”96180-79-9”) OR (TS=”microcystin-LF” OR TS=”154037-70-4”) OR (TS=”microcystin-LR” OR TS=”101043-37-2”) OR (TS=”microcystin-LY” OR TS=”123304-10-9”) OR (TS=”microcystin-RR” OR TS=”111755-37-4”) OR (TS=”microcystin-YR” OR TS=”101064-48-6”) OR TS=”microcystin-LW” OR TS=”[Asp3]MCRR” OR TS=”Asp3,Dhb7]MCRR” OR TS=”MCWR”) OR ((TS=”nodularins” OR TS=”nodularin”) OR (TS=”nodularin-R” OR TS=”118399-22-7”) OR TS=”Nodularin-Har” OR TS=”Nodularin-V (motuporin)” OR TS=”[D-Asp1]NOD” OR TS=”[DMAdda3]NOD” OR TS=”[dhb5]NOD” OR TS=”[Glu4(Ome)]NOD” OR TS=”[MeAdda3]NOD” OR TS=”[(6Z)-Adda3]NOD”) OR (((TS=”anatoxins” OR TS=”anatoxin”) OR (TS=”anatoxin-a” OR TS=”64285-06-9”) OR TS=”homoanatoxin-a” OR TS=”dihydroanatoxin-a” OR TS=”2,3-epoxy-anatoxin-a” OR TS=”4-hydroxy-anatoxin-a” OR TS=”4-oxo-anatoxin-a” OR TS=”dihydrohomoanatoxin-a” OR TS=”Anatoxin-a(s)” OR TS=”(guanitoxin (GNT))”) AND (PY= 2015-2021)) OR (TS=”Cylindrospermopsin” OR (TS=”7-epicylindro-spermopsin” OR TS=”143545-90-8”) OR TS=”7-deoxycylindrospermopsin” OR TS=”7-deoxy-desulfo-cylindrospermopsin” OR TS=”7-deoxy-desulfo-12-acetylcylindrospermopsin”) OR (TS=”BMAA” OR TS=”(beta-Methylamino-L-alanine)”) OR ((TS=”saxitoxin” OR TS=”saxitoxins”) OR (TS=”stx-dihydrochloride” OR TS=”35554-08-6”) OR TS=”Gonyaulax-toxin”) OR (TS=”Carbamate” OR TS=”NeoSTX” OR TS=”GTX1” OR TS=”GTX2” OR TS=”GTX3” OR TS=”GTX4” OR TS=”GTX5 (B1)” OR TS=”GTX6 (B2)”) OR (TS=”N-sulfocarbonyl”) OR (TS=”Decarbamoyl” OR TS=”dcSTX” OR TS=”dcNEOSTX” OR TS=”dcGTX1” OR TS=”dcGTX2” OR TS=”dcGTX3” OR TS=”dcGTX4”) OR (TS=”Deoxydecarbamoyl” OR TS=”doSTX” OR TS=”doGTX2” OR TS=”doGTX3”)	26,098

Topic 5 Database Search: Web of Science (April 2022)

Date of Search: April 14, 2022

- Anatoxins: January 2015 to April 2021
- BMAA: no limit to April 2021
- Cylindrospermopsin: January 2014 to April 2021
- Microcystins: January 2014 to April 2021
- Nodularins: no limit to April 2021
- Saxitoxins: no limit to April 2021

Fields Searched: Topic

Cyanotoxin	Search Strategy for Web of Science	Results (Number of Titles)
Anatoxins	((TS=”anatoxins” OR TS=”anatoxin”) OR (TS=”anatoxin-a” OR TS=”64285-06-9”) OR TS=”homoanatoxin-a” OR TS=”dihydroanatoxin-a” OR TS=”2,3-epoxy-anatoxin-a” OR TS=”4-hydroxy-anatoxin-a” OR TS=”4-oxo-anatoxin-a” OR TS=”dihydrohomoanatoxin-a” OR TS=”Anatoxin-a(s)” OR TS=”(guanitoxin (GNT))”) AND (DOP=2015-01-01/2021-04-13)	327
BMAA	(TS=”BMAA” OR TS=”(beta-Methylamino-L-alanine)”) AND (DOP=1800-01-01/2021-04-13)	562
Cylindrospermopsin	(TS=”Cylindrospermopsin” OR (TS=”7-epicylindro-spermopsin” OR TS=”143545-90-8”) OR TS=”7-deoxycylindrospermopsin” OR TS=”7-deoxy-desulfo-cylindrospermopsin” OR TS=”7-deoxy-desulfo-12-acetylcylindrospermopsin”) AND (DOP=2014-01-01/2021-04-13)	465
Microcystins	((TS=”microcystins”OR TS=”microcystin”) OR (TS=”microcystin-LA” OR TS=”96180-79-9”) OR (TS=”microcystin-LF” OR TS=”154037-70-4”) OR (TS=”microcystin-LR” OR TS=”101043-37-2”) OR (TS=”microcystin-LY” OR TS=”123304-10-9”) OR (TS=”microcystin-RR” OR TS=”111755-37-4”) OR (TS=”microcystin-YR” OR TS=”101064-48-6”) OR TS=”microcystin-LW” OR TS=”[Asp3]MCRR” OR TS=”Asp3,Dhb7]MCRR” OR TS=”MCWR”) AND (DOP=2014-01-01/2021-04-13)	3,401
Nodularins	((TS=”nodularins” OR TS=”nodularin”) OR (TS=”nodularin-R” OR TS=”118399-22-7”) OR TS=”Nodularin-Har” OR TS=”Nodularin-V (motuporin)” OR TS=”[D-Asp1]NOD” OR TS=”[DMAdda3]NOD” OR TS=”[dhb5]NOD” OR TS=”[Glu4(Ome)]NOD” OR TS=”[MeAdda3]NOD” OR TS=”[(6Z)-Adda3]NOD”) AND (DOP=1800-01-01/2021-04-13)	779
Saxitoxins	(((TS=”saxitoxin” OR TS=”saxitoxins”) OR (TS=”stx-dihydrochloride” OR TS=”35554-08-6”) OR TS=”Gonyaulax-toxin”) OR (TS=”Carbamate” OR TS=”NeoSTX” OR TS=”GTX1” OR TS=”GTX2” OR TS=”GTX3” OR TS=”GTX4” OR TS=”GTX5 (B1)” OR TS=”GTX6 (B2)”) OR (TS=”N-sulfocarbonyl”) OR (TS=”Decarbamoyl” OR TS=”dcSTX” OR TS=”dcNEOSTX” OR TS=”dcGTX1” OR TS=”dcGTX2” OR TS=”dcGTX3” OR TS=”dcGTX4”) OR (TS=”Deoxydecarbamoyl” OR TS=”doSTX” OR TS=”doGTX2” OR TS=”doGTX3”)) AND (DOP=1800-01-01/2021-04-13)	18,221
Total Unique References		**22,756**

Topic 5 Database Search: Scopus (April 2021)

Date of Search: April 14, 2021

- Anatoxins: January 2015 to present
- BMAA: no limit

Search Strategy for Scopus	Results (Number of Titles)
((TITLE-ABS-KEY ({microcystins}) OR TITLE-ABS-KEY ({microcystin})) OR (TITLE-ABS-KEY ({microcystin-LA}) OR TITLE-ABS-KEY ({96180-79-9})) OR (TITLE-ABS-KEY ({microcystin-LF}) OR TITLE-ABS-KEY ({154037-70-4})) OR (TITLE-ABS-KEY ({microcystin-LR}) OR TITLE-ABS-KEY ({101043-37-2})) OR (TITLE-ABS-KEY ({microcystin-LY}) OR TITLE-ABS-KEY ({123304-10-9})) OR (TITLE-ABS-KEY ({microcystin-RR}) OR TITLE-ABS-KEY ({111755-37-4})) OR (TITLE-ABS-KEY ({microcystin-YR}) OR TITLE-ABS-KEY ({101064-48-6})) OR TITLE-ABS-KEY ({microcystin-LW}) OR TITLE-ABS-KEY ({[Asp3]MCRR}) OR TITLE-ABS-KEY ({Asp3,Dhb7]MCRR}) OR TITLE-ABS-KEY ({MCWR})) OR ((TITLE-ABS-KEY ({nodularins}) OR TITLE-ABS-KEY ({nodularin})) OR (TITLE-ABS-KEY ({nodularin-R}) OR TITLE-ABS-KEY ({118399-22-7})) OR TITLE-ABS-KEY ({Nodularin-Har}) OR TITLE-ABS-KEY ({Nodularin-V (motuporin)}) OR TITLE-ABS-KEY ({[D-Asp1]NOD}) OR TITLE-ABS-KEY ({[DMAdda3]NOD}) OR TITLE-ABS-KEY ({[dhb5]NOD}) OR TITLE-ABS-KEY ({[Glu4(OMe)]NOD}) OR TITLE-ABS-KEY ({[MeAdda3]NOD}) OR TITLE-ABS-KEY ({[(6Z)-Adda3]NOD})) OR (((TITLE-ABS-KEY ({anatoxins}) OR TITLE-ABS-KEY ({anatoxin})) OR (TITLE-ABS-KEY ({anatoxin-a}) OR TITLE-ABS-KEY ({64285-06-9})) OR TITLE-ABS-KEY ({homoanatoxin-a}) OR TITLE-ABS-KEY ({dihydroanatoxin-a}) OR TITLE-ABS-KEY ({2,3-epoxy-anatoxin-a}) OR TITLE-ABS-KEY ({4-hydroxy-anatoxin-a}) OR TITLE-ABS-KEY ({4-oxo-anatoxin-a}) OR TITLE-ABS-KEY ({dihydrohomoanatoxin-a}) OR TITLE-ABS-KEY ({Anatoxin-a(s)}) OR TITLE-ABS-KEY ({(guanitoxin (GNT))})) AND (PUBYEAR > 2014)) OR (TITLE-ABS-KEY ({Cylindrospermopsin}) OR (TITLE-ABS-KEY ({7-epicylindro-spermopsin}) OR TITLE-ABS-KEY ({143545-90-8})) OR TITLE-ABS-KEY ({7-deoxycylindrospermopsin}) OR TITLE-ABS-KEY ({7-deoxy-desulfo-cylindrospermopsin}) OR TITLE-ABS-KEY ({7-deoxy-desulfo-12-acetylcylindrospermopsin})) OR (TITLE-ABS-KEY ({BMAA}) OR TITLE-ABS-KEY ({beta-Methylamino-L-alanine)})) OR ((TITLE-ABS-KEY ({saxitoxins}) OR TITLE-ABS-KEY ({saxitoxins})) OR (TITLE-ABS-KEY ({stx-dihydrochloride}) OR TITLE-ABS-KEY ({35554-08-6})) OR TITLE-ABS-KEY ({Gonyaulax-toxin})) OR (TITLE-ABS-KEY ({Carbamate}) OR TITLE-ABS-KEY ({NeoSTX}) OR TITLE-ABS-KEY ({GTX1}) OR TITLE-ABS-KEY ({GTX2}) OR TITLE-ABS-KEY ({GTX3}) OR TITLE-ABS-KEY ({GTX4}) OR TITLE-ABS-KEY ({GTX5 (B1)}) OR TITLE-ABS-KEY ({GTX6 (B2)})) OR (TITLE-ABS-KEY ({N-sulfocarbonyl})) OR (TITLE-ABS-KEY ({Decarbamoyl}) OR TITLE-ABS-KEY ({dcSTX}) OR TITLE-ABS-KEY ({dcNEOSTX}) OR TITLE-ABS-KEY ({dcGTX1}) OR TITLE-ABS-KEY ({dcGTX2}) OR TITLE-ABS-KEY ({dcGTX3}) OR TITLE-ABS-KEY ({dcGTX4})) OR (TITLE-ABS-KEY ({Deoxydecarbamoyl}) OR TITLE-ABS-KEY ({doSTX}) OR TITLE-ABS-KEY ({doGTX2}) OR TITLE-ABS-KEY ({doGTX3}))	29,314

- Cylindrospermopsin: no limit
- Microcystins: no limit
- Nodularins: no limit
- Saxitoxins: no limit

Fields Searched: Title, Abstract, and Keywords

Topic 5 Database Search: Anatoxins 2014 (December 2021)

Date of Search: December 28, 2021

- Anatoxins: 2014

Fields Searched

- PubMed: All Fields
- Web of Science: Topic
- Scopus: Title, Abstract, and Keywords

Database	Search Strategy for Database	Results (Number of Titles)
PubMed	((("anatoxins"[tw] OR "anatoxin"[tw]) OR ("anatoxin-a"[tw] OR "64285-06-9"[rn]) OR "homoanatoxin-a"[tw] OR "dihydroanatoxin-a"[tw] OR "2,3-epoxy-anatoxin-a"[tw] OR "4-hydroxy-anatoxin-a"[tw] OR "4-oxo-anatoxin-a"[tw] OR "dihydrohomoanatoxin-a"[tw] OR "Anatoxin-a(s)"[tw] OR "(guanitoxin (GNT))"[tw]) AND (2014 [dp]))	28
Web of Science	(((TS="anatoxins" OR TS="anatoxin") OR (TS="anatoxin-a" OR TS="64285-06-9") OR TS="homoanatoxin-a" OR TS="dihydroanatoxin-a" OR TS="2,3-epoxy-anatoxin-a" OR TS="4-hydroxy-anatoxin-a" OR TS="4-oxo-anatoxin-a" OR TS="dihydrohomoanatoxin-a" OR TS="Anatoxin-a(s)" OR TS="(guanitoxin (GNT))") AND (PY= 2014))	47
Scopus	(((TITLE-ABS-KEY ({anatoxins}) OR TITLE-ABS-KEY ({anatoxin})) OR (TITLE-ABS-KEY ({anatoxin-a}) OR TITLE-ABS-KEY ({64285-06-9})) OR TITLE-ABS-KEY ({homoanatoxin-a}) OR TITLE-ABS-KEY ({dihydroanatoxin-a}) OR TITLE-ABS-KEY ({2,3-epoxy-anatoxin-a}) OR TITLE-ABS-KEY ({4-hydroxy-anatoxin-a}) OR TITLE-ABS-KEY ({4-oxo-anatoxin-a}) OR TITLE-ABS-KEY ({dihydrohomoanatoxin-a}) OR TITLE-ABS-KEY ({Anatoxin-a(s)}) OR TITLE-ABS-KEY ({(guanitoxin (GNT))})) AND (PUBYEAR = 2014))	48
Total Unique Studies		62

Topic 5 Database Search: Gray Literature

Date of Search: See "Date Searched" column

- No limit for any cyanotoxin

Source	Search Strategy for Source	Results (Number of Titles)	Date Searched
EPA Chemicals Dashboard ToxVal database	Searched for each cyanotoxin by provided CAS RN or name(s)	10	5/12/2021

Source	Search Strategy for Source	Results (Number of Titles)	Date Searched
ECHA registration dossiers	Searched for each cyanotoxin by provided CAS RN or name(s)	12	5/13/2021
EPA ChemView database	Searched for each cyanotoxin by provided CAS RN or name(s)	0	5/13/2021
NTP CEBS database	Searched for each cyanotoxin by provided CAS RN or name(s)	4	5/13/2021
OECD SIDS HPV	Searched for each cyanotoxin by provided CAS RN or name(s)	0	5/13/2021
ECOTOX database	*Search does not work	-	-
ATSDR	Looked for cynatoxins of interest in listed profiles	0	5/19/2021
NAS	Searched site for each cyanotoxin of interest	0	5/19/2021
NCI	Searched for each cyanotoxin by provided name(s)	0	6/02/2021
FDA	Searches for each cyanotoxin by provided name(s)	3	6/02/02021
NIEHS	Microcystin OR Nodularin OR anatoxin OR Cylindrospermopsin OR BMAA OR Saxitoxin OR Carbamate OR N-sulfocarbonyl OR Decarbamoyl OR Deoxydecarbamoyl Limited search results to National Institute of Environmental Health Sciences	5	5/25/2021
WHO/IARC	Microcystin OR Nodularin OR anatoxin OR Cylindrospermopsin OR BMAA OR Saxitoxin OR Carbamate OR N-sulfocarbonyl OR Decarbamoyl OR Deoxydecarbamoyl	0	5/25/2021
Health Canada	Searched for each cyanotoxin by provided name(s)	9	5/25/20215
CalEPA	Microcystin OR Nodularin OR anatoxin OR Cylindrospermopsin OR BMAA OR Saxitoxin OR	11	5/26/2021

Source	Search Strategy for Source	Results (Number of Titles)	Date Searched
	Carbamate OR N-sulfocarbonyl OR Decarbamoyl OR Deoxydecarbamoyl		
Australian Gov. Department of Agriculture, Water, and the Environment	Microcystin OR Nodularin OR anatoxin OR Cylindrospermopsin OR BMAA OR Saxitoxin OR Carbamate OR N-sulfocarbonyl OR Decarbamoyl OR Deoxydecarbamoyl	1	5/27/2021
Water Research Australia	Microcystin OR Nodularin OR anatoxin OR Cylindrospermopsin OR BMAA OR Saxitoxin OR Carbamate OR N-sulfocarbonyl OR Decarbamoyl OR Deoxydecarbamoyl	25	5/27/2021
European Food Safety Authority	Microcystin OR Nodularin OR anatoxin OR Cylindrospermopsin OR BMAA OR Saxitoxin OR Carbamate OR N-sulfocarbonyl OR Decarbamoyl OR Deoxydecarbamoyl	1	6/02/2021
USGS	Searched for each cyanotoxin by provided name(s)	80	6/03/2021
CDC MMWR	Searched for each cyanotoxin by provided name(s) FYI – Can use the search bar at this link to search only within MMWR: https://www.cdc.gov/mmwr/mmwr_trends.html	12	06/14/2021
Total Unique References			**171**

Topic 5 Title/Abstract Screening Criteria and Tagging

Two separate individuals screened the available literature based on the populations, exposures, comparators, and outcomes (PECO) criteria and supplemental material categories in the tables that follow. Studies were "included" if they met PECO criteria or fell into one of the supplemental material categories. In cases where screener 1 and 2 disagreed, a third screener was consulted to provide conflict resolution.

Populations, Exposures, Comparators, and Outcomes (PECO) Criteria

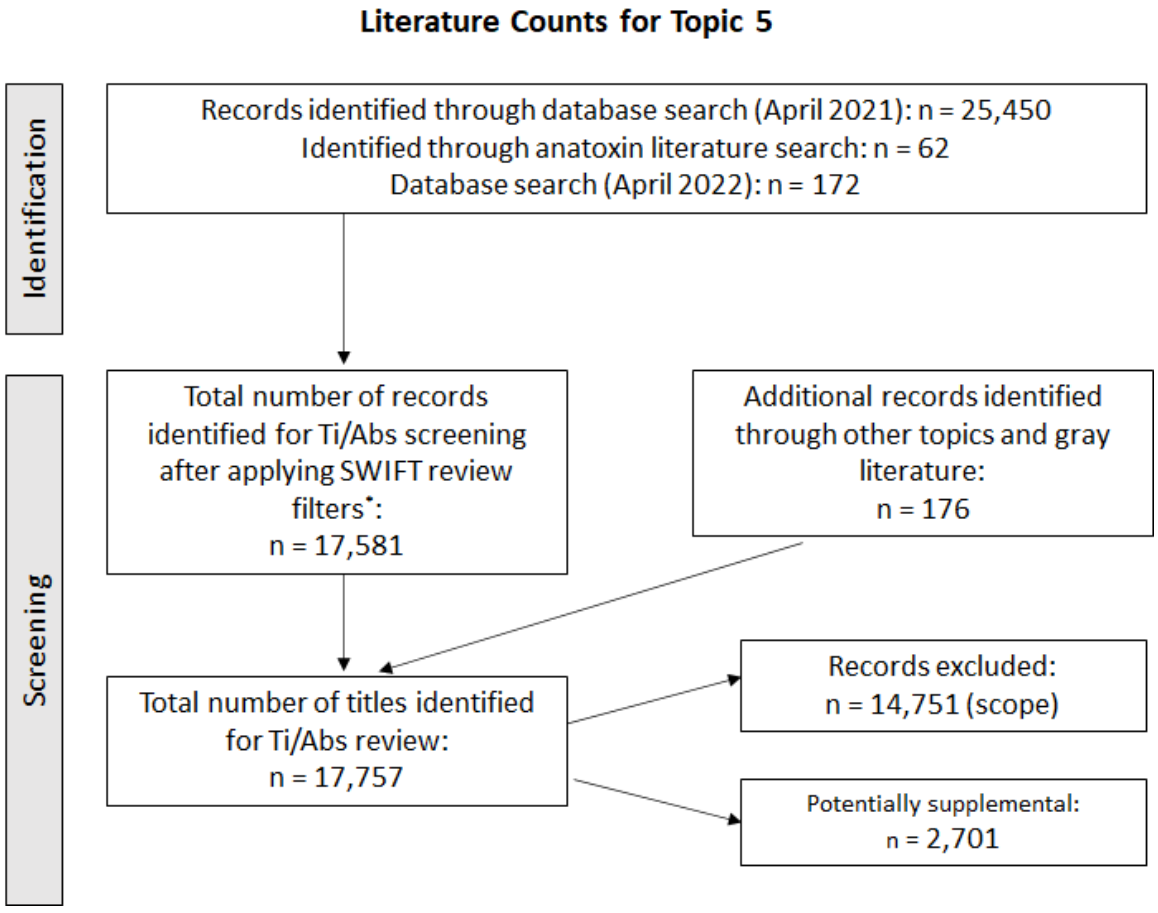
PECO Element	Description
Populations	**Human:** Any population and lifestage (occupational or general population, including children and other sensitive populations). **Animal:** Non-human mammalian animal species (whole organism) of any lifestage (including preconception, in utero, lactation, peripubertal, and adult stages). Examples include: rat, mouse, rabbit, guinea pig, hamster, monkey, dog, mink. -Studies of transgenic animals will be tracked as mechanistic studies under "potentially relevant supplemental material." *-Studies on alternative animal models and aquatic animal species will be tracked as nonmammalian models system under "potentially supplemental material."* *-In vitro studies (including human or animal cells, tissues, or organs (not whole animals); bacteria, nonmammalian eukaryotes) will be tracked as mechanistic information under "potentially supplemental material."*
Exposures	Relevant forms: Harmful algal blooms or their associated toxins (all forms of: anatoxins, BMAA, cylindrospermopsin, microcystins, nodularins, and saxitoxins) Metabolites used to estimate exposures **Human and Animal:** Any exposure to oral, inhalation, dermal, intraperitoneal, or intravenous injection, intravenous by dialysis routes of >1-day duration, or any duration assessing exposure during reproduction or development. Studies will also be included if biomarkers of exposure are evaluated (e.g., measured chemical or metabolite levels in tissues or bodily fluids, anatoxin-a levels in human blood) but the exposure route is unclear or likely from multiple routes. Exposure measured in water (e.g., fresh, salt, surface) soil, fish or shellfish, dietary supplements, edible plants *will be tracked as "potentially supplemental material."*
Comparators	**Human:** A comparison or referent population exposed to lower levels (or no exposure/exposure below detection limits), or exposure for shorter periods of time, or cases versus controls, or a repeated-measures design. Case reports or case series of >3 people will be considered to meet PECO criteria, while case reports describing findings in 1–3 people will be tracked as "potentially relevant supplemental material." **Animal:** A concurrent control group exposed to vehicle-only treatment and/or untreated control (control could be a baseline measurement e.g., acute toxicity studies of mortality, or a repeated-measures design).
Outcomes	All health outcomes (cancer and noncancer). In general, endpoints related to clinical diagnostic criteria, disease outcomes, histopathological examination, or other apical/phenotypic outcomes are considered to meet PECO criteria and prioritized for evidence synthesis over outcomes such as biochemical measures.

Categories of Potentially Relevant Supplemental Material

Category	Evidence
Mechanistic information	Studies reporting measurements related to a health outcome that inform the biological or chemical events associated with phenotypic effects, in both mammalian and nonmammalian model systems, including in vitro, in vivo (by any route of exposure, includes transgenic models), ex vivo, and in silico studies. Genotoxicity tests are considered "mechanistic." Studies where the chemical is used as a laboratory reagent generally do not need to be tagged (e.g., as a chemical probe used to measure antibody response).
Toxicokinetic (ADME)	Toxicokinetic (absorption, distribution, metabolism, and excretion; ADME) studies are primarily controlled experiments, where defined exposures usually occur by intravenous, oral, inhalation, or dermal routes, and the concentration of particles, a chemical, or its metabolites in blood or serum, other body tissues, or excreta are then measured. These data are used to estimate the amount absorbed (A), distributed (D), metabolized (M), and/or excreted (E) through urine, breath, feces. • The most informative studies involve measurements over time such that the initial increase and subsequent concentration decline is observed, preferably at multiple exposure levels. However, data collected from multiple tissues or excreta at a single time-point also inform distribution. • ADME data can also be collected from human subjects who have had environmental or workplace exposures that are not quantified or fully defined. However, to be useful such data must involve either repeated measurements over a time-period when exposure is known (e.g., is zero because previous exposure ended) ***or*** time- and subject-matched tissue or excreta concentrations (e.g., plasma and urine, or maternal and cord blood). • ADME data, especially metabolism and tissue partition coefficient information, can be generated using in vitro model systems. Although in vitro data may not be as definitive as in vivo data, these studies should also be tracked as ADME. For large evidence bases it may be appropriate to separately track the in vitro ADME studies. *Studies describing environmental fate and transport or metabolism in bacteria are not tagged as ADME.
Classical Pharmacokinetic (PK) Model Studies, or Physiologically based Pharmacokinetic (PBPK) Model studies	**<u>Classical Pharmacokinetic (PK) or Dosimetry Model Studies</u>:** Classical PK or dosimetry modeling usually divides the body into just one or two compartments, which are not specified by physiology, where movement of a chemical into, between, and out of the compartments is quantified empirically by fitting model parameters to ADME (absorption, distribution, metabolism, and excretion) data. This category is for papers that provide detailed descriptions of PK models, that are not a PBPK model. • The data are typically the concentration time-course in blood or plasma after oral and or intravenous exposure, but other exposure routes can be described. • A classical PK model might be elaborated from the basic structure applied in standard PK software, for example to include dermal or inhalation exposure, or growth of body mass over time, but otherwise does not use specific tissue volumes or blood flow rates as model parameters. • Such models can be used for extrapolation like PBPK models, although such use might be more limited.

Category	Evidence
	Physiologically based Pharmacokinetic (PBPK) or Mechanistic Dosimetry Model Studies: PBPK models represent the body as various compartments (e.g., liver, lung, slowly perfused tissue, richly perfused tissue) to quantify the movement of chemicals or particles into and out of the body (compartments) by defined routes of exposure, metabolism and elimination, and thereby estimate concentrations in blood or target tissues. • Usually specific to humans or defined animal species; often a single model structure is calibrated for multiple species. • Some mechanistic dosimetry models might not be compartmental PBPK models but predict dose to the body or specific regions or tissues based on mechanistic data, such as ventilation rate and airway geometry. • A defining characteristic is that key parameters are determined from a substance's physicochemical parameters (e.g., particle size and distribution, octanol-water partition coefficient) and physiological parameters (e.g., ventilation rate, tissue volumes); that is, data that are independent of in vivo ADME data that are otherwise used to estimate model parameters. • Chemical-specific information on metabolism (e.g., Vmax, Km) or other molecular processes (e.g., protein binding) might be obtained by fitting the model to in vivo ADME data or determined from in vitro experiments and extrapolated to in vivo predictions. • They allow extrapolation between species, routes of exposure, or exposure durations and levels; that is, they do not just quantify ADME for specific experiments to which they have been fitted.
Nonmammalian model systems	Studies in nonmammalian model systems (e.g., fish, birds, *C. elegans*). Ecotoxicity studies, such as those on daphnia, algae, or aquatic plants, will be excluded unless they are relevant to human health outcomes.
Non-PECO routes of exposure	Exposure measured in water (e.g., fresh, salt, surface) soil, fish or shellfish, dietary supplements, edible plants.
Exposure characteristics (no health outcome assessment)	Exposure characteristic studies include data that are unrelated to health outcomes, but which provide information on exposure sources or measurement properties of the environmental agent (e.g., demonstrate a biomarker of exposure).
Mixture studies	Mixture studies are not considered to meet PECO criteria unless they use methods that allow investigation of the exposure of interest by itself, rather than only evaluating effects of the whole mixture. Methods used to assess investigation of the exposure by itself may not be clear from the abstract, in particular for epidemiology studies. When unclear, the study should be advanced to full text review to determine eligibility.
Case reports	Case reports of ≤3 subjects that describe health outcomes after exposure.
Records with no original data	Records that do not contain original data, such as other agency assessments, informative scientific literature reviews, editorials, or commentaries.
Conference abstracts/abstract only	Records that do not contain sufficient documentation to support study evaluation and data extraction.

Topic 5: PRISMA Diagram



*Filters were applied to identify animal toxicology, epidemiology

Appendix B. Summaries of Studies Reviewed by EPA

This appendix includes summaries of studies that EPA found during the literature searches for four topics, including:

1. Advances in molecular microbial source tracking (MST) for recreational water applications.
2. Advances in molecular methods.
3. Advances in quantitative microbial risk assessment (QMRA) and epidemiological studies.
4. Characterization of children's risk from exposure to recreational water contaminated by fecal contamination.

Only studies that passed the scope and study quality criteria (see Appendix A for the Literature Search Strategies) are included in the table below. There are studies that are cited in the five-year review that are not included in this table because those studies were for different topics or were provided by subject matter experts independent of the literature search.

Table B-1. Brief Summary of Studies for Topics 1 through 4

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Abello et al. (2021)	Laguna Lake, Philippines	MST (culture and molecular methods)	Human, cattle, swine	*E. coli*	A library-independent method of microbial source tracking was used to isolate *E. coli* and detect heat-labile toxin (LTIIA), heat-stable II (STII) genes, and human biomarker mitochondrial DNA (mtDNA) NADH gene in samples. Authors encouraged the use of *E. coli* LTIIa and STII toxin genes for MST because they are host associated and do not require large water sample volumes for detection. Although fecal contamination at the study site was attributed to agricultural sources (cow and pig), the mtDNA was found to be a geographically stable marker that was not influenced by diet (when compared to fecal indicator bacteria).
Abia et al. (2016)	South Africa	QMRA	Human	*E. coli*, *V. cholerae*, *Salmonella* spp. and *Shigella* spp.	QMRA was conducted for the Apies River, Gauteng, South Africa. Water samples were evaluated for *E. coli* levels and the presence or absence of *V. cholerae*, *Salmonella* spp. and *Shigella* spp. The study used the rate of detection for calculating the probability of infection. Based on information from the literature, the authors assumed that 8% of *E. coli* counts were pathogenic and estimated a probability of infection for pathogenic *E. coli*. Ingestion rates used were 1 mL, 50 mL, and 100 mL to represent different types of exposure, from incidental ingestion to intentional ingestion.
Ahmad et al. (2017)	NA – not environmental sampling	Method development	NA – spiked samples	*Enterococcus faecalis* (ATCC, 19433); *E. coli* K12 strain C3000; Set of six specific LAMP primers (F3, B3, FIP, BIP, LF, and LB) for each target gene	Using laboratory spiked samples, this study demonstrated that Most Probable Number – Loop-Mediated Isothermal Amplification (MPN-LAMP) can be used to quantify bacteria in water using PCR without performing DNA extraction. MPN-LAMP was demonstrated in both 25 µL standard reaction volumes and in 1 µL microchip reactions. Direct DNA amplification from bacterial cells would eliminate sample processing steps and reduce the complexity of gene analysis instruments. The authors suggest that MPN-LAMP has the potential to replace the conventional MPN method and would be valuable for use with simple, sensitive, and rapid (25 minutes) integrated

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
					gene analysis systems with sample-in-answer-out capability.
Ahmed et al. (2016a)*	Brisbane River, Brisbane Australia; Freshwater creek in Kissimmee Florida	MST (culture, qPCR)	Avian	*E. coli*, Fecal coliforms, *Enterococcus* spp., *Helicobacter* spp. associated GFD marker	The host specificity and prevalence of *Helicobacter* spp. GFD marker (avian-associated) was evaluated by testing fecal samples from both avian and non-avian host groups. Using qPCR, mean concentrations of GFD marker were found to be two orders of magnitude higher in avian samples than non-avian samples. GFD marker was detected in water samples collected in both Brisbane, Australia and Kissimmee, Florida. The authors concluded the GFD marker is highly specific to avian host groups and could serve as an effective marker to detect the presence and amount of avian fecal pollution in environmental waters. Strong correlations between *E. coli* and *Enterococcus* were observed in water samples from both sampling locations, however concentrations of these fecal indicator bacteria did not correlate with the concentrations of the avian-associated GFD marker in samples from these locations.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Ahmed et al. (2016b)	Samples collected in USA, Canada, Singapore, France, Bangladesh, Kenya, and Belgium	MST (PCR, qPCR)	Human, cattle, deer, swine, canine	Molecular markers: HF183, HF134, HuBac, BacHum-UCD, BacH, Human-Bac1, HumM2, HumM3, *B. thetaiotaomicron*, α-1.6-mannanase, gyrB	Available research on MST methods that utilize *Bacteroidales*/*Bacteroides*-associated genes as markers of sewage pollution were reviewed. The advantages and disadvantages of associated PCR-based methods used for MST were discussed. The available literature supports the use of the HF183 marker as it is well characterized with respect to host sensitivity and specificity and presence in sewage, worldwide. The available literature indicates that HumM2 and HumM3 require more study. Limitations associated with employing a one-marker-one-assay approach for MST included challenges tied to using qPCR for evaluating complex environmental water samples.
Ahmed et al. (2016c)	Brisbane River, Australia	Method development – qPCR	Wildlife, human	Laboratory seeded human adenoviruses (HAdVs, target Hexon) and human polyomaviruses	Recovery efficiencies were compared for human adenoviruses (HAdVs) and human polyomaviruses (HPyVs) from 10-L river water samples seeded with raw human wastewater (100 and 10 mL) using hollow-fiber ultrafiltration (HFUF) and glass wool filter (GWF) methods. The HFUF method provided better recovery for HAdVs and HPyVs compared to the GWF method. The mean recovery efficiencies using HFUF were 36% (HAdVs) and 90% (HPyVs), whereas the mean recovery efficiencies of HAdVs and HPyVs in this study for GWF ranged from 1.3% to 3.4%. Another advantage of HFUF is that the filters are readily available (also used in dialysis treatment of patients).

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Ahmed et al. (2018a)	Australia (recreational beaches, not specified)	MST (qPCR); QMRA	Human	MST markers: *Bacteroides* HF183, *Methanobrevibacter smithii nifH,* HAdV, HPyV, pepper mild mottle virus (PMMoV). Reference pathogens: norovirus, adenovirus	QMRA modeling was used to interpret MST markers. Fresh untreated and secondary treated sewage were seeded into filter-sterilized recreational water. Five qPCR MST markers, *Bacteroides* HF183, *Methanobrevibacter smithii nifH*, HAdV, HPyV, and PMMoV were evaluated to determine at what concentrations of these MST markers reflected a significant health risk from exposure to fresh untreated or secondary treated sewage in beach water. Using NoV and HAdV as reference pathogens to anchor the risk level for QMRA, the authors determined the HF183 concentration above 3.22 x 10^3 genome copies (GC) in 100 mL samples represents a risk above the GI illness benchmark value (0.036).
Ahmed et al. (2018b)	Hillsborough River, Tampa, Florida, USA	MST (qPCR)	Human	CrAssphage, HF183	Host sensitivity and specificity was determined for crAssphage marker in environmental river water seeded with eight composite human samples (untreated sewage). Method limit of quantification, process limit of quantification, and DNA recovery efficiency using TaqMan qPCR was determined. The mean concentrations of the crAssphage marker were within the same order of magnitude as HF183 concentrations. The authors concluded the crAssphage marker is highly sensitive, but less specific (cross reacts with poultry litter).
Ahmed et al. (2019)	Lake Parramatta, Sydney, Australia	MST (qPCR); Method development	Human	MST markers: *Bacteroides* HF183, crAssphage CPQ_056	A duplex qPCR assay that allows for simultaneous quantification of *Bacteroides* HF183 and crAssphage CPQ_056 was developed. Multi-lab validation was conducted using archived water samples collected from Lake Parramatta in Sydney, Australia during a dry-weather event and two storm events with gauged sewage overflow. The simultaneous quantification of two marker genes minimized false-negative results. The performance characteristics of the duplex qPCR assay were similar to its simplex counterparts. When compared to published simplex qPCR assay results, the duplex qPCR assay generated highly reproducible data with high sensitivity and accuracy.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Ahmed et al. (2020)	Davidson Park, Gymea Bay, Hen and Chicken Bay, Lake Parramatta, Sydney, Australia	MST (qPCR); Method development	Human	*Bacteroides* HF183, crAssphage CPQ_056, and PMMoV	Interlaboratory agreement on qPCR-based testing for *Bacteroides* HF183, crAssphage CPQ_056, and PMMoV from samples collected from estuarine and freshwater locations was investigated. An approximately 74% agreement over qualitative co-detection when compared to non-co-detection between laboratories was found. However, laboratories were unable to produce comparable quantitative results. The authors underscore the importance of standardized protocols, laboratory equipment, sample processing strategies, and appropriate quality controls to further improve the accuracy and precision of qPCR-based MST tracking of microbial gene markers.
Arnold et al. (2016)	United States: Great Lakes region; Southern CA; Gulf Coast, Eastern Seaboard, Puerto Rico	Epi – multiple cohort studies	Human; point source sewage discharge and urban runoff	*Enterococcus* EPA 1600, IDEXX, EPA 1611	This analysis of 13 prospective cohorts pooled from marine and freshwater beaches in the United States found increased risk at the highest *Enterococcus* levels, which supports their use as monitoring tools. Children under the age of 10 had higher water exposure, GI risk, and illness burden. Attributable illness estimates illustrated that attaining EPA water quality guidelines is protective of public health.
Arnold et al. (2017)	San Diego, CA, USA	Epi – cohort study	Human; storm runoff	*Enterococcus* species US 1600, fecal coliforms 9222D, total coliforms 9222B	A longitudinal cohort study of surfers in CA waters to evaluate wet weather health impacts was conducted. There was a consistent increase in acute illness incidence rates between unexposed, dry-weather, and wet-weather exposure periods. Fecal indicator bacteria were strongly associated with illness only during wet weather periods.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Augustine et al. (2020)*	Boquerón Beach, Puerto Rico	Epi-cohort study	Human; Publicly Owned Treatment Works (POTW)	*Enterococcus* USEPA 1600	A rapid population-based, salivary antibody screening method was used to monitor hepatitis A virus (HAV) immunoconversions in a population of beachgoers at Boquerón Beach, Puerto Rico, where directly monitoring pathogens in the water was unfeasible. Water samples indicated good water quality with levels of *Enterococcus* below the U.S. EPA RWQC. There were no statistically significant associations between any of the demographic or exposure risk factors tested and only 1.43% of the participants who provided three samples were found to have HAV immunoconversions. The study did not link FIB levels to illness.
Aw et al. (2019)*	Michigan, USA	Method development (qPCR)	Human	*E. coli*	The performance of 21 laboratories was reported. The labs had varying experience in meeting proposed, standardized data quality acceptance (QA) criteria for EPA's draft qPCR Method C for *E. coli*. QA criteria for the method failed to meet in 24% of the 376 test samples analyzed. Of these failures, 39% came from two of the "new" laboratories. Deviations from recommended procedures for the storage and preparation of reference and control materials likely contributed to QA failure. The study demonstrated the feasibility of multiple laboratories implementing this qPCR method.
Ballesté et al. (2018)	Spain	MST (qPCR, culture,	Sewage and wastewater from abattoirs (human, ruminant, porcine, and poultry waste)	*E. coli*, enterococci, *Bifidobacterium* spp. markers: BifHM, BifCW and BifPL, *Bacteroidales* spp. markers: HF183, Rum2Bac, and Pig2Bac	Decay rates of MST markers for human, ruminant, porcine, and poultry waste were assessed in dialysis bags filled with diluted wastewater from different sources and kept in an outdoor water tank during winter and summer. A higher variability among T90 values of the different MST markers in winter was observed, whereas similar T90 values were detected in summer indicating a stronger effect of environmental parameters during winter. The different decay trends for FIB observed in human and animal faecal pollution sources is a key issue when natural inactivation rates are used to model the kinetics of the faecal pollution in a water catchment. Incorporating the MST molecular markers would facilitate microbial faecal pollution modeling.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Beale et al. (2017)	Brisbane River and Queensland, Australia	Method development (culture, PCR)	Human	*E. coli*, enterococci, other freshwater bacteria identified using metagenomics	Water samples from five sites along the Brisbane River, Queensland, Australia (rural and urban downstream locations) were collected. The study utilized metagenomics sequencing and reported the top 17 orders of bacteria using Venn Diagrams. The metagenomics output indicated a presence of high levels of freshwater bacteria such as Burkholdariales and lower levels of Actinomycetes and Rhodospirillae in the upstream sites. In contrast, the population levels reversed in downstream sites, affected by salinity, pH, and oxygen availability changes. Human interference was indicated by the increasing populations of Actinomycetes (BR3), including fecal bacteria and Pseudomonadales (BR4and BR5). The study also evaluated community metabolomics. The majority of the identified metabolites were sugars, fatty acids and amino acids. Secondary metabolites such as perillyl alcohol, lithocholic acid and phytol were also observed.
Benjamin-Chung et al. (2017)	Southern California, Alabama, and Rhode Island, USA	QMRA/Epi	Human point and non-point sources, urban runoff (varied by location, some had no known contamination sources)	Male-specific and somatic coliphage, enterococci	Six prospective cohort studies at coastal beaches in the United States using enterococci as water quality indicator were evaluated. Associations between enterococci levels and GI illness were observed only under human-impacted conditions. The cumulative incidence ratios for a 1 $\log_{10}$ increase of enterococci was 1.21 (95% 1.01, 1.46).

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Blanch et al. (2020)	NA – review article	Method development (culture, "fast culture methods," molecular methods)	NA – review article	MSC and Somatic coliphages	Somatic coliphage and MSC detection and enumeration methods were reviewed with the aim of outlining improvements of standardized techniques. Weaknesses in standardized techniques included long operating times, complex procedures, and readability of plates. The authors described several new approaches for fast and feasible coliphage detection including plaque assay improvements, lysis detection in liquid media, application of molecular methods, and electronic sensors. Lysis detection in selective media was underscored as being a specific, fast, and feasible approach to enumerating coliphages in contaminated waters.
Boehm (2019)	Mostly U.S. sewage samples	QMRA	Human; untreated sewage	reference pathogens: *Salmonella, Campylobacter, E. coli* 0157:H7, *Cryptosporidium, Giardia,* Norovirus, Adenovirus; Indicators: somatic coliphage, MSC	QMRA was used to estimate the levels of somatic and MSC in sewage impacted recreational waters that correspond to the 32/1,000 GI illnesses from exposures to a suite of bacteria, viruses, and protozoa. The risk-based water quality threshold for somatic and MSC was 60 PFU per 100 mL and 30 PFU per 100 mL, respectively, for fresh sewage contamination. The thresholds decrease as the contamination ages because, on average, coliphages decay more quickly than norovirus, the pathogen that contributes the most to risk.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Boehm and Soller (2020)	Mostly U.S. wastewaters	QMRA	Human and animal (fresh and aged sewage and gull feces)	Reference pathogens included *Salmonella* (CFU), *Campylobacter* (MPN), *E. coli* O157:H7, *Cryptosporidium* (oocysts), *Giardia* (cysts), norovirus (gene copies), and adenovirus (infectious unit). Indicators included HF183 (gene copies) and enterococci (CFU)	QMRA was used to estimate a risk-based water quality threshold for human marker HF183 in ambient waters corresponding with the health benchmark of 32 illnesses/1,000 recreators for different contamination scenarios. The threshold of 525 HF183 copies/100 mL was estimated for human contamination of unknown age assuming a mix of aged sewage and in the range of 1 to 525 HF183 copies/100 mL when gull contamination was present.
Boehm et al. (2018)	Global	QMRA, review, meta-analysis	Human; Untreated sewage of known age	Reference pathogens: *Salmonella* spp, *Campylobacter, E. coli* O157:H7, *Cryptosporidium, Giardia,* norovirus. Indicator: HF183	A systematic review, meta-analysis and QMRA were conducted to evaluate decay rate constants for pathogens and fecal contamination indicators (HF183). The decay rate constants were used in a risk assessment to evaluate ingestion exposure to water polluted with untreated sewage of different ages, using the human fecal marker HF183.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Bonadonna et al. (2019)	NA – review article	Method development (culture, molecular methods)	NA – review article	Various – review article	This review discussed the advantages and disadvantages of a wide array of analytical methods for monitoring microbial water quality. These methods include rapid culture-based methods (chromogenic and fluorogenic substrates), cultivation-independent detection methods (ATP cell viability luciferase assay, flow cytometry, biosensors), microbial identification by mass spectrometry (matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF)), and molecular techniques (real-time PCR, digital PCR, nucleic acid sequence-based amplification (NASBA), DNA microarray, loop-mediated isothermal amplification (LAMP), next generation sequencing (NGS): amplicon sequencing, whole-genome sequencing and metagenomics). The review concluded that ideal real-time analysis cannot be routinely achieved, but recent developments have made it possible to detect many indicator organisms and pathogens in water within a few hours or in the same day.
Bortagaray et al. (2020)	Santa Lucia River and Uruguay River in region between Argentina, Brazil, and Uruguay	Children	Sewage, agriculture activities (livestock, horticulture, and fruit production)	Group A Rotavirus (RVA)	This study detected, quantified, and assessed risk of infection and illness for RVA in the watersheds of the Santa Lucia and Uruguay rivers. The authors performed qPCR on surface water samples and QMRA for people that use surface waters from the aforementioned rivers, including children. Both rivers had comparable risks of infection and illness, as RVA was consistently detected in surface waters.
Brooks and Field (2017)	Corvallis, Oregon, USA	MST (qPCR)	Cattle	*E. coli*, MST markers: GenBac3, CF128, Rum2Bac, CowM2, CowM3	Decay profiles for culturable *E. coli*, *Bacteroidales* genetic markers (GenBac3, CF128, Rum2Bac), and cattle markers (CowM2, CowM3) were generated. DNA samples were amplified using qPCR to quantify marker concentrations. The study results are useful for future work studying the decay of faecal MST markers, e.g., identifying the limitations of highly specific but less prevalent markers like CowM2 and CowM3.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Brown et al. (2017a)	California, USA	QMRA, MST (qPCR)	Human, gull	*Bacteroides dorei*, MST markers: HF183, CAT	Human and gull-associated fecal markers were used as indicators in a QMRA estimating the probability of illness due to swimming in contaminated recreational water. The authors detected human markers in most of the treated effluent samples and at lower concentrations compared to raw sewage. Given the concentrations of HF183 in treated effluents, treated effluent is unlikely to be responsible for HF183 in ambient waters above 10^4 copies per 100 ml.
Brown et al. (2017b)	California, USA	QMRA, MST (qPCR)	animal feces, gull	16S rRNA gene copies of *Catellicoccus marimammalium* (CAT) through qPCR	There are no guidelines for interpreting measured concentrations of CAT genes in recreational waters. The authors collected 37 gull fecal samples, measured the CAT gene concentrations, and integrated the measurements in a QMRA framework using dose-response functions from reference pathogens. They estimated that when the level of CAT surpasses 4 × 10^6 copies per 100 mL of water, the median predicted illness exceeds three illnesses per 100 swimmers.
Brumfield et al. (2021)	Creek in urban watershed, USA	MST (qPCR, whole metagenome sequencing); Method development	Human, avian, dog, ruminant	*E. coli*, enterococci, MST markers: HF183, BacR287, Rum2Bac, DG3, GFD, Entero1a, EC23S857	Culture for FIB, qPCR amplification of FIB and host-associated genetic markers, and whole metagenome sequencing (WMS) were used to detect, identify, and enumerate bacteria, archaea, fungi, protists, and viruses in an urban watershed before and after a storm event. Fecal contamination from multiple sources (human, avian, dog, and ruminant), as well as FIB, enteric microorganisms, and antibiotic resistance genes increased demonstrably after a storm event. The addition of qPCR and WMS to traditional techniques may provide enhanced characterization and improved understanding of microbial pollution sources in ambient waters.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Bushon et al. (2017)	Little Blue River, Kansas City, Missouri, USA	MST (culture, qPCR)	Human, cats, chickens, cows, deer, dogs, geese, horses, mice, rabbits, wild turkeys	*E. coli*, MST markers: AllBac, HF183, BacCan, BoBac	Culture and qPCR methods were used to analyze water samples in the Little Blue River in Missouri over a 7-year period for concentrations of *E. coli* and human, canine, and ruminant-associated markers. The authors used microbiological and hydrological data to rank the streams contributions of bacteria. The results showed that stormwater contained high levels of *E. coli* and, in certain tributaries, high levels of human, canine, and ruminant markers. The methodologies used in this study may prove useful in prioritizing remediation in different sub-basins.
Byappanahalli et al. (2018)	Sleeping Bear Dunes National Lakeshore, Benzie County, Michigan, USA	Method development (culture, qPCR)	Not reported	*E. coli*, enterococci	This study compared culture methods (EPA Method 1603 for *E. coli* and EPA Method 1600 for enterococci) to qPCR-based methods (modified EPA Method 1611 qPCR for *Enterococcus* and Chern et al., 2011 qPCR for *E. coli*) at coastal beaches and rivers located at the Sleeping Bear Dunes National Lakeshore in Michigan. Overall, the culture-based and qPCR-based results for both indicators were correlated in this study, leading authors to conclude that qPCR may be a viable alternative method to the culture-based method for monitoring water quality on public lands. The results indicated that qPCR methods would have resulted in fewer water quality advisories, but the increased benefit of same-day results provided by qPCR provides better protection overall. [Chern, E.C., S. Siefring, J. Paar, M. Doolittle, and R.A. Haugland. 2011. Comparison of quantitative PCR assays for *Escherichia coli* targeting ribosomal RNA and single copy genes. Lett. Appl. Microbiol. 52:298–306.]
Cao et al. (2016)	USA	Method development; MST (qPCR, duplex dPCR);	Human (spiked)	*Enterococcus* spp., HF183	A method for duplex droplet digital PCR, which measures enterococci and the human marker HF183 simultaneously, was reported. They also demonstrated that ddPCR is more resistant to inhibition by humic acid than qPCR. qPCR had almost no amplification at 5 ng/uL of humic acid, whereas ddPCR quantification was still within the 95% confidence level above 15 ng/uL humic acid.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Cao et al. (2018a)*	Escondido, Marie Canyon, and Topanga, California, USA	MST (qPCR)	Human (spiked)	HF183, BacR287	A standardized HF183/BacR287 qPCR method was used to establish a human fecal contamination score (HFS) in 42 samples across three marine surface water sites in coastal California. The authors showed that site prioritization with HFS is feasible. They also observed that sampling intensity and the number of qPCR replicates affect HFS estimates' reliability. The effort could help future users design studies aiming for optimal HFS performance.
Cao et al. (2018b)	USA	MST (droplet digital TM PCR); Method development	Human (spiked)	*Enterococcus* spp., HF183	This study provided a complete method protocol for Droplet Digital™ PCR, which allows for simultaneous testing for a general microbial water quality indicator (*Enterococcus* spp.) and a human-associated fecal marker (HF183) in environmental waters from marine and freshwater beaches. This method offers the opportunity to maintain qPCR's advantages while addressing qPCR's major imitations, which include the need for standard curves and the impact of inhibition on method performance.
Chandler et al. (2017)	Massachusetts, USA	Method development (RT-PCR)	Fecal contamination (source not specified)	F-specific RNA (FRNA) coliphages, enteric viruses	An anion exchange resin-based system to concentrate FRNA coliphages and enteric viruses, followed by RNA isolation and RT-PCR detection was developed. The performance of the anion exchange resin-based methodology was tested using 65 environmental marine and freshwater samples considered to be fecally contaminated. The method facilitated detection of FRNA coliphages in 61.5% samples, which provides evidence of viability of this system to concentrate FRNA coliphages in water. This system provides a rapid, ease to use, and inexpensive method to concentrate coliphages in water, and it is particularly useful in places that need frequent sampling to monitor water status as well as to identify the contamination sources.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Chen et al. (2020)	Beijing, China	MST (qPCR)	Human, dog, bird	*Bacteroidetes*, MST markers for dog, bird, humans (not specified)	Surface water was sampled near Beijing Normal University during four rain events. The relationships between the copy number of fecal microbes and other environmental factors including the rainfall time, total amount of rainfall, number of antecedent drying days, runoff amount, peak flow rate, and duration of rainfall were assessed. The number of antecedent drying days was the key factor for dog fecal pollution, while human fecal pollution was impacted by more factors. The bird source had a weak correlation with the runoff volume and had no correlation or even a negative correlation with other environmental factors.
Chyerochana et al. (2020)	Tha Chin River, Thailand	MST (culture, qPCR); Method development	Human	Enterococci, *E. faecalis* phages (strains AIM06 and SR14)	EPA Method 1611 (*Enterococcus* qPCR) and enterococci culture method (EPA Method 1600) were used to measure bacteriophages of *E. faecalis* strains AIM06 and SR14 from freshwater of the Tha Chin River in Central Thailand. AIM06 and SR14 phages were present at similar levels as other human-specific bacteriophages of *E. faecalis* isolated and detected in other geographical regions and in environmental water samples. Both phages were present in 92.9% of freshwater samples. Culture and qPCR method results showed strong correlation with human-specific DNA markers (Kendall's tau = 0.600) as well as moderate correlation between each other (Kendall's tau = 0.445). The correlation between culture and qPCR was found to be only moderate due to intrinsic difference in enterococci specificity. qPCR detected genes found in all enterococci species, whereas the culture method is specific to certain species including *E. faecalis*. Double-layer agar assay method to detect enterococci phages indicated continuing fecal pollution with no significant level of variance between stations. The authors concluded that AIM06 and SR14 phages are low-cost MST tools for pollution source identification in freshwater and coastal water.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Cloutier and McLellan (2017)	Lake Michigan, Wisconsin, USA	MST (culture, qPCR)	Human, gull, ruminant	*E. coli*, MST markers: Entero, Lachno2, HB (human *Bacteroides*), Gull2, BacR	Recreational water at six Wisconsin beaches was assessed. The gull-associated *Catellicoccus marimammalium* (Gull2) marker was found in over 80% of water samples, regardless of *E. coli* levels. Human-associated *Bacteroides* (HB) and *Lachnospiraceae* (Lachno2) were detected in only 2.4% of water samples collected under baseflow and post-rain conditions but produced a robust signal after a combined sewage overflow, despite low *E. coli* concentrations. Microcosm studies to assess decay found that Gull2, HB, and Lachno2 qPCR signals were reduced twice as quickly as those from *E. coli* and enterococci and approximately 20% faster than signals from culturable *E. coli*.
Codello et al. (2021)	Hawkesbury-Nepean River, Sydney, Australia	MST (qPCR)	Human	MST markers: uidA (*E. coli*), HF183, Lachno3	A weight-of-evidence approach was used to identify untreated sewage contamination in the Hawkesbury-Nepean River in Sydney, Australia. The authors used a qPCR method targeting markers for human *E. coli*, *Bacteroidales*, and *Lachnospiraceae*. They found that using multiple indicators was more effective than using individual techniques, overcoming their independent limitations (e.g., *E. coli* detection).
Cormier and Janes (2016)	NA – spiked samples	Method development (qPCR)	NA – spiked samples	Human adenovirus (HAV), MS2 phage	A method to concentrate HAV from seawater using zeolite was developed to aid rapid detection. Artificial seawater was inoculated with HAV and MS2 and filtered with zeolite. The viruses were detected using qPCR. Zeolite was able to concentrate HAV and MS2 from artificial seawater with 99% and 90% efficiencies, respectively.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Crain et al. (2021)	San Diego County, California, USA	Method development (ddPCR, qPCR, culture: Enterolert)	Urban runoff, human (varied with location, some samples were not impacted by known contamination sources)	*Enterococcus* spp.	This study compared a ddPCR method (Cao et al., 2018), to both an EPA-approved culture method SM9230D (Enterolert) and an EPA-approved qPCR method (EPA Method 1609.1). Findings supported the conclusion that ddPCR readouts align closely with Enterolert MPN for identifying FIB exceedance levels of *Enterococcus* spp. in coastal waters of San Diego, CA. Findings also suggested that ddPCR and genomic 23S rDNA could be effectively employed as an alternative indicator for beach management decisions in Southern California.
Crank et al. (2019)	Adenovirus data from Spain, data for PMMoV from multiple countries. All other data are from the United States	QMRA	Fresh untreated domestic wastewater (no specific study location)	Reference pathogens: *Salmonella*, *Campylobacter*, *E. coli* O157:H7, *Cryptosporidium*, and *Giardia*. Viral water quality indicators included: CrAssphage and PMMoV	A web-based QMRA model that evaluates adults' risk of illness from incidental ingestion of recreational water polluted by fresh wastewater was developed. CrAssphage and PMMoV were used as viral indicators of human waste to estimate the risk of illness in the aforementioned scenario, comparing indicator concentrations to several viral pathogens' concentrations and using the pathogen dose-response functions. The model is accessible through a web-based interface and can be adapted to other indicators or pathogens.
Crank et al. (2020)	Italy	MST (qPCR); Method development	Human	crAssphage (CPQ56), human polyomavirus (HPyV), human bocavirus (HBoc), hepatitis E virus (HepE)	CrAssphage abundance was assessed in 156 Italian wastewater samples. CrAssphage correlation with other molecular viral markers (human bocavirus, Hepatitis E virus, and human polyomavirus) was reported. CrAssphage was present in 150 out of 156 samples, with a 96% overall detection rate. This is notable, as prior studies have shown a 100% crAssphage detection rate in untreated wastewater. Most WWTPs represented in this study are in populous, urban areas, and so results presented were representative of a large mixture of diets and potential fecal pathogens in wastewater.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Cui et al. (2017)	Olympic Forest Park, Beijing, China	Method development (next generation sequencing, qPCR)	Human	Various pathogens, including marker genes of pathogenic *Aeromonas* spp., *Salmonella* spp., *M. Avium*, *P. aeruginosa*	16S rRNA gene targeted next generation sequencing and qPCR were used to analyze pathogen diversity and quantify pathogens in samples collected from waterbodies in Beijing Olympic Forest Park. The authors used these methods to provide a more comprehensive picture into the bacterial pathogen diversity in wastewater. While qPCR provides a quicker result with better specificity and sensitivity, its detection is limited to the scope and targets of its surveyors and does not capture the total complexity of the environment.
DeFlorio-Barker et al. (2018)*	Multiple locations in the U.S.: California, Alabama, Mississippi, South Carolina, Indiana, Michigan, Ohio, Maine	Children; Epi/Exposure	Not explicitly defined	Did not target microorganisms or genes; estimated recreational water exposure through ingestion	Data from 12 prospective cohorts with a total sample size of 68,685 subjects were pooled. Stochastic modeling was conducted to estimate the range of incidental water volume ingestion by beachgoers (children and adults). The authors identified that children are more likely to have greater exposures by spending more time in the water and direct contact with algae and sand (compared to adults). Children, specifically ages 6 to 12 years, swallow more water per swimming event than any other age group (median of 36 mL; 90th percentile = 150 mL). In comparison, adults ≥35 years old swallow 9 mL (90th percentile = 64 mL) per swimming event. Additionally, on average, males swallow more water than females per event. This puts these individuals at a higher risk for becoming ill after swimming at recreational beaches compared to adults.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Derx et al. (2021)	Vienna, Austria	MST; QMRA	Human, pig, ruminant, duck	*Cryptosporidium*, *Giardia*, *E. coli*, unspecified MST markers for pig, ruminant, human, duck, cow, bird	QMRA was conducted using measured concentrations of human, ruminant, pig, and bird-associated MST markers as well as *E. coli* in a Danube wetland area. The scenarios investigated included: (i) the impact of river discharges into the backwater channel (allochthonous sources), (ii) the resuspension of pathogens from animal fecal deposits in inundated areas, and (iii) the pathogen release from animal fecal deposits after rainfall (autochthonous sources). The study is an integrative modeling approach for determining the transfer rates of pathogens from diverse fecal sources into alluvial wetlands during storm events and floods. Although surface water was considered, the focus was safe drinking water supply.
Deshmukh et al. (2016)	NA – review article	Method development (various)	NA – review article	*E. coli*, *Shigella* spp., *Salmonella* spp., *Vibrio parahaemolyticus, Pseudomonas aeruginosa, Bacillus anthracis, Brucella abortus, C. botulinum, Coxiella burnetii, Francisella tularensis, Rickettsia prowazekii, C. perfringens, Staphylococcus aureus, V. cholerae, Vibrio alginolyticus, Yersinia pestis*	This review summarized the current state of rapid methods for the monitoring and detection of waterborne bacterial pathogens. The nucleic acid-based, immunology-based, and biosensor-based detection methods studied in this review were found to be sensitive, specific, time-effective, and important in prevention and diagnosis of waterborne bacterial diseases.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Devane et al. (2019)	Avon River, Christchurch, New Zealand	MST (culture, qPCR)	Human, dog, wildfowl	*E. coli*, coliphage, *Campylobacter* measured using culture; MST markers: GenBac3, HumM3, B. adol, HumBac, HF183, Bac708R primers, canine-associated sources (referred to as a dog marker, not specified)	MST markers (GenBac3, HumM3, HumBac (HF183-Bac708R); *Bifidobacterium adolescentis*, wildfowl and canine-associated markers), fecal indicator bacteria (*E. coli*), and pathogens (*Campylobacter*) were detected in sewage-contaminated river water collected in New Zealand. Human-associated qPCR markers were found in water samples collected after known sewage discharge events. The authors reported that the concentration of the *B. adol* and HumBac PCR markers were in general, tenfold lower than the general fecal PCR marker, GenBac3, and the HumM3 PCR marker was approximately two orders of magnitude lower than the other two human PCR markers.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Dorevitch et al. (2017)	Lake Michigan, Chicago, Illinois, USA	Method development (culture, qPCR)	Noted that the Chicago River system protects Lake Michigan and its beaches from point sources of fecal pollution	*E. coli, Enterococcus faecalis*	Water samples (n = 1,796) collected in 2015 and 2016 at nine Lake Michigan beaches in Chicago, Illinois were analyzed by *E. coli* culture (Colilert method) and *Enterococcus* qPCR (EPA Methods 1609 and 1609.1). Unlike many other studies that compare culture and qPCR results from the same water sample (same day), this study was interested in what data would actually be available for beach management decisions. From the standpoint of deciding whether qPCR or culture methods should be used for daily public notification purposes, it is the agreement between Day0 culture BAV and Day1 qPCR BAV (not Day0 vs. Day0) that matters. This study looked at the two pieces of information available to beach managers on a given day: *Enterococcus* qPCR results of samples collected that morning and *E. coli* culture results of samples collected the previous day. The study found that if the Day1 qPCR result not been available, Day0 culture results would have triggered unnecessary beach advisories 24% of the time ("false alarms"). And, on 4.7% of the beach-days, Day0 culture results would have resulted in a "failure to act" (meaning, failure to trigger advisories) when advisories were needed (based on exceedance of the Day1 qPCR BAV). At one beach, "failure to act" would have occurred 6.8% of the time. Overall, Day0 culture results were more likely to result in a "failure to act" (n = 32) than a "correct advisory" (n = 13). Additionally, this study took chance into account. The 71.3% concordance of Day1 qPCR and Day0 culture beach actions can be explained entirely by chance. The authors conclude that no meaningful agreement was observed between beach management actions driven by *Enterococcus* qPCR results versus *E. coli* culture results and that there is little scientific rationale for continued *E. coli* culture testing of beach water in this setting.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Duan et al. (2016)	Three rivers (Jialing River in Chongqing and the Han River in Hanzhong and The Wenxian River in Jiaozuo), a pond in Beibei, Chongqing, and seawater in Yantaim, China	MST	swine, human, chicken, duck, cow, dog	*Faecalibacterium* 16SrRNA gene sequences	qPCR detection of genetic markers within the 16S rRNA gene of *Faecalibacterium* were used for the identification of swine fecal contamination in surface waters in China. Using 454 pyrosequencing, the authors obtained a total of over 146,000 bacterial sequences and assessed their specificity to swine fecal waste. Two PCR primer sets, PFB-1 and PFB-2, had no cross-reaction with other animal samples, though PFB-2 was more sensitive than PFB-1. PFB-2 was detected in concentrations ranging from 100 to 10,000 copies per 100 mL of water in sites near swine farms.
Dufour et al. (2021)*	NA	Review	NA	NA	This review summarizes methods used for enumeration of fecal indicators in recreational water from the 1930s to 2021.
D'Ugo et al. (2019)	Castel Giubileo, Mezzocamino, Albano Lake, Aniene, Pertusillo Lake, Rome, Italy	MST (RT-PCR, qPCR); Method development	Human, unspecified agricultural animals	Human adenovirus (ADVTot = 51 types of human adenoviruses, ADV41, ADV40); human enterovirus, HEV, HAV; mammalian orthoreoviruses; norovirus (GI, GII)	qPCR was used to analyze samples collected from lakes and rivers in Italy. The study targeted significant enteric viruses and results were compared to poliovirus 1 as a reference virus. No evidence of inhibition was observed in the samples. All samples showed the presence of at least one viral species, with the most frequently detected being ADVTot and ADV41. Human enterovirus was not detected in any water samples. From the study, it was suggested that human fecal contaminants are prevalent in all kinds of surface waters. While controls showed recovery of MS2 phage (similar in size to Human Enteroviruses) in spiked water samples, caution must be taken when concentrating human enteroviruses such as poliovirus, coxsackievirus, and echovirus because they might be too small to be recovered in the filtration processes.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Egorov et al. (2018)*	Lawrence, Massachusetts, USA	Children	Not reported, presumed human sewage	NA	This study found no association between infection with noroviruses and swimming in natural waterbodies (rivers, lakes, or oceans). Swimming in natural waterbodies was associated with 2.33 adjusted odds ratio (95% confidence interval 0.35; 15.4) of immunoconversion to *Cryptosporidium*. An association was not found between immunoconversion to norovirus (GI or GII) and swimming in natural waterbodies. Swimming in all types of natural waterbodies was only reported among 3% of the study participants (n= 165) likely because the survey was administered in the fall. The authors concluded that the salivary antibody assay can serve as a valuable tool for assessing these risk factors.
Eregno et al. (2016)	Norway	QMRA	Human, sewer overflows	Indicators: *E. coli*. Reference pathogens: *Giardia*, *Cryptosporidium*, Norovirus, *Salmonella*, and *Campylobacter*	A hydrodynamic water quality model was coupled with QMRA to estimate the risk of infection from swimming in marine waters following a rainfall event with combined sewer overflows. Of the simulated bacteria, protozoa and virus infection risks, the virus risk dominated, as represented by norovirus, and exceeded the infection health benchmark of 19 illnesses per 1,000 recreators in the days after the rainfall event.
Fan et al. (2017)	Southeastern China	MST (qPCR)	Human, swine, cow, goat, sheep, chicken, duck, goose, dog	HF-183	This study sought to identify unique genetic sequences for bacteria that were specific to swine fecal sources present in natural water samples collected in southeastern China. Genome fragment enrichment (GFE) was determined to be useful in differentiating between different fecal metagenomes. Two GFE sequences in *Bacteriodales*-like organisms were determined to be strongly swine-specific for sources of fecal contamination when testing natural waters with qPCR for type of organism responsible for fecal contamination.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Fang et al. (2018)	Beijing, China	Method development (next generation sequencing, qPCR)	Human	*E. coli, Salmonella enterica, Aeromonas* spp., *Mycobacterium avium, Pseudomonas aeruginosa*	Next generation sequencing was used to determine the diversity of genera containing pathogens, and qPCR was used to assess the presence of genes from *E. coli, Salmonella enterica, Aeromonas* spp., *Mycobacterium avium*, and *Pseudomonas aeruginosa*. The authors recommended using combinations of culture-dependent methods in order to provide comprehensive information concerning pathogen diversity and distribution.
Farkas et al. (2018)	North Wales, United Kingdom	Method development (RT-qPCR, qPCR, PGM-MB)	Human	Adenovirus type 40, JC and BK polyomavirus, Norovirus genotype I and II, Sapovirus genotype I, Hepatitis A and E viruses	A variety of enteric viruses were monitored by RT-qPCR, qPCR, and porcine gastric mucinconjugated magnetic beads (PGM-MB) at freshwater and marine shores in the United Kingdom. PCR-based approaches do not address viral integrity and infectivity, hence the need to evaluate viral degradation using the PGM assay. The results were consistent in diluted nucleic acid extracts, hence inhibition during detection was unlikely. The study used EPA Method 1615 for the quantification of enteric viruses and the authors concluded that their data can be applied to inform predictive models for the transport of enteric viruses in water and to improve current viral risk assessment.
Federigi et al. (2019)	Global	Review/QMRA	Human and multiple animals (not specified)	various	PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines were used to review QMRAs of recreational waters published between 2003 and 2018. Found research gaps: 1) lack of epidemiological data on the main pathogens of concern in specific geographic areas; 2) lack of site-specific water quality data; 3) little consideration of recovery efficiency or infectivity of pathogens; 4) lack of consideration of host-specific factors (like previous immunity or immunodeficient); 5) lack of QMRA model validation.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Feng and McLellan (2019)	Milwaukee, Wisconsin, USA	MST (qPCR); Method development	Humans, pigs, dogs, cats, deer, cows, gulls, chickens, human	*Bacteroides*	qPCR of the V6 and V4V5 regions of *Bacteroides* were used to specifically target sewage-derived *Bacteroides*. The V6 and V4V5 regions contained human fecal sequences that were not part of the HF183 cluster, making these two regions appropriate for detecting sewage-based *Bacteroides*. The authors also suggested that qPCR and next generation sequencing could be used together for source tracking.
Ferguson et al. (2019)	Florida and Texas, USA	Children	Not reported, focus was on oil contaminants	NA	The behavior of children ages 1 to 6 years old was studied at recreational marine beaches in Florida and Texas. The study found that age was the most relevant factor in how and where children play (i.e., dry sand, intertidal area, water) and that hygiene practices like washing hands before eating at the beach varied by region. The data from this study can give insight into microbial exposures children might be exposed to in these locations.
Ferguson et al. (2021)	Florida and Texas, USA	Children	Not reported, focus was on oil contaminants	NA	A virtual timing device was used to collect very detailed activity data for children at beaches. The authors found that children (1 to 6 years) spend the most time at the beach in the seawater through videotaping children and observing their beach activities. Children were observed to spend the majority of time wading in the intertidal zone and seawater. The time spent wading was 38.9% for children 0 to 24 months, 37.6% for children 25 to 48 months, and 45.1% for children greater than 48 months. Older children were observed to spend more time digging and running and less time sitting than the younger children.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Fu et al. (2021)	Singapore	Method development (MPN-LAMP, qPCR)	Fecal contamination (source not specified)	*Enterococcus* spp., *E. coli*	A rapid on-chip gene quantification method was developed based on loop-mediated isothermal amplification (LAMP) PCR and using a polymethyl methacrylate (PMMA) microchip. This method does not require the use of expensive qPCR instruments. Using the MPN-LAMP assay, target genes of fecal indicator bacteria (*Enterococcus* and *E. coli*) were quantified in environmental water samples collected from a beach and a freshwater reservoir in Singapore. Results obtained using the MPN-LAMP approach and qPCR were correlated, demonstrating that the MPN-LAMP method can be used for microbial quality monitoring in areas that have limited resources.
García-Aljaro et al. (2019)	NA – not environmental sampling	MST (qPCR)	Human, ruminant, porcine, poultry	NA – review article	This review article summarized the types of microbes found in sewage and used for MST. Detection of sewage microbes was often done using qPCR, and microbes such as *Bacteroidales*, *Biffidobacterium* spp., enterococci, Firmicutes, Proteobacteria, human adenovirus, and bacteriophages were detected. In addition, this review summarized detection of FIB that were used as surrogates for pathogens in testing sewage. It was noted that composition of microbes in sewage was dependent upon factors such as geographic location and ambient temperature. Detection of the origins of fecal contamination using both MST molecular methods and live organisms by culture-based methods were advocated in many water management situations.
Gibson et al. (2017)	Beaver Lake Watershed and Beaver Lake Reservoir, Arkansas, USA	MST (qPCR)	Human, cow, poultry	*E. coli*; MST markers: HumM2, CL	Detection of host-associated markers by qPCR was compared with *E. coli* concentrations from several locations within the Beaver Lake Watershed in Arkansas. While the HumM2 (human) and CL (poultry) markers correlated with *E. coli* concentrations, they did not indicate conclusively the fecal contamination source needed for MST. The location of sampling, rain events, and the season were important variables affecting host-associated marker detection. The authors suggest that

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
					similar trends could exist for other human-made reservoirs.
Gitter et al. (2020)	Brazos River Basin, Texas, USA	MST; QMRA	Human, cattle, other wildlife	*E. coli*	Human, cattle, and wildlife MST markers were measured in a freshwater tributary in Texas. QMRA was conducted to estimate pathogen dose using MST to identify the proportion of *E. coli* load from each source. Human health risk contributed the most risk despite contributing the least to the bacterial concentration.
Gonçalves et al. (2018)	Bay of Koper Gulf of Trieste, Adriatic Sea	Method development (RT-qPCR)	Human	Rotaviruses, noroviruses	Enteric viruses from coastal waters were concentrated using methacrylate monolithic chromatographic and quantified using RT-qPCR. Rotaviruses and noroviruses were monitored in an area in the northern Adriatic Sea impacted by human fecal sources and at a nearby bathing area. The study demonstrated that CIM C4 hydrophobic interaction columns combined with RT-qPCR provide an efficient and consistent tool to monitor enteric viruses from coastal environments.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Gosselin-Théberge et al. (2016)	Southern Quebec, Canada	Method development (qPCR, dPCR)	Avian	*Campylobacter* spp.	This review evaluated real-time (qPCR) assays targeting *Campylobacter*, a significant public health concern associated with recreational water exposure in developing countries. The authors compared nine published qPCR assays: three for thermotolerant *Campylobacter* spp. targeting the 16S rRNA and six for *C. jejuni* targeting different genes. *Campylobacter* spp. isolates collected from recreational water samples in Quebec, Canada were used. Three *C. jejuni*-specific assays demonstrated good specificity and sensitivity when tested. The authors selected two assays targeting *Campylobacter* spp. and *C. jejuni* to compare DNA concentration estimation, using spectrophotometry and digital PCR (dPCR), in order to calibrate standard curves (SC) for greater accuracy of qPCR-based quantification. Differences in the quantification of *Campylobacter* isolates between qPCR assays were observed and method-specific bias in standard curve preparation was also identified.
Graciaa et al. (2018)	35 U.S. states and 1 territory (Guam)	Outbreaks	Human, non-human	NA	This review summarized the outbreaks associated with untreated recreational water voluntarily reported to CDC during 2000-2014 from 35 U.S. states and Guam. Outbreaks resulted in at least 4,958 cases of disease and two deaths. Among the 95 outbreaks with a confirmed infectious etiology, enteric pathogens caused 80 (84%); 21 (22%) were caused by norovirus, 19 (20%) by *E. coli*, 14 (15%) by *Shigella*, and 12 (13%) by *Cryptosporidium*.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Green et al. (2019)	Onondaga Creek, New York, USA	MST (qPCR, culture)	Human, ruminant, dog	Fecal coliforms, MST markers: HF183, Rm2Bac, DG3, Entero1	Samples were collected during dry-weather conditions in the Onondaga Creek in New York. The authors used qPCR-based assays targeting human (HF183), ruminant (Rum2Bac), and canine (DG3) markers. qPCR was used to enumerate *Enterococcus* (Entero1) and culture was used to enumerate fecal coliforms to assess overall fecal contamination. The authors reported that ruminant contaminants are likely not major contributors to the high levels of observed FIB in urban areas during dry weather and these contaminants were likely significantly degraded, diluted, and deposited during in-stream transport. Instead, *Enterococcus* and human marker concentrations dominated urban locations.
Gutiérrez-Cacciabue et al. (2016)	Vaqueros River and La Caldera River, Argentina	Method development (culture, qPCR)	spiked river water	*E. coli*, enterococci (*E. faecalis*)	*E. coli* and enterococci were spiked into water samples collected from two Argentinian rivers and enumerated by culture methods and qPCR. The primary focus of this study was to assess the impact of sunlight inactivation on indicator bacteria present in freshwater. *E. faecalis* detection by qPCR showed that the persistence of DNA was higher than that of culturable cells. Sunlight also accelerated DNA decay and solid particles present in the water column provided a protective role, thereby reducing decay.
Hata et al. (2016)	Japan	Method development (IC-RT-PCR-MPN)	Human, animal	Male-specific RNA phages	Male-specific RNA phages genotypes were characterized using integrated culture (IC)-RT-PCR-MPN. The method described allows quantitative analysis of male-specific RNA phages combining culture and molecular techniques (through MPN) and was tested in different seasons. The method differentiates between infectious and non-infectious viral indicators, which may improve risk assessments. Surface water samples were evaluated by three different methods: the IC-RT-PCR–MPN method, conventional RT-qPCR, and conventional plaque assays.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Haugland et al. (2021)*	Michigan, USA	Method development (culture, qPCR)	Human	*E. coli*	A statewide survey was conducted in Michigan to compare culture-based methods and EPA Draft Method C for enumeration of *E. coli* in a variety of locations and water sources. This large-scale comparison analyzed 6,965 samples collected from 101 recreational sites. The hypothetical exercise to evaluate the frequency of water impairments based on theoretical qPCR thresholds corresponding to the *E. coli* water quality standard for culture methods suggested that the methods may provide the same beach notification outcomes over 90% of the time. Results from this study suggest that a statewide, multi-site threshold value may be feasible for Method C in Michigan despite site-to-site variability in its relationship to *E. coli* culture methods. The authors also indicate that confirmation is needed to determine whether corresponding theoretical Method C thresholds utilized in the study represent an equivalent public health protection compared to established culture *E. coli* water quality standards.
He et al. (2016)	Qianhuang and Xueyan, China	MST (qPCR, PCR)	Human, pig, bovine, goat, dog, chicken, duck, goose, cormorant	MST markers: H-ND6, H-ND5, BacH, HF183, *B. adolescentis*, Pig-2 Bac, L. amylovorus, P-CytB, P-ND5	The sensitivities and specificities of microbial and mitochondrial DNA (mtDNA) markers were evaluated using PCR and qPCR methods. The study collected fecal samples from humans, pigs, and seven other species and surface water samples from the Taige River and Taihu Lake. The authors concluded that human-associated microbial DNA markers were inferior indicators compared to the human mtDNA markers. The results suggest the use of H-ND6, H-ND5, and Pig-2-Bac for better fecal source tracking.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Hofstra et al. (2019)	Global	QMRA	Unspecified	*Cryptosporidium*	An analysis framework that includes large-scale waterborne pathogen concentrations, burden of diarrheal disease, and modeled global burden of disease for waterborne cryptosporidiosis was presented. Results included a global heat map of cryptosporidiosis and fecal coliform levels in surface waters and disease burden expressed in DALYs. Although the scenario was drinking surface water and not recreational exposures, the study is innovative because two large-scale models, WorldQual for fecal coliform and GloWPa for *Cryptosporidium*, were used for modeling loads and in-stream concentrations. These concentration data were used with exposure and dose-response information to estimate risk and burden of disease. Gaps and opportunities for the further development of the framework were highlighted.
Holcomb and Stewart (2020)	NA – review article	Method development	NA – review article	NA – review article	This is a review of indicators and methods for assessing water quality. Methods were classified into three stages of development, late, middle, and early. Culture-based methods for *E. coli* and enterococci, coliphages, and *Bacteroides* bacteriophages were classified as late-stage development, meaning they are the most developed. Molecular detection of *E. coli* and enterococci, *Bacteroides* HF183, HumM2, PMMoV, crAssphage, BacCow, BacCan, avian GFD, Pig-2-Bac, Noroviruses, rotaviruses, *Salmonella* spp., *Campylobacter* spp., and *Cryptosporidium* spp. were classified as middle stage development. Antimicrobial resistant bacteria and resistance genes were classified as early-stage development.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Hsu et al. (2017)	Olentangy River Wetland Research Park, Columbus, OH, USA	MST (qPCR, culture)	Human, avian, ruminants	*E. coli*, *Arcobacter*, *Campylobacter*, Shiga toxin-producing *E. coli*, antibiotic resistance (tetracycline, tetQ and sulfonamide, sul1), MST markers: HF183, GFD, Rum2Bac	Water samples were collected across the urban wetlands in central Ohio from June 2013 to June 2014 and tested with culture-based (*E. coli*) and qPCR methods. No significant reductions in antibiotic resistance genes across the wetlands were observed in this study. The wildlife activity, specifically the presence of Canada Geese and White-Tailed Deer, contributed to potential bacterial pathogens in the water. At two impoundments where geese management was applied, total and fecal coliforms levels were three times lower compared to an unmanaged site. Reductions in fecal indicator *E. coli* from inflow to outflow showed a seasonal difference.
Jennings et al. (2018)	San Francisco, California, USA	MST (qPCR, culture)	Human	Enterococci, MST markers: HF183, BacR287	Traditional (culturable enterococci, cENT) and molecular (qPCR-enterococci, qENT and human-associated marker, HF183/BacR287) methods were used to investigate marine waters. All three indicators showed seasonal variation. The current California standard for cENT yielded nearly twice as many exceedances as the qENT standard given in USEPA's 2012 RWQC. Combined sewer discharges were not important predictors of indicator levels typically measured in weekly monitoring samples. Precipitation and solar insolation were predictors of cENT in weekly samples, while precipitation and water temperature were predictors of HF183/BacR287 and qENT.
Jiang et al. (2018)	Waller Creek, Austin, Texas, USA	Method development (LAMP)	Human	*Bacteroides* HF183	A field-ready nucleic acid diagnostic platform, which relied on loop-mediated isothermal amplification (LAMP) of human-associated *Bacteroides* HF183 genetic markers and oligonucleotide strand exchange (OSD) probes was developed. The platform was tested using environmental water samples (i.e., local creeks and ponds) in Texas and spiked with lab-grown recombinant *E. coli* pHF183, primary filtered raw sewage, or fresh canine/feline feces. The authors concluded the platform could detect as few as 17 copies/mL within 80 minutes. The assay was concluded to be a cheap, easy-to-use, and rapid method to

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
					identify human fecal contamination (*Bacteroides* HF183) without off-target signals from canine/feline feces.
Jikumaru et al. (2020)	Japan	Method development (dPCR)	Not reported	STEC, *C. jejuni, E. coli, Shigella, Salmonella*	Water samples from nine rivers in Japan were collected and evaluated by dPCR for stx 1 and stx2 of STEC, hipO of *C. jejuni*, ipaH of *Shigella*/EIEC, invA of *Salmonella*, and uidA of *E. coli.* Among the five pathogen genes tested, only stx2 of STEC and hipO of *C. jejuni* were detected in the water samples. The other genes (stx1of STEC, ipaH of *Shigella*/EIEC, and invA of *Salmonella*) were not detected. The authors found that the abundance of fecal indicator bacteria such as *E. coli* does not necessarily correlate with the density of pathogens.
Jogi et al. (2020)	Anne Canal, Estonia	Method development (immunosensor, culture, qPCR)	Human	*E. coli*, total coliforms	An *E. coli* immunosensor was compared to culture and qPCR methods using samples from bathing water (Anne Canal, Estonia). The median biosensor results were about four times higher than the qPCR results and 40 times higher than the results of microbiological cultivation.
Joosten et al. (2017)	Netherlands	Epi/cohort study	Human, sewer overflows	*E. coli*, enterococci, norovirus G1 and G2	A prospective cohort study was conducted to assess risk factors for health complaints (GI illness, respiratory illness, and skin) associated with an acute gastrointestinal illness outbreak after a canal swimming event in two cities in the Netherlands in 2015. An increased risk of acute gastrointestinal illness was found for swimmers compared to non-swimmers in canal waters recently contaminated by heavy rainfall. Five out of seven stool samples tested positive for norovirus G1 and one of three water samples tested positive for norovirus G1. Two water samples tested positive for norovirus G2. *E. coli* and enterococci levels were below EU thresholds days prior to the event and the morning of the event, however in the afternoon on the day of the even *E. coli* levels exceeded the EU threshold.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Kabiri et al. (2016)	Salt River, Verde River, Central Arizona Project canal system, Gilbert, Arizona, USA	MST (PCR)	Human, cat, cow, dog, duck, fish, gull, pig	*Bacteroides* spp. (including *B. dorei*)	*Bacteroides* 16S rRNA was detected by PCR in water samples. After constructing cladograms of the data, *B. dorei* of human origin was clearly present and separated from other *Bacteroides* species from other animal origins. The authors claim that *B. dorei* is a strong marker for MST for human fecal contamination. They further state that this method provides another piece to a "toolbox" approach at MST of ambient surface waters.
Kinzelman et al. (2020)	Racine, Wisconsin, USA	MST (culture, qPCR)	Human	*E. coli*, enterococci	FIB along North Beach (Racine, Wisconsin, United States) were evaluated using culture-based and molecular methods. The authors concluded that emerging technologies, like 16S rRNA gene sequencing, are needed to characterize contaminant sources in urban environments. The sequencing helped to identify different point sources like the retention/infiltration storm water basin.
Kolm et al. (2017)	Austria	Method development (qPCR)	Human, animal	*Enterococcus* (15 specific strains)	An *Enterococcus* helicase-dependent amplification (HDA) assay using a set of 15 enterococcal target strains and 15 non-enterococcal reference strains was evaluated using environmental surface water samples. The assay produced results with a high level of agreement with EPA qPCR Methods 1611 and 1609. The authors concluded that the assay is a suitable candidate for additional development and can be performed on a simple heating block and without the use of a thermocycler.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Kolm et al. (2019)	Austria	MST (qPCR), Method development	Cattle, sheep, goat, deer, and other ruminant and non-ruminants	BacR	This study analyzed 20 single ruminant (target) and 20 single non-ruminant (non-target) fecal suspensions in surface water from the Danube River. The authors evaluated a BacR helicase-dependent amplification (HDA) assay and a BacR strip test to compare to qPCR methods. They concluded that their HDA assay resulted in comparable sensitivity, specificity, and limits of detection to qPCR. Unlike qPCR the strip test requires only a heating block to amplify the target gene. The reaction mixture is applied directly to the test strip which detects and displays the amplification products by marker-specific hybridization probes via an on-strip colorimetric reaction. The entire assay takes 2 hours, does not require extensive practical training, and is low cost.
Kongprajug et al. (2019)	Tha Chin River, Thailand	MST (PCR, qPCR)	Human, animal	Markers: GenBac3, HF183/BFDrev, Pig-2-Bac, Bac3	PCR and qPCR results were compared from 48 water samples from 12 sampling stations along the Tha Chin River. Universal markers (both PCR and qPCR) were detected in all samples, indicating persistent and continuing fecal contamination. There was 87.5%–100% agreement between the MST-PCR and MST-qPCR. The authors suggest that traditional presence/absence PCR is a screening step that requires less technical skills and has lower costs.
Kongprajug et al. (2020)	Tha Chin River, Thailand	MST (qPCR); Method development	Human, animal	Markers: GenBac3, HF183/BFDrev, CPQ-056, Pig-2-Bac, Bac3qPCR	Five qPCR assays, GenBac3, human-associated HF183/BFDrev and CPQ-056, swine-associated Pig-2-Bac, and cattle-associated Bac3qPCR, were evaluated using the LinRegPCR model. Freshwater samples from the Tha Chin River in Thailand were spiked with human sewage and non-human fecal samples. The LinRegPCR approach improved identification of sources. Overall, the authors emphasized the need for a standardized data analysis protocol for interlaboratory consistency and comparability. The LinRegPCR model is a standard curve-independent qPCR analysis, which does not involve the limit of detection (LOD), limit of quantification (LOQ), or did not quantify (DNQ). Non-detection by this method is deemed zero when no or poor amplification

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
					efficiency is observed, thus eliminating the incorporation of nondetected data. LinRegPCR analysis has a demonstrated advantage for analyzing data obtained at low target concentrations.
Korajkic et al. (2019)*	Tampa and Tierra Verde, Florida, USA	MST (qPCR)	Animal	Markers: GenBac3, Entero1a, EC23S857, Rum2Bac, CowM2, CowM3	qPCR was used to estimate decay of general fecal indicator bacteria (GenBac3, Entero1a, EC23S857) and MST markers (Rum2Bac, CowM2, CowM3). The study found that water type is the most influential factor for their decay. The higher the salinity, the faster the bacteria decayed. Discrepancies in water type decay suggested that osmotic stress induced by increased marine salinity and associated ionic content had a similar influence on all fecal indicators measured in this study, irrespective of the analytical technique (e.g., cultivation or qPCR).
Kuhn et al. (2018)	Denmark	Children; Epi/case-control	Not reported	NA	A prospective case-control study was conducted among Danish persons ages 1 to 30 years old to identify risk factors for campylobacteriosis. For all cases (adults and children; N = 556 cases, 2,117 controls) the model showed an increased risk of infection with bathing in fresh water (OR = 5.1), contact to beach sand (OR = 1.8), owning a pet dog with diarrhea (OR = 4.6), and eating minced beef (OR = 2.6) or chicken (OR = 2.5). The model for children ages 1 to 5 (N = 125 cases, 321 controls) showed increased risk of infection with bathing in paddling pool (OR = 13.6).

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Lane et al. (2020)	Michigan	Method development (qPCR)	Not reported	*E. coli*	This study compared EPA Draft Method C results between 10 Michigan labs and EPA's National Exposure Research Laboratory across 82 recreational sites. The beach management decision (i.e., remain open or issue an advisory or closure) agreed in 94% of the samples analyzed. The authors also note that one important finding from this study is that EPA Draft Method C can be effectively taught to and implemented by lab personnel with minimal qPCR experience. When individual years were assessed, the Michigan labs' precision became progressively better in consecutive years, indicating increasing skill with the method. The study also suggested that further research on inhibition mitigation is needed. Although the inhibition rate overall was low (217 out of the 3,580 paired data points (6%) had quality control failures), samples from one laboratory were unusable throughout most of the 2017 summer because of inhibition. The study also compared qPCR-based beach management decisions to Colilert-18®-based decisions. Michigan determined that 1.863 $\log_{10}$ target gene copies per reaction is equivalent to 300 *E. coli* per 100 mL, which is referred to as the 'regulatory equivalent' value (EV). This value is used for beach notifications. Utilizing EPA Draft Method C about 7% of samples (240 of 3,166) exceeded the EV. This was marginally higher than the 4% of samples reported by the Michigan Department of Environment, Great Lakes and Energy that exceeded the Colilert-18® Michigan recreational water quality criteria between 2016 and 2018.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Lapen et al. (2016)	Canada	QMRA	Human (treated wastewater), agriculture	*Cryptosporidium*	*Cryptosporidium* oocyst concentration and species/genotype data were collected from three surface water surveillance programs in two river basins in Ontario, Canada. QMRA estimated that the risk of infection was one order of magnitude lower when only the human infectious species/genotypes were included in the modeling. *Cryptosporidium* seasonality in water samples appeared to match the seasonality of human infections from *Cryptosporidium* in the study regions.
Leifels et al. (2016)	Germany	Method development (qPCR)	Human	Human adenovirus, enterovirus, rotavirus	This study assessed the utility of pretreatment using ethidium monoazide (EMA) and propidium monoazide (PMA), two dyes that are capable of penetrating the damaged or compromised capsid of inactivated viruses and binding to viral nucleic acids. The authors collected urban river water samples in Germany and assessed whether dye treatment is a suitable approach to improve the ability of qPCR to distinguish between infectious and non-infectious human adenovirus, enterovirus, and rotavirus A. The authors found that pretreatment EMA-/PMA-qPCR succeeded in removing false positive results and that this approach could provide a tool for improving the efficacy of molecular quantification methods and reduce overestimation of viral load in environmental samples.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Li et al. (2016)	Tampa and St. Petersburg, Florida, USA	MST	Human, cow, sheep, swine, avian	*E. coli*, *Enterococcus*, *Bacteroidales, S. aureus, S. enterica*, human polyomavirus, human adenovirus, human norovirus	An MST microarray was used to simultaneously detect genes for multiple pathogens and indicators of fecal pollution in freshwater, marine water, sewage-contaminated freshwater and marine water, and treated wastewater. A linear relationship between gene copies determined by qPCR versus microarray fluorescence was found, indicating the semiquantitative nature of the MST microarray. Whole-genome amplification (WGA) increased gene copies from ultrafiltered samples thus increasing the sensitivity of the microarray. These results indicate that ultrafiltration coupled with WGA provides sufficient recovery of nucleic acids for detection of viruses, bacteria, protozoa, and antibiotic resistance genes by the microarray in applications ranging from beach monitoring to risk assessment.
Li et al. (2018)*	Ohio, USA	MST (qPCR)	Human	MST markers: EC23S857, Entero1 GenBac3, HF183, HumM2	This study assessed the capacity of periphyton (complex mixture of algae, microbes, inorganic sediment, and organic matter that is attached to submerged surfaces in most flowing freshwater systems) to trap genetic markers from the water column. After wastewater effluent was pumped into the periphyton mesocosm, genetic markers were detected in periphyton at frequencies up to 100% (EC23S857, Entero1, and GenBac3), 59.4% (HF183), and 21.9% (HumM2) confirming sequestration from the water column.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Li et al. (2019)*	Trask, Kilchis, and Tillamook rivers and tributaries, Oregon, USA	MST (qPCR); Method development	Human, cow, canine, avian	*E. coli*, Markers: HF183/BacR287, HumM2, CowM2, CowM3, Rum2Bac, DG3, DG37, GFD	A large-scale field study in the Tillamook Bay Watershed, Oregon was conducted to assess the feasibility of implementing standardized fecal source identification qPCR methods with established data acceptance metrics. A total of 602 water samples were collected over a 1-year period at 29 sites along the Trask, Kilchis, and Tillamook rivers and tributaries. Using HF183/BacR287 and HumM2 human-associated qPCR results, geographic information system land use, and *E. coli* monitoring data, the authors concluded that elevated *E. coli* levels may be linked to specific pollution sources and land use activities in the Tillamook Bay Watershed. The study also revealed key issues regarding qPCR data interpretation including the importance of confirming method performance with local reference fecal samples and utilizing appropriate censored data analysis strategies.
Li et al. (2021)*	Ohio, Wisconsin, Indiana, USA	MST (qPCR), Method development	Human, ruminant, canine, avian	Somatic and male-specific coliphage, enterococci, *E. coli*, MST markers: HF183/BacR287, HumM2, Rum2Bac, DG3, GFD	Previously reported paired measurements of cultivated somatic and male-specific coliphage, enterococci, *E. coli*, and genetic markers indicative of human, ruminant, avian, and canine fecal sources were used in this analysis. Due to a large number of non-detection and below-detection data points, the authors determined weighted fecal scores, which is a Bayesian censored data analysis approach. It compares the average density of a host-associated genetic marker between two groups of samples using all data measurements without the need to fabricate any Cq values. A limitation of the fecal score approach is the requirement to group samples together which prevents higher resolution assessment of temporal or site-specific variability.
Liang et al. (2021)	Beijing, China	MST (qPCR, FEAST)	Human, swine, canine, equine, donkey, bovine, sheep, goat, chicken, duck, goose, pigeon, fish	MST markers: HF183-1, HF183-2, BacH, BacHum, Pig2-Bac, Rum-2-Bac, AV4143	MST methods based on molecular markers and machine learning programs were applied together to distinguish the fecal inputs from multiple sources. Along with qPCR, the community-based FEAST (fast expectation–maximization microbial source tracking) program was applied to estimate multiple potential sources and the relative contributions of various fecal inputs at the same time.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Liao et al. (2016)	Virginia, USA	QMRA	Human, non-human	Indicator: *E. coli*. Reference pathogens: *Campylobacter jejuni, E. coli* O157:H7, *Cryptosporidium* spp., *Giardia lambia, Salmonella enterica*, and Norovirus	This study linked a watershed-scale FIB fate and transport model frequently used in existing TMDL processes Hydrologic Simulation Program–Fortran (HSPF, version 12.2) with QMRA for comparison of estimated risks to regulatory benchmarks. The results indicate that total human illness risks were consistently higher than the regulatory benchmark of 36 illnesses per 1,000 recreators for the study watershed, even when the predicted FIB levels were in compliance with the *E. coli* geometric mean standard of 126 CFU/100 mL. Sanitary sewer overflows were associated with the greatest risk of illness.
Lim et al. (2017)	Baby Beach in Dana Point, Orange County, California	QMRA	Human, seagull, pig, cow	Enterococci	Historical enterococci measurements at Baby Beach in Dana Point, Orange County, California were used to evaluate risks from before and after improvements were made in dry-weather runoff diversion and installation of a media filtration system near the main storm drain. A source-apportionment QMRA based on parameters from previously published QMRAs was used. During dry weather, the median recreational waterborne illness risk at this beach was below the U.S. EPA recreational water quality criteria (RWQC) of 36 illness cases per 1,000 bathers. During wet weather, the median recreational waterborne illness risk predicted by the QMRA depended on the assumed level of human waste associated with stormwater; the risk was below the EPA RWQC illness risk benchmark 100% of the time provided that <2% of the FIB in stormwater are of human origin.
Linke et al. (2021)	National Park Lake Neusiedl, Austria	Method development (qPCR)	Human	Markers: *Enterococcus*, HF183/BacR287	qPCR methods targeting a *Enterococcus* genetic marker and the human-associated MST assay HF183/BacR287, were used to test the performance of a modified DNA extraction protocol on freshwater samples from an Austrian lake. The authors concluded that no universal DNA extraction protocol or general rule to improve extraction efficiency can be provided. The amount of suspended material and its composition affect the amount of additives required to improve the extraction efficiency.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Liu et al. (2017)	NA	MST; Method development	raw sewage	*Bacteroidales, E. coli*	This study included microcosm experiments to evaluate microbial decay in untreated seawater. Data from a previous publication that evaluated decay of cow fecal markers was compared to human fecal bacteria decay rates. The authors concluded that tracking of the relative concentration between *Bacteroidales* DNA markers and culturable *E. coli* can differentiate pollution that is relatively fresh from one that has aged and could be useful in episodic pollution events.
Martzy et al. (2017)	Austria	Method development (LAMP, qPCR)	Human, animal	*Enterococcus* spp.	The authors developed the LAMP assay as an alternative to qPCR in identifying microbial contamination in fresh water sources such as springs and surface water. They found that their assay was equally sensitive, more specific, and faster at identifying microbes in water sources than qPCR methods.
Mattioli et al. (2021)*	Pennsylvania, USA	MST (qPCR, culture, genome sequencing)	Human	MSC and Somatic coliphages, total coliforms, *E. coli*, enterococci, MST markers: HF 183/ BFDrev, genome sequencing: norovirus GI and GII	An overloaded septic system at a Pennsylvania event center and campground was the source of human fecal contamination in a nearby creek and swimming area that resulted in several guests developing GI illnesses after exposure. This is a real-word example of how MST could be used to track fecal contamination and make recommendations to protect public health.
McGinnis et al. (2018)	Cobbs and Tacony Creek, Philadelphia, Pennsylvania, USA	MST (qPCR and RT-qPCR)	Human	human polyomavirus (HPoV), PMMoV, adenovirus, enterovirus, norovirus genogroups I and II, *Campylobacter*, *Salmonella*, enterohemorrhagic *E. coli*, fecal coliforms, HF183	Fecal indicator organisms, human fecal markers (HF183), and pathogens were measured at two sites in a freshwater urban creek. The authors distinguished which organisms and/or markers could be used as indicators of recent combined sewage overflows (CSOs). In particular, human *Bacteroides* was found to be the most specific, and total coliforms were found to be the most sensitive indicators. *E. coli*, PMMoV, and HPoV did not show consistent correlations with recent CSO and rainfall events.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Ming et al. (2020)	Xinghai, China	QMRA, MST (qPCR)	Human non-point source	Pathogens: *Aeromonas hydrophila, Listeria monocytogenes, Pseudomonas, Clostridium perfringens, Salmonella, Shigella dysentery, Campylobacter jejuni, E. coli* O157:H7, *Streptococcus faecalis, Aeromonas caviae, S. aureus, Vibrio vulnificus, Vibrio cholera* (not O1), *Vibrio arahaemolyticus, Vibrio alginolyticus*. Indicator: human *Bacteroidales*	Human *Bacteroides* and 15 typical bacterial pathogens were measured using culture-dependent and independent methods in surface seawater samples from Xinghai, China beaches. The authors concluded that enterococci may not accurately indicate fecal source pollution, and human *Bacteroides* should be proposed for beach monitoring.
Monteiro et al. (2021)	Lisbon, Portugal	MST (culture, qPCR)	Humans, pig, poultry, cattle, dog	*E. coli*, enterococci, coliphages, Markers: CWMit, PLMit, PGMit, HMMit and HAdV, GB-124	Fecal contamination sources were determined by analyzing fecal indicators (*E. coli*, enterococci, and coliphages) as well as culture, and culture-independent, source-associated markers. Sampling sites were highly impacted by fecal contamination from both human and livestock sources. There were also seasonal and physicochemical parameters that influenced correlations between fecal indicators.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Mosnier et al. (2018)	French Guiana	Children; Outbreaks	human	NA	A cryptosporidiosis outbreak among children aged between 4.5 and 38 months living in a remote area along the Maroni River in French Guiana was reported. The clinical features, epidemiology, and state of current investigations for the largest documented outbreak of cryptosporidiosis in French Guiana were described. Stool samples were screened for pathogens. Bacterial culture and turbidity analysis of water distribution sources of the area was performed. The outbreak reflected multifactorial dynamics of waterborne disease among children cases including human behavior such as playing, bathing, and eating, and probable immunity.
Napier et al. (2017)*	Cleveland, Ohio, St. Joseph Michigan, Michigan City and Portage, Indiana, Fairhope, Alabama, Warwick, Rhode Island, USA	MST (culture, qPCR)	Human	*Bacteroides* MST markers: BsteriF1, BuniF2, HumM2, HF183	The presence of four human-associated *Bacteroides* markers (HF183, BsteriF1, BuniF2, and HumM2) were assessed for association with self-reported gastrointestinal illness, diarrhea, and respiratory illnesses. This study used data from the NEEAR study, which involved 12,060 adult visitors of six beaches across the United States. Less reliable associations were observed between illness and the presence of *Bacteroides* markers than expected. The authors concluded that quantitative measures of fecal pollution using *Bacteroides*, rather than presence-absence, may be necessary to accurately assess human risk specific to the presence of human fecal pollution.
Napier et al. (2018)*	Cleveland, OH, Portage, IN, Michigan City, IN, St. Joseph, MI, Biloxi, MS, Fairhope, AL, Warwick, RI, USA	Method development (qPCR)	Human	*Enterococcus* spp.	This analysis using data from the NEEAR study involving 12,060 adult visitors to six U.S. beaches reported that with the exception of bisphenol A and cholesterol, no chemicals were found to be consistently associated with an increase of GI illness. Interaction contrast estimates were imprecise, particularly for chemicals that were infrequently detected (e.g., acetaminophen).

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Nappier et al. (2019)*	NA – review article	Method development (qPCR)	NA – review article	*E. coli, Enterococcus* spp.	This review article included information on qPCR methods interference controls reported in 16 papers for *Enterococcus* spp. and 13 papers for *E. coli*. EPA Method 1609, EPA Method 1611, EPA Method A, and Scorpion methods were reported in studies to detect and quantify *Enterococcus* spp., and EPA Method C, Scorpion method, and other methods were applied for *E. coli*. Low levels of interference were reported except in tropical marine waters in Hawaii. Use of *E. coli* qPCR should be considered on a site-specific basis, whereas use of EPA Method 1609 for *Enterococcus* spp. detection is appropriate when the required and suggested controls are employed.
Nieuwkerk et al. (2020)*	NA – review article	Method development (isothermal amplification methods)	NA – review article	NA – review article	This review article summarized applications and performance metrics for most common isothermal amplification methods (IAMs) including helicase-dependent amplification (HAD), loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), nucleic acid sequence-based amplification (NASBA), and rolling circle amplification (RCA). The sensitivity, specificity, LOD, and comparison with PCR and inhibition were reported for each IAMs. The authors concluded that some reported advantages of IAMs may lack empirical evidence and more head-to-head studies of qPCR and IAM methods that include performance metrics and assess susceptibility to inhibition in environmental samples are needed. IAMs may be superior tools to inform spatiotemporal distributions of undesirable microorganisms in the environment.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Olds et al. (2018)	Milwaukee, Wisconsin, USA	MST (culture, qPCR)	Human, ruminant	*E. coli,* enterococci, total fecal coliforms, MST markers: HB, Lachno2, other ruminant-specific markers	*E. coli*, enterococci, and total fecal coliforms using human and ruminant-associated indicators were tested at freshwater (estuary and river) locations in Milwaukee, Wisconsin. The authors found that contamination in surface waters were at levels above the acceptable risk for recreational use. Their findings quantified hazards in exposure pathways from rain events and illustrated the additional stress that climate change may have on urban water systems.
Oliveira et al. (2016)	Rio de Janeiro, Brazil	MST (culture, PCR, qPCR)	Human	*E. coli, Methanobrevibacter smithii* (nifH gene)	The authors enumerated *E. coli* using Colilert, detected *Methanobrevibacter smithii* by conventional PCR, and detected and quantified the nifH gene of *M. smithii* by TaqMan-based qPCR. Forty-six percent of sampled beaches presenting *E. coli* levels in compliance with Brazilian water quality guidelines showed nifH gene between 5.7 × 10^9 to 9.5 × 10^11 copies, thus revealing poor correlation between the two approaches. The authors suggest that the nifH gene could be used, in combination with other markers, for source tracking studies to measure the quality of marine ecosystems.
Oliveira et al. (2020)	Girona, Spain; Jena, Germany	Method development (LAMP, PCR, qPCR)	Human, animal, non-impacted	genetic markers: lacZ (total coliform detection) and uidA (*E. coli* marker)	A multiplex LAMP coupled to an Au-nanoprobe colorimetric assay was used for water quality assessment. The approach was validated in 22 impacted and non-impacted lake and river water samples collected in Spain and Germany using standard PCR and qPCR detecting and quantifying two specific marker genes – lacZ and uidA. The integration of mLAMP and Au-nanoprobe colorimetric methods revealed good sensitivity and specificity without adding to assay complexity. This methodology significantly reduced the reaction time while allowing for an easier translation to field context and simple screening of on-site contaminations.

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Osunmakinde et al. (2018)	NA – review article	Method development	NA – review article	NA – review article	This paper reviewed metagenomic techniques for quantifying human enteric viruses as a method for monitoring water quality in Africa. Both qPCR and NGS were highly useful in monitoring water quality but factors such as cost, training, and equipment necessary for these techniques present challenges to some African countries. The authors suggest more efforts should be directed at instituting water quality monitoring programs that utilize technologies such as qPCR and NGS in countries with emerging economies.
Pawar et al. (2019)	Pune district, Satara district, Raigad district, Maharashtra, India	MST (RT-PCR); Method development	Avian	Avian influenza virus	RT-PCR was conducted on two avian influenza viruses (human pathogenic H5N1) spiked in reservoir and sea water, from Maharashtra India. The authors compared precipitation with potassium aluminum sulphate and milk powder to a virus concentration method using erythrocytes. The precipitates were positive for the presence of the virus using RT-PCR and viable as shown by the growth of virus in embryonated chicken eggs. The method might be suitable for the detection of avian influenza viruses from different environmental water sources and can also be applied during outbreak investigations.
Pedrosa de Macena et al. (2021)	Rio de Janeiro, Brazil	Method development (qPCR)	Human	35 enteric pathogens (using 71 target genes)	The TaqMan array card (TAC) method was used to assess 35 enteric pathogens (using 71 target genes) simultaneously in a lagoon system in Rio de Janeiro, Brazil. TAC results identified 17 enteric pathogens including 4/5 viral species investigated, 8/15 bacteria, 4/6 protozoa and 1/7 helminths. The authors concluded the TAC methodology is a useful molecular tool for the rapid screening of microbiological contamination.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Pendergraph et al. (2021)	Absaroka Beartooth Wilderness, Montana, USA	MST (culture, ddPCR); Method development	Human, animal	*Bacteroides, E. coli*, total coliforms	Water from 23 wilderness lake outlets and streams in Montana were assessed with culture-based FIB and ddPCR for a general *Bacteroides* marker and a human *Bacteroides* marker. The highest *E. coli* CFUs were found in lake outlets that are popular recreational water sources and accessible to stock animals and human foot traffic. In some cases, samples tested positive for the presence of *E. coli*, but human-derived *Bacteroides* were not detected. The authors concluded that the results point to other possible sources of fecal contaminants, including animals (wild or domesticated).
Petcharat et al. (2020)	Thailand	MST (qPCR); Method development	Human	crAssphage	This study spiked phosphate buffered solution (PBS), seawater, freshwater, and influent and effluent from a wastewater treatment plant with crAssphage to evaluate recovery by qPCR. Recovery of crAssphage in PBS was 0.36. The river and one beach had no indigenous crAssphage detected. A second beach had low levels of indigenous crAssphage. Environmental water with no or low background of crAssphage showed no loss in the recovery process. Evaluating recovery efficiencies in samples with high crAssphage backgrounds posed a challenge due to the inability to prepare crAssphage spiked samples with high concentrations.

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Phelan et al. (2019)	New Jersey, USA	MST (qPCR); Method development	Human, animal (horse, goose, dog)	Markers: HF18328, BacHum29, HoF597	The results of amplicon sequencing were compared to established qPCR methods for fecal markers in library and field samples from the Navesink River in New Jersey to determine the sources of fecal pollution in samples with elevated culturable indicator organisms. To determine the microbial community present in the fecal library and surface water samples, amplicon sequencing (Illumina MiSeq, 300 bp, paired end) was performed targeting the V3–V4 region of the 16S rRNA gene. A separate fecal library from researchers in Australia was created using the 115 fecal sequences from human or sewage (human feces, septic, raw sewage, secondary sewage), avian (bird, common miner, chicken, duck), dog, horse, cat, bat, agricultural mammals (cow, goat, pig, sheep), and wild mammals (possum, rabbit, rat). SourceTracker (a Bayesian statistical technique that relates 'sink' microbial community structure to 'source' libraries) was run without tuning using the Australian fecal library as sources and the Navesink fecal library and surface water samples as sinks. Results of this study indicate that fecal indicator organisms were elevated in the Navesink river at the time of study and sources include human/sewage, avian, and horse manure.

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Polkowska et al. (2018)	Western Finland	Children; Outbreaks	Human	Indicators: *E. coli*, intestinal enterococci, cyanobacteria, algae. Pathogens: norovirus, and adenovirus	A retrospective cohort study was conducted using an internet-based survey to study an outbreak of acute gastroenteritis in Tampere Finland. Cases were from the general population and included children who visited beaches at four separate lakes. Risk factors included getting water into mouth while swimming and playing at the beach. Norovirus was found in 19 stool samples. All water samples from implicated beaches had acceptable values of FIB and were negative for norovirus. Closure of swimming beaches in Tampere, advice on hygienic precautions and rapid outbreak alerts were effective at controlling swimming in areas affected by the norovirus outbreak. The results suggest a need for new indicators of water quality for norovirus and development of evidence-based recommendations regarding timing of safe reopen of recreational water venues associated with outbreaks.
Poma et al. (2019)	Argentina	QMRA	Unspecified	Pathogens: Human enterovirus and norovirus	The risk of infection was calculated using: (a) two methodological approaches to find the distributions that best fit the data sets, (b) four different exposure scenarios (primary contact for children and adults and secondary contact by spray inhalation/ingestion and hand-to-mouth contact), and (c) five alternatives for treating censored data. The risk of infection for norovirus GII was much higher than enterovirus. The authors suggest that in most cases the use of the half-LOD approach is appropriate for QMRA modeling.
Powers et al. (2020)	Corpus Christi, Texas, USA	MST (ddPCR)	Human, animal (gull and canine)	*Enterococcus*, HF183	Three host-associated markers tested in this study were detected in all of the water samples. Gull was the most prevalent, followed by human, then canine. This study also found that there was no correlation between enterococci and source-associated human and animal markers, meaning the use of elevated enterococci concentrations to inform beach advisories may not be best practice in urbanized subtropical bays.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Prado et al. (2018)	Sao Paulo, Brazil	MST (culture)	Human	*Bacteroides fragilis* GB-124 bacteriophages	A culture-based, double-layer agar technique was used to enumerate *Bacteroides fragilis* GB-124 bacteriophages in wastewater, secondary effluent, and reclaimed water. Lower concentrations of bacteriophages were detected in reclaimed water but they were still detectable. The authors indicated that monitoring for bacteriophages was low cost and relatively simple to perform, so bacteriophage quantification was encouraged for MST of a variety of water types.
Purnell et al. (2020)	Southeast England	QMRA	Wastewater effluent, without disinfection	Adenovirus, *Salmonella*, *Cryptosporidium*	The authors indicate that advanced treatment to remove adenovirus, S*almonella,* and *Cryptosporidium* would be required to avoid further deterioration of water quality at the studied river sites. The QMRA model results demonstrated the potential for pathogen reduction through augmentation with reclaimed water in rivers heavily impacted by de facto reuse practices.
Rachmadi et al. (2016)	NA – review article	MST	Human	NA	Human polyomaviruses were human-associated and concentrations did not vary seasonally. In addition, the density of human polyomaviruses correlated positively with the density of other enteric viruses in water samples. While there were some limitations involved (human pathogenicity, dsDNA, and persistence in the environment), the authors suggested that human polyomaviruses were a useful tool for MST of human fecal contamination in water.
Raj et al. (2020)	Ghana	Children	Human	*E. coli*	The SaniPath Exposure Assessment Tool is an open source online tool (https://tool.sanipath.org/) where users enter behavioral survey data and *E. coli* quantification data for up to up to nine pathways (drinking water, bathing water, surface water, ocean water, open drains, floodwater, raw produce, street food, and public or shared toilets). The tool uses Bayesian analyses to estimate the percentage of the population exposed and the mean dose of fecal exposure for children and adults. The tool provides visual representations of the output. The SaniPath Tool supports public health evidence-based

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
					decision-making for urban sanitation policies and investments.
Reses et al. (2018)	Minnesota and Colorado, USA	Epi/case-control	Not Reported, suggested human and animal fecal contamination	NA	This study provides evidence for transmission of *Giardia* via contaminated water or food. Drinking water from a river, lake, stream, or spring was identified as a strong risk factor for giardiasis. Waterborne outbreaks and sporadic cases of giardiasis have been previously associated with the consumption of untreated water from natural bodies of water including streams and rivers.
Reyneke et al. (2017)	South Africa and Namibia	Method development (qPCR)	Not reported	*L. pneumophila, S. typhimurium, P. aeruginosa*	This study optimized ethidium monoazide (EMA) and propidium monoazide (PMA) concentrations for pretreatment of qPCR reactions. Water samples spiked with five waterborne bacterial pathogens were used. 6 uM EMA and 50 uM PMA were identified as the optimal dye concentrations for the rapid identification and quantification of multiple intact opportunistic pathogens in ambient water.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Rocha et al. (2019)	USA	Method development (culture, qPCR)	Human	Total coliforms, fecal coliforms, enterococci, *E. coli*, markers: gadAB, uidA, blaCTX-M, and intI1	This study evaluated whether antibiotic resistance genes could be used as measures of FIB. Total coliforms, fecal coliforms, and enterococci were quantified by culture methods and enterococci (16S rRNA and 23S rRNA) and *E. coli* (uidA and gadAB) by qPCR along with antibiotic resistance genes sul1, sul2, tet(A), tet(O), bla-OXA-1, bla-CTX-M, and intl1. Regression analyses comparing the abundance values of cultivable bacteria and gadAB, uidA, blaCTX-M, and intI1 indicated that these genes may be suitable biomarkers for total coliforms, beta-lactam resistant subpopulations, and their possible role in the dissemination of antibiotic resistance in the environment.
Rosiles-González et al. (2019)	Mexico	Health Study	Not Reported	Somatic and MSC, norovirus strains GI and GII, and human adenovirus	This study provided a baseline of the distribution, concentrations, and diversity of noroviruses and human adenoviruses in aquifers from Mexico. During rainy season, 70%–80% of the samples contained somatic coliphages. MSC were detected in 80% of the samples. During the dry season, 60% of samples had detectable somatic coliphages using *E. coli* bacterial strains. MSC were detected in 30% of the samples during the dry season. The highest concentration of somatic and MSC was observed during the rainy season at site 2 which was a freshwater sinkhole located in Cancun. The enteric viruses tested were found to be more prevalent during the rainy season and during the rainy season 70% of the sampling sited resulted in detection of at least one type of virus. This is compared to only 40% during the dry season. Overall, norovirus was more frequently detected than human adenoviruses.
Rothenheber et al. (2018)	Wells, Maine, USA	MST	Human, animal (gull, dogs, birds, ruminants)	Enterococci	Elevated enterococcal levels were reflective of a combination of increased fecal source input, environmental sources, and environmental conditions. Enterococci densities in the estuarine and marine waters were strongly influenced by particle-associated enterococci and mammalian fecal sources. For freshwater systems, sediments acted as a reservoir for enterococci.

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					For these reasons, the authors suggested using an encompassing approach to MST.
Rudko et al. (2020)	Michigan, USA	Method development (qPCR)	Human, animal	18S avian schistosomes, toxigenic (mcyE gene) cyanobacteria, HF183	Community-based monitoring (CBM) of freshwater for avian schistosomes (18S), toxigenic cyanobacteria (mcyE gene), and HF183 *Bacteroides* was compared to laboratory performed qPCR for the same samples. CBM partners were provided with training video and a written protocol detailing all steps from DNA extraction to results analysis. In addition, two in-person trainings were provided. Water samples were collected in Michigan and portions were shipped to Alberta for secondary laboratory analysis for comparisons. CBM partners ran 985 qPCR samples over 2 years. The field equipment, Open qPCR thermocycler, has a higher limit of detection than the laboratory Applied Biosystems (ABI) thermocyclers. Although HF183 was detected in 54/237 samples by the lab and only detected in one field assay, the level of HF183 was between 15-35 copies/5 µL. This level is below the HF183 gene copy number (210 GC/5 µL) that would exceed the EPA benchmark for GI illness. The authors concluded that the detection limit of the Open qPCR thermocycler is sufficient for potential outbreak scenarios but may not be sufficiently low for detection of leaking septic or other source tracking efforts. The authors indicated that it is important to work with the CBM partners to understand their specific monitoring questions and critically appraise if CBM qPCR is capable and appropriate to answer the questions.

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Saeidi et al. (2018)	Singapore	MST (RT-qPCR)	Human	PMMoV	PMMoV was characterized using RT-qPCR and viral metagenomics at seven freshwater waterbody locations with varied land use in Singapore between 2014–2016. They found a correlation between PMMoV and urban land use weightage (rho=.728, $p < 0.01$) in comparison to FIB (rho = 0.583; $p < 0.01$). The authors also observed a correlation between their PMMoV qPCR data and viral metagenomics data ($0.588 < rho < 0.879$; $p < 0.01$), suggesting that viral metagenomics could be used to estimate specific microbial indicators during water quality monitoring.
Sagarduy et al. (2019)	France, South Atlantic Coast	Method development (reverse transcription qPCR)	Human	*E. coli*, enterococci	Microbial decay experiments were conducted in controlled microcosms and in situ transparent dialysis bags in sea water, estuarine water, and freshwater locations along the South Atlantic Coast of France. The decay of sewage-sourced enterococci and *E. coli* culturable cells and their associated molecular markers (16S rRNA) was quantified by reverse transcription qPCR. Decay rate models were also developed based on the laboratory experiments. The decay rate model predicted quantification compared reasonably well with the in situ observed quantification. Sunlight had the largest influence on the culturable bacteria although it did not influence decay of the genetic markers.
Saingam et al. (2018)	Hawaii, USA	Method development		*invA* gene of *Salmonella*	False-negative PCR and qPCR reactions were created using serial dilutions of laboratory-prepared *Salmonella* genomic DNA and then analyzed directly by NGS. NGS was able to detect invA sequences in false-negative PCR and qPCR reactions. The ability of PCR-NGS to identify false negatives was confirmed under more environmentally relevant conditions using *Salmonella*-spiked stream water and sediment samples. The PCR-NGS approach was applied to 10 urban stream water samples and detected invA sequences in eight samples that would be otherwise deemed *Salmonella* negative using PCR or qPCR.

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Schang et al. (2016)	Australia	Method development (culture, qPCR)	Human	*E. coli*, coliforms	IDEXX methods for enumeration of *E. coli* and coliform densities were compared with the results of three alternative approaches for monitoring fecal indicator organisms: the TECTA™ system, U.S. EPA Method 1611 (a qPCR method for enumerating enterococci), and next generation sequencing (NGS) in surface waters. When comparing methods, the authors considered cost and analysis time in addition to how the enumeration results were correlated. All of the alternative approaches produced statistically significant correlations with IDEXX results, but there were differences in cost and time. U.S. EPA Method 1611 had significant disadvantages such as highly technical analysis and higher operational costs (330% of IDEXX) and even higher costs (3,000% of IDEXX) and analysis time (300% of IDEXX) were found for NGS.
Schets et al. (2018)	The Netherlands	Outbreaks	Human	Indicator: *E. coli*. Pathogens: Norovirus, *Campylobacter*	A recreational water-associated outbreak of norovirus infections affecting at least 100 people in the Netherlands occurred in August 2012. Patients, mostly children, became ill with gastroenteritis 1–6 days (median 2 days) after exposure. The results of this investigation, combined with detection of norovirus GI in stool samples of patients and sandy samples collected at the lake, suggested that exposure to the recreational lake resulted in the outbreak. The most likely source of contamination was determined to be an infected human and the authors concluded that active communication about human shedding of viruses during and after diarrheal is needed along with guidance to refrain from swimming when contamination is suspected.
Schets et al. (2011)	The Netherlands	Children	NA	NA	This study evaluated 742 bathing related outbreaks (between 1991–2007) involving 5,623 patients at all 641 official bathing sites in the Netherlands. Health endpoints included gastroenteritis, skin, ear, eye, leptospirosis, and other. This paper indicated that skin conditions and gastroenteritis were the two most common health

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
					endpoints in outbreaks in the Netherlands between 1991 and 2007.
Schoen et al. (2020)	United States	QMRA	human point source	crAssphage (CPQ_056), *Bacteroides* spp. (HF183/BacR287 and HumM2), and human polyomavirus (HPyV)	The QMRA predictions in this study provided useful information to compare potential indicators and to explore different recreational water quality monitoring scenarios based on a series of assumptions and uncertainties. The methods used in this work can be used to establish risk-based thresholds for alternative indicators.
Seidel et al. (2017)	North Rhine-Westphalia and Lower Saxony, Germany	MST (qPCR); Method development	Human, cow, dog, pig, deer, badger	Markers: AllBac, HF183, BacCow	This study targeted host-specific markers in *Bacteroidales* bacteria for subgroups associated with human and ruminant fecal matter. Two amplicon sizes were compared and applied to water samples from three different freshwater sites in western Germany. The proportion of intact cells dropped by up to 38% when the longer sequence was targeted. The B-57addition of dimethyl sulfoxide (DMSO) improved the efficiency of PMA treatment. The authors concluded that the comparison of signal decay by qPCR reactions targeting sequences of different lengths might present a methodological tool to shed more light on the role of DNA disintegration and DNA damage in MST signal decay.

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Seruge et al. (2019)	Island of O'ahu, Hawaii, USA	Method development (culture, qPCR)	Human	Enterococci, *Clostridium perfringens*	Culture (EPA Method 1600 and Enterolert) and qPCR (EPA Method 1611) methods were tested at beaches and canals in Hawaii. Method 1611 was compromised due to coralline sand which interfered with DNA extraction. Sample process controls indicated that the PCR reaction was not inhibited. In addition to enterococci, the study also assessed *Clostridium perfringens* enumerated using culture methods. There was good agreement among methods for beach management decisions when qPCR samples were not compromised. The authors concluded that samples for EPA Method 1611 should not be collected close to shore where particles are suspended or there are visible milky plumes.
Shanks et al. (2016)*	Ohio, USA	MST (qPCR); Method development	Human	HF183/BacR287, HumM2	This study specified conditions for data acceptance derived from a multiple laboratory data set using standardized procedures for human-associated fecal source identification quantitative real-time PCR. The validity of qPCR data acceptance criteria for measurement of genetic marker concentrations in reference DNA material and freshwater sources using standardized HF183/BacR287 and HumM2 qPCR protocols was assessed. Lab-to-lab, replicate testing within a lab, and random error for amplification inhibition and sample processing controls were specified. Other data acceptance measurements included extraneous DNA contamination assessments and calibration model performance (correlation coefficient, amplification efficiency, and lower limit of quantification). This work is important for the transition of qPCR methods from a research tool into a standardized protocol with a high degree of confidence in data quality across laboratories.

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Sheth et al. (2016)	Door County, Wisconsin, USA	Method development (culture, qPCR)	Human, animal	*E. coli*, enterococci	Concentrations of *Enterococcus* in samples collected along freshwater beaches along Lake Michigan in Door County, WI were evaluated using qPCR and compared with *E. coli* detected using culture-based methods. The most differences were seen for qPCR *E. coli* compared to *E. coli* culture-based beach notification decisions. Detection of numbers of enterococci were closer for Enterolert and *Enterococcus* qPCR (EntTaq), but there was still disagreement of numbers between both methods. The authors suggested studying what conditions might affect the difference in numbers of enterococci and *E. coli* between Colilert/Enterolert and qPCR, including ambient conditions and sanitary surveys. The authors noted that molecular techniques such as qPCR can be done successfully in small laboratories with personnel with minimal molecular biology training but cautioned that each beach community should consider the impact of utilizing rapid methods for beach management decisions prior to adoption.
Shoults et al. (2021)	Borden Park, Edmonton, Canada	QMRA	Human non-point source (accidental fecal contamination)	Spiked water with MS2 bacteriophage, *E. faecalis*, and baker's yeast surrogates and *E. coli*. Reference pathogens: norovirus, C*ampylobacter*, *Cryptosporidium*, *Giardia*	A reverse QMRA was conducted for a natural swimming pool. Of the four reference pathogens used (*norovirus, Campylobacter, Cryptosporidium*, and *Giardia*), only norovirus exceeded the median risk benchmark. *Campylobacter* was the only other reference pathogen that exceeded the risk benchmark at the estimated 95th percentile.

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Shrestha and Dorevitch (2019)	Lake Michigan, Chicago, Illinoi, USA	Method development (culture, qPCR)	Not reported	*E. coli*, enterococci	EPA draft Method C (*E. coli* qPCR) was compared to *E. coli* culture (Colilert) and *Enterococcus* qPCR (EPA Method 1609.1) in 288 water samples collected from eight of Chicago's Lake Michigan beaches. Method C demonstrated acceptable performance characteristics, but was prone to low level DNA contamination, possibly originating from assay reagents derived from *E. coli* bacteria. The study found that lag *E. coli* culture results, e.g., samples collected Monday with results available Tuesday, were not associated with either *E. coli* culture results ($r = 0.12$; $p = 0.08$) or *Enterococcus* qPCR results ($r = 0.09$; $p = 0.17$) for Tuesday. This indicates that *E. coli* culture results available to a beach manager on a given day (from samples cultured the prior day) are not predictive of current water quality. In contrast, Method C was most strongly statistically significantly associated with same-day Colilert® results ($r = 0.83$; $p < 0.0001$). And, in addition, statistically significant correlations were also observed between *Enterococcus* qPCR and Method C and between *Enterococcus* qPCR and same-day Colilert® results (both $r = 0.67$, $p < 0.0001$). The study also utilized two different approaches for estimating potential *E. coli* qPCR BAV thresholds: 1) EPA's Site-Specific Alternative Recreational Criteria Technical Support Materials for Alternative Indicators and Methods (Alternative Methods TSM) and 2) a receiver operating characteristic (ROC) analysis. Potential BAV thresholds differed substantially, ranging from 200.9 calibrator cell equivalents (CCE)/100 mL (ROC analysis, *Enterococcus* qPCR BAV as the reference) to 1,000 CCE/100 mL (Alternative Methods TSM analysis, *Enterococcus* qPCR BAV as the reference). The authors suggest that ROC analysis, which optimizes sensitivity and specificity and generates a dichotomous cut point should be given consideration for generating BAVs, rather than relying on the linear relationship between the two sets of continuous

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
					variables as is currently provided by the Alternative Methods TSM.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Shrestha and Dorevitch (2020)	Great Lakes, USA	Methods (qPCR)	NA	*Enterococcus*	This article describes a qPCR-based beach monitoring program in which a central laboratory tested water samples from up to 20 beaches per day, seven days per week, and reported the results to the public by noon.
Sips et al. (2020)	The Netherlands	Children; Outbreaks	Human, waterfowl	*E. coli,* enterococci, cyanobacteria, Norovirus GI and GII	A norovirus outbreak in a natural playground in the Netherlands was investigated. The Public Health Service received 21 case notifications via the national online reporting system and an additional 100 cases (mostly children) were identified following an online query of the local health-related Facebook platform. This approach indicated that public health monitoring could serve as an early warning system. Water samples and fecal samples from humans and birds were tested. The results indicated that human introduction of norovirus was the most likely cause of the outbreak. Although waterfowl feces could have contributed to the outbreak, no evidence for transmission via waterfowl was found.
Sivaganesan et al. (2019)	NA – spiked samples	Method development (qPCR)	Not reported	*E. coli*	This study included 21 laboratories that evaluated standardized water samples, to determine data quality acceptance criteria values for laboratories wishing to establish their own comparable laboratory-specific criteria for Draft EPA Method C or other qPCR methods used in monitoring programs. After evaluating a total of 18 shared surface water samples, including ambient, low spike, and high spike for six different locations, the quality acceptance criteria for Draft EPA Method C method resulted in a 24% failure rate. The two newest laboratories included in the study were responsible for 39% of the failures. It was determined that the quality acceptance failures were due to inconsistencies in storage and preparation of reference materials. Variability between laboratories was the greatest contributor to overall method variability. The study concluded that it is technical feasible for multiple laboratories to implement Draft Method C or other qPCR water quality monitoring methods with similar data quality acceptance criteria, but

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
					additional practice and/or assistance may be valuable, even for some more generally experienced qPCR laboratories.
Soller et al. (2016)	Puerto Rico	QMRA	WWTP and recreators	Adenovirus (culture), Enterovirus (culture), *Cryptosporidium Giardia, Salmonella enterica, E. coli* 0157:H7 (qPCR)	A QMRA was conducted to estimate the GI Illness at Boquerón Bay, Puerto Rico to improve interpretation of a recreational water epidemiological study. The QMRA estimated an event risk of two illnesses per 1,000 recreation events, which was below the level that the epidemiological study was designed to detect. The QMRA findings provided a feasible explanation for the lack of relationship between fecal indicator organisms and swimming-related illness during the study.
Soller et al. (2017)	California, USA	QMRA	Urban river discharges	Norovirus, Adenovirus, *Campylobacter jejuni, Salmonella enterica*	QMRA was used to predict the GI illness risk to surfers at marine beaches in Southern California impacted by urban stormwater. The predicted GI illness was dominated by norovirus risk and varied between 0.6 and 36.0 illnesses per 1,000 recreators, depending on the norovirus dose-response selected. The QMRA results were in broad agreement with epidemiological results from the same location.
Somnark et al. (2018)	Thailand	MST (PCR)	Human, cattle, swine	*Bacteroidales* (BacUni EP, HF183/BFDrev EP, Pig-2-Bac EP, Bac3)	The performance of modified endpoint PCR assays in detecting *Bacteroidales* in agricultural watersheds in central Thailand was evaluated. The BacUni EP, HF183/BFDrev EP,Pig-2-Bac EP, and Bac3 assays demonstrated potential for MST of general and human-, swine-, and cattle-derived fecal pollution.
Staley and Edge (2016)	Toronto, Canada	MST (culture, qPCR, PCR)	Human, ruminant, gull, dog	*E. coli*, MST markers: genBac, Bac32, HF183, CowM2, DG37, Gull2, qGull4, CF128	MST markers for human, cow, gull, and dog were used to identify sources of fecal contamination at beaches in the Toronto region. They found that the human (HF183), cow (CowM2), and dog (DG37) markers had good host-specificity, but the gull markers (Gull2 and qGull4) and ruminant endpoint PCR marker (CF128) amplified other species.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Staley et al. (2016)	Toronto, Canada	MST (culture, qPCR)	human, ruminant, gull, dog	*E. coli*, MST markers: Bac32, GenBactF3, HF183, CF128, CowM2, Gull2, DH37, DG37	*E. coli* and ampicillin resistant *E. coli* as well as MST markers for general *Bacteroidales* spp. (Bac32, GenBactF3), human (HF183), ruminant/cow (CF128, CowM2), gull (Gull2), and dog (DH37, DG37) were assessed in the Humber River and one of its tributaries in Toronto, Canada. Human and gull fecal source markers were detected at all sampled sites. The authors also observed a higher correlation between the presence of ampicillin resistant *E. coli* and human qPCR marker than culturable *E. coli*, suggesting that ampicillin resistant *E. coli* might act as a better indicator of human sewage contamination in this watershed.
Staley et al. (2018a)	Toronto, Canada	MST (culture, qPCR)	Human, gull	*E. coli*, HF183, qGull4	MST, chemical source tracking (CST), and environmental DNA (eDNA) sequencing were used to confirm that storm water was an important source of human fecal contamination. However, the authors indicated that MST-qPCR methods have limitations in that each individual assay can only detect a singular known target. The authors concluded that eDNA could be a good supplement to MST-qPCR and CST for profiling sources of fecal contamination.
Staley et al. (2018b)	Great Lakes, Canada	MST (dPCR, qPCR); Method development	Human, gull	HF183, qGull4	qPCR for human HF183 marker and the gull qGull4 marker were compared to digitalPCR duplexed to assess both markers at the same time. The authors found that the dPCR multiplex assay was more sensitive and capable of detecting fecal contamination than was undetected by qPCR assays. The cost per multiplexed dPCR was equivalent to the cost of running single-target qPCRs for two targets, making dPCR a cost-effective alternative to qPCR.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Stokdyk et al. (2016)	NA – spiked samples	Method development (qPCR)	Not reported	*Salmonella*, adenovirus, poliovirus	This study developed an approach for determining qPCR LOD. The approach limited the number of analyses required and was amenable to testing multiple genetic targets simultaneously (i.e., spiking a single sample with multiple microorganisms). An LOD determined this way can facilitate study design, guide the number of required technical replicates, aid method evaluation, and inform data interpretation.
Sunger et al. (2019)	United States, hypothetical freshwater beach	QMRA	Treated wastewater effluent	Adenovirus, Norovirus, *Cryptosporidium, Giardia, Salmonella, Campylobacter jejuni*	QMRA and literature values of pathogen densities in raw sewage were used to simulate the risk of illness from swimming in freshwater impacted by secondarily treated wastewater effluent aged 1 day. The estimated average total risk due to the reference pathogens was similar to FIB-based estimates and was dominated by viral risk. The risk evaluated captured the likelihood of gastrointestinal illnesses only and did not address the overall health risk potential of recreational waters with respect to other disease endpoints.
Symonds et al. (2016)	Miami-area, Florida, USA	MST (qPCR, RT-qPCR, culture)	Human (point and non-point sources)	Enterococci, MST markers: BacHum, CowM2, DogBact, HF183, HPyV, PMMoV	Surface water samples were collected from inlets and coastal sites in Florida. Human sources of fecal pollution were detected at all sampling sites following analysis of culturable enterococci and a suite of MST markers (BacHum, CowM2, DogBact, HF183, HPyV, PMMoV) detected using qPCR and RT-qPCR. All sites met the 2012 U.S. EPA RWQC, despite detection of these wastewater-associated MST markers. PMMoV were only correlated with human-associated MST markers, indicating that PMMoV RT-qPCR can act as an efficient human-associated marker and should be included in the MST toolbox.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Symonds et al. (2017)	Gulf of Nacoya, Costa Rica	MST (culture, RT-qPCR)	Human, gull, cow, dog, bird, horse	*E. coli,* enterococci, MST markers: BacCow, BacCan, DogBac, PMMoV, GFD, Gull2, HorseBac, HoF, HF183, HPyV, PF	Surface water samples were collected and analyzed from a shellfish harvesting area in the Gulf of Nacoya, Costa Rica. *E. coli* was enumerated from these samples using culture and RT-qPCR. The authors also evaluated the sensitivity and specificity of 11 MST-PCR assays, associated with cows (BacCow), dogs (BacCan, DogBac), domestic wastewater (PMMoV), general avian (GFD), gulls (Gull2), horses (HorseBac, HoF), humans (HF183, HPyV), and pigs (PF). Human/domestic wastewater-associated markers and low concentrations of FIB were determined using molecular methods, indicating sufficient microbial water quality for shellfish harvesting. The authors found that *E. coli* was always detected using MPN methods, and that *E. coli* and Entero1a were only detected by qPCR in 81% and 41% of the samples, respectively.
Symonds et al. (2018)	USA	MST	NA	PMMoV	This literature review discusses PMMoV as an indicator for fecal MST. The review determined that there are needs for new indicators closely related to enteric viruses that could replace or supplement current monitoring tools. According to the authors, PMMoV is a proven domestic wastewater tracer and a promising indicator and index virus for enteric viruses which makes PMMoV a good candidate for efficient molecular-based microbial water quality monitoring.
Thulsiraj et al. (2017)	Tijuana, Mexico	MST (qPCR)	Human, gull, dog, horse	MST markers: HF183, Gu112, DogBac, HoF597	Propidium monoazide (PMA) was used to inhibit amplification of DNA from dead cells during PCR. The modified MST approach was used to identify fecal contamination from humans, dogs, horses, and gulls in freshwater creek and coastal seawater. The technique was successful in inhibiting amplification of dead cells' DNA in freshwater that receives treated wastewater effluent. Horse- and gull-associated markers were detected in 4% and 8% of samples tested, respectively. The human- and dog-associated markers were positive in 74% and 63% of watershed samples and 92% and 75% of storm drain samples, respectively.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Toubiana et al. (2021)	Marseille, France	MST (qPCR)	Human, gull, dog, horse	*Catellicoccus marimammalium*, MST markers: HF183, DF475, HoF597	Seawater samples were collected from a busy beach at Marseille, France and MST was used to confirm that humans were the main fecal source, while horses, dogs, and gulls have little contribution to fecal pollution at the study site. The authors indicated that high attendance at beaches is an important factor to be considered to prevent public health risks.
Unno et al. (2018)	NA – review article	MST (qPCR, next generation sequencing); Method development	NA – review article	NA	This review summarized the use of qPCR as a traditional MST method and discussed the potential of next generation sequencing (NGS)-based MST approaches. One NGS method defines operational taxonomic units (OTUs) that are specific to a fecal source, (e.g., humans and animals or shared among multiple fecal sources) to determine the magnitude and likely source of fecal pollution. The other method uses SourceTracker, a program using a Bayesian algorithm, to determine which OTUs have contributed to an environmental community based on the composition of microbial communities in multiple fecal sources. The geographical variability of the fecal material should be taken into account when using SourceTracker. For example, fecal sources from Florida could not be identified when tested against libraries from Minnesota, California, and Australia. However, including sources from other geographic regions in the library did not compromise the detection of sources in spiked samples once the native sources were included in the source library.
Urban et al. (2021)	Cambridge, United Kingdom	Method development (16S rRNA sequencing)	NA	Not specified	This study sequenced water samples from the River Cam in Cambridge, UK. The study indicates that portable nanopore sequencing technology is simple, fast, and cost-effective. The study complemented DNA analyses with physicochemical measurements to depict the hydrological core microbiome and fine temporal gradients.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Vadde et al. (2019a)	Tiaoxi River and Taihu watershed, China	MST (next generation sequencing); Method development	Human, chicken, cow, dog, duck, goose, pig	*Bacteroides, Prevotella, Blautia, Faecalibacterium, Dorea, Acinetobacter, Aeromonas, Arcobacter, Brevundimonas, Enterococcus, Escherichia-Shigella, Streptococcus*	The microbial population of the Tiaoxi River water was characterized using next generation sequencing (NGS) paying particular attention to detection of bacteria of fecal origin. For water samples (n = 45), a total of 39,192 OTUs were generated ranging from 564 to 1292 OTUs. The Ribosomal Database Project classifier categorized all the OTUs of water into 20 bacterial phyla, however, their relative abundance varied with the type of sample. NGS was shown to be useful for preliminary investigations of water to detect bacteria of fecal origin including potential pathogens. Shared OTUs between water samples and chicken, pig, and human fecal samples ranged from 4.5% to 9.8% indicating the presence of avian, pig, and human fecal contamination in the Tiaoxi River. The authors suggested that *Faecalibacterium* could be used as a human-associated target for MST using qPCR. NGS was shown to be useful for preliminary investigations of water to detect bacteria of fecal origin including potential pathogens. NGS could be used in conjunction with other methods for MST including qPCR.
Vadde et al. (2019b)	Tiahu Lake, Tiaoxi River, and Changxing River, China	MST (qPCR)	Human, pig, chicken, cow, duck, goose, industrial	*E. coli, Bacteroidales, C. jejuni, Leptospira* spp., *Shigella*, MST markers: acUni, GenBac, BacHum, HF183, Pig-2-Bac, GFD, AV4143, mapA, LipL32, ipaH, stx2, eae	The use of MST for fecal contamination was evaluated in the Taihu watershed which was impacted by human, animal, and industrial pollution. The study indicated that BacUni, HF183, Pig-2-Bac, and GFD markers as detected by qPCR were best at identifying the source of contamination and differentiating between human and fecal contamination. Five bacterial pathogens were monitored. Of these pathogens, *Campylobacter jejuni* was found in avian fecal contamination, *Shigella* in human fecal contamination, and *E. coli* (STEC) in either human or pig fecal contamination. These pathogen results correlated with the fecal marker data obtained using qPCR.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Van Abel et al. (2017)	Gauteng province, South Africa	QMRA	Not reported	norovirus	A QMRA was conducted to estimate risk of swimming, boating, and playing in river waters in South Africa using norovirus as reference hazard. Risks varied based on recreational use (swimming, boating, or playing) and on the dose-response model selected for the modeling.
Vergara et al. (2016)	Singapore	QMRA	Urban non-point sources	norovirus, adenovirus	A QMRA was conducted to estimate the GI illness risk from primary and secondary contact recreation in an urban catchment in Singapore using field measurements of norovirus and human adenovirus. The higher prevalence and illness risk of norovirus supported the use of norovirus as a reference pathogen for QMRA in recreational waters in Singapore.
Verhougstraete et al. (2020)	Sao Paulo, Brazil	QMRA; Children	Poorly treated sewage	enterococci	Adjusted risk difference models (excess gastrointestinal illness per swimming event) were determined for children (<10 years of age) and non-children (≥10 years of age) across five Brazil beaches using epidemiological data collected in the UK and Brazil. The risk models indicated that children in Brazil have twice the risk of gastrointestinal illness than non-children. Elevated enterococci levels resulted in an excess of 96 cases of NGI cases per 1,000 swimming children. Enterococci levels are higher in Brazil than in the primary UK study (Kay et al., 1994) that informed WHO beach guideline values. The study found that distinct enteric pathogen profiles exist in tropical settings as well as in settings with minimal wastewater treatment, highlighting the importance of regionally specific guideline development.
Vincent-Hubert et al. (2021)	France	Method development (qPCR)	Human, animal	Viruses, bacteria	The authors used passive water sampling and qPCR to detect viruses and bacteria from two sites in an estuary of the French Atlantic coast. The method allowed for detection of microorganisms at low or variable concentrations, and observation of the seasonality of microorganisms.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Vital et al. (2017)	Vietnam	Method development (qPCR)	Human, animal	*E. coli*	*E. coli* enumerated by qPCR in urban canal samples were significantly lower than *E. coli* enumerated by conventional culture methods for the wet season. The authors conclude that qPCR may be impacted by sources of runoff based on surveyed samples.
Wade et al. (2018)	Puerto Rico	Epi/cohort study	Treated wastewater, recreators, diffuse sources	norovirus GI.1 (Norwalk virus) and GII.4 (VA387 variant)	Saliva samples were collected as part of the U.S. EPA Boquerón Beach epidemiology study to tested for IgG responses to norovirus GI.1 and GII.4. There was a strong association between swimming and asymptomatic norovirus infection. The findings implicate wastewater impacted recreational water as potentially important transmission pathway for norovirus infection.
Walker et al. (2017)	England	Method development (qPCR)	NA	*E. coli:* ybbW gene	A new quantitative real-time PCR assay was developed to detect the ybbW gene sequence, which was found to be 100% exclusive and inclusive (specific and sensitive) for *E. coli.* Of the 87 *E. coli* strains tested, 100% were found to be ybbW positive, 94.2% were culture positive, 100% were clpB positive and 98.9% were uidA positive.
Wang et al. (2016)	Northern California, USA	Method development (dPCR, qPCR, culture)	Human, animal	Enterococci	This study used dPCR, qPCR, and culture methods to evaluate chip-based dPCR for quantifying enterococci. The authors took 24 samples from multiple surface waters in California (marine and freshwater) and observed a consistent quantification capability in dPCR compared to qPCR. They also observed that at realistic concentrations, dPCR had lower variability (narrower 95% CI) than qPCR. There were similar levels of inhibition by humic acid, but less inhibition by calcium for dPCR compared to qPCR.
World Health Organization 2018	EU	Review	NA	Enterococci and *E. coli*	The European Union Bathing Water Directive (2006/7/EC) was reviewed, and it was recommended that the two indicators in use (enterococci and *E. coli*) as well as the four levels within the current classification system (excellent, good, sufficient, and poor) should be retained.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Wu et al. (2017)	Cuihu Lake in Kunming, China	MST (culture, qPCR)	Human, birds, cat, rabbit, pig, goat, horse, dog, squirrel, hedgehog	*Catellicoccus marimamalium, E. coli, Bacteroidales, Campylobacter* spp., *Helicobacter*	A novel genetic marker targeting *Catellicoccus marimamalium* was tested for MST of seagull fecal contamination. Higher levels of the *C. marimamalium* marker correlated with overwintering of seagulls at Cuihu Lake, China. Levels of *C. marimamalium* dropped when seagulls were not present, but a detectable amount of the marker remained after seagull migration. An MST scheme involving the *C. marimamalium* marker, detected using qPCR, with human-associated *Bacteroidales* and FIB could be useful for assessing potential human health risks.
Wu et al. (2020)	NA	QMRA/MST (qPCR)	raw sewage, secondary treated effluent WWTP, cattle, seagulls	Markers: *Catellicoccus* assay, BacCow, HF183, *Enterococcus*. Pathogens: norovirus, adenovirus (40/41), *Campylobacter, Salmonella, E. coli* O157:H7, *Cryptosporidium, Giardia*	Risk-based thresholds (RBT) (corresponding with 36 illnesses per 1,000 swimmers) were determined for a hypothetical waterbody contaminated by a continuous loading of both human and non-human fecal contamination. Results indicated that a larger difference in decay rates between pathogen and indicator leads to an increased miscalculation of gastroenteritis risk compared to calculations that do not account for differential decay. Given the continuous loading scenario, the difference in RBT between fresh and aged contamination was less for human marker HF183 in the human contamination scenarios than for animal markers in the animal contamination scenarios. The median RBT for human contamination was roughly 3.5 to 3.7 $\log_{10}$ gene copies/100 mL for HF183.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Wymer et al. (2021)	Four Great Lakes and three marine beaches (NEEAR study)	Method development (qPCR, culture)	one or more sources of treated sewage effluents which were generally in compliance with local and federal water quality guidelines	enterococci	The variability of enterococci measured by culture and qPCR was assessed for the NEEAR beaches. The 27-hour variance of changes in CFU was significantly higher than the three-hour variance of changes in CCE. This illustrated the inherent magnitude of uncertainty in predictability of next-day CFU results compared to a faster measurement method (qPCR). The authors concluded that even if it were possible to obtain culture results within 3 hours, as it is for qPCR, CFUs would be a less reliable predictor of conditions later in the day than would be CCE. The levels of the molecular indicator were more consistent throughout the day between 8:00 am and 3:00 pm.
Xie et al. (2017)	Baltic Sea, India, United Kingdom, USA, Israel, China, Portugal, Poland, South Africa	Method development (qPCR)	NA – review article	*Salmonella, Vibrio, Staphylococcus, Candida,* Hepatitis A virus, among other targets	This study reviewed multiple techniques to detect microbial contamination in coastal waters, including qPCR. The authors discussed interference on the success of qPCR and the importance of having high-quality template for qPCR assays.
Xue et al. (2018)	Lake Pontchartrain, Louisiana, USA	MST (culture, qPCR)	Human, cow	*E. coli,* total coliforms, *Enterococcus*, MST markers: HF183, CowM3	Surface water samples from Lake Pontchartrain, Louisiana were evaluated to identify sources of fecal contamination. Fecal indicator bacteria (i.e., *E. coli*, total coliforms, and *Enterococcus*) were measured using culture and qPCR-based assays and the genetic markers HF183 and CowM3 were enumerated using qPCR. *E. coli* was detected in 90.6% (culture) and 97.5% (qPCR) of water samples. *Enterococcus* was detected in 95.8% (culture) and 91.8% (qPCR) of water samples. The relationship between *E. coli* and *Enterococcus* results observed for both qPCR and culture methods was statistically significant. The HF183 marker was detected in 94.3% of water samples (149 of 158). Daily precipitation levels were graphed with microbial results and the authors concluded that rainfall events might introduce large amounts of fecal bacteria into the lake.

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Xue et al. (2019)	Alabama, USA	MST (qPCR)	Cow	CowM2, CowM3	Two cattle-associated *Bacteroidales* fecal markers, CowM2 and CowM3, were used to assess MST in ambient water. CowM3 had a lower limit of detection (LLOD) and lower limit of quantification (LLOQ) than CowM2, suggesting CowM3 was a more reliable marker to use for MST. The method developed was useful in MST studies and standardizing comparison of different labs' results.
Young 2016	Epi/Review	Review	various	NA	This study reviewed epidemiological evidence for the association between marine bathing and infectious disease. Numerous studies demonstrated an increased risk of gastrointestinal illness associated with marine swimming compared to non-swimming. An association between levels of FIB and illness among swimmers was found in some studies while other studies indicated that traditional FIB may not be predictive of human health impacts when human waste is not the predominant source of pathogens.
Yuan et al. (2018)	Missouri, USA	Method development (qPCR)	NA – spiked samples	*E. coli*	Freshwater samples were collected from multiple sources around Missouri and spiked with fecal *E. coli* strains. Prior to qPCR, the spiked samples were treated with propidium monoazide (PMA), a dye that can inhibit the amplification of DNA from dead cells, allowing for the detection and quantification of only viable cells present in the sample. The authors reported no significant differences among the PMA-qPCR assay and two other standard culture-based methods for detection of viable *E. coli* (including EPA Method 1603).

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Zeki et al. (2021)	Istanbul, Turkey	MST (culture, qPCR)	Human	Fecal coliforms, enterococci, *Bacteroides* (BT-a)	An MST study was conducted on an urban estuary, Golden Horn, Istanbul, Turkey, which was subject to decades of industrial and urban pollution and then years of rehabilitation. The study compared membrane filtration results to qPCR results, and both methods confirmed that despite rehabilitation of the estuary, there was still sewage intrusion. The *Bacteroides* marker (BT-a) was detected in 30% of the samples and had a positive correlation with culture and qPCR-based enterococci concentrations.
Zhang and Ishii (2018)	Sapporo, Japan, Minneapolis, Minnesota, USA	Method development (qPCR)	NA	*Pseudogulbenkiania* spp., *E. coli* O157:H7, *S. Typhimurium, Campylobacter jejuni, Listeria monocytogenes*	A novel sample process control (SPC) strain was created by genetically engineering a *Pseudogulbenkiania* sp. strain. And a new TaqMan qPCR assay was developed to quantify the strain. Environmental water samples were co-spiked with four different pathogens, including *E. coli* O157:H7, and the SPC strain. The estimated pathogen densities were not significantly different from the inoculated pathogen densities. This approach could be used to quantify FIB, multiple pathogens, and the SPC strain simultaneously in high throughput in environmental water samples.
Zhang et al. (2020)	China	MST (qPCR)	Studied fecal samples collected from humans and various animals	BacH, BacHum, SYBR-HF183, Hum2, Hum163, CPQ_056, CPQ_064; Pig-q-Bac, Pig-2-Bac, Lamylovorus, P.ND5, Rum-2-Bac, Bac708, BacCow, GFD	The sensitivity, specificity, and magnitude of a variety of human, ruminant, and poultry MST markers in fecal samples in China was tested. The authors found evidence that a higher mean concentration of markers increased the likelihood of observing true positive signals for target hosts (above the limit of detection). *Bacteroidales* markers exhibited greater sensitivity and higher concentrations compared to other bacterial/viral markers, but had low specificity; thus, multiple markers might be necessary.
Zimmer-Faust et al. (2018)	Northwestern Baja California, Mexico	MST (culture, qPCR, Inv-IMS/ATP)	Human, non-human	Enterococci, MST markers: HF183, *B. theta*	An inversely coupled (Inv-IMS/ATP) viability-based assay for rapid evaluation and screening of surface water was developed. There was a high correlation between the human genetic marker, *B. theta*, measured by Inv-IMS/ATP and measurements of this marker and HF183 using qPCR (r=0.76 for HF183, and r=0.82 for *B. theta*).

Study Name (*Denotes EPA Authors)	Location	Study Type (Search Topic)	Contamination Sources	Water Quality Metrics	Summaries
Zimmer-Faust et al. (2021)	Punta Bandera to Silver Strand State Beach, California, USA and Mexico	MST (culture, ddPCR); Method development	Human	Enterococci, MST markers: HF183, dENT, Lachno3	Water samples were collected along the coast of the United States-Mexico border. The authors used culture (Enterolert) and molecular methods (ddPCR for gene quantification, and next generation sequencing for microbial community analysis) as MST tools. A significant gradient of human fecal contamination from the San Antonio de los Buenos wastewater treatment plant was observed. This work increases the understanding of the fate and transport of wastewater treatment plant pollution.

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