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Standardizing Methods with QA/QC Standards for Investigating the Occurrence and Removal of Antibiotic Resistant Bacteria/Antibiotic Resistance Genes (ARB/ARGs) in Surface Water, Wastewater, and Recycled Water

Standardizing Methods with QA/QC Standards for Investigating the Occurrence and Removal of Antibiotic Resistant Bacteria/Antibiotic Resistance Genes (ARB/ARGs) in Surface Water, Wastewater, and Recycled Water

Prepared by:

Krista Liguori, Ishi Keenum, Benjamin Davis, Erin Milligan, Lenwood S. Heath, Amy Pruden
Virginia Tech

Jeanette Calarco, Valerie J. Harwood
University of South Florida

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For more information, contact:
The Water Research Foundation

1199 North Fairfax Street, Suite 900	6666 West Quincy Avenue	
Alexandria, VA 22314-1445	Denver, Colorado 80235-3098	www.waterrf.org
P 571-384-2100	P 303-347-6100	info@waterrf.org

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Research Team

Principal Investigators:

Amy Pruden, PhD
Virginia Tech

Lenwood S. Heath, PhD
Virginia Tech

Valerie J. Harwood, PhD
University of South Florida

Project Team:

Ishi Keenum, PhD
Virginia Tech

Krista Liguori, MSPH
Virginia Tech

Benjamin Davis, PhD
Virginia Tech

Erin Milligan
Virginia Tech

Gabriel Maldonado Rivera
Virginia Tech

Anna Kurowski
Virginia Tech

Jeanette Calarco
University of South Florida

WRF Project Subcommittee or Other Contributors

Anthea Lee, PhD
Metropolitan Water District of Southern California

Ed Topp, PhD
Agriculture and Agri-Food Canada

Emily Garner, PhD
West Virginia University

Jean McLain, PhD
University of Arizona

WRF Staff

John Albert, MPA
Chief Research Officer

Lyndsey Bloxom
Research Program Manager

Julie Minton
Research Program Manager

Erin Swanson
Research Program Manager

Erin Partlan
Research Program Manager

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Acronyms and Abbreviations

ADA	Ampicillin-dextrin agar
AGISAR	Advisory Group on Integrated Surveillance of Antimicrobial Resistance
AMR	Antimicrobial resistance
ANOSIM	Analysis of similarities
APHA	American Public Health Association
ARB	Antibiotic-resistant bacteria
ARG	Antibiotic resistance gene
CARD	Comprehensive antibiotic resistance database
CDC	US Center for Disease Control and Prevention
CECs	Contaminants of emerging concern
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CN	Cetrimide and nalidixic acid (agar)
ddPCR	Droplet digital polymerase chain reaction
DO	Dissolved oxygen
EMBARK	Establishing a monitoring basement for antibiotic resistance in key environments
ESBL	Extended-spectrum beta-lactamase
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FPKM	Fragments per kilobase of transcript per million mapped reads
GC	Gene copies
GSP	Glutamate starch polyethylene
HDPE	High density polyethylene
HRSD	Hampton roads sanitation district
HT-PCR	High throughput polymerase chain reaction
ISO	International Organization for Standardization
JPIAMR	Joint programming initiative on antimicrobial resistance
LEfSE	Linear discriminant analysis effect size
LOD	Limit of detection
LOQ	Limit of quantification
MAGs	Metagenome-assembled genomes
MDR	Multi-drug resistant
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments

MLS	Macrolide-lincosamide-streptogramin
mTEC	Membrane-thermotolerant *Escherichia coli* agar
NARMS	National Antimicrobial Monitoring System
NARS	National Aquatic Resource Survey
ND	Non-detect
NGS	Next-generation sequencing
NMDS	Non-metric multidimensional scaling
NRSA	National monitoring programs of rivers and streams
NTC	Non-template control
ORD	Office of Research and Development
PAC	Project advisory committee
PACCARB	U.S. Presidential advisory committee on combating antibiotic resistant bacteria
PCoA	Principal Coordinate analysis
PCR	Polymerase chain reaction
PI	Principal investigator
QA	Quality assurance
QAPP	Quality assurance project plan
QC	Quality control
qMeta	Quantitative metagenomics
QMRA	Quantitative microbial risk assessment
qPCR	Quantitative polymerase chain reaction
RPKM	Reads per kilobase of transcript, per million mapped reads
SARG	Structured antibiotic resistance gene database
SOP	Standard operating procedure
SWAM	Surface water antimicrobial resistance monitoring
TBX	Tryptone bile X-glucuronide
UK	The United Kingdom of Great Britain
US	The United States of America
USDA	United States Department of Agriculture
USEPA	U.S. Environmental Protection Agency
USF	University of South Florida
VBNC	Viable but nonculturable
VT	Virginia Tech
WARD	Water Antibiotic Resistance Database
WBE	Wastewater-based epidemiology
WGS	Whole genome sequencing
WHO	World Health Organization
WRF	The Water Research Foundation
WWTP	Wastewater treatment plant

Executive Summary

ES.1 Key Findings

The overarching objective of this study was to develop a framework to standardize methods for monitoring antibiotic resistance in wastewater, recycled water, and surface water. The following summarizes the key findings of this effort, which were derived from a high-level literature review, an expert survey, a systematic literature review, an expert workshop, field and lab validation of standard operating procedures (SOPs), and development of a web-based repository and analytical tool.

Culture, quantitative polymerase chain reaction (qPCR), and metagenomic sequencing are extensively applied in the scientific literature for characterizing antibiotic resistance in water samples but vary in the maturity of the methods and the interpretation of the data these methods generate.

Culturing of *Escherichia coli* is a good starting point for standardization because of its clinical relevance, survival in the environment, and existing prominence in regulatory monitoring. Furthermore, sensitive and specific selective-differential media are readily available. The World Health Organization (WHO) recently published the Tricycle Protocol of a standard method (WHO, 2021) for monitoring extended-spectrum beta-lactamase (ESBL)-producing *E. coli* as an idealized target that is relevant across the One Health spectrum.

Other culture-based targets could provide additional perspective, such as *Enterococcus* spp. as a Gram-positive organism as well as *Pseudomonas aeruginosa*, *Acinetobacter* spp., and *Aeromonas* spp. as organisms that survive and grow in the environment (and thus may provide more sensitive targets for detecting evolution and transfer of antibiotic resistance genes (ARGs)). Although existing culture methods can reliably quantify *Enterococcus* spp., additional effort would be needed to develop any of the latter four organisms as standard methods for monitoring antibiotic resistance in the water environment.

qPCR provides sensitive quantification of ARGs and other key genes across a microbial community, avoiding culture bias. It also provides a medium-technology approach accessible to an increasing number of water utilities. Metagenomics is gaining momentum and promise as a future application, but there are numerous aspects of analytical methods and data analysis to consider if it is to be standardized as a method for routine monitoring.

The SOPs developed here for culturing of presumptive ESBL *E. coli* and qPCR analysis of *sul*1 and *int*I1 build on existing standard or well-vetted methods and technologies and will be feasible for implementation by water utilities. The SOPs were validated between two labs for analysis of replicate wastewater, recycled water, and surface water samples from five water utilities and yielded comparable results. The Water Antibiotic Resistance Database (WARD) was further developed through this project and will serve as a forum for SOP updates, data sharing, data analysis, and discussion.

ES.2 Background and Objectives

Antibiotic resistance is one of the greatest human health threats of our time, crippling the efficacy of antibiotics for treating and preventing deadly bacterial infections. According to the U.S. Centers for Disease Control (CDC), antibiotic-resistant bacteria (ARB) and antimicrobial resistant fungi cause more than 2.8 million infections and 35,000 deaths in the United States each year (U.S. CDC, 2021). Numerous national and international reports highlight the need for a comprehensive strategy to combat the spread of antibiotic resistance, including investing in research to better understand the environment's role in the evolution and dissemination of antibiotic resistance to human pathogens. The WHO, EU Global Action Plan, and U.S. National Action Plans all call for a One Health (i.e., humans-animals-environment) framework to address antibiotic resistance, particularly identifying concerns about wastewater treatment plants (WWTPs) as key recipients and disseminators of antibiotics, ARB, and ARGs (The White House, 2015; WHO, 2015; European Commission, 2017). The present moment is a critical juncture in history for the U.S. water industry and The Water Research Foundation (WRF) to provide leadership in the environmental surveillance of antibiotic resistance. Concerns are high regarding water reuse; multiple treatment barriers are being emplaced to safeguard against contaminants of emerging concern (CECs) and other contaminants but have not been rigorously evaluated in terms of their efficacy for removal of ARBs/ARGs.

A major impediment to addressing concerns about the role of the water environment as a recipient, source, and pathway for the spread of antibiotic resistance is the lack of standardized methods for monitoring ARBs and ARGs. The specific objectives of this research were to:

- Incorporate input from literature reviews, expert surveys, and an expert workshop to develop a framework for antibiotic resistance monitoring of wastewater, recycled water, and surface water that aligns methods and targets with corresponding monitoring objectives
- Seek expert workshop input in the development of SOPs for the identified priority targets and validate the SOPs through an interlaboratory comparison of samples provided by representative water utilities
- Develop a web-based forum for sharing the SOPs and any associated updates, data sharing, data analysis, and discussion of user experiences

ES.3 Project Approach

A high-level literature review was first carried out to identify common analyses used to measure various dimensions of antibiotic resistance in the water environment. It was found that culture, qPCR, and metagenomic sequencing strategies were most commonly applied. Then, an online survey was conducted to narrow down a list of methods and targets for further consideration. The survey captured recommendations from over 100 U.S. and international experts in the field. A systematic literature review was carried out to further evaluate the performance of the methods, quantitative ranges in various water matrices, and pros and cons for development as standard methods for monitoring of wastewater, recycled water, and surface water. The survey and literature review informed draft SOPs, which were further evaluated at an expert workshop. Over 50 experts attended the workshop, including

representatives from academia, U.S. environmental and public health agencies, the WHO, consultants, and U.S. water utilities. A decision tree was developed during this process to aid in aligning the monitoring objective with the selected target and method. SOPs for culturing presumptive ESBL *Escherichia coli* and qPCR of *sul*1 (sulfonamide resistance gene) and *int*I1 (integrase gene) were further refined and validated through an inter-lab study comparing analysis of water samples collected by five U.S. utilities. The WARD database and analytical tool was developed and made freely available on the web to facilitate data sharing and analysis.

ES.4 Results

Culture-, qPCR-, and metagenomic-based methods for monitoring antibiotic resistance in the environment were commonly encountered in the literature but differed greatly in consistency of approach, maturity, and agreement on interpretation of data. It was clear from the literature reviews, expert survey, and expert workshop that no *one* method or target comprehensively captures all dimensions of antibiotic resistance that may be of interest for a monitoring program. Therefore, a framework was developed that aligns specific targets and methods with specific monitoring objectives (Figure ES-1).

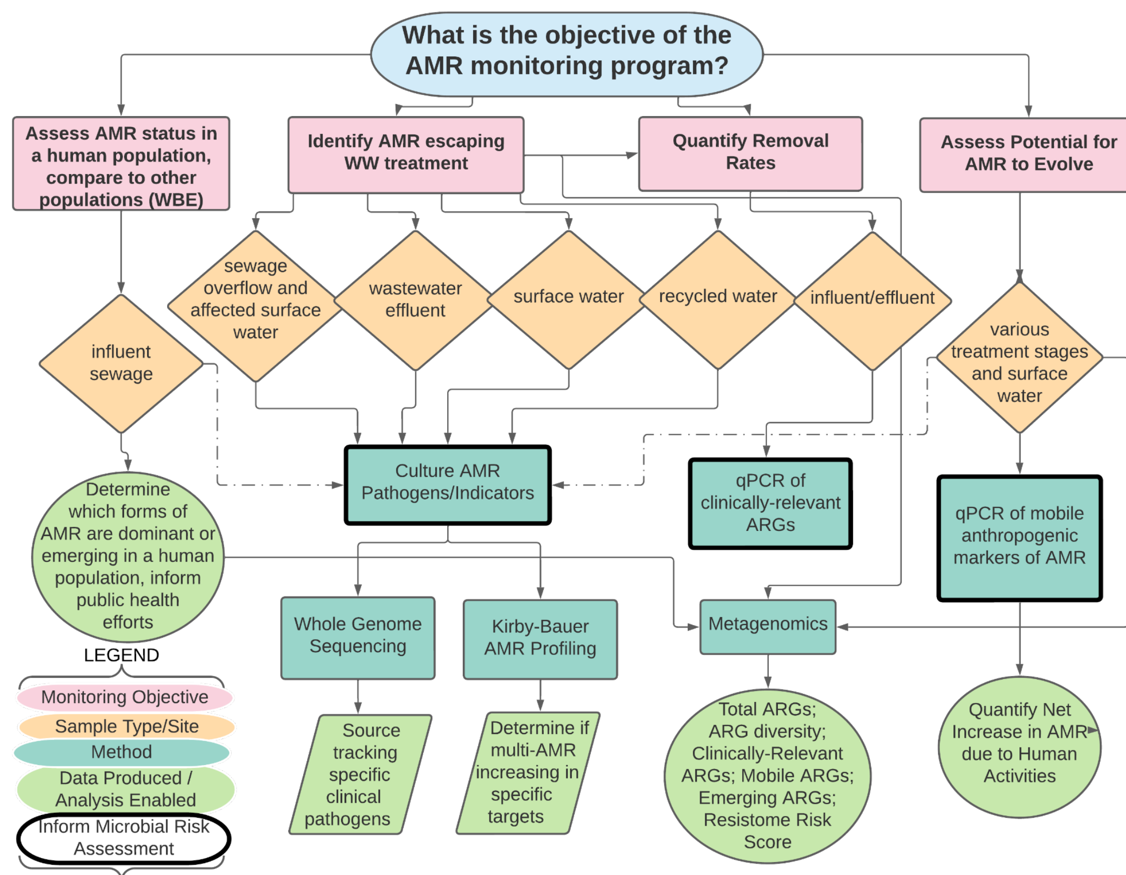

Figure ES-1. Framework for Antibiotic Resistance Monitoring of Wastewater, Recycled Water, and Surface Water Developed through Project 5052.

Emphasizing alignment of methods with monitoring objectives (WBE: Wastewater-based epidemiology)
Source: Liguori et al., 2022 Available under Creative Commons License Attribution-NonCommercial-NoDerivatives 4.0 International

E. coli was identified as a good starting point because of its clinical relevance, survival in the environment, existing prominence in regulatory monitoring, and because the WHO recently developed the Tricycle Protocol for standardized monitoring of ESBL *E. coli* across One Health environments. Other culture-based targets could provide additional perspective. *Enterococcus* spp. is a Gram-positive organism that would provide insight into resistance to distinct suites of antibiotics and also benefits from existing regulatory frameworks and standard methods. *Pseudomonas aeruginosa*, *Acinetobacter* spp., and *Aeromonas* spp. survive and grow in the environment and may provide more sensitive targets for detecting evolution and transfer of ARGs. However, additional effort would be needed to standardize methods for monitoring these latter four organisms in water samples.

qPCR was identified as a medium-tech approach accessible to an increasing number of water utilities. It also provides sensitive quantification of ARGs and other key genes across a microbial community, thus avoiding culture bias. In particular, *sul*1 and *int*I1 were identified as good targets for capturing inputs of anthropogenic sources of antibiotic resistance and potential for co-occurrence of mobile forms of multi-antibiotic resistance. Additionally, *bla*CTX-M was identified as an ARG of high clinical concern worthy of monitoring.

Workshop attendees expressed substantial enthusiasm around metagenomics because this type of analysis can capture multiple ARGs and other genes of interest without the need for prior selection of targets. Efforts will be needed to standardize metagenomics and improve comparability of data, with particular emphasis on: implementing benchtop controls to exclude and account for contamination and validate the workflow, sequencing at sufficient depth to capture targets of interest on a matrix-specific basis, including internal standards to enhance quantitative capacity, and analyzing data in a fashion that supports data sharing and comparability (e.g., common denominators for normalization and agreed upon databases).

SOPs were further refined for culture of presumptive ESBL *E. coli* with cefotaxime as the selective antibiotic and qPCR of *sul*1 and *int*I1. The *E. coli* quantification method is derived from the USEPA method (Method 1603) using mTEC agar; it was found to be comparable to the WHO Tricycle protocol using TBX agar and to yield reproducible results between labs. qPCR assays similarly produced measurements of gene copies/volume that were within 1-log difference between the two laboratories. WARD was demonstrated as a user-friendly tool for sharing protocols and for uploading and accessing data and associated analysis.

ES.5 Benefits

National and international recognition of the need to develop monitoring systems for antibiotic resistance in wastewater and affected environments is growing. However, standardization of methods is needed to ensure that generated data are comparable. The literature reviews, expert survey, and expert workshop conducted through Project 5052 served to inform a comprehensive framework for selecting targets and methods according to the monitoring objectives. Input was incorporated from others engaged in similar efforts (including representatives from the WHO and the U.S. National Antimicrobial Monitoring System (NARMS)) to support generation of data that are comparable at national and global scales. Efforts were also made to ensure that the SOPs developed through this effort will be feasible

for U.S. water utilities by building on existing standard methods and considering available infrastructure and resources. The framework developed can guide utilities in proactively addressing growing concerns about antibiotic resistance being raised by the public and the scientific community. Guidance is provided for low-tech (culture), medium-tech (qPCR), and high-tech (metagenomics) methods. In particular, monitoring sewage for markers of antibiotic resistance carried by the community (i.e., wastewater–based epidemiology) was identified as a promising entry point, along with developing a better understanding of how advanced treatment trains applied for water reuse can minimize ARB and ARGs in the treated water. Additionally, the research team developed the WARD website, database, and analytical tool to facilitate data sharing and as a forum for accessing updated SOPs and discussing user experiences. The SOPs and overall framework developed here will guide water utilities and other interested parties in meeting the need for antibiotic resistance monitoring of wastewater, recycled water, and surface water.

ES.6 Related WRF Research

- Critical Evaluation and Assessment of Health and Environmental Risks from Antibiotic Resistance in Reuse and Wastewater (4813)
- Occurrence, Proliferation, and Persistence of Antibiotics and Antibiotic Resistance During Wastewater Treatment (4887)
- Treatment Processes for Removal of Wastewater Contaminants (1474)
- Fate of Antibiotic Resistant Genes (ARGs) and Antibiotic Resistant Pathogens in Full-Scale Activated Sludge Processes and the Optimization of Activated Sludge Processes for Reduction of ARGs (5028)
- Fate and Impact of Antibiotics in Slow-Rate Biofiltration Processes (4135)

CHAPTER 1

Introduction

1.1 Background

Antibiotic resistance is one of the greatest human health threats of our time, crippling the efficacy of antibiotics for treating and preventing deadly bacterial infections. Antibiotic resistance refers to the ability of bacteria to survive antibiotic treatment, an ability encoded by their antibiotic resistance genes (ARGs). In 2019, the United States Centers for Disease Control (US CDC) released an updated report, indicating that antibiotic-resistant bacteria (ARB) and antimicrobial resistant fungi cause more than 2.8 million infections and 35,000 deaths in the US each year, up from 2 million infections and 23,000 deaths estimated by the CDC in 2013 (US CDC, 2019b). Although the threat of increasing antibiotic resistance has until recently been primarily associated with hospitals and clinics, community-acquired infections originating outside the clinic have also been on the rise (US CDC, 2019b). Numerous national and international reports highlight the need for a comprehensive strategy to combat the spread of antibiotic resistance, including investing in research to better understand the role that the environment can play in the evolution and dissemination of antibiotic resistance to human pathogens (Hernando-Amado et al., 2019; EU Commission, 2020). In particular, the United Nations recently issued a report of Emerging Issues of Environmental Concern, citing the need to understand the role of the water environment in the dissemination of antibiotic resistance as a top priority (UNEP, 2019).

The World Health Organization (WHO), European Union (EU) Global Action Plan, and US National Action Plans all call for a One Health (i.e., humans-animals-environment) framework in addressing antibiotic resistance, identifying linkages with water sanitation and a need for corresponding surveillance (European Commission 2017; Federal Task Force on Combating Antibiotic-Resistant Bacteria, 2020; WHO, 2015). In the United Kingdom (UK), Establishing a Monitoring Baseline for Antimicrobial Resistance in Key Environments (EMBARK) is an international program by the Joint Programming Initiative for Antimicrobial Resistance (JPIAMR), which aims to identify baseline levels of environmental AMR by region and types of resistance, and thereafter standardize methods for surveillance and monitoring of those resistances worldwide (Garland et al., 2019; Keely et al., 2022). In the US, the Federal Task Force on Combating Antibiotic-Resistant Bacteria has recognized the need for environmental research and monitoring to engage in a One Health approach (Federal Task Force on Combating Antibiotic-Resistant Bacteria, 2020) and recently the US CDC launched a multi-year initiative to fund research on antibiotic resistance, including topic areas focused on water and wastewater-related sources (US CDC, 2019b). Correspondingly, the US Environmental Protection Agency (USEPA) began incorporating ARGs into their national monitoring programs of rivers and streams (NRSA) (Keely et al., 2022). The CDC, US Department of Agriculture (USDA), and the US Food and Drug Administration (FDA) are collaborating on the National Antimicrobial Resistance Monitoring System (NARMS), which ultimately will synergize with existing federal infrastructure, including NRSA, to address AMR from a truly One-Health approach. The state

of California has also taken a proactive approach in exploring the possibility of monitoring ARBs/ARGs in recycled water along with other contaminants of emerging concern (CECs) (California State Water Resources Control Board, 2019; California State Water Resources Control Board, 2016).

The present moment is a critical juncture in history for the US water industry and the Water Research Foundation (WRF) to provide leadership in the environmental surveillance of antibiotic resistance. It is now well-established that wastewater treatment plants (WWTPs) are a recipient of antibiotics, ARBs, and ARGs and there is concern that they are not intentionally designed to remove these CECs and that their release into the environment could pose a distinct human health hazard. Several studies have now identified WWTP effluents to be a source of ARBs/ARGs to surface water, including work led by our team that identified a significant correlation of the *sul*1 ARG in the Poudre and S. Platte River watersheds to upstream capacities of WWTPs (Garner et al., 2016). Concerns are particularly elevated in the context of water reuse, where multiple treatment barriers are being emplaced to safeguard against CECs and other contaminants but have not been rigorously evaluated in terms of their efficacy for removal of ARBs/ARGs.

However, a major impediment to moving forward in addressing concerns about the role of the water environment as a recipient, source, and pathway for the spread of antibiotic resistance is lack of standardized methods for monitoring ARBs and ARGs. Herein is presented work performed in partnership with WRF, water utilities, consultants, and other relevant experts and stakeholders to reach consensus on ARB/ARG monitoring methods relevant to the water industry and to develop corresponding standard operating procedures (SOPs), quality assurance/quality control (QA/QC) guidelines, and data quality criteria.

1.2 Overview

Antibiotic resistance is a growing global health threat that calls for collaboration across multiple disciplines and sectors in order to prevent its spread and to maintain antibiotics as a life-saving resource for future generations. In particular, research over the last 15-20 years has shown that successful strategies to combat the spread of antibiotic resistance require consideration of environmental reservoirs, pathways of exposure for humans and animals, and opportunities for horizontal gene transfer in aquatic environments (Pruden et al., 2006; Hujibers et al., 2015; Pruden et al., 2013; Ashbolt et al., 2013; Kristiansson et al., 2011; Burgmann et al., 2018). The receipt, treatment, and release of antibiotics, ARBs, and ARGs to and from WWTPs is of particular interest. Figure 1-1 provides a summary of these pathways by which antibiotics and antibiotic resistance may be circulated in the aquatic environment, especially as they relate to WWTP inputs and outputs, and highlights key monitoring locations and objectives of interest.

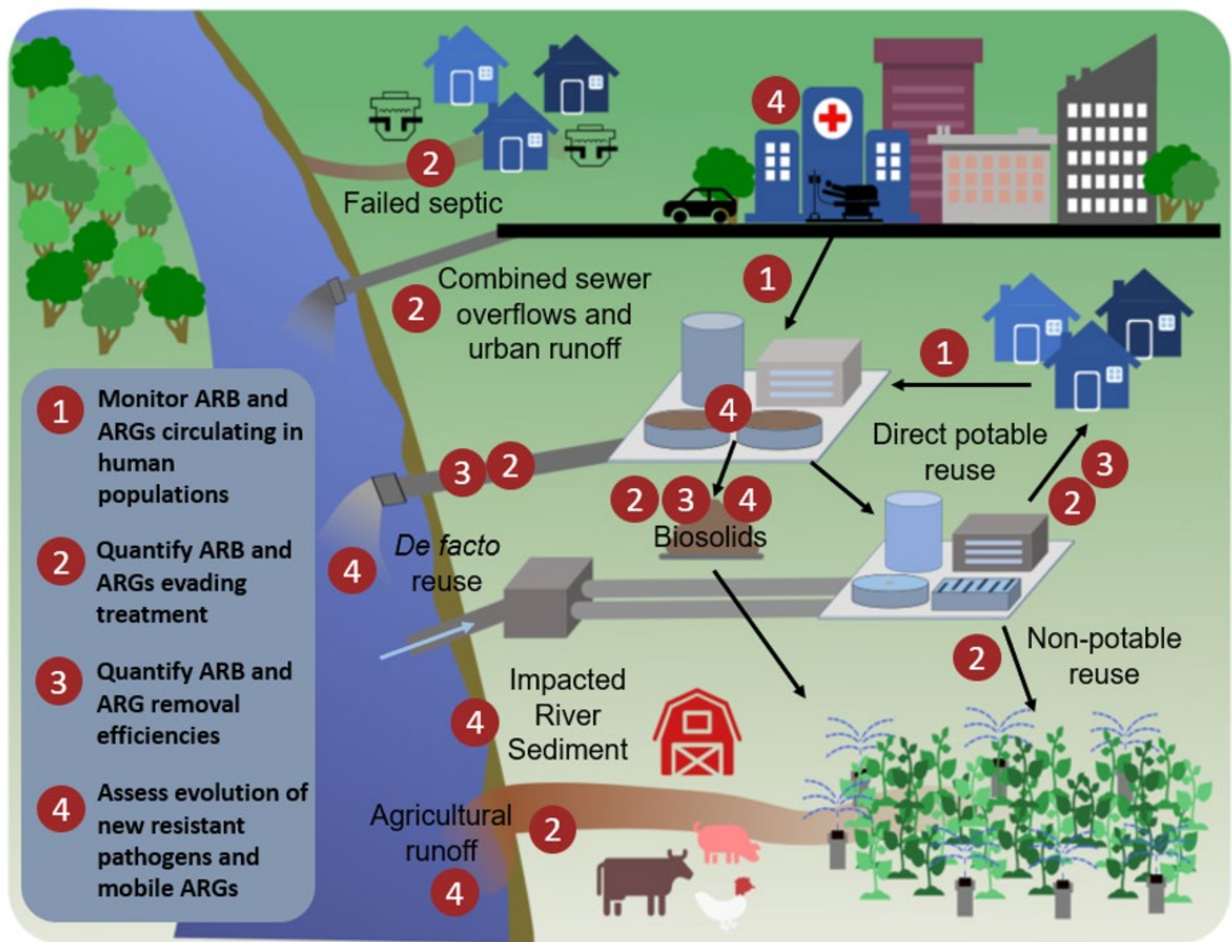

Figure 1-1. Key Transmission Pathways for Antimicrobials, Antibiotic Resistant Bacteria (ARB), and Antibiotic Resistance Genes (Args) in the Aquatic Environment as They Relate to WWTP Inputs and Outputs.
Key monitoring locations and corresponding monitoring objectives are indicated and correspond numerically
Source: Liguori et al., 2022. Available under Creative Commons License Attribution-NonCommercial-NoDerivatives 4.0 International

The need to establish environmental monitoring of antibiotic resistance as part of a One Health approach to addressing the spread of antibiotic resistance is increasingly being recognized (Aarestrup & Woolhouse, 2020; Pruden et al., 2021; National Academies of Sciences, Engineering, and Medicine, 2022). However, a critical challenge to realizing this goal is the need for agreed upon standard methods. Various culture, quantitative polymerase chain reaction (qPCR), and metagenomic sequencing techniques have been widely applied across the scientific literature, but are lacking in the comparability needed to support comparison across studies, to reliably inform risk assessment, and to inform broader comparative and epidemiological studies needed to address key knowledge gaps and support broader conclusions. Water Research Foundation Project 5052 provided a key opportunity to build consensus across the research community with respect to targets and methodologies for antibiotic resistance monitoring. A survey was conducted of 105 international experts in order to narrow down a list of culture-, qPCR-, and metagenomic-based targets for monitoring. Subsequently, the team compiled a comprehensive literature review and convened an expert workshop in order to engage in

discussion and further refine these targets and begin to consider key aspects of corresponding monitoring protocols. Of special consideration in evaluating current methods included:

- sensitivity
- specificity
- degree of existing standardization
- clinical relevance
- quantitative capacity
- relevance for risk assessment models
- affordability
- feasibility
- overall potential to achieve monitoring objectives and to address key research questions for a wide variety of stakeholders

Draft SOPs were accordingly developed based on the literature review and expert survey. Both the literature review and draft SOPs were evaluated at an expert workshop. The workshop took place virtually over a 2-week period (May 2021) and was attended by 49 (43 US, 6 international) experts representing academia (17), industry (8), federal governmental agencies (e.g., USEPA, FDA, USDA) (13), state and local governmental organizations (2), United Nations agencies (WHO) (1), and water utilities (9). Invited presentations focused on important context to consider, such as what was learned through the development of the WHO Tricycle Protocol for ESBL *E. coli* and the need to consider how suitable the various targets and measures are for human health risk assessment. Notably, partnering with Water Research Foundation Project 4813 (Hamilton 2018) helped the team to gain specific guidance on selecting targets relevant to human health risk assessment. A presentation from Hamilton 2018 was included at the beginning of the workshop program to frame further discussion of targets and methods in the context of risk assessment. Also on the first day of the workshop program, Jorge Matheu gave a presentation to share lessons learned by the WHO in the development of the Tricycle Protocol for standardized monitoring One Health monitoring of antibiotic resistant *Escherichia coli.* Other presentations given by our team members focused on the high points of each proposed target: *E. coli* culture, *Enterococcus* spp. culture, environmentally-adapted pathogen culture (i.e., *Aeromonas* spp., *Pseudomonas aeruginosa*, *Acinetobacter baumannii*), qPCR of anthropogenic sources of antibiotic resistance (*sul*1, *int*I1, *tet*A), qPCR of clinically-relevant ARGs (*bla*CTX-M, *van*A), and metagenomics. The full workshop program is available in the Appendix (Appendix A2).

As this research effort progressed and input from experts was considered and synthesized, it became apparent that no single method can adequately capture all aspects of antibiotic resistance that may be of interest in a given water sample. Participants also judged that priority should be placed on targets and methods that are informative to human health risk assessment. Bearing this in mind, a detailed decision tree was developed to help guide researchers, regulators, water utility staff, or other users, in the selection of methods and targets contingent upon monitoring objectives. The specific objectives that are likely to drive a water quality monitoring program targeting antibiotic resistance include the following:

1. Assess AMR status in a human population and compare to other populations (wastewater-based epidemiology (WBE))
2. Identify AMR escaping wastewater treatment
3. Quantify removal rates
4. Assess potential for AMR to evolve

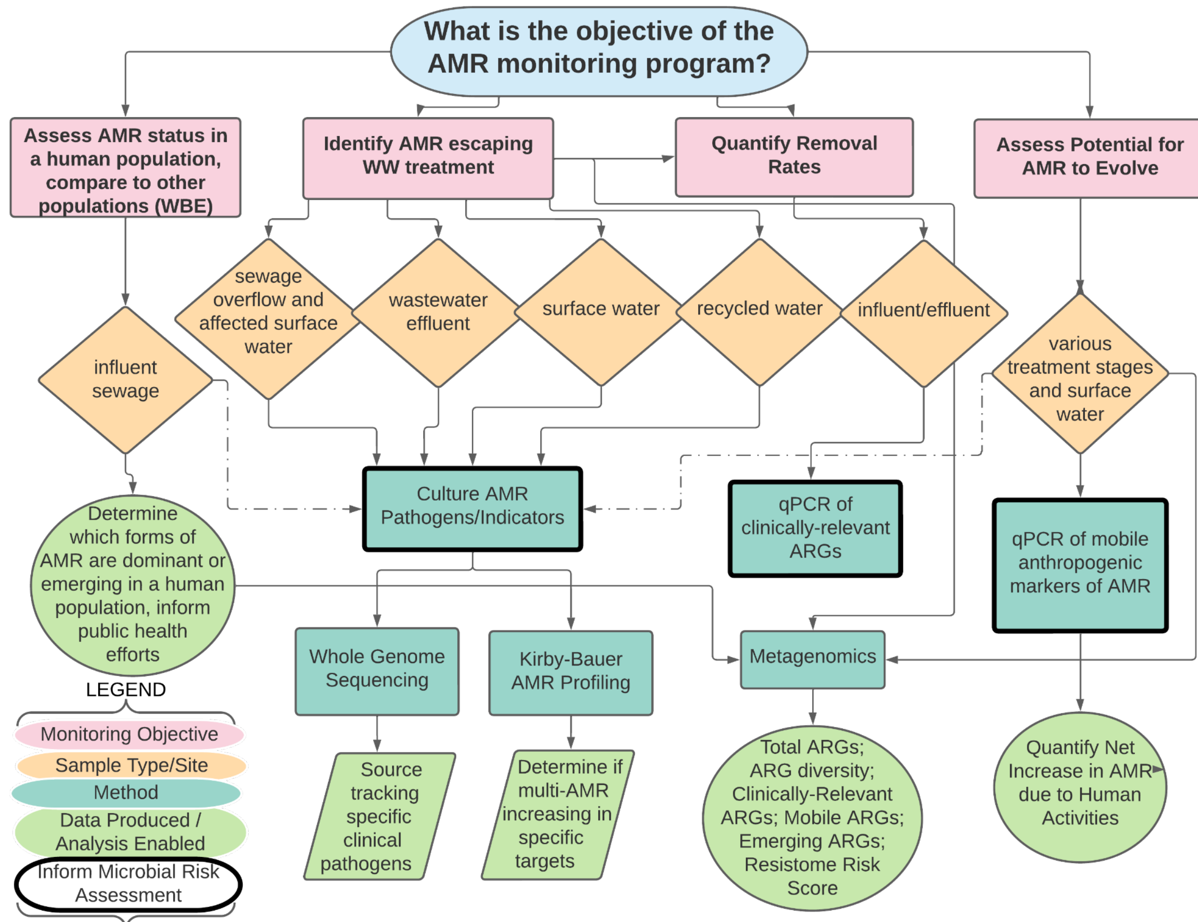

Figure 1-2. Decision Tree for Selecting Culture-, qPCR, or Metagenomics-Derived Monitoring Methods for Antibiotic Resistance Monitoring of Wastewater, Recycled Water, or Surface Water, Depending on the Monitoring Objective.

Dashed lines indicate the potential for enhanced realization of research objectives when molecular methods are coupled with culture. (WBE- wastewater-based epidemiology). This decision tree was developed through input from an expert survey, systematic literature review, and expert workshop

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Figure 1-2 above summarizes the decision tree for selecting targets and methodologies for monitoring antibiotic resistance in water environments developed through this effort (Liguori et al. 2022). This framework was published as an open-access article in *Environmental Science and Technology*, with the aim of encouraging others to adopt common targets, methods, and approaches and thus broadening the comparability of data being produced across the field (Liguori et al. 2022).

SOPs were further refined based on feedback from the expert workshop. Pilot-testing was accomplished by analyzing WWTP, recycled water, and surface water samples collected and shipped by partnering water utilities. Six utilities were selected to represent geographically diverse sites across the US (Virginia, Florida, Georgia, Nevada, California). Samples were shipped to and analyzed independently at our laboratories in Virginia Tech in Blacksburg, Virginia and University of South Florida in Tampa, Florida. The resulting data were compared across labs in order to evaluate the repeatability of the results. The SOPs and quality assurance project plan (QAPP) were further refined according to lessons learned during the pilot testing.

In addition to the antibiotic resistance monitoring framework manuscript (Liguori et al., 2022), five manuscripts were prepared from the literature review that was conducted as part of Project 5052. Calarco et al. (2023) focuses on *E. coli*; Davis et al. (in revision for *Water Research*) focuses on *Enterococcus* spp.; Milligan et al. (in revision for *Current Environmental Health Reports*) focuses on *Aeromonas* spp., *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*; Keenum et al. (2022) focuses on qPCR of *sul*1, *int*I1, *tet*A, *bla*CTX-M, and *van*A; and Davis et al. (in review for *Critical Reviews in Environmental Science and Technology*) focuses on metagenomics. These have been, or will be, published in open access format to encourage consistency in methods and data collection across the community. The generation of comparable data will be critical towards achieving broader, globally-coordinated monitoring objectives, such as (Pruden et al. 2021):

- Informing policy through integrated One Health surveillance
- Identifying drivers of antibiotic resistance through large, comparable longitudinal datasets
- Identifying epidemiological links between the environment, humans, and animals
- Providing data needed to inform risk assessment models and target regulatory limits
- Identifying hotspots for evolution and spread of AMR
- Identifying treatment technologies that most effectively mitigate AMR spread
- Informing human and animal medicine regarding which antibiotics will be most effective at population-specific scales

Ultimately sharing of data can help support the ability to address broader monitoring objectives and research questions, such as those indicated above. Encouraging sharing of data and protocols is also a critical aspect of Project 5052. Accordingly, the final task of Project 5052 was the development of a website, Water Antibiotic Resistance Database (WARD), where users can find the latest SOPs and QAPPs and engage in online discussion forums for feedback. User-friendly templates were developed to encourage upload and sharing of data and metadata (e.g., antibiotic measurements, metal measurements, temperature, pH, geographical coordinates).

This report summarizes the key products of Project 5052. The following chapters describe the findings of the expert survey, provide a high-level synthesis of the findings of the systematic literature reviews, and summarize the recommendations of the expert workshop. Then the results of pilot-testing the culture and qPCR SOPs and an overview of WARD are presented. The final SOPs and QAPP are included in the Appendix.

CHAPTER 2

Prioritizing Potential Targets and Methods through a High-Level Literature Review and Expert Survey

2.1 Introduction

Antibiotic resistance is a looming public health threat, with an estimated 2.8 million antimicrobial-resistant infections in the US each year (US Centers for Disease Control (CDC), 2019). Given that sewage contains a confluence of antibiotics, ARB, ARGs, and bacterial pathogens, wastewater systems are of increasing interest for antibiotic resistance surveillance (Pruden et al., 2018a; Bürgmann et al., 2018; Rizzo et al., 2013). Key monitoring objectives were outlined in Chapter 1 (Figures 1-1 and 1-2). Here they are listed in further detail, with specific benefits of standardization noted:

- Assess AMR status in a human population and compare to other populations (WBE)
  - Standardization facilitates comparison at various scales, i.e., from the community to worldwide
- Identify AMR escaping wastewater treatment
  - Standardization would allow comparison across WWTP effluents to determine if there are specific ARB/ARGs of concern that tend to escape treatment
  - Standardization would facilitate comparison of ARBs and ARGs detected in surface waters with those in WWTP effluents
  - Standardization would help to improve confidence in ARB/ARG numbers used for human health risk assessment models
- Quantify removal rates
  - Standardization will help to improve quantification of the removal of ARBs and ARGs by specific wastewater and recycled water treatment processes, which could help to inform improved treatment design in the future
  - Evaluation of the treated WWTP effluent and recycled water to assess the efficacy of the treatment processes
- Assess potential for AMR to evolve
  - Standardized methods would facilitate comparisons of where resistance measures are “high” or “low” and thus where to prioritize mitigation efforts
  - Standardization would facilitate comparisons across WWTPs, water reuse systems, and impacted surface waters globally to help identify potential hot spots where new forms of ARBs are likely to evolve (e.g., where there are high rates of horizontal gene transfer, elevated numbers of ARGs of clinical concern, and high levels of pathogens)

A major challenge to achieving such objectives is that a plethora of methods for measuring antibiotic resistance in water samples have been applied and reported in the literature (Berendonk et al., 2015; Gupta et al., 2020) and they are often not comparable due to inconsistencies such as species identification/confirmation, use of antibiotics in the primary selection step, confirmation of the specificity of molecular methods, and units used for data

analysis. Standardized methods for monitoring antibiotic resistance in water and wastewater are needed in order to ensure that the data collected are meaningful and comparable across studies. In Chapter 2, a description is provided of construction, dissemination and expert analysis conducted to narrow down a list of targets and methods that are suitable candidates for standard methods of monitoring antibiotic resistance in water.

There have been numerous calls for standardization of methods for environmental antibiotic resistance monitoring (Berendonk et al., 2015; Pruden et al., 2018a; Hujibers et al., 2019). Unfortunately, no available methods can address all possible monitoring objectives. Thus, the ultimate choice of ARB/ARG target and methodology will depend on the priority of the questions posed by researchers and regulators as well as cost and resource constraints (Huijbers et al., 2019). In order to help guide this choice, major influential review articles in the field were evaluated as a starting point (Aarestrup and Woolhouse, 2020; Woegerbauer et al., 2020; Nappier et al., 2020; Huijbers et al., 2019; Nnadozie and Odume, 2019; Pruden et al., 2018a; Bürgmann et al., 2018; Ashbolt et al., 2018; Yang et al., 2018; Hong et al., 2018; Christou et al., 2017; Berendonk et al., 2015; Gillings et al., 2014; Rizzo et al., 2013). This helped to narrow down the potential list of targets and to begin refining their potential alignment with research objectives. Then, this information was used to develop and launch a survey of experts in the field. The survey was conducted online, via Qualtrics and reached 105 international experts spanning various academic and research fields, state and federal governments, consulting, and water/wastewater utilities.

The following paragraphs provide a brief summary of the key strengths and limitations of culture-, qPCR-, and metagenomic-based approaches for monitoring antibiotic resistance in water environments based on our initial non-systematic high level literature review, which started with the major review articles cited above and the references cited therein. Then, a description of the expert survey design is provided, and finally the key results and findings are presented.

2.1.1 Culture-Based Methods

Culture-based methods are attractive because a target can be selected with known clinical relevance (e.g., taxonomic groups containing human pathogens), methods are fairly well standardized for defining clinical antibiotic resistance levels (e.g., EUCAST (2021), CLSI (2021), Kirby-Bauer (Hudzicki et al., 2009)), and by definition, the measured target is viable. Further, isolates in pure culture can be further characterized by analyses such as multidrug-resistance testing, sequence-based typing, and whole genome sequencing, which can aid in identification of ARG-carrying plasmids, delineation of phylogenetic relationships among strains, and surveillance for specific resistant strains. Culture-based targets also are most amenable to informing human health risk assessment models, such as quantitative microbial risk assessment. A challenge, however, is that there are numerous genera/species found in wastewater and surface water environments that could be informative targets, while there is also typically a high level of background microorganisms that can interfere with the isolation methods. No one target can comprehensively capture the antibiotic resistance status of a given environment.

Fecal indicator bacteria such as fecal coliforms, *E. coli*, and *Enterococcus* spp., are obvious candidates due to the long history of regulatory monitoring in water and wastewater systems and correspondingly, the high level of standardization for existing methods. Recently, standard methods have been proposed for extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae (Marano et al., 2020) and *E. coli* (JPIAMR, 2019). *Enterococcus* could potentially be a good complement as a Gram-positive organism, thus representing a distinct suite of antibiotics for which AMR is a concern. *Enterococcus* is also a required target for regulatory compliance of both drinking water and wastewater quality in the EU (European Environment Agency, 2020), and recreational water quality in the US (USEPA, 2012), and therefore there are several existing standard methods that could be adapted for AMR monitoring.

On the other hand, bacterial targets commonly present in sewage that have broader niches in the environment than traditional fecal indicators, such as *P. aeruginosa, Acinetobacter baumannii,* and *Aeromonas* spp. are also important to consider. Because such targets survive and grow in the environment, they may be more informative indicators of AMR that is acquired through mutation or horizontal gene transfer of ARGs taking place during sewage collection, wastewater treatment, or in the receiving environments. However, these organisms are typically not required targets for regulatory purposes and there are fewer available existing standardized methods from which to draw from.

Table 2-1. Overview of Culture's Strengths and Weaknesses

	Strengths	Weaknesses
Culture-Based Methods	Clinical Relevance	One target species provides a narrow perspective on AMR
	Clinical resistance levels are established and standardized	Isolating a pure culture from environmental samples can be difficult if media lack specificity
	Target is viable	Best practices require additional steps to confirm isolate phylogeny (genus or species) and antibiotic resistance
	Isolated targets can be further characterized	In depth genomic analysis (or genotyping) may be out of reach for routine monitoring
	Can be directly input to human health risk assessment models	

2.1.2 qPCR-Based Methods

qPCR-based methods began to gain frequent usage in the mid-2000s, as this technology became widely available and studies began to reveal striking patterns of elevated ARGs in anthropogenically-impacted aquatic environments (Pruden et al., 2006; Schwartz et al., 2003; Volkmann et al., 2004; Czekalski et al., 2014; LaPara et al., 2011; Knapp et al., 2010). A benefit of qPCR is that it provides an integrated measure across multiple bacterial species representing the complex microbial community's characteristic of water environments. qPCR can also

provide precise quantitation, over a broad detection range, which is an attractive feature for monitoring and informing human health risk models. Because qPCR is based on precise matching of primers and probes with DNA sequence targets, there is often less ambiguity in a positive result than there often is with culture-based techniques, which can be susceptible to contamination and growth of non-target organisms. A challenge again, however, is selecting which ARGs to target.

Several studies have now identified robust correlation of the sulfonamide resistance gene, *sul*1, to anthropogenic inputs (Pruden et al., 2012; Davis et al., 2020; Lee et al., 2021). Similarly, class 1 integrons (typically tracked by targeting the corresponding *int*I1 integrase gene) are also highly indicative of anthropogenic "pollution." Because integrons function to capture and mobilize genes present in corresponding cassettes, which commonly contain multiple ARGs, class 1 integrons are of further interest as indicators of multi-antibiotic resistance and the potential for ARG mobility (Gillings et al. 2015).

Thousands of known ARGs are reported in currently-available public databases (CARD, DeepARG) and could also be valuable and informative monitoring targets. There is growing understanding that ARGs vary in their behavior in aquatic environments, e.g., surveys across WWTPs show that some ARGs are removed throughout a treatment train, while others increase, and others are more sensitive to the specific treatment processes at play (Majeed et al., 2021; Ashbolt et al., 2018). There are many factors to consider in selecting ARG target(s) for research or monitoring programs, including:

- Level of clinical relevance (e.g., CDC threat level or WHO characterization as "access", "watch" or "urgent" (CDC, 2019b; WHO, 2019)
- Is it typically located on a mobile genetic element (MGE), such as a plasmid, transposon, or an integron (i.e., is it capable of moving across bacterial populations and species via horizontal gene transfer?)
- Is it readily detected in the environment? (Or is it so rare that most sampling efforts will yield null results?).

Recently, high throughput qPCR arrays that include hundreds of ARG targets have been developed, reducing the need to choose only a small handful of ARGs for monitoring (Ishii, 2020). Other markers, such as MGE and pathogen-specific genes, can also be included. However, the high throughput qPCR instrumentation is not widely available and detection limits are relatively high, which is a drawback for environmental monitoring. Another variation of qPCR that is emerging is droplet digital qPCR (ddPCR), which is considered to be more precise than qPCR because it provides absolute quantification (Taylor et al., 2017), i.e., does not require a standard curve, which also makes it more comparable across labs. ddPCR is also thought to be less affected by inhibitors and to provide a lower limit of detection (LOD). However, simultaneous performance of several assays in one analysis (multiplexing) is subject to many methodological constraints in ddPCR, as it is in qPCR. The capital equipment cost of ddPCR is also much higher than qPCR, as a qPCR instrument can presently be purchased for ~$30,000 USD, whereas a ddPCR instrument can cost more than $100,000, especially with automated droplet generators. Costs of reagents and supplies for qPCR and ddPCR are comparable (Yang et

al., 2014). Most qPCR assays are expected to be translatable to ddPCR as it gains more widespread application (Keenum et al., 2022).

Table 2-2. Overview of qPCR's Strengths and Weaknesses

	Strengths	Weaknesses
qPCR-Based Methods	Measures genes across multiple bacterial species	Have to choose a reasonable number of gene targets
	Precise quantitation	Can only look for known genes; no ability to detect or quantify new or emerging ARGs
	Specificity can be readily confirmed by probes and melt curves.	Can be used in human health risk assessment, but requires assumptions (e.g., proportion functional/ viable) and cannot be directly linked to a specific host organism
	Medium technological requirements	Cannot confirm the viability of host organisms nor determine host-DNA from "free DNA"
	Broad detection range	Dependent on the availability of well-validated/developed qPCR assays for the selected gene target
		Requires specialized training beyond that used for culture, which is common across utilities

2.1.3 Metagenomics-Based Methods

Shotgun metagenomic sequencing is carried out through direct extraction of DNA, fragmentation, and application of next-generation DNA sequencing (NGS) to obtain millions of reads representing the bacterial community associated with environmental samples of interest. In principle, metagenomics is a highly promising means to circumvent biases associated both with culture and the need to select targets *a priori*, as is necessary for qPCR, as it can directly access a very broad sweep of genes in a given sample. Genes of interest can be identified by annotating resulting DNA sequences using publicly-available databases such as CARD, DeepARG-db, ResFinder, MEGARes, or SARG to identify ARGs, while other databases can be applied to identify MGEs and profile bacterial phylogeny (Garner et al., 2021). Assembling the sequences into contigs can further aid in identifying putative linkages between ARGs, MGEs, and pathogens.

Metagenomic analysis presents theoretical and logistical challenges. For example, the cost may be prohibitive to achieve the sequencing depth needed to access the ARG/MGE targets of interest and very rare genes are unlikely be detected (Gweon et al., 2019; Zaheer et al., 2018). Data analysis and interpretation are very active areas of research, but this methodology must be standardized if data from separate studies are to be compared ((Bengtsson-Palme et

al., 2017; Angers-Loustau et al., 2018; Gupta et al., 2020). Finally, as is true with any molecular method, viability cannot be determined via metagenomics.

Table 2-3. Overview of Metagenomics' Strengths and Weaknesses

	Strengths	Weaknesses
Metagenomics-Based Methods	No need to identify targets *a priori* (i.e., non-target approach)	Much more expensive than qPCR or culturing
	Allows the detection and characterization of ARGs, MGEs, and microbiome simultaneously	Requires deep sequencing (which is more costly) to detect rare targets (i.e., high limit of detection)
	Linkages between ARGs, MGEs, and pathogens can be determined	Cannot confirm the viability of host organisms
	Sequencing data can be stored, shared, and reanalyzed as new targets emerge, and analyses evolve	Lack of standardized approaches for methods and analysis impedes comparability
	Sample preparation (i.e., DNA extraction) for sending to external labs for sequencing is relatively simple	Requires specialized training and knowledge for data analysis and interpretation
		Long turnaround time; not suitable for rapid feedback or day-to-day process optimization needs

2.2 Methods

2.2.1 Survey Design

To further narrow a short list of targets and methods of interest identified in the high level literature review, an expert survey was conducted. The survey was designed and deployed using Qualtrics management software (Qualtrics, Provo, UT). A variety of text entry, multiple choice, slider, rank order, and matrix table questions were employed in the 19-question survey. In addition to the 19 core questions, six 'display' questions, i.e., additional questions that may or may not pop-up for a participant based on their answer to a previous question, were incorporated. An application for IRB approval was submitted and evaluated and the study was classified as exempt according to IRB—20-659. A copy of the survey is provided in the Appendix (Appendix A1).

The survey first captured information about the participants and the organizations that they represented and assessed participant confidence and expertise in environmental AMR monitoring. Participants provided information about any AMR methods currently used, their familiarity with culture-, qPCR-, and metagenomic-based methods, and their opinions regarding ideal attributes for future methods. Finally, participants ranked a variety of factors with respect to their importance for standardizing AMR monitoring of water and wastewater systems.

2.2.2 Target Expert List and Recruiting

Experts were identified via multiple avenues, with the aim of attaining representation across environmental disciplines and gaining insight across continents and professional fields. Special attention was given to ensure inclusion of US water industry representatives, e.g., those who had volunteered to assist with research sponsored by the Water Research Foundation. The initial list started with collaborators known to the principal investigators and their professional networks. Attendee lists from recent relevant professional conferences were compiled, including: The Environmental Dimensions of Antimicrobial Resistance International Conference, The UNC Water Microbiology Conference, and the Gordon Research Conference on Microbiology of the Built Environment, as well as those subscribed to an AMR email listserv maintained by Dr. Ed Topp (Agriculture and Agri-Food Canada). Further, snowball sampling was employed, in which each participant was encouraged to suggest or invite their colleagues and others to participate in the survey. Students were considered ineligible to participate in the survey and were removed from the list. Using this approach, a list of 327 individuals was compiled and emailed the survey.

Invitations were sent via email utilizing the Distribution function within Qualtrics. Invitations were sent with a standardized email format, including information about the project, the survey objectives, a link to the survey, contact information, and IRB disclosure and contact information. Reminder emails were sent 7-14 days after the initial invitation, depending on time of initial email (average 10.25 days). Reminder emails contained the deadline for submission, the survey link, and the IRB disclosure and contact information. All individuals invited received a standardized invitation email and a standardized reminder email afterwards; timing of these emails was dependent upon when the individual was added to the Invited Expert list.

2.2.3 Results

Three hundred twenty-seven (327) experts were identified and emailed the survey, and 105 surveys were returned. The majority of participants represented academic institutions or universities (67%, n=70). Thirteen of the participants worked for government and/or regulatory organizations, nine participants worked for water or wastewater utilities, seven worked in water engineering and/or consulting, four worked at research institutes, and one participant worked in the pharmaceutical industry at the time of survey. Organizations employing the participants were located in North America (n=52), Europe (n=38), Asia (n=11), and Africa (n=4).

Participants were asked to select the job title that most closely fit their role in their organization. Seventy percent of respondents self-reported as Principal Investigator (PI), eight percent as Manager, about five percent as Post-Doctoral Researcher, and one percent as Laboratory Technician (n=1). The remaining eighteen percent identified as “Other.” Upon review of the text entries for respondents choosing “Other,” almost all indicated a title of scientist or researcher, with the exception of one (1) consultant and one (1) corporate employee.

To gain higher resolution on participant expertise, respondents selected from a dropdown list of environments that they specialize in or have worked with, with unlimited selections.

Wastewater led, with 85 selections (25%), followed by surface water (n=79), reuse/recycled water (n=40), drinking water (n=39), soil (n=31), manure (n=28), livestock/animals (n=18), human clinical (n=16), and other (n=11). The "other" written responses referred to seawater (n=2), sediments (n=2), groundwater (n=2), wildlife (n=1), coral reef (n=1), stormwater (n=1), and biofilms (n=1).

Participants were queried with respect to which aquatic environment(s) (surface water, recycled water, wastewater, drinking water) they are currently monitoring, testing, or researching. The majority of participants work with wastewater and surface water. When split out by continent of their organization, similar patterns were observed (Figure 2-1), i.e., most participants identified as working with wastewater and/or surface water, a portion worked with drinking water, and a small subset worked in water reuse.

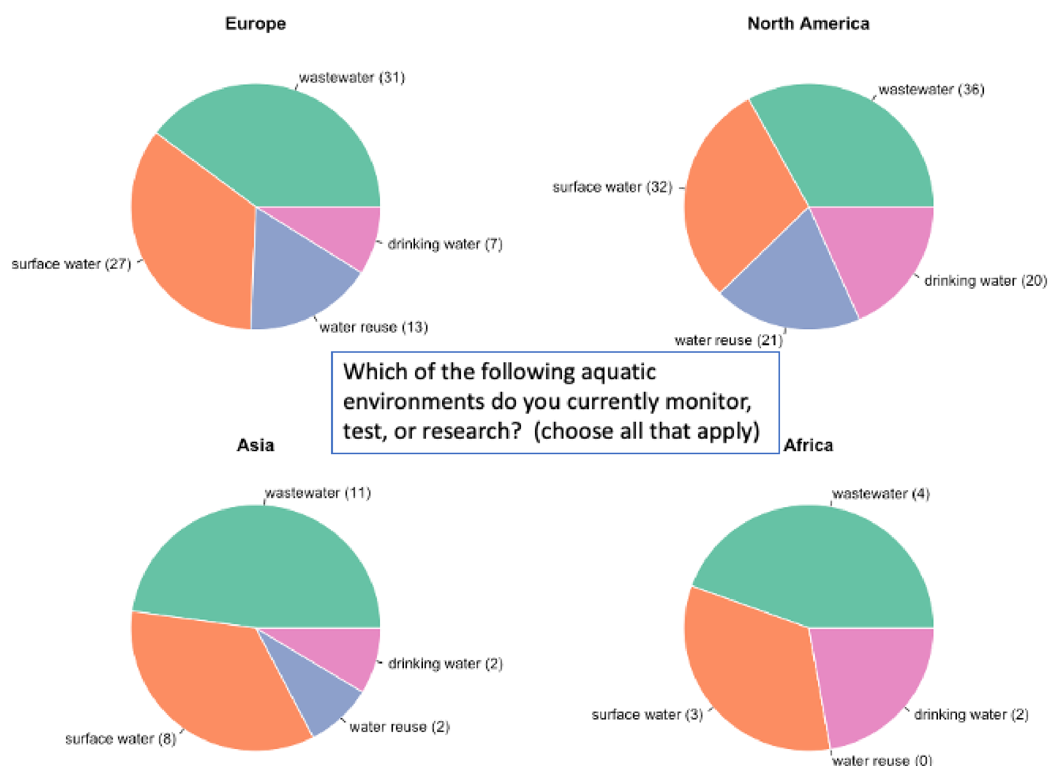

Figure 2-1. Water Environments Studied by the Expert Participants Across Continents

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Survey participants were asked questions specific to eight (8) main methodologies considered in this survey: PCR, qPCR, ddPCR, IDEXX, metagenomics, qPCR array or microfluidic qPCR, membrane filtration, and culturing fecal coliforms.

qPCR was the most commonly used method by the groups represented in this study (n=85). Membrane filtration is a commonly used method for concentration water matrices before bacterial culturing and quantification. Membrane filtration was the second most common (n=79) method used of the eight surveyed. Detection of fecal coliforms (n=78) and

PCR (n=78) were the next most frequently-utilized methods. Metagenomics, ddPCR, IDEXX, and microfluidic or array qPCR were the least commonly used in-house methods, although the actual metagenomic sequencing is typically outsourced (n=66).

Eighty-six survey respondents (82%) reported that they had an understanding of culture-based methods. *E. coli*, enterococci, and Enterobacteriaceae were consistently selected as the most frequent in-house culture-based targets. These three targets were also indicated as having been tested in their labs within the last 12 months, and indicated as the top three options for AMR monitoring. Survey participants were asked which culture targets they believed to be the best option for standardized AMR monitoring of water environments (**Figure 2-2**).

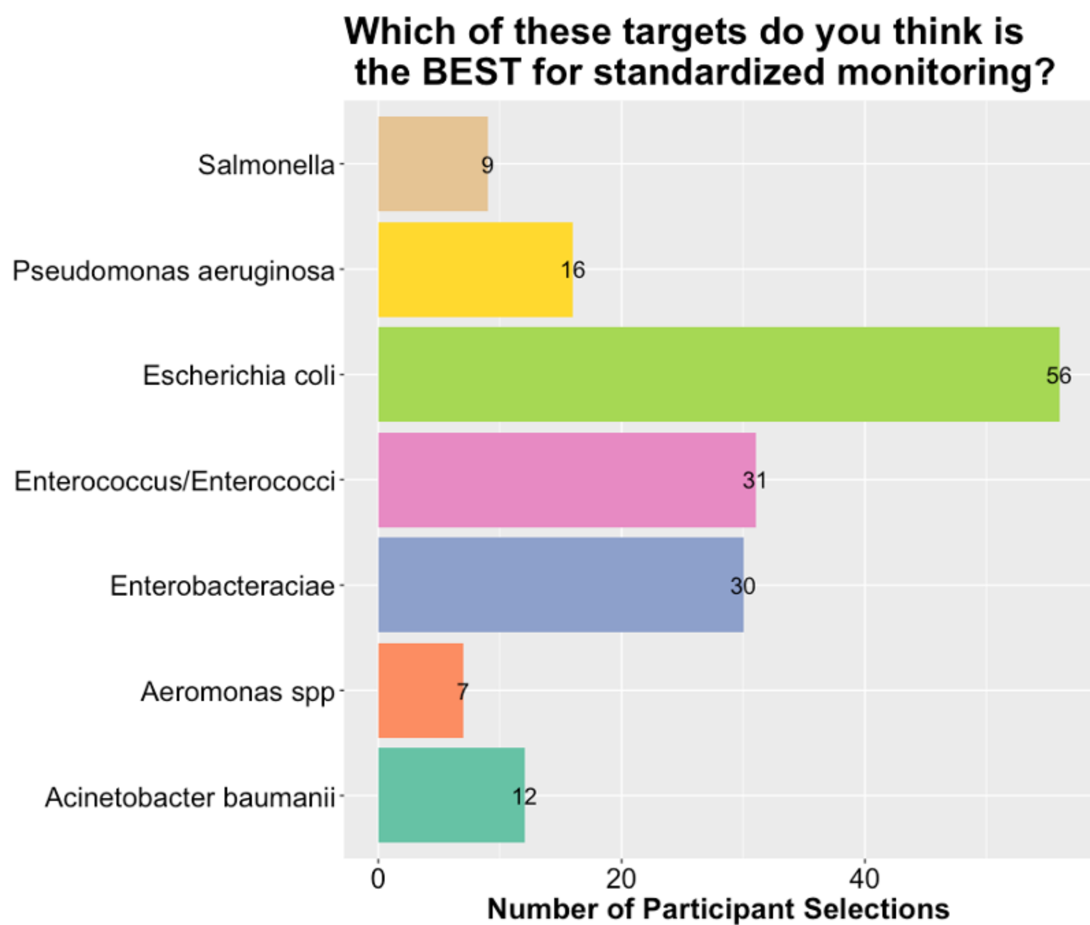

Figure 2-2. Preferred Target Bacteria for Standardized Culture-Based Monitoring of AMR Of Water Environments.

Participants could select up to two targets

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Eighty-nine survey respondents (85%) reported that they had an understanding of qPCR. The characteristics ranked most important for monitoring were genes reported to occur in human pathogens and ARGs with clinical relevance. Fifty-four respondents (51%) reported that they were familiar with high-throughput qPCR/multi-array approaches. Experts indicated that specificity, sensitivity, and quantitation are the most important characteristics for development

of a strong qPCR array method. Survey participants were asked which qPCR targets they believed to be the best option for AMR monitoring of water environments and the results are summarized in Figure 2-3.

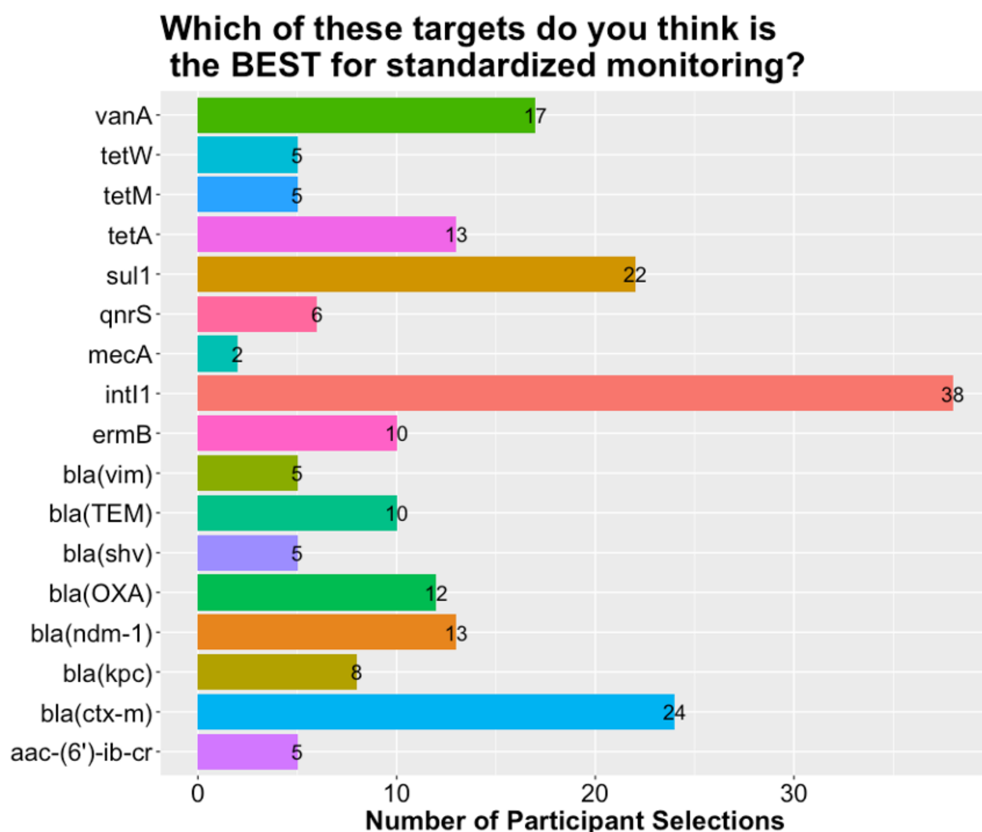

Figure 2-3. Preferred Target Genes for qPCR-Based Monitoring of AMR of Water Environments.
Participants could select up to three targets
Source: Liguori et al., 2022. Available under Creative Commons License Attribution-NonCommercial-NoDerivatives 4.0 International

When asked for familiarity around metagenomic sequencing, 81% of survey respondents reported that they or their organizations had an understanding of bioinformatic analysis (**Figure 2-4**).

Does your organization have capacity for or are you familiar with bioinformatic analysis of next generation DNA sequencing data?

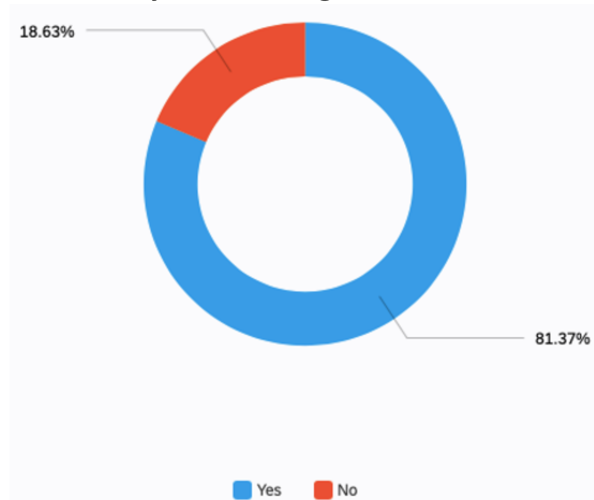

Figure 2-4. Experts' and/or Their Organizations Familiarity with Next-Generation Sequencing Data Analysis
Source: Liguori et al., 2022. Available under Creative Commons License Attribution-NonCommercial-NoDerivatives 4.0 International

Participants were asked to rank sequencing-derived metrics for next-generation sequencing (NGS) data for AMR monitoring, with 1 being the most important and 9 being the least important. The data are represented here (**Table 2-4**) using the overall score each metric received, thereby sorted by least important to most important (highest to lowest score). Clinical relevance scored as the highest-priority for designing an metagenomic analysis workflow, followed by mobility of ARGs.

Table 2-4. Experts' Ranked Preference

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Please rank the following with respect to next generation DNA sequencing-derived metrics for AMR monitoring	Average Ranking score (1 being most important)
Clinically-Relevant ARGs	3.82
Mobile ARGs (e.g., databases specifically tailored to ARGs that are known to be mobile and exclude intrinsic ARGs)	4.63
Total ARG Relative Abundance (e.g., normalized to 16S rRNA genes or RPKM)	5.15
ARGs Occurring on Contiguous DNA Strand that is Taxonomically-Classified as Pertaining to a Genus Known to Contain Human Pathogens	5.46
Total Mobile Genetic Elements (e.g., plasmids, transposons, and integrons)	5.64
ARGs Occurring on Contiguous DNA Strand with Mobile Genetic Elements	5.64
Emerging ARGs (i.e., bioinformatically- or functionally-predicted ARGs that have not yet been reported in the clinic)	6.09
ARGs reported to occur frequently in water systems	6.15
Total ARG Absolute Abundance (e.g., ARGs/mL)	6.33
Total ARG Diversity (e.g., Shannon or Chao Index)	6.73

Participants were asked how many assays is reasonable to expect water utilities and other relevant organizations to carry out for AMR monitoring of water environments (**Figure 2-5**).

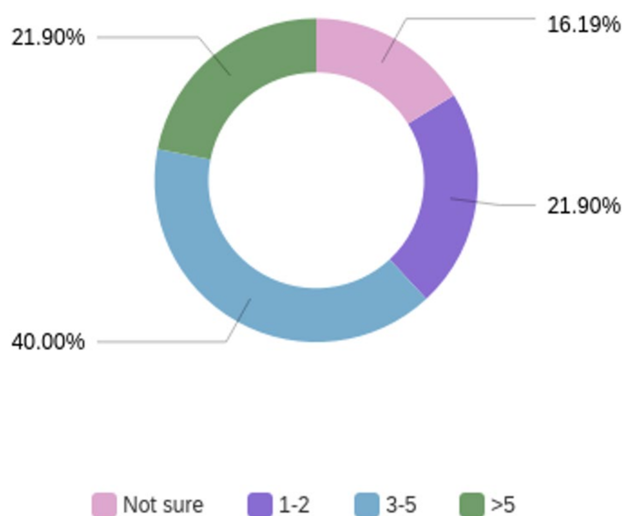

Figure 2-5. Experts' Recommendations Regarding the Number of Targets that is Reasonable to Recommend for AMR Monitoring of Water Environments.

Participants could select from four bins: 1-2, 3-5, >5, or not sure.

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Experts were also surveyed about factors of importance in AMR monitoring standard methods. Results indicated that ability to inform a human health risk assessment, relevance to human health, and a quantifiable target were the most important factors to the experts completing the survey (Figure 2-6). Timeliness of results, low technical skill requirement, and precedence of a standardized method tended to rank lower.

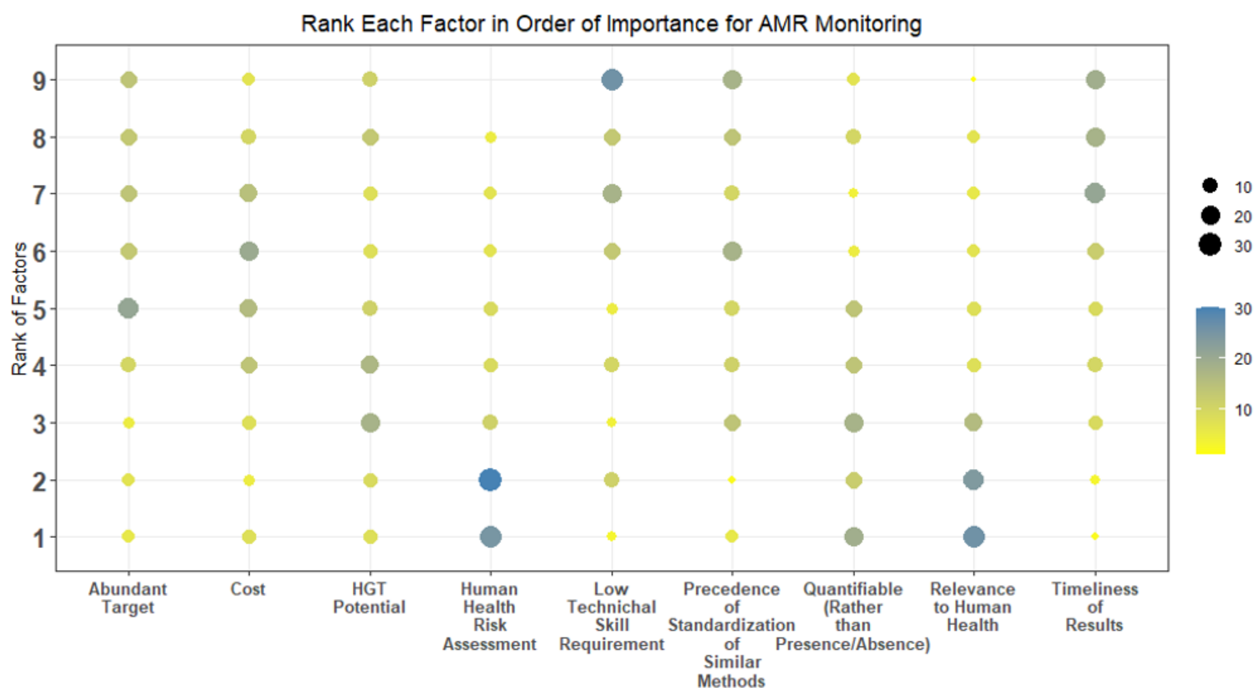

Figure 2-6. Expert Ranking of Factors of Importance for AMR Monitoring of Water Environments, with 1 being most important and 9 being least important. The color scale and size of circle indicate the number of respondents selecting the indicated ranking (y-axis) for each factor (x-axis).
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The barriers to uptake of a new or proposed monitoring method were collected via comments and open-ended feedback forms. The main barriers mentioned include cost, skill/training/labor requirement, insufficient sensitivity, high detection limit, insufficient quantitation, uncertainty around relevance of results and how to analyze or interpret the results, inhibition of the PCR, difficulty selecting meaningful targets, matrix interference, inability to identify host of gene, general lack of information, legislation, and a lack of standardization.

2.3 Discussion

Stakeholder input and buy-in is an essential aspect in the development of standardized methods. Here stakeholders were engaged early on and at detail-level, in order to assess the state of the field and their goals for the future of monitoring. The team attempts here to establish the main opportunities and barriers for development and implementation of standard methods in order to inform the design of methods that can be widely incorporated into existing infrastructures for a better antibiotic resistance monitoring and comparison platform. Based on the feedback provided from the expert survey and workshop, it was possible to prioritize targets and methods that are widely suitable and applicable for utilities, academics, government researchers, industry scientists and the like. Taking into consideration the feedback from experts on best target genes and organisms for antibiotic resistance monitoring, a systematic literature review was subsequently conducted focused specifically on the following targets for the purpose of antibiotic resistance monitoring of wastewater, recycled water, and surface water:

- *E. coli*
- *Enterococcus* spp.
- *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Aeromonas* spp. (organisms that readily grown in environmental niches)
- qPCR of *int*I1, *sul*1, *tet*A, *bla*CTX-M, and *van*A
- Metagenomic profiling of ARGs

CHAPTER 3

Literature Review

3.1 E. coli

3.1.1 Overview

A culture-based target with human clinical relevance and adequate concentration in wastewater for ready detection could provide quantification of viable ARB that can also directly inform quantitative microbial risk assessment. Antibiotic-resistant *Escherichia coli* are particularly problematic agents of urinary tract, diarrheagenic disease, and other life-threatening infections (Boczek et al., 2007; Croxen & Finlay, 2010; Nataro & Kaper, 1998). *E. coli* is a prime target to consider for antibiotic resistance monitoring in the water environment because it is already widely monitored for regulatory purposes and standard methods are already in existence and have been widely validated (International Organization for Standardization, 2012a, 2012b; Merck KGaA, 2020; USEPA Method 1603). Multiple standardized culture methods have been developed for quantification of culturable *E. coli*. Notably, the WHO recently released a standard method that targets cefotaxime-resistant (presumptive ESBL) *E. coli* for the purpose of One Health antibiotic resistance monitoring, as the first effort at standardization of an idealized target for One Health monitoring of antibiotic resistance. *E. coli* is ideal for this purpose because it is relevant to monitoring human clinical, agricultural, food production, wastewater, and aquatic environments (World Health Organization, 2021). The WHO protocol is widely referred to as the WHO Tricycle Protocol (with the Tricycle referring to the three domains of One Health: human, animal, environment). Multiple protocols for monitoring antibiotic resistant *E. coli* in aquatic environments, including the Tricycle Protocol, were considered.

3.1.2 Literature Review Strategy

A systematic literature review was conducted to assess and compare the methods used to isolate and profile antibiotic resistant *E. coli* in wastewater, recycled water, and surface water. Topic searches for articles that (1) assessed antibiotic resistance, (2) focused on wastewater, recycled water, or surface water environments, (3) used culture-based methods, and (4) targeted the organism of interest were used as follows:

(1) TS = (“antibiotic resistan*” OR “antimicrobial susceptibility” OR “antimicrobial resistan*” OR “drug resistan*” OR “multi-drug resistan*” OR “resistome” OR ”ARG” OR “antibiotic resistan* gene”)

(2) TS = (“wastewater” OR “reclaimed water” OR “recycled water” OR “water recycled” OR “non-potable recycled” OR “greywater” OR “hospital wastewater” OR “surface water” OR “sewage” OR “wastewater treatment plant” OR “filtration” OR “direct potable recycled” OR “indirect potable recycled” OR “river” OR “watershed” OR “lake” OR “pond” OR “recreational water” OR “influent” OR “effluent” OR “aquatic” OR “water quality” OR “de facto recycled”).

(3) TS = (“culture” OR “dis* diffusion” OR “isolat*” OR "membrane filtrat*" OR "spread plating" OR "IDEXX" OR "Colilert" OR "Colilert-18" OR "Colisure" OR "Enterolert" OR "Pseudalert" OR "Enterolert-E")

(4) TS = (“Escherichia coli” OR “*E. coli*”)

Studies were excluded if they focused specifically on biosolids, drinking water, phage mediation, or ballast water. Studies that did not evaluate antibiotic resistance in isolates, used isolates of unknown origin, pre-enriched samples prior to initial culture, or did not provide culture details were also excluded. Aquaculture and other animal farming studies were excluded except for cases where surface water was under direct influence of animal wastewater. The review was conducted in Web of Science and targeted papers that were published between 2000 to 2020, ultimately recovering 144 articles. One key aspect to any culture-based monitoring protocol is sample collection and concentration, i.e., what volume of sample to collect to ensure likelihood of capturing the intended target and which method was used to concentrate the sample so that it can be inoculated onto selective media. Membrane filtration was the most common sample concentration technique encountered in the literature (91.1% of articles).

3.1.3 Performance of Selective-Differential Media

The selective media chosen is also a key consideration to any culture-based technique. The literature review revealed that the four most frequent isolation media utilized were modified mTEC (USEPA method 1603), Chromocult Coliform, TBX (WHO Tricycle Protocol), and CHROMagar ECC. The sensitivity and specificity of these four media are summarized in Table 3-1.

Table 3-1. Sensitivity and Specificity of the Four Most Frequently Used Media to Isolate *E. Coli* in Wastewater and Surface Water.

Media	Sensitivity[a]	Specificity[b]	Environment(s) Tested	Reference
Modified mTEC	95%	94%	Marine and freshwater	(USEPA, 2002)
	94.1%	97.5%	Secondary wastewater	
	96.1%	98.5%	Disinfected wastewater	
Chromocult Coliform	93.8%	97.4%	Drinking water spiked with contaminated river water	(Lange et al., 2013)
	100%	100%	Sewage	(Maheux et al., 2017)

	91.8%	97.3%	Fecal samples in pre-reduced buffered peptone water	(Finney et al., 2003)
TBX	90%	89.1%	Wastewater	(Vergine et al., 2017)
TBX + 1 mg/L cefotaxime	95.7%	100%	Sewage, agricultural waste, surface water	(Koltun, 2018)
CHROMagar ECC	95%	95.7%	Surface water and spiked drinking water	(Brenner et al., 1993)

[a] Sensitivity = true positive/(true positive + false negative)
[b] Specificity = true negative/(true negative + false positive)

3.1.4 Selection of Antibiotic(s)

In order to adapt existing standard methods for *E. coli* culture for the purpose of antibiotic resistance monitoring, it is necessary to identify an antibiotic of interest to include in the culture media. This is often referred to as a primary selective antibiotic. Plating an environmental sample on selective-differential media with and without a primary selective antibiotic allows for the calculation of percent resistance of the *E. coli* population, along with the standard CFU/100 mL of total *E. coli* that is typically reported for regulatory requirements. Fifty-six (38.9%) of the articles reported isolation in the presence of antibiotics. The most frequently used isolation antibiotics were cefotaxime (16.1%), ciprofloxacin (14.3%), ampicillin (14.3%), and tetracycline (10.7%). This usage aligns with the WHO's Tricycle Protocol, which recommends including 4 µg/mL cefotaxime as the primary selective antibiotic. Cefotaxime is desirable because it is a 3rd generation cephalosporin that is widely used to identify ESBL-producing *E. coli* strains.

ESBL *E. coli* are classified as serious antibiotic-resistant threats by CDC (CDC, 2019b). The WHO Tricycle Protocol highlighted the importance of this organism and uses four tenets as their rationale for selecting the organism: 1) highly variable rates and prevalence of ESBL *E. coli* colonization in humans in and between countries; 2) prevalence in farm animals is variable and antibiotic usage in the food chain may indicate some human morbidity linked to ESBL *E. coli*; 3) interventions leading to decreased exposure to antibiotics have been followed by decreased ESBL *E. coli* rates; and 4) ESBLs confer resistance to critically-important antimicrobial drugs (World Health Organization, 2021). Other enzyme classes such as carbapenamases can produce cephalosporin-resistant phenotypes, therefore confirmation of the resistance mechanism requires testing presumptive ESBL isolates for sensitivity to clavulanic acid (WHO, 2021). In practice, many clinical laboratories omit confirmation of presumptive ESBL isolates, restricting confirmatory testing to epidemiological and infection control applications (Castanheira et. Al., 2021; CLSI, 2016).

Many studies went beyond testing for resistance for the primary selective antibiotic and surveyed resistance to multiple antibiotics. The majority of studies first isolated *E. coli* on media

without antibiotics and subsequently used Kirby-Bauer disk diffusion (66.7%) for phenotypic antimicrobial susceptibility testing. Ciprofloxacin, tetracycline, and ampicillin resistance were the most frequently assayed for isolates recovered from wastewater, while tetracycline, gentamicin, and ampicillin resistance were the most frequently assayed for isolates recovered from surface water. One challenge for data comparability is the need to apply consistent benchmarks for antibiotic susceptibility, intermediate resistance, and full resistance. There are two widely cited sets of guidelines for this purpose: the European Committee on Antimicrobial Susceptibility Testing (EUCAST) "Breakpoint Tables for Interpretation of MICs and Zone Diameters" and the Clinical and Laboratory Standards Institute (CLSI) "Performance Standard for Antimicrobial Testing". The most notable difference between the EUCAST and CLSI guidelines is the elimination of intermediate resistance as an interpretative category in the EUCAST guidelines, which is replaced with "susceptible, increased exposure" for measurements falling between sensitive and resistant breakpoints. In the WHO Tricycle Protocol, either guideline recommendation can be followed.

3.1.5 Confirmation of Species

Given the diverse microbial communities characteristic of the water environment, there is a high likelihood that non-target organisms will also grow on selective media. Thus, confirming isolation of *E. coli* at the species level is critical. Most studies in which *E. coli* colonies were confirmed used PCR (37.1%) assay with specificity for *E. coli* (e.g. *uidA, tuf*, or *mdh* genes), followed by MALDI-TOF (17.7%), API 20E (16.1%), and biochemical testing (9.7%) as the most frequently used methods. PCR-based approaches for confirmation are widely used and accessible to most labs, making this method an attractive candidate for use in standardization.

3.1.6 Media and Antibiotics Used in This Study

A key consideration in the development of an SOP for ESBL *E. coli* that is suitable for US water utilities' use in antibiotic resistance monitoring is whether to directly adopt the WHO's Tricycle Protocol. One challenge is that the Tricycle protocol employs TBX media, whereas existing USEPA method 1603 that is widely applied for regulatory monitoring employs modified mTEC. Thus, there could be a trade-off between recommending a method that would be easy for US water utilities to adapt (USEPA method 1603) versus producing data that will be globally comparable (WHO Tricycle Protocol). An adaptation of the USEPA method 1603 was used for analysis of ESBL *E. coli* by including 4 µg/mL of cefotaxime in modified mTEC media, and to compare the results with the WHO protocol (TBX +4 µg/mL of cefotaxime). If the results are comparable, then this would help to ease the adoption of antibiotic resistance monitoring by US water utilities, while also yielding globally comparable data.

The SOP for modified mTEC-based ESBL *E. coli* monitoring is available in Appendix B2. Comparison of this SOP with the WHO Tricycle Protocol is described in Chapter 5.

3.2 Enterococcus spp.

Enterococcus spp. present several advantages for waterborne monitoring of antimicrobial resistance. Like *E. coli*, it is a fecal indicator that is commonly monitored for regulatory purposes. Thus, there is already an array of standard methods available for monitoring *Enterococcus* spp. in

wastewater and surface water (USEPA, 2006a, 2006b; Rice & Baird, 2017). Also, like *E. coli*, *Enterococcus* spp. are relevant to One Health monitoring. In the US and abroad, *Enterococcus* spp. have emerged as prevalent etiological agents of dangerous antibiotic resistant infections, such as septicemia, endocarditis, and surgical site infections. In particular, vancomycin resistance among the genus has been designated as a "serious" threat level by the CDC and "high" priority level by the WHO. *Enterococcus* spp. are also commonly monitored in food products, e.g., tigecycline and erythromycin-resistant *Enterococcus* spp. are regularly monitored in retail meat as part of the NARMS program (Karp et al., 2017). *Enterococcus* spp. are also a logical choice to complement the WHO Tricycle program because they are Gram-positive organisms and thus capture distinct antibiotic-resistant genotypes and phenotypes than Gram-negative *E. coli*.

Here a systematic literature review was conducted to assess the strengths and weaknesses of common workflows applied for the isolation and characterization of antibiotic-resistant enterococci from wastewater, recycled water, and surface water. The review targeted literature relating to antibiotic-resistant *Enterococcus* spp. monitoring in the environment and was carried out in Web of Science (2000-2020) with the key search terms: "water*" & "enterococc*" & "antibiotic resist*" (Davis et al., in revision). Over 105 peer-reviewed articles from 33 countries across 6 continents were identified. Key information, such as *Enterococcus* selective media, antibiotics used for susceptibility testing, and methods for genotyping resistant isolates was extracted from the papers to enable comparison of workflows across studies.

Antibiotic-resistant *Enterococcus* present many advantages as a potential target for monitoring AMR in the environment: they are abundant in human and animal feces, persist extra-enterically, have several environmental niches, and are easily cultured. *Enterococcus* have been targeted for decades for water quality monitoring in the US and EU and several standardized culture methods have been developed for their enumeration in water and wastewater. Although the environmental monitoring of antibiotic-resistant enterococci has been recognized by national and international organizations, lack of procedural standardization has hindered generation of universally comparable data needed to implement an integrated AMR surveillance program.

Ideally, a standard method for enumerating both total (generic) and antibiotic-resistant *Enterococcus* from diverse aquatic matrices would balance the sensitivity (i.e., low LOD), specificity (i.e., avoid detecting other types of bacteria), and the throughput needed for both large-scale and routine monitoring efforts. Logistical considerations are also warranted, such as the ability to employ the proposed assay in low-tech labs with reagents, materials, and techniques that are economically feasible. In this regard, standard methods put in place for recommended monitoring of saline and recreational freshwaters in the US and abroad can be leveraged and modified to meet these criteria. These assays include the USEPA Methods 1106.1 and 1600 for ambient waters and wastewaters, the International Organization for Standardization Methods 7899-1 and 7899-2, and Method 9230 (A-D) as part of the American Public Health Association's "Standard Methods for the Examination of Water and Wastewater". These assays represent three distinct techniques: membrane filtration, multiple tube fermentation, and defined substrate techniques (e.g., Enterolert™). Among these, membrane filtration is the most appropriate for isolating individual colonies for downstream antibiotic resistance genotyping and phenotyping. Leveraging the extensive QA/QC employed in these

standard assays, and the standard antibiotic susceptibility testing provided by the CLSI, could further facilitate adoption of proposed SOPs by US water utilities and other stakeholders.

Based on the literature review, a further evaluation of the three distinct approaches for enumerating antibiotic resistant *Enterococcus* spp. from aquatic matrices was considered, viewing them as three distinct frameworks (Figure 3-1):

- *Enterococcus* spp. population-level surveys
- Targeted monitoring for specific antibiotic resistant phenotypes
- Recovery of low concentration or viable but non-culturable (VBNC) populations

Population level surveys serve to provide ecologically relevant insights into the "true" distribution of *Enterococcus* spp. and their genotypes and phenotypes by plating on selective media in the absence of a primary selective antibiotic and collecting isolates at random for characterization. However, this approach is akin to finding a 'needle in a haystack' considering the number of isolates needed to detect phenotypes of interest with statistical significance.

Targeted monitoring is much more efficient and is advantageous when the phenotype of interest is known beforehand (e.g., high-level vancomycin resistance). Targeted approaches include an antibiotic (e.g., vancomycin) in the primary selective medium. Including vancomycin in the primary selective medium is also advantageous because *Enterococcus* spp. are intrinsically resistant to several antibiotics, most of which are not used to treat enterococcal infections. Thus, the targeted approach can also produce data that are more clinically relevant. To generate universally compatible data types, generic enterococci can be enumerated in parallel, thus allowing resistant colonies to be reported as a percent of the total enterococci population. This is analogous to the approach recommended by the WHO Tricycle program for ESBL *E. coli* monitoring.

It is important to be aware that both the population-level and the target approach are prone to overlooking some clinically-relevant phenotypes. For example, resistance to vancomycin, ampicillin, teicoplanin, oxazolidinone-linezolid, daptomycin, quinupristin/ dalfopristin, and tigecycline often exist at restrictively low concentrations in the environment. Also, *Enterococcus* spp. of interest may be VBNC, which is a common outcome of engineering controls, such as disinfection. In such cases, it could be of value to directly enrich for the phenotype of interest using highly concentrated broths spiked with low/intermediate levels of the relevant antibiotic. This can help both to decrease the LOD while also encouraging the growth of VBNC organisms. However, if this route is taken, then the resulting data will be qualitative, not quantitative as the enrichment step precludes the ability to quantify the absolute number of *Enterococcus* spp. in the original sample.

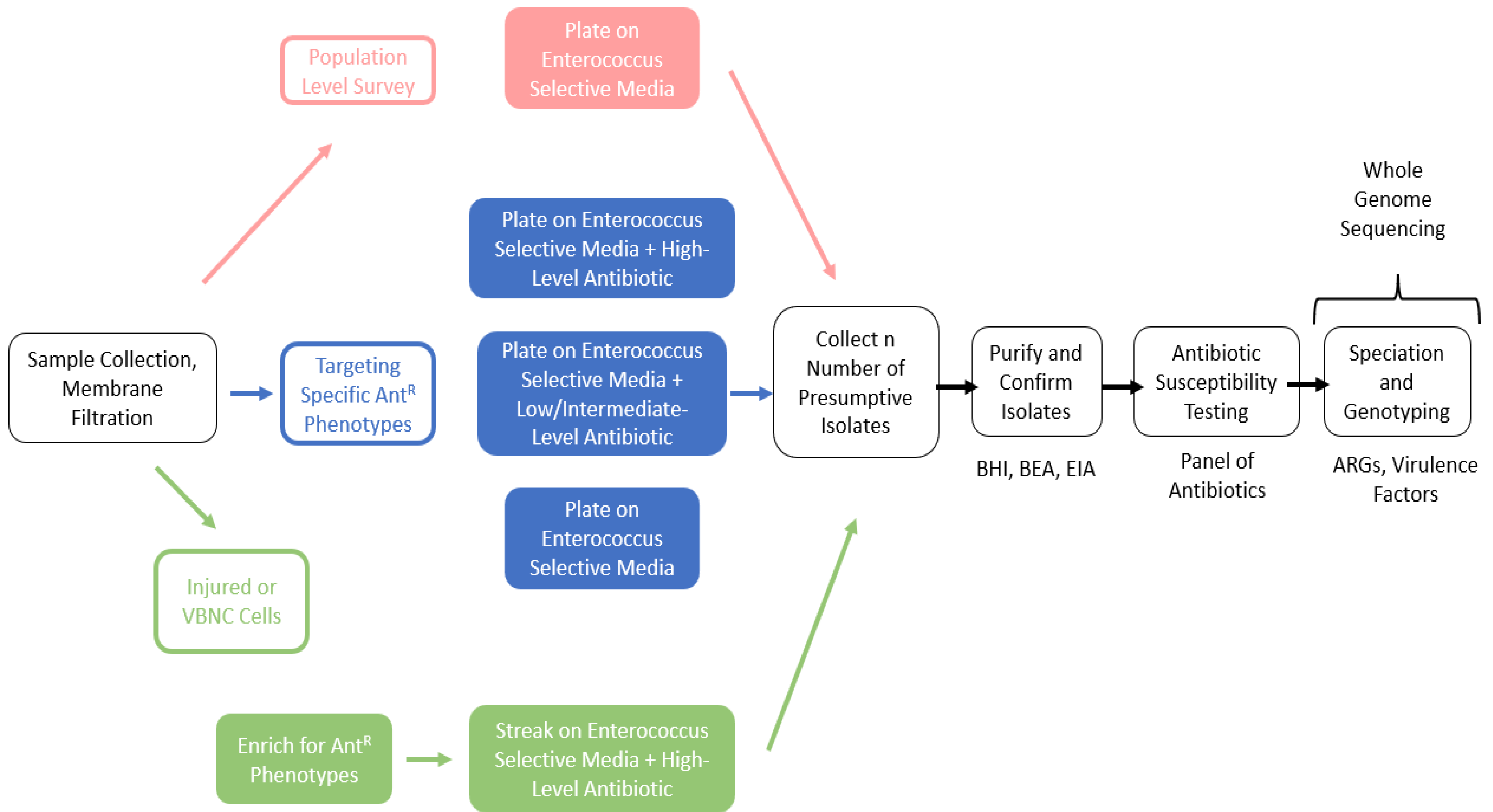

Figure 3-1. Workflows for Monitoring Antibiotic-Resistant *Enterococcus* in the Environment.

[1]Note that pre-enrichment for resistance phenotypes (injured or VBNC cells) prevents their quantification. [2]Antibiotic susceptibility testing of subsampled colonies often includes original selective antibiotic to confirm full "resistant" classification. [3]Whole genome sequencing is recommended for the most accurate speciation and comprehensive genotyping for global isolate comparisons. Ant^R = antibiotic resistance, VBNC = viable but non-culturable, BHI=brain-heart infusion, BEA=bile esculin azide, EIA=esculin iron agar, ARGs = antibiotic resistance genes

Once a collection of isolates has been generated, a myriad of approaches exist for speciation and genotyping. Because there are dozens of *Enterococcus* spp. with varying potential for pathogenicity, and all of these can carry a large diversity of ARGs, speciation and genotyping is necessary for comprehensive and informative monitoring programs. PCR was found to be the most widely applied and accessible method to both speciate and screen for ARGs and virulence factors, and several published assays demonstrate high specificity. Because the 16S rRNA gene has been found to be less discriminatory between closely related enterococci, targeted genes for speciation included atpA (alpha subunits of ATP synthase), groESL (chaperonin), pheS (phenylalnyl-tRNA synthase alpha subunits), and sodA (superoxide dismutase) (Jackson et al., 2004; Sanderson et al., 2019). The vancomycin resistance genes *van*A, *van*B, *van*C1, and *van*C2-3 (Dutka-Malen et al., 1995) were the most common ARGs screened, and virulence factors associated with surface adhesion and biofilm formation were prioritized (*esp, agg, gelE*). When feasible, whole genome sequencing will provide the most accurate and comprehensive characterization of isolates and provide the level of genomic detail necessary for assessing the relative hazard that individual isolates pose to human and ecological health (Gouliouris et al., 2018; Zaheer et al., 2020). Public sharing of whole genome sequences will further aid in the generation of libraries for tracking the evolution of the genus over time as clonal complexes traverse the globe and escape WWTPs.

A draft SOP for antibiotic resistant *Enterococcus* spp. monitoring was developed as part of Project 5052 and shared with the expert workshop participants (Chapter 4). Based on the literature review, mEI amended with vancomycin was identified as a good starting place to complement the WHO Tricycle Protocol for ESBL *E. coli.* Vancomycin-resistant *Enterococcus* spp. are important nosocomial pathogens in the U.S. and resistant isolates frequently display acquired multidrug resistance, increasing the value of tracking the evolution of the vancomycin-resistance phenotype across space and time. Additionally, mEI was shown to be the most sensitive and specific media across standard membrane filtration assays due to addition of the indoxyl-β-D-glucoside chromogen (Table 3-2), increasing the efficiency of any proposed SOP. Researchers should be wary, however, because intermediate vancomycin resistance is common among clinically-irrelevant *Enterococcus* spp. in the environment and may add noise to data collection. Ultimately, the draft *Enterococcus* spp. SOP was not selected for further validation but could be a useful starting place for others wishing to do so. Most of the sample collection and preparation steps described in the Project 5052 SOP for *E. coli* would be applicable to monitoring of *Enterococcus*.

Table 3-2. Sensitivity and Specificity of *Enterococcus* Selective Media Used in Standard Membrane Filtration Assays

Media	Sensitivity[a]	Specificity[b]	Environment(s) Tested	Reference
mEI	100%	97.3	Pure Cultures	(Maheux et al., 2009)
	82.4%	-	Marine	(Ferguson et al., 2005)
	100%	-	Surface	(Nishiyama et al., 2015)
	94.9%	-	Surface; Wastewater; Marine	(Ferguson et al., 2013)
	94.5%	-	Surface; Wastewater; Marine	(Ferguson et al., 2010)
	93.9%	-	Surface; Wastewater; Marine	(Messer and Dufour, 1998)
mEnterococcus	88.2%	91	Pure Cultures	(Pagel and Hardy, 1980)
	97.5%	-	Marine	(Dionisio and Borrego, 1995)
	94.2%	-	Marine	(de Oliveira and Watanabe Pinhata, 2008)
	88.5%	-	Surface	(Levin et al., 1975)
	90.2%	-	Surface; Wastewater; Marine	(Adcock and Saint, 2001)
Slanetz-Bartley	74.4%	78%	Pure Cultures	(Pagel and Hardy, 1980)
	93.8%	-	Marine	(Audicana et al., 1995)
	92.7%	-	Marine	(Tiwari et al., 2018)
	95.3%	-	Surface	(Łuczkiewicz et al., 2010)
	93.8%	-	Surface; Wastewater	(Fricker and Fricker, 1996)

[a]Sensitivity = true positive/(true positive + false negative)
[b]Specificity = true negative/(true negative + false positive)

3.3 Environmentally-Adapted Pathogen Targets: *Pseudomonas*, *Acinetobacter*, and *Aeromonas*

Other bacterial targets that are not fecal indicator bacteria could also be of value for culture-based monitoring of antibiotic resistance in water environments. Opportunistic bacterial pathogens that can persist and grow in environmental niches have a greater chance of their evolution being influenced by the environment itself (Blasco et al., 2008). For example, bacteria that are commonly present in WWTP effluents that can also persist and grow in aquatic

receiving environments have a greater chance of engaging in horizontal gene transfer with the diverse array of microbes that they encounter, both acquiring and transferring ARGs along the way (von Wintersdorff et al., 2016; Dey et al., 2020). Further, pathogens with environmental niches broaden the environmental dimension of One Health monitoring efforts, which has generally been lacking until now (Chapter 1), including relevance to wastewater, recycled water, animals, and humans (Rizzo et al., 2013; Ghosh et al., 2021; Patel et. al 2013). Monitoring data of this nature can help to identify broader trends in antibiotic resistance, by facilitating comparison across multiple compartments. Studying these organisms in culture provides the advantage of being able to confirm their viability while also being able to further characterize resistance phenotypes and genotypes.

A systematic literature review was conducted to evaluate the current state of culture-based methods employed for monitoring clinically-relevant ARB with environmental niches in the environment. Based on the high-level literature review and expert survey (Chapter 2), the focus turned to three candidate targets: *Acinetobacter*, *Aeromonas*, and *Pseudomonas*. All three of these targets are clinically important in the context of antibiotic resistance. For example, these bacteria may cause opportunistic infections in vulnerable populations. Though *Acinetobacter* spp. and *Pseudomonas aeruginosa* are typically associated with severe nosocomial infections, community-acquired infections are increasingly being recognized (Kanj and Sexton 2022). Severe community-acquired pneumonia, for example, is often associated with *Acinetobacter baumannii* in tropical environments (Kanafani and Kanj 2022; Anstey et al. 1992). *P. aeruginosa* infection can be expected in most cases of skull base osteomyelitis, a life-threatening progression of otitis externa, commonly known as “swimmer’s ear'', which also has the potential for drug resistance and clinical recurrence (Schaefer and Baugh 2012). Sepsis due to *Aeromonas hydrophila* following leech therapy has been linked to emerging fluoroquinolone resistance (Patel et al. 2013). All are also prevalent in aquatic environments, and a number of existing isolation methods exist that could potentially be adapted for standardized monitoring of antibiotic resistance in wastewater, recycled water, and surface water (US Environmental Protection Agency 2001, International Organization for Standardization 2006, Eaton et al., 2005). A systematic Web of Science search was carried out, spanning 2000-2020, with the following tiered key word search:

(1) TS = (“antibiotic resistan*” OR “antimicrobial susceptibility” OR “antimicrobial resistan*” OR “drug resistan*” OR “multi-drug resistan*” OR “resistome” OR ”ARG” OR “antibiotic resistan* gene”)

(2) TS = (“wastewater” OR “reclaimed water” OR “recycled water” OR “water reuse” OR “non-potable reuse” OR “greywater” OR “hospital wastewater” OR “surface water” OR “sewage” OR “wastewater treatment plant” OR “filtration” OR “direct potable reuse” OR “indirect potable reuse” OR “river” OR “watershed” OR “lake” OR “pond” OR “recreational water” OR “influent” OR “effluent” OR “aquatic” OR “water quality” OR “de facto reuse”)

(3) TS =(“culture” OR “dis* diffusion” OR “isolat*” OR "membrane filtrat*" OR "spread plating" OR "IDEXX" OR "Colilert" OR "Colilert-18" OR "Colisure" OR "Enterolert" OR "Pseudalert" OR "Enterolert-E")

(4) TS =("*Acinetobacter*" OR "*A. baumannii*" OR "*Aeromonas*" OR "*Pseudomonas*" OR "*P. aeruginosa*").

Based on these criteria, 50 papers were included in the review, with nine targeting *Acinetobacter* spp., 24 targeting *Aeromonas* spp., and 19 targeting *Pseudomonas* spp.

Acinetobacter spp., *Aeromonas* spp., and *Pseudomonas* spp. were found to be widely encountered in aquatic environments across the globe. These bacterial taxa are known to have highly plastic genomes that are readily modified by horizontal gene transfer. Many ARGs located on one MGE, often associated with increased virulence, can be acquired through horizontal transfer and integrated into the host chromosome of *Acinetobacter*, *Aeromonas*, and *Pseudomonas* spp. (Fernández-Bravo & Figueras, 2020; Finley et al., 2013; Partridge et al., 2018). The CDC antibiotic resistance threats priority list includes MDR P. aeruginosa as serious and carbapenemase-producing *Acinetobacter* as urgent (CDC 2019). The categories concerning, serious, and urgent were first defined by the CDC in the 2013 Antibiotic Resistance Threats Report, with the latter two requiring more monitoring and prevention efforts. Serious threats cause significant antibiotic resistant infections but may have low incidence rates or reasonably available therapeutic options, whereas urgent threats may have rising incidence rates and few available therapeutic agents (CDC 2013). Recently, carbapenemase-producing *A. baumannii* strains have emerged that can harbor resistance to nearly all conventional antibiotics and that, in some cases, are virtually impossible to treat (Finley et al., 2013; Santajit & Indrawattana, 2016).

Table 3-3 summarizes the media that were most commonly found to be applied for isolating these organisms from water samples. A challenge for these organisms, relative to *E. coli* and *Enterococcus* spp., is that the methods are not as well validated for application to water samples. Relative to clinical samples, for which many of these media were originally developed, there are numerous interfering non-target bacteria that can grow on the selective media and confound the results.

Table 3-3. Summary of Frequently Used Selective Media and Standardized Methods for the Culture of *Acinetobacter* spp., *Aeromonas* spp., and *P. aeruginosa* from Water and Wastewater

Acinetobacter spp. (9 studies)	**Frequently Reported Media**			
	Media	**Number of Citations (%)**	**Number of Wastewater Isolates Recovered**	**Number of Surface Water Isolates Recovered**
	CHROMagar *Acinetobacter*	6 (67)	5	1

Aeromonas spp. (24 studies)	**Reported Standardized Methods**			
	Method	**Media**		**Intended Matrices**
	USEPA Method 1605	Ampicillin dextrin + vancomycin (ADA-V)		Finished water
	Frequently Reported Media			
	Media	**Number of Citations (%)**	**Number of Wastewater Isolates Recovered**	**Number of Surface Water Isolates Recovered**
	GSP agar	7 (29)	4	3
	ADA group	6 (25)	6	2

Pseudomonas aeruginosa (15 studies)	**Reported Standardized Methods**			
	Method	**Media**		**Intended Matrices**
	ISO Method 16266	Cetrimide nalidixic acid (CN) agar		Finished water
	APHA Method 9213 E-F	M-PA-C		Surface and finished waters
	Frequently Reported Media			
	Media	**Number of Citations (%)**	**Number of Wastewater Isolates Recovered**	**Number of Surface Water Isolates Recovered**
	Cetrimide	6 (40)	6	1
	CN agar	5 (33)	3	5
	M-PA-C	1 (6.7)	1	1

Acinetobacter spp. isolates were largely cultured using the commercial chromogenic media CHROMagar *Acinetobacter* (89% of *Acinetobacter* studies). All but one of the studies using CHROMagar *Acinetobacter* (77% of *Acinetobacter* studies) included the addition of 15-mg/L of the cephalosporin antibiotic cefsulodin sodium hydrate to suppress the growth of *Pseudomonas* and *Aeromonas* species as well as the proprietary “MDR supplement” CR102, which selects for carbapenem-resistant strains.

Isolation of *Aeromonas* spp. has been standardized for drinking water, as described in the USEPA Method 1605 (USEPA, 2001). The purpose of this method is mainly to test the biological stability of the water, i.e., *Aeromonas* spp. as a nuisance heterotrophic organism. The isolation medium recommended by USEPA Method 1605 is ampicillin-dextrin agar with vancomycin (ADA-V). It is not recommended to use ampicillin-containing media in clinical settings because

some pathogenic species of *Aeromonas* are sensitive to ampicillin, and therefore will not grow on the media (Morris & Horneman, 2022). The two most frequently used selective media for *Aeromonas* were ADA agar with antibiotics to suppress non-target growth (ADA group) and glutamate starch phenol red agar (GSP). A comparison of the two media is needed to assess their performance with surface water and wastewater samples.

Nearly 80% of the studies that focused on *Pseudomonas* spp. isolated specifically for the pathogen P. aeruginosa. Isolation of P. aeruginosa in finished water has been standardized by the ISO Method 16266 (International Organization for Standardization, 2006), while isolation from natural and finished surface waters is standardized by the APHA Method 9213 E-F in the "Standard Methods for the Examination of Water and Wastewater" (Eaton et al., 2005). ISO Method 16266 recommends the use of *Pseudomonas* agar base with the addition of cetrimide and nalidixic acid (CN agar), while APHA Method 9213 E-F recommends the use of M-PA-C agar. The two most frequently reported media were cetrimide agar and CN agar.

All the studies included in the systematic review confirmed the genus of their isolates at a minimum. However, many of the papers reviewed unfortunately lacked reporting of phylogenetic confirmation rates, i.e., the frequency of false-positive results (only 16% of studies reported this). This makes comparison of media performance difficult to track. Further speciation and characterization of strains can further be of value for antibiotic resistance monitoring, especially to account for intrinsic resistance or variable resistance rates among species. However, many of the methods reported for isolate speciation are known to be reliant on inaccurate and out of date databases.

There are no standardized methods for the culture of any of the three organisms from wastewater, nor are there any existing standardized culture methods for recovery of *Acinetobacter* spp. from any water environment. This is a fundamental stumbling block to development of standardized methods for targeting any of these three organisms for the purpose of monitoring antibiotic resistance in wastewater, recycled water, or surface water environments. As a next step to moving towards standardization of these targets, it is recommended that the performance of GSP agar be compared to the USEPA Method 1605 for isolation of *Aeromonas* spp. in surface and wastewater matrices. GSP agar may be suitable for isolation of *Aeromonas* and *Pseudomonas* spp. simultaneously, however more work is needed to validate this aspect on environmental water samples. ISO Method 16266 has shown to be a promising method for isolating *P. aeruginosa* from surface water and can be systematically evaluated for use on unfinished surface water and wastewater. Draft SOPs for the target organisms were developed and evaluated at the expert workshop (Chapter 5), but were not selected for further evaluation. More work is needed in developing and validating methods for the isolation of *Acinetobacter* spp. from the environment.

3.4 qPCR Targets

qPCR has been applied broadly over the past two decades to directly quantify ARGs in various environmental matrices (manure, soil, water, air, etc.). qPCR helps to avoid some of the biases associated with culture-based methods, because it theoretically captures the ARG target of interest carried across the bacterial community, and not just the particular organism subject to

culture. qPCR also provides much quicker results than culture-based methods, requiring only a few hours to concentrate the sample, extract DNA and perform the analysis. DNA extractions obtained from the water samples can also be preserved in the freezer for extended time periods, making it possible to return to the same sample for repeated analysis. Depending on the concentration method and other factors, qPCR can be a very sensitive technique and produce quantitative data over several logs, without the guesswork associated with selecting dilutions, as is required for culture-based methods. qPCR can be particularly useful for comparative studies or for quantifying removal rates (i.e., Chapter 1, Monitoring Objective 3). A challenge with qPCR data is that they are less relevant to risk assessment models than culture-based targets, but efforts are underway to incorporate qPCR data for this purpose (Hamilton et al.).

A critical first step to developing standard methods for qPCR-based monitoring of ARGs is selecting agreed upon targets. A systematic literature review was conducted in order to identify ARGs best suited for monitoring in the water environment. First, Ashbolt et al. (2018), who recently compiled a comprehensive summary of occurrence and trends for 50 ARGs monitored across various WWTP reported in the literature, was carefully considered. This work was published as a book chapter as part of the Global Water Pathogens Project (GWPP, 2015). Similarly, Nnadozie and Odume (2019) synthesized the gene abundances reported in the literature for ARGs conferring resistance to a variety of antibiotics (sulfonamides, tetracyclines, aminoglycosides, beta-lactams, chloramphenicols, macrolide-lincosamide-streptogramin (MLS)) in freshwater sources (rivers, ponds, and lakes). The CDC recently identified five clinically-relevant ARGs often carried by pathogenic ARB posing serious health threats that encode ESBLs: *bla*CTX-M, *bla*KPC, *bla*OXA-48, *bla*NDM, and *bla*VIM (CDC, 2019a). Further, the team took into consideration the targets under investigation in the National Aquatic Resource Survey (NARS) recently launched by the USEPA: *int*I1, *sul*1, *tet*W, *bla*TEM, *bla*KPC, *van*A, and MCR-1 (Keely, 2019). Finally, the recommendations based on the expert survey (Chapter 2) were considered.

As suggested by Ashbolt et al. (2018), Berendonk et al. (2015), Pruden et al. (2018b) and in combination with feedback from the expert survey on prioritizing ARG targets (Liguori et al., 2022), the team opted to evaluate a combined monitoring framework that considers clinically-relevant ARGs (*bla*CTX-M and *van*A), anthropogenically sensitive ARGs (*sul*1, *tet*A), and an MGE commonly associated with carriage of multiple ARGs, ARG mobility, and anthropogenic inputs (*int*I1). Such a framework could serve to identify potential "hot spots" for evolution and dissemination of antibiotic resistance, while also capturing ARGs that are of immediate concern in terms of human health risk. Thus, the five recommended qPCR targets that were further evaluated were:

- *int*I1 (Class 1 integron commonly indicative of anthropogenic sources and mobile forms of multi-antibiotic resistance)
- *sul*1 (Encodes resistance to sulfonamides, the first class of antibiotics to be mass produced for human medicine. *sul*1 shows strong correlations with human inputs and is typically carried by class 1 integrons)
- *tet*A (Encodes resistance to tetracyclines, a very widely used class of antibiotics in humans and livestock, and tends to be associated with anthropogenic sources)

- *bla*CTX-M (Encodes ESBLs and is noted as a “serious” concern in CDC threat list.)
- *van*A (Encodes resistance to vancomycin, which is a last-resort antibiotic used to treat Gram positive infections, and is listed as “serious” in CDC threat list)

Various aspects of methodology, quality assurance, and data expression in protocols must be addressed in order to ensure that qPCR measurements are representative and comparable across studies. Using the proposed gene targets, a systematic literature review was conducted in order to identify currently prevalent workflows for ARG monitoring (Keenum et al., 2022). 117 peer-reviewed studies were identified which met search criteria for application of assays to the analysis of wastewater, recycled water, or surface water and systematically assessed the corresponding protocols with respect to the following: sample collection and concentration, DNA extraction, primer/probe specificity, amplification conditions, amplicon length, evaluation of PCR inhibition, and limits of detection and quantification. There was substantial variation in the qPCR workflows applied, from sample collection to data analysis. However, there were also many commonalities and opportunities to standardize the methods. Gene copy numbers reported across studies were compared by assay and water matrix (Figure 3-2) to help narrow down qPCR protocols for the development of SOPs to those targets that were detectable in the target matrix. Due to the varied nature of the samples that were going to be assessed, SOPs were developed for gene targets that would be most widely detectable across water matrices.

Overall only 59% of studies reported any of the recommended QA/QC guidelines as stated in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). The lack of reporting of LOD and LOQ values specifically is problematic as it means these studies cannot be further implemented in downstream risk assessment models. Findings indicated that the choice of DNA extraction kit, surprisingly, did not significantly impact the quantified abundance of ARGs in the target water environments, however, the choice of gene assay did. Evaluation of PCR inhibition was applied in 59% of studies. Dilution of DNA extracts was the most common method, and was employed in 60% of studies that noted inhibition testing. While dilution of the template DNA is an effective strategy for assessing inhibition when gene targets are abundant (Wilson, 1997), it effective when gene targets can be easily diluted below the limit of detection. In this case, standards that are spiked into samples and run as multiplex assays with the primary assay (internal standards) or standards spiked into separate assays containing the sample nucleic acid are better options (Green et al 2014).

We identified the common objectives for conducting ARG monitoring and were able to effectively reflect how this would modify ARG selection and quantification methodology. This is reflected in the qPCR SOP available in the Appendix (Appendix B3). Standardizing the detection and quantification of ARGs in various water samples will facilitate comparison across studies, including global assessment of antibiotic resistance levels in various systems and identification of hot spots of concern where ARGs are persisting, amplifying, and/or mobilizing

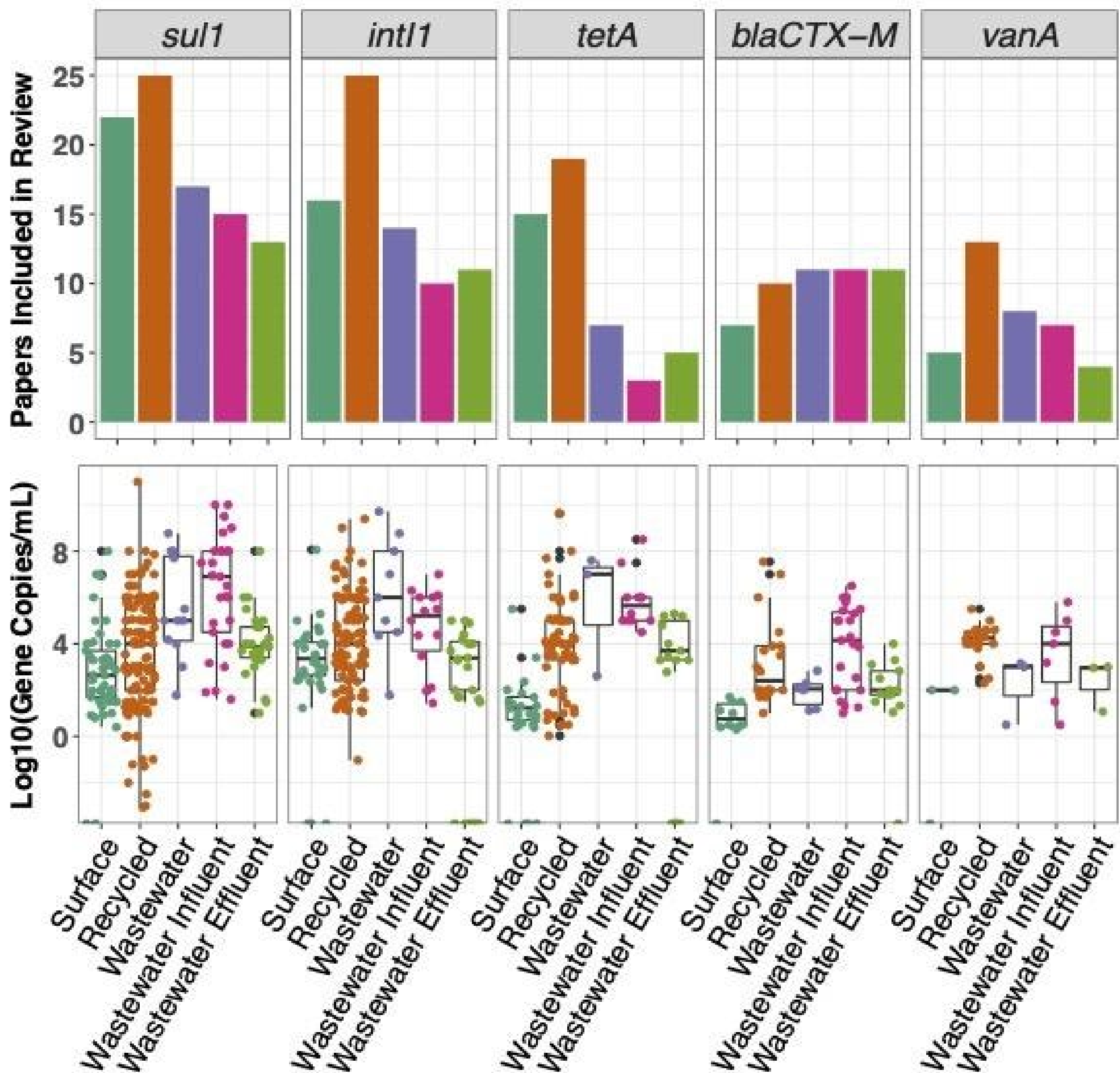

Figure 3-2. Summary of qPCR Literature Review.

(A) Number of Articles that Met the Search Criteria for Each Gene by Water Matrix (Surface, Recycled Water, and Wastewater). Articles that examined more than one water matrix are double counted. (B) Target Gene Concentrations Measured in Each Water Matrix Based on Data Extracted across the Studies in a That Reported as gc/mL. Box plots indicate the median, first, and third quartiles and whiskers extend no more than 1.5 times the inter-quartile range

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3.5 Metagenomic Based Monitoring of Antibiotic Resistance

Shotgun metagenomic sequencing (i.e., metagenomics) entails the use of NGS to capture the collective genomic information across a microbial community inhabiting a sample of interest. The technique has emerged as a powerful approach for in-depth surveys of bacterial

communities in various environmental samples, including wastewater, recycled water, and surface water environments (Hendriksen et al., 2019; Garner et al., 2018; Lee et al., 2020). Metagenomic sequencing data can be compared against publicly-available databases to identify genes of interest, such as ARGs. Metagenomics is a highly promising tool for antibiotic resistance monitoring in the environment because it has the potential to survey all known and putative ARGs in a given sample (i.e., the "resistome") simultaneously, in one analytical step, without *a priori* identification of gene targets. This capability circumvents the requirement to select a target of interest in advance of analysis, which is a distinct drawback of both culture- and qPCR-based approaches described above.

Metagenomic methods are generally less developed and standardized for work in environmental matrices relative to culture- and qPCR-based methods. However, the promise of metagenomics is widely recognized across the research community as a comprehensive monitoring tool for antibiotic resistance and efforts are underway to consider means of advancing its application for this purpose (Aarestrup and Woolhouse, 2020). For example, as part of the EMBARK project supported by the JPIAMR, an ambitious combination of genomic and metagenomic data paired with machine learning and mathematical modeling is proposed to track the risk of resistance development across environmental sectors.

We conducted a systematic review of the literature to identify papers wherein metagenomics was specifically applied for the purpose of monitoring resistomes in wastewater, recycled water, and surface water. A Web of Science search was conducted, spanning 2000-2020, with the following keywords: "metagenomic*" or "next-generation sequenc*" & "antibiotic resist*" & "*water*". 95 articles were identified that ultimately met our search criteria and evaluated their corresponding workflows from sampling to bioinformatics and data analysis approaches. Based on this review, here are summarized key observations regarding strengths and limitations of metagenomic monitoring of antibiotic resistance in water environments and outline a suggested path forward for future standardization.

Table 3-4 summarizes the key advantages and disadvantages of a metagenomic approach to antibiotic resistance monitoring of aquatic environments, along with a recommended path forward for standardization.

Table 3-4. Limitations and Recommendations for Metagenomics-Based Monitoring

Limitation	Recommendations
Much more expensive than qPCR or culturing	-Robust experimental and sampling design that focuses on key comparisons of interest and does not include extraneous samples -Wait for sequencing technology costs to decrease to an affordable level (NIH, 2021)
Requires deep sequencing (which is more costly) to detect rare targets	-Develop guidance on necessary sequencing depth for monitoring objective, to obtain needed data without the excess cost
Cannot directly confirm the viability of host organisms	-Employ messenger RNA sequencing to assess gene expression (meta transcriptomics) -Employ PMA or EMA to focus on DNA from intact cells (*these methods are in continued validation/development) -Perform pre-enrichment steps to favor dominance of DNA from viable organisms of interest
Lack of common approaches for methods and analysis impedes comparability	-Compare effect of selected normalization denominators on conclusions drawn from comparisons within and between data sets -Develop guidance and pipelines to standardize data analysis and reporting to improve comparability

One major advantage of the metagenomic approach to antibiotic resistance monitoring is that the DNA extracts can be frozen (-80 °C is ideal) for many years and re-analyzed at a later date. Similarly, another advantage is that once an NGS library has been generated, it can be shared to public repositories where it can be analyzed and reanalyzed to query additional targets as they become known to the scientific and medical communities. This can greatly facilitate data sharing and meta-analysis and support evaluation of larger, global research questions (Chapter 1), such as resistome trends across time and space. However, it will be critical for such endeavors to ensure that shared sequencing data are representative and comparable.

Unfortunately, among the articles identified in the systematic review, there was vast variation in the protocols applied for generating metagenomic sequencing data with little application of experimental controls to verify the quality of generated data. Specifically, guidance is needed with respect to sampling design, sample preservation and storage, DNA extraction techniques, library preparation, sequencing depths, bioinformatic approach and the use of experimental controls (Figure 3-3). Table 3-4 additionally proposes measures that can be taken to identify the current limitations identified for metagenomic analysis. Key aspects of these components of the metagenomic workflow are discussed in the following sub-sections.

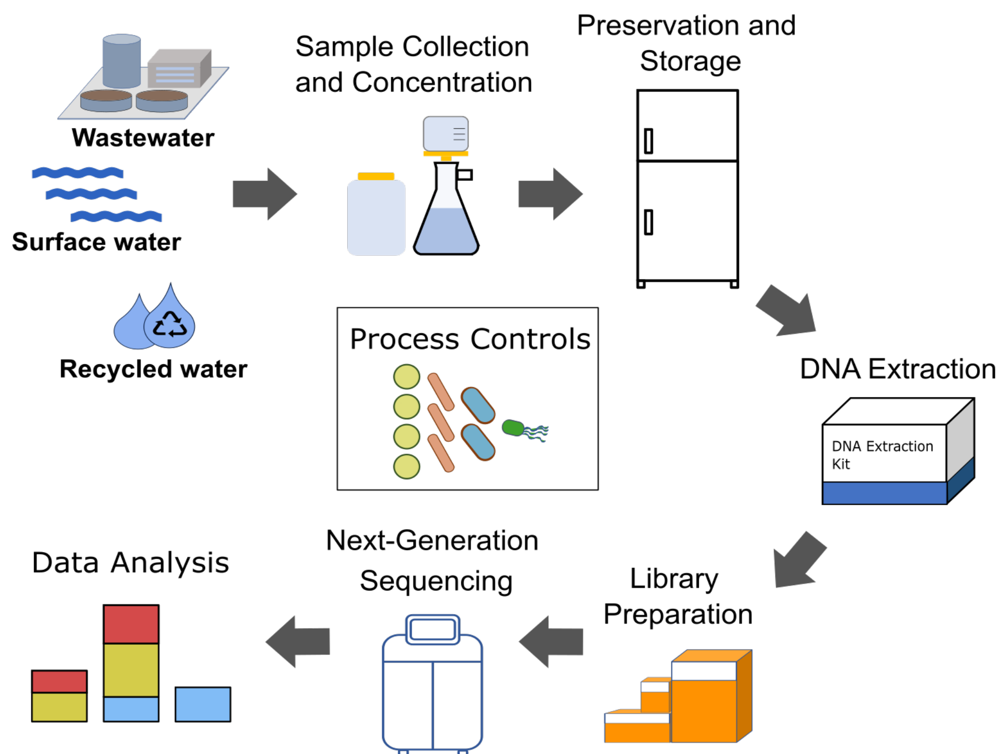

Figure 3-3. Overview of Major Steps Involved in Metagenomic Investigations of Aquatic Environments. Process controls are centered in the middle as they can be used to characterize contamination or bias along the entire workflow.

3.5.1 Sampling Approach

Generation of quality metagenomic data starts with sampling design. Ideally, sampling locations, volumes, and methods should be selected in a manner that is representative of the environment of interest and preserves the corresponding microbiota of the original sample. Across the 95 articles identified in this review, nearly all samples were taken as grab samples. These were typically collected to assess the dynamics of or impacts to the system of interest in cross-sectional study designs. For example, many studies employed grab sampling to compare various stages of wastewater treatment and upstream and downstream of discharge points to examine the effects of anthropogenic inputs to surface water (Chu et al., 2018; Zhang et al., 2019). Composite sampling is occasionally done, e.g., with integrated samplers that periodically collect samples of a specified volume over a set period of time (typically 24 hours), which are then combined for further time- or flow-weighted analysis. Composite sampling is increasingly being applied to WBE, i.e., the monitoring of public health threats in influent sewage with time (Augusto et al., 2022). In this case, composite sampling helps to increase the probability of capturing the target of interest, especially if it is transient. However, it is important to be aware that composite sampling requires costly and cumbersome samplers and is not appropriate if the intention is to capture diurnal stochastic variation in microbiome or resistome composition.

Sample processing and preservation is also important to consider. As is the case for culture-based methods, best practice is to maintain the samples on ice, to minimize microbial activity, and to concentrate the sample as soon as possible, ideally within the same day. Concentrating

onto a 0.2 µm or 0.5 µm filter was the most common method for capturing microbes from the aqueous samples for further analysis. A comparative study in which samples were collected in Hong Kong and Virginia and subject to various storage and shipment conditions prior to being swapped for analysis found that storing filters containing concentrated cells in 50% ethanol solution prior to shipping and storage at -20 °C adequately preserved the microbial community such that they could not be distinguished by taxonomic or resistome composition following metagenomic sequencing (Li et al., 2018).

3.5.2 DNA Extraction

Ideally, DNA extraction serves to obtain the DNA across all microbes present in the sample so that it can then be subject to NGS. However, it is widely known that no DNA extraction method obtains all DNA present in a sample and that there are also biases in the DNA that is obtained (Tan & Yiap, 2009). Even lysing of both Gram-negative and Gram-positive organisms is especially problematic, because the latter are characterized by thicker cell walls that must be penetrated to access the DNA. On the other hand, more aggressive extraction to obtain Gram positive DNA can damage the DNA from Gram negative organisms. Li et al. reported that extraction protocols applied to wastewater that employ bead-beating result in recovery of microbial community DNA that is characterized by higher diversity (Li et al., 2018). Based on this, DNA extraction kits that employ bead beating were recommended. Another consideration is obtaining DNA that is relatively pure and free of impurities that could inhibit downstream metagenomic sample preparation. Notably, Li et al. compared the Fast DNA Spin Kit for Soil (MP Biomedicals), PowerSoil DNA Isolation Kit (MoBio), and the Fecal DNA MiniPrep (Zymo Research) extraction kits, noting that Fast DNA Spin Kit for Soil yielded the highest concentration and purity of DNA. Across the 95 studies examined for this study, DNA extraction was performed almost exclusively with commercial kits employing bead beating and silica spin columns for subsequent purification and removal of PCR inhibitors. Specifically, the most common kit used was the Fast DNA Spin Kit for Soil, followed by the DNeasy water and soil kits. As noted above, a key advantage of metagenomic sequencing is that DNA extracts can be stored frozen (ideally at -80 °C) for later analysis.

3.5.3 Sequencing Platform, Library Preparation, and Sequencing Depths

Illumina sequencing (Illumina, Inc., San Diego, CA), which produces short reads of up to 300 bp (depending on platform and chemistry), currently dominates the NGS market. Accordingly, only four of the 95 studies examined did not use Illumina sequencing for profiling resistomes in the wastewater, recycled water, and surface water samples. A disadvantage of Illumina sequencing is that the resulting reads can be too short to capture the full gene length or neighboring genes of interest. For example, it is often desired to determine if the ARGs are present on MGEs or are carried by pathogens, which could be confirmed by annotating genes adjacent to the ARG on the DNA sequence. For such purposes, long-read sequencing is more suitable. Nanopore (Oxford, UK) and PacBio (Menlo Park, CA) are currently the dominant technologies for long-read sequencing and can produce read lengths upwards of 2 Mb. The trade-off with long-read sequencing is that it cannot achieve the same sequencing depths as short read sequencing and therefore rare sequences can be overlooked (Che et al., 2019). Only four articles that met the search criteria employed long read sequencing, but it is expected that momentum will gain in the application of long read sequencing for AMR monitoring of water environments due to the

advantages of longer sequences and also the ease of use of the Nanopore technology, in particular. Note that short reads can be assembled to produce longer sequence information, i.e., contigs and scaffolds (and in some cases even metagenome-assembled genomes (MAGs), but there are a wide variety of assembly algorithms and all introduce error into the analysis (Brown et al., 2021). These sources of assembly error are primarily due to the inherent complexity of environmental microbiomes and there are currently no recommended paths towards correcting for them.

Library preparation is highly dependent on the sequencing platform selected, as there are a variety of commercial kits available that are compatible for each technology, many of them manufactured by the sequencing company. Little research is available on biases and differences introduced into the sequencing data as a result of the library preparation method chosen. One key aspect to pay attention to is whether the kit employs a PCR amplification step (e.g., Nextera XT Library Preparation Kit). This is often necessary for samples with low DNA yield (e.g., < 1 ng), but will introduce PCR bias into the resulting library. Thus, kits employing amplification should only be employed if necessary, e.g., when less than 1 ng of total DNA is available for sequencing.

Sequencing depth is another critical choice that must be made when designing a metagenomic-based monitoring program. Coverage, i.e., the proportion of the total microbiome captured, varies directly with sequencing depth. Deeper sequencing increases coverage and is more likely to capture rare sequences, such as an emerging ARG of interest, but incurs significantly greater cost per sample. Currently, commercial labs charge about $18,000 per Illumina NovaSeq 6000 S2 flow cell (150 bp chemistry) with accompanied library preparation, which typically generates 1000 Gb of sequencing data. Thus, if choosing between running 1-96 samples on a NovaSeq flow cell, the cost would be $187.5-$18,000 to generate 10-1000 Gb of sequencing data per sample, respectively. It is important to consider that these estimates include the library preparation service and costs can be cut significantly if libraries are generated in-house. Data was extracted across the 95 articles and found that sequencing depth varied widely across studies, with an average depth of 7.1 Gb and coverage of 60.8% (Figure 3-4). Thus, it can be said that, as typically employed in the scientific literature to date, metagenomic sequencing will mainly only capture dominant ARGs in the microbial community. The authors of the Nonpareil software (Rodriguez et al., 2018) used here to calculate metagenomic coverage recommend a minimum coverage of 60% to achieve accurate representation of microbial diversity in individual samples. Generally, deeper sequencing (e.g., ≥ 10 Gb) will be necessary to reliably achieve ≥ 60% coverage and capture rare ARGs of interest, however, this will be dependent on the inherent complexity of individual aquatic environments and the implications of individual research objectives.

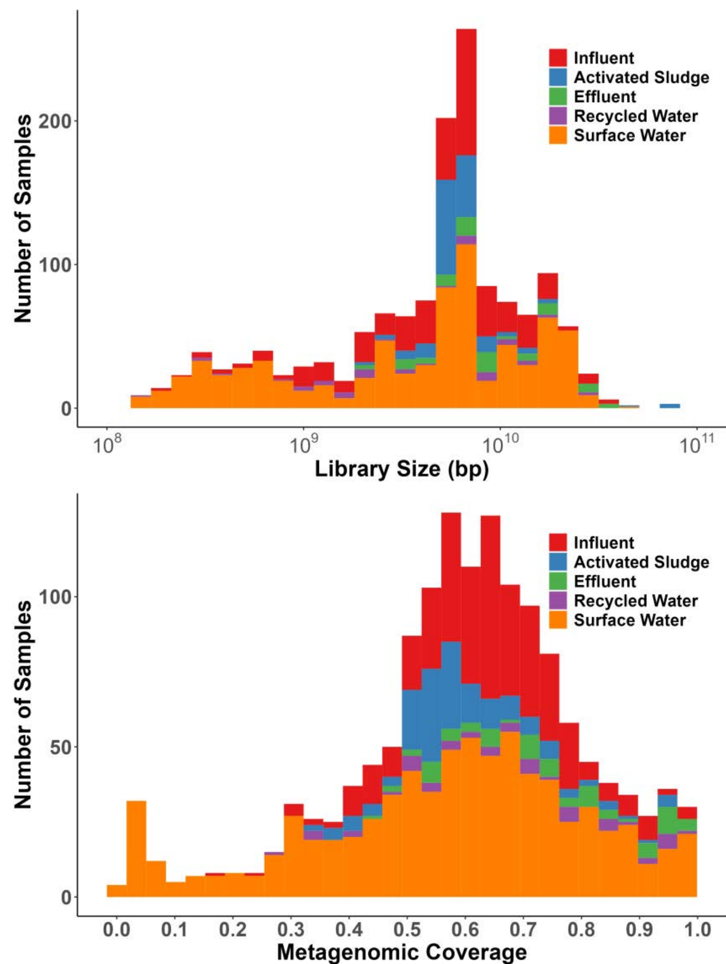

Figure 3-4. Sequencing Depth and Coverage by Water Matrix.

All publicly available Illumina sequencing data from the 95 reviewed articles was downloaded from the Sequence Read Archive (n=1440) and analyzed using Nonpareil. The y-axes represent the density of individual metagenomes occurring at that depth or coverage factor.

Data Source: Rodriguez et al., 2018

3.5.4 Bioinformatic Analysis, Data Normalization, and Quantitative Capacity

Detecting genes of interest from metagenomic data requires alignment of sequencing data against a relevant database. This is a major limiting factor for cross-study comparisons because there are numerous ARG databases to choose from. The most common databases for ARG annotation were: the Comprehensive Antibiotic Resistance Database (CARD; 38 articles), the Structured Antibiotic Resistance Gene database (SARG; 14 articles), ResFinder (9 articles), and DeepARG-db (5 articles). Databases were used independently, or sometimes they were combined. Manual curation was also common in earlier studies to address issues such as some of the ARGs in databases being incurred by a point mutation in a housekeeping gene, which cannot be distinguished from sequencing error. However, such issues have been addressed in the most recent version of CARD, for example, by the option to exclude such genes. Using common databases, where curation occurs centrally and the version is tracked, is important to support comparability of the data. Annotating MGEs has been more challenging, as commonly used databases (e.g., ACLAME) are not comprehensively curated or updated. Taxonomic

annotation is relatively more straightforward as it is widely applied across the microbial ecology field. Kraken2/Bracken (Wood et al., 2019; Lu et al., 2017), Centrifuge (Kim et al., 2016), and GTDB-TK (Chaumeil et al., 2020) are commonly employed for taxonomic annotation. An important cautionary note, however, is that rarely is there sufficient resolution in sequencing data, even when assembled, to be confident in the annotation of human pathogens. Thus, usually, such detections are reported as “taxa known to contain human pathogens” or “pathogen-like sequences”.

Detecting genes based on alignment to databases further requires annotation criteria. Annotation criteria are based on sequence homology cutoffs, such as percent amino acid identity, e-value, and bit score. This is a key choice that will directly affect comparability across studies because the absolute number of detected ARGs in a given library is directly governed by these threshold values. Newer databases now recommend cutoffs, or employ them as default, for distinct protein models that will help avoid such guesswork. Machine learning algorithms for annotating ARGs present another alternative to arbitrary cut-offs that is gaining traction (Arango-Argoty et al., 2018).

Once metagenomic sequencing data are obtained, there are seemingly infinite ways to analyze the data. One challenge is that the size of the sequencing library obtained is dependent on a variety of factors, including targeted sequencing depth and stochastic variation, and therefore annotated genes cannot directly be normalized to the volume of sample originally analyzed (e.g., gene copies/mL). For this reason, metagenomic data are typically reported in relative abundance units. This is achieved by normalizing the data to an internal (e.g., library size) or an external (e.g., a database of single-copy genes) factor. Common units of relative abundance encountered in the literature for resistome analysis include; ARGs/16S rRNA gene copies (21 articles), ARGs/*rpoB* gene copies (12 articles), fragments per kilobase million (FPKM, 11 articles), or a parts-per-million (ppm, 10 articles) normalization. This variance in reporting units is a challenge to producing universally comparable data. Based on an analysis of publicly-available metagenomic data associated with the 95 articles, the consistency and comparability of different normalization methods were compared by their ability to eliminate sequencing bias (i.e., the more sequencing data that is generated, the higher the total ARG abundance) which masks biologically relevant insights (Figure 3-5). Based on this analysis, it was found that normalization to the *rpoB* gene was optimal, because it occurs as a single copy gene and provides a per-cell-normalized estimate of carriage of ARGs (Davis et al., in review). Encouragingly, trends were highly similar when normalizing to the historically more common denominator of 16S rRNA genes. The FPKM normalization strategy also effectively eliminates sequencing bias more effectively than ppm and is an attractive alternative for researchers wanting an internal normalization strategy.

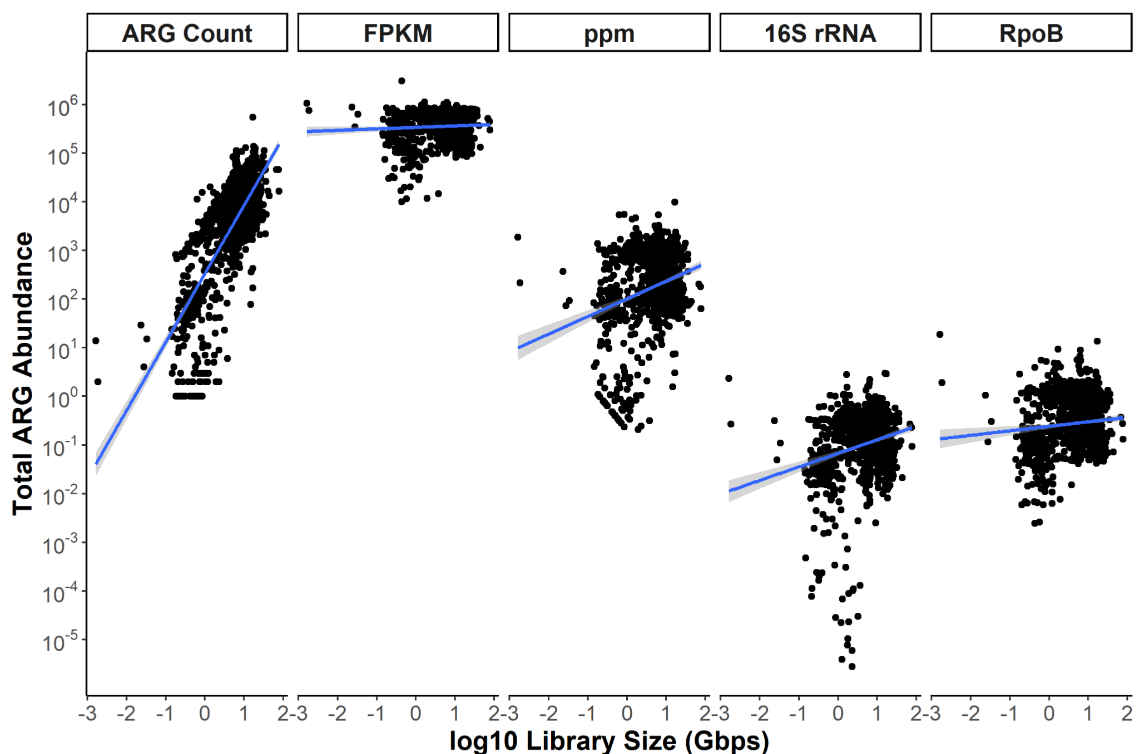

Figure 3-5. Analysis of Different ARG Count Normalization Strategies to Eliminate "Sequencing Bias" (i.e., the more sequencing data that is generated, the higher the total ARG abundance). The "ARG Count" column represents unnormalized data that highlights the effect of sequencing depth on the recovery of additional ARGs. All publicly available Illumina sequencing data from the 95 reviewed articles was downloaded from the Sequence Read Archive (n=1440) and are represented.

Once ARGs are annotated and normalized, there are numerous analysis and visualization options (Majeed et al., 2021). Figure 3-6 provides several examples, while Table 3-5 lists several common analysis and visualization approaches and the example research questions that they can address. For example, non-metric multidimensional scaling (NMDS) analysis is useful as a data reduction tool, truncating resistomes into single points in 2-D or 3-D space based on matrix-tabulating their relative dissimilarities. The result is that resistomes that cluster can readily be identified as "similar" and those that do not as "dissimilar." Such comparisons can further be supported by statistical tests such as Analysis of Similarities (ANOSIM) and others used in the field of ecology. Stacked bar charts of total annotated ARGs, classified according to the class of antibiotic to which they encode resistance, are also a common representation of the data and can usually be compared using parametric or nonparametric statistics, as appropriate. Another option is specifically filtering for clinically-relevant ARGs of interest, such as those encoding ESBLs, carbapenem resistance, vancomycin resistance, and colistin resistance. ExtrARG is a publicly-available tool that specifically identifies discriminatory ARGs, i.e., ARGs that differentiate samples of interest, using an extremely randomized tree analysis (Gupta et al., 2019). MetaCompare specifically notes long-read or assembled short-read data to identify the extent to which ARGs occur on MGEs (ARG + MGE) and are carried by potential human pathogens (ARG + MGE + pathogen) and produces a relative "resistome risk" score (Oh et al., 2018). This score can be used to compare samples representing different sampling locations and prioritize where there is greatest concern for new forms of resistance to evolve

and where mitigation efforts may be particularly warranted. Similar analyses can be used to examine the mobility potential of individual ARGs through the use of co-occurrence or correlation network analysis.

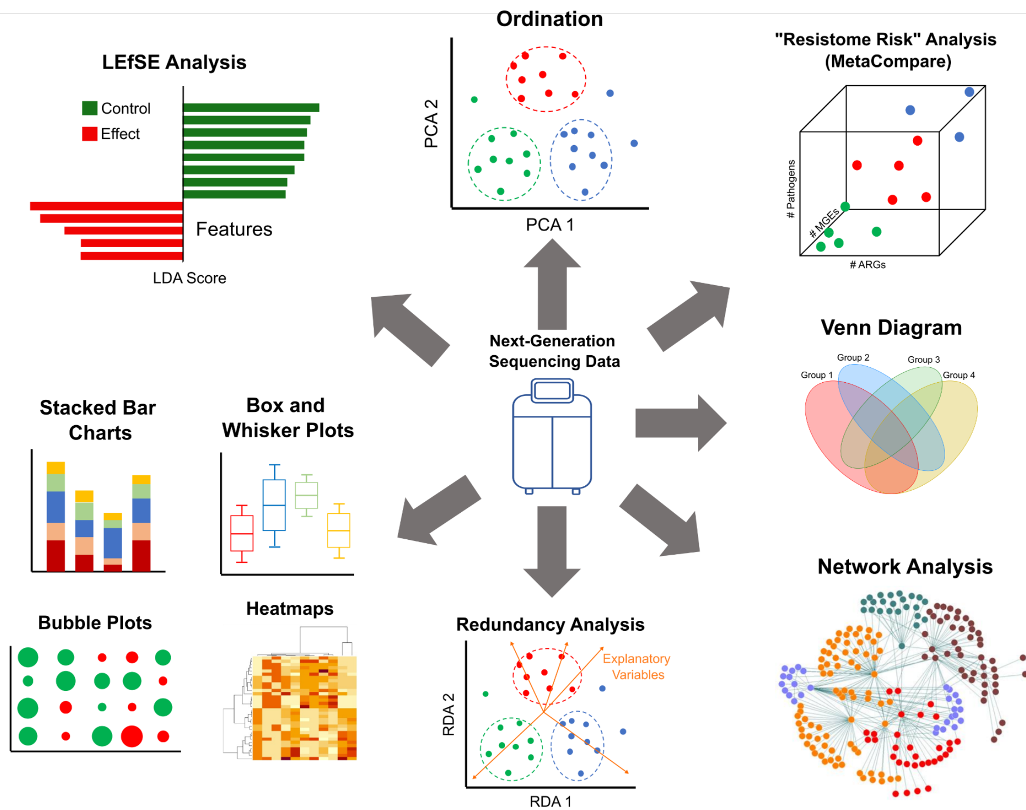

Figure 3-6. Common Analysis and Visualization Techniques Used to Investigate Environmental Resistomes Using Metagenomics.

Example research questions and analysis approaches are presented in Table 3-5.

Table 3-5. Common Research Questions Posed and Analysis and Visualization Strategies Used for Metagenomic Based Investigations of Aquatic Resistomes

Research Question	Analysis Approach	Common Visualization Strategy
Are ARGs being removed by an engineering control?	[1] Examine change in abundance of total ARGs, individual drug classes, and individual ARGs	Stacked bar charts, box-and whisker plots, bubble plots, heat maps
How do my sample compositions compare to each other?	[1] Ordination with dissimilarity matrix (NMDS, PCoA)	Two-dimensional ordination plots colored by sample factors
What ARGs characterize an aquatic environment?	[1] Determine the ARGs that are common to all samples and those that are unique (LEfSE, ExtrARG)	Linear discriminant analysis effect size plots, Venn diagrams
Which biotic or abiotic factors are driving ARG abundance?	[1] Redundancy analysis	Two-dimensional ordination plots with explanatory vector overlays
How do I determine the hazard ecology of my environment?	Risk assessment models (MetaCompare, ARG Ranker)	Plotting samples in 3-D "Risk Space"
Which ARGs are associated with mobile genetic elements or pathogens?	*De novo* assemble sequence reads; annotate for ARGs, MGEs, and pathogens; assess co-occurrence patterns. Correlation networks	Network analysis

[1] Note that each analysis begins with annotating reads to an ARG database and normalizing counts to an internal (FPKM) or external (*rpoB* gene copies) factor

3.5.5 Quantitative Metagenomics (qMeta)

The status quo application of metagenomics for antibiotic resistance monitoring to date has been poorly suited to informing human health risk assessment. In particular, the lack of strict quantitation and consequential reporting of relative abundance units is a drawback. However, recent studies have demonstrated that metagenomics does in fact have quantitative capacity (Crossette et al., 2021; Li et al., 2021; Hardwick et al., 2018). With the addition of internal reference standards, it is possible to adapt metagenomics as a quantitative approach (qMeta).

qMeta can be achieved through minor adaptation to the metagenomic sequencing protocol. Specifically, exogenous nucleic acids that have minimal potential homology to DNA present in the DNA extract are spiked in just before library preparation and sequencing. These exogenous reference standards are then quantified in the generated metagenomic libraries and compared to known spiked-in concentrations, generating a gene target quantity on a "copy per volume" basis. This approach has been demonstrated for manure and pure culture sample types, and would be worthy of evaluation for analysis of wastewater, recycled water, and surface water samples.

3.5.6 Achieving the Full Potential for Metagenomic Monitoring of Antibiotic Resistance in Aquatic Environments: Call for Improved Quality Control in Metagenomic Sequencing and Analysis

It is clear that metagenomics has vast potential as a tool for monitoring antibiotic resistance in the water environment. However, in order to achieve this potential, consensus is needed in the research community regarding protocols for the production and analysis of metagenomic data. Several best practices, summarized above, were identified as part of the Project 5052 effort. In particular, ensuring that the sequencing platform and depth are appropriate to achieve the monitoring objectives is key. Maintaining consistent protocols and reporting all aspects of workflows in accompanied metadata is also key to ensuring comparable data. Additionally, given that metagenomic sequencing is prone to contamination, including experimental process controls, in the form of blanks, mock communities, or exogenous cell spike-ins is critical to ensure the integrity of the data on a per-experiment basis. These types of controls were unfortunately universally lacking across the identified studies and will be essential in ensuring the comparability and representativeness of generated sequencing data. In addition to including these types of process and sequencing controls, beginning to include standards for producing quantitative data would greatly advance the field and facilitate the incorporation of generated data into emerging human health risk assessment models.

CHAPTER 4

Expert Workshop

4.1 Overview and Purpose

Midway through Project 5052, in May 2021, the project team conducted a 4-day virtual workshop to obtain input on the direction of Project 5052 from key experts representing academia, industry, government, and water utilities. In particular, the team sought input on the prioritization of targets and methods to be recommended to the U.S. water industry and regulatory community for monitoring of antibiotic resistance in wastewater, recycled water, and surface water. At this point in the project, a summary of the expert survey (Chapter 2), the high-level literature review (Chapter 2), draft systematic literature reviews of the short list of targets (Chapter 3), and draft SOPs for the short list of targets (Appendices) were provided. Also included were a number of presentations apprising the participants of the state of the science and other parallel efforts with similar goals. The workshop format set the stage with presentations summarizing the state of the science and enabled participants to fully evaluate the progress of the project. The team sought specific advice with respect to further prioritization of targets and key recommendations for corresponding SOPs.

4.2 Workshop Participants

A total of 49 external attendees participated in the workshop; 43 US-based and 6 internationally based. Among the 49 participants were 9 representatives of US water utilities, 8 representatives from industry, 17 representatives from academia, 13 representatives from federal governmental organizations, 2 representatives from state and local governmental organizations, and one representative from the World Health Organization. In addition to the PIs, the WRF Project Manager and the Project Advisory Committee (PAC) also participated. Additionally, several Virginia Tech (VT)-based and University of South Florida (USF)-based students and collaborators helped to organize logistics of the workshop and to facilitate break-out group discussions.

A survey launched at the beginning of the workshop asking individuals to identify which sector they best represented. Among respondents (n=27), 22.2% chose University, 18.5% chose Public Health, and 14.8% chose Wastewater. Following these, the next three most common selections were: Recycled Water, Surface Water/Natural Environment, Food, Research, and Government. The polling software also allowed the addition of “write-in” responses, and several participants took that option to add Utility, Drinking Water, and Recycled/Reclaimed Water to the list. The following is the full participant list and affiliation (Table 4-1):

Table 4-1. Workshop Participant List

Expert Workshop Participant	Affiliation	Role
PROJECT 5052 TEAM		
Jeanette Calarco	University of South Florida	Student Workshop Organizer
Benjamin Davis	Virginia Tech	Student Workshop Organizer
Valerie (Jody) Harwood	University of South Florida	Workshop Co-Chair
Lenwood Heath	Virginia Tech	Co-PI
Ishi Keenum	Virginia Tech	Student Workshop Organizer
Krista Liguori	Virginia Tech	Student Workshop Organizer
Erin Milligan	Virginia Tech	Student Workshop Organizer
Amy Pruden	Virginia Tech	Workshop Co-Chair
WRF & PAC		
Emily Garner	West Virginia University	PAC Member/Speaker
Anthea Lee	Metropolitan Water District of Southern California	PAC Member
Jean McLain	University of Arizona	PAC Member
Erin Swanson	Water Research Foundation	WRF Project Manager
Ed Topp	Agriculture and Agri-Food Canada	PAC Member
CONSULTANTS		
Kati Bell	Brown and Caldwell	Participant
Zia Bukhari	American Water	Panel Discussant
Walter Jakubowski	WaltJay Consulting	Participant
Mark LeChevallier	Dr. Water Consulting LLC	Participant
Tanja Rauch-Williams	Carollo	Panel Discussant
Jeff Soller	Soller Environmental, LLC	Participant
WATER UTILITIES		
Daniel Gerrity	Southern Nevada Water Authority	Panel Discussant
Raul Gonzalez	Hampton Roads Sanitation District (HRSD)	Speaker
Gertjan Medema	KWR Watercycle Research Institute, The Netherlands (Hamilton 2018 Collaborator)	Participant
Gaya Ram Mohan	Gwinnett County Department of Water Resources	Participant
Bina Nayak	Pinellas County Utilities	Panel Discussant
Daniel R. Quintanar	City of Tucson Water Department	Participant
ENVIRONMENTAL & PUBLIC HEALTH AGENCIES		
Jorge Matheu Alvarez	World Health Organization (WHO)	Speaker
Mark Borchardt	USDA	Participant
Kim Cook	USDA	Participant
Lisa Durso	USDA	Participant
Alison Franklin	USEPA	Participant
Jay Garland	USEPA	Speaker
John Griffith	Southern California Coastal Water Research Project	Panel Discussant

Mark Ibekwe	USDA	Participant
Scott Keely	USEPA	Participant
Amy E. Kirby	CDC	Participant
Sharon Nappier	USEPA	Participant
Andrea Ottesen	FDA	Participant
ACADEMICS		
Nicholas Ashbolt	Southern Cross University, Australia	Speaker
Johan Bengtsson-Palme	University of Gothenburg, Sweden	Speaker
Thomas Berendonk	Institute Hydrobiology at the Technische Universität Dresden, Germany	Speaker
Connor Brown	Virginia Tech	Student Facilitator
Suraj Gupta	Virginia Tech	Student Facilitator
Kerry Hamilton	Arizona State University (Hamilton 2018 PI)	Speaker
Satoshi Ishii	University of Minnesota	Speaker
Jennifer Jay	University of California, Los Angeles	Participant
Sunny Jiang	University of California, Irvine	Participant
Ayella Maile-Moskowitz	Virginia Tech	Student Facilitator
Jade Mitchell	Michigan State University	Participant
Kara Nelson	U.C. Berkeley	Participant
Mark Sobsey	Gillings School of Global Public Health University of North Carolina	Participant
Lauren Stadler	Rice University	Participant
Peter Vikesland	Virginia Tech	Participant
Liqing Zhang	Virginia Tech	Participant

During the end-of-day surveys, participants ranked themselves with respect to their relative level of expertise in the methods discussed that day. Table 4-2 summarizes the landscape in self-judged expertise in the methods discussed.

Table 4-2. Participant Self-Reported Familiarity with Topic Areas Covered at Workshop

The topic of "_" as an AMR Monitoring Target:	Expert	Very Familiar	Somewhat Familiar	Beginner	Not at all familiar
E. coli (n=27)	3	11	11	2	0
Enterococcus (n=25)	0	11	9	5	0
Environmentally-Relevant Organisms (n=25)	0	9	12	4	0
qPCR (n=11)	3	14	6	2	0
HT-qPCR (n=11)	1	3	4	2	1
Metagenomics (n=11)	2	3	5	1	0

4.3 Workshop Content and Format

The expert workshop took place on four days spread out over two weeks in May of 2021 (May 18, 20, 25, 27), with each session being about three hours in duration. The workshop was conducted virtually using Zoom web-conferencing (Video Communications, Inc. San Jose, CA). A workshop packet was prepared that contained the workshop agenda, the high-level literature review, the expert survey results, and drafts of the systematic literature reviews, as summarized in Chapters 2 and 3. The literature reviews focused on the following candidate monitoring methods/targets:

- *E. coli* culture
- *Entercoccus* spp. culture
- *Pseudomonas* spp., *Aeromonas* spp., and *Acinetobacter* spp. culture
- qPCR targeting *int*I1, *sul*1, *tet*A, *van*A and *bla*CTX-M
- Metagenomic profiling of resistomes

Draft SOPs for each of the five above methods/targets were developed by the Project 5052 team and provided in the workshop packet. Participants were emailed a pdf of the workshop packet three weeks prior and were instructed to familiarize themselves with the materials prior to the workshop. The workshop was organized according to the following themes:

- Overview and purpose of antibiotic resistance monitoring in wastewater, recycled water, and surface water
- Framing environmental monitoring of antibiotic resistance in a human health risk context
- Coordinating with national and international efforts to develop standard methods for antibiotic resistance monitoring in the environment: NARMS, The WHO Tricycle Protocol, and EMBARK
- Pros and cons of culture-based fecal targets for monitoring
- Pros and cons of culture-based targets with environmental niches for monitoring
- Pros and cons of qPCR and high throughput qPCR-based monitoring
- Pros and cons of metagenomic-based monitoring

- Feasibility for adoption by US Water Utilities

The workshop itself consisted of presentations by the WRF Project 5052 team members, invited external presentations, interactive break-out sessions, large group discussions, and a closing panel discussion at the end focusing on recommendations for US Water Utilities. Break-out groups focused on five topics:

1. What key questions can be answered by surveillance of antibiotic resistance in wastewater, recycled water, and surface water?
2. What are the advantages and challenges to fecal indicator bacteria (i.e., *E. coli* and *Enterococcus*) as culture-based antibiotic resistance monitoring targets?
3. What are the advantages and challenges to environmentally-relevant bacteria (e.g., *Pseudomonas aeruginosa*, *Aeromonas* spp., *Acinetobacter baumanii*) as culture-based AMR monitoring targets?
4. How should qPCR-based ARG targets be prioritized for AMR monitoring?
5. What is the potential for metagenomic-based AMR monitoring of water environments and which metrics and approaches are most meaningful and amenable to standardization?

Break-out session discussions were guided by specific prompts, guided by the discussion leader. Break out groups devoted specifically to the evaluation of SOPs considered factors such as degree of existing standardization, method sensitivity, specificity, clinical relevance, quantitative capacity, relevance for risk assessment models, affordability, feasibility and overall potential to achieve monitoring objectives and to address key research questions for a wide-variety of stakeholders. Discussions also solicited input on meta data that should be collected and how it should be analyzed, especially for environmental fate, water treatment, and human health risk assessment modeling purposes. Breakout groups were recorded to facilitate later analysis.

Each break-out discussion was followed up with polls that allowed participants to rank priorities and targets that were discussed and to provide other feedback. At the end of each workshop session, polls were also launched for more general feedback. On the final day, a closing online survey solicited integrated evaluation and rankings across the targets as a whole and focused on recommendations for US water utilities. Table 4-3 provides the Workshop Agenda.

Table 4-3. Expert Workshop Agenda

Time (EDT)	Tues, May 18	Thurs, May 20	Tues, May 25	Thurs, May 27
1:00 PM	Welcome! Workshop Charge, Dr. Amy Pruden	Review Survey Results from Tuesday	Review Survey Results from Thursday	Review Survey Results from Tuesday
1:05 PM				
1:10 PM	Workshop Overview, Krista Liguori	Breakout Session 3: Discuss *Enterococcus* spp. SOP	High Throughput qPCR, Dr. Satoshi Ishii	Utility Perspective on AMR Monitoring, Dr. Raul Gonzalez
1:15 PM				
1:20 PM	Expert Survey Results, Dr. Amy Pruden			
1:25 PM				
1:30 PM	Breakout Session 1: Purpose of Monitoring		Group feedback on qPCR-based targets	Panel Discussion: What is Feasible for Utilities? Drs. Zia Burkharia, Dan Gerrity, Bina Nayak, Raul Gonzalez, Tanja Rauch-Williams, John Griffith
1:35 PM		Group feedback on fecal culture targets		
1:40 PM				
1:45 PM		Environmentally-relevant culture targets, Dr. Nick Ashbolt	Poll 3: qPCR-based targets	
1:50 PM	Poll 1: Purpose of monitoring		- BREAK -	
1:55 PM	- BREAK -		Metagenomic AMR monitoring/ EMBARK, Dr. Johan Bengtsson-Palme and Dr. Thomas Berendonk	
2:00 PM	Risk Assessment, Dr. Kerry Hamilton	- BREAK -		
2:05 PM		Environmental Organisms, Erin Milligan		
2:10 PM				
2:15 PM		Breakout Session 4: Discuss *Aeromonas/ Pseudomonas/ Acinetobacter* SOP	Metagenomics-based Monitoring, Dr. Emily Garner	What next? Dr. Amy Pruden
2:20 PM	WHO and Tricycle Surveillance, Dr. Jorge Matheu			
2:25 PM				
2:30 PM			Metagenomics Lit Review, Benjamin Davis	Final Survey and Ranking
2:35 PM				
2:40 PM	Fecal Targets, Dr. Valerie (Jody) Harwood	Group feedback on environmentally-relevant culture methods	- BREAK -	Social
2:45 PM				
2:50 PM		Poll 2: Culture-based targets	Breakout Session 6: A path towards standardized metagenomic monitoring of AMR	
2:55 PM		- BREAK -		
3:00 PM	- BREAK -	NARMS Environmental Working Group, Dr. Jay Garland		
3:05 PM	*E. coli* as an AMR Monitoring Target, Jeanette Calarco			
3:10 PM				
3:15 PM	Breakout Session 2: Discuss *E. coli* SOP		Group feedback on metagenomics	
3:20 PM		qPCR -based AMR monitoring, Ishi Keenum		
3:25 PM				
3:30 PM		Breakout Session 5: qPCR SOPs	Poll 4: Metagenomics	
3:35 PM			Social	
3:40 PM				
3:45 PM	*Enterococcus* spp. as an AMR Monitoring Target, Benjamin Davis			
3:50 PM				
3:55 PM	Day 1 Closing Remarks	Day 2 Closing Remarks	Day 3 Closing Remarks	
4:00 PM	End of Day Survey	End of Day Survey	End of Day Survey	

4.4 Workshop Themes and Findings

4.4.1 What Key Questions Can Be Addressed by Surveillance of Antibiotic Resistance in Wastewater, Recycled Water, and Surface Water?

The workshop opened with an overview of the goals and objectives of Project 5052 and a presentation of a draft of the Figure 1-2 decision tree for selecting methods and targets for antibiotic resistance monitoring of aquatic environments, depending on the monitoring objectives. A break-out group followed in which the decision-tree was discussed. Discussions were centered around the question, *if your organization were to conduct environmental AMR monitoring, what would your organization's primary goal(s) be*? Workshop participants were in strong agreement that the questions, goals, and motivations behind AMR monitoring should be established early on in a monitoring program. Results of the poll that followed the discussion indicated that human health risk assessments and informing public health activities were the main drivers (Figure 4-1). Other responses included assessing treatment efficacy in removal of AMR, an interest in basic research, assessing the potential for AMR to adapt and/or spread, informing policy, and generating expertise. It was also noted that often the goal is simply to address concerns raised by the public. One key outcome of this early discussion was that no singular method can address all monitoring objectives, and thus it was recommended to proceed with recommendations for improving and standardizing all three categories of methods (culture, qPCR, metagenomics) within the context of the decision tree.

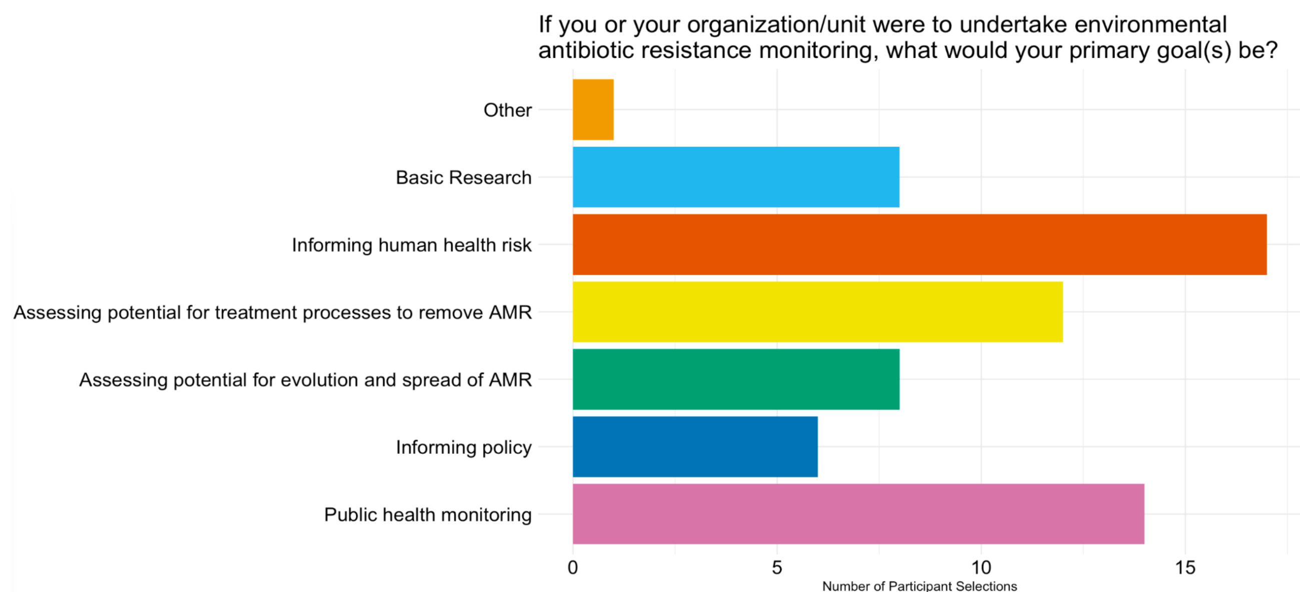

Figure 4-1. Primary Goal Motivating Organizations to Undertake AMR Monitoring.
When asked which primary goal would motivate their organization or unit to undertake AMR monitoring, most participants chose either informing human health risk assessment or public health monitoring.
Source: Liguori et al., 2022. Available under Creative Commons License Attribution-NonCommercial-NoDerivatives 4.0 International

Notably, participants particularly saw value in WBE as a key motivator to initiate antibiotic resistance monitoring in the water sector. WBE entails testing the influent sewage for disease agents with the purpose of gaining insight into the prevalence of carriage within corresponding populations served by the sewer shed. The benefits of WBE have been demonstrated for polio

and most recently have gained popularity for monitoring of the SARS-CoV-2 virus. In terms of antibiotic resistance, WBE could serve to inform with respect to carriage of ARB and ARG of interest by populations served by WWTPs (Pruden et al., 2021). Metagenomics in particular was viewed as a promising method for comprehensively addressing public health threats via WBE, but culturing and characterization of individual pathogens of concern was also seen to be of value.

Quantifying removal of AMR throughout the wastewater treatment process and identifying types of AMR of clinical concern that might evade that treatment process were also considered of significant importance to US water utility monitoring objectives. In particular, it was viewed that the experience with advancing water reuse treatments to address CECs could be adapted to address antibiotic resistance. It was also noted that there were many lessons learned from the water reuse experience with CECs that could inform efforts to address antibiotic resistance within the US water sector. Along this vein, a number of individuals expressed that the real driving force behind utilities participating in AMR monitoring would be some type of statutory requirement, regulation or incentive. Otherwise, motivating utilities to participate on a broad scale may be challenging. Still, many proactive water utilities might participate voluntarily, just as they have for WBE of COVID.

4.4.2 Framing Environmental Monitoring of Antibiotic Resistance in a Human Health Risk Assessment Context

The second presentation was delivered by Dr. Kerry Hamilton (Arizona State University), who serves as PI on Water Research Foundation Project 4813 "*Critical Evaluation and Assessment of Health and Environmental Risks from Antibiotic Resistance in Reuse and Wastewater"* (Hamilton 2018). This provided a key opportunity to link the two projects. The presentation provided an overview of the human health risk assessment framework for waterborne sources of antibiotic resistance that had been developed thus far as part of Hamilton 2018. In particular, it was noted that it is important to consider multiple exposure routes for such sources, including ingestion, skin contact, and inhalation. Also, a key challenge to modeling the human health risks of antibiotic resistance is that bacteria are able to acquire resistance, especially through horizontal gene transfer. Dr. Hamilton discussed the adaptation of quantitative microbial risk assessment (QMRA) to consider horizontal gene transfer and the team's recent efforts to adapt QMRA to qPCR-based monitoring of ARGs, such as *sul*1. Culture-based measurements of ARBs can most readily be used to inform QMRA.

Other risk modeling efforts being developed through Hamilton 2018, besides QMRA, were also described. For example, Bayesian modeling may be particularly suitable for identifying critical control points in a given treatment train where there are vulnerabilities to the proliferation of ARB, through selective pressure or horizontal gene transfer of ARGs. Bayesian modeling can likewise be used for identifying which wastewater and water reuse treatment processes most effectively attenuate antibiotic resistance. An advantage of Bayesian modeling is that it is compatible with a variety of data types, and provides a means to integrate both ARB and ARG measurements with a variety of water quality and other metadata to provide a comprehensive understanding of the system of interest. Metagenomic data are currently more challenging to incorporate into existing risk models, but efforts are underway to incorporate it into various

Bayesian approaches (Gupta et al., 2021). One approach being investigated by Hamilton 2018 is the "relative resistome risk" approach, in which metrics are derived from metagenomic sequencing data to estimate the relative proportion of ARGs that co-occur with MGE and pathogen sequences. This analysis is performed with online tools, such as MetaCompare and ARG Ranker. It was noted that it is difficult to translate such information into quantitative assessments of human health risk, but that it can be useful to assess efficacy of treatment processes in the relative reduction of the "risk" of contributing to the evolution and dissemination of antibiotic resistance (Chapter 1, Monitoring Objective 4).

Finally, Dr. Hamilton commented on the Hamilton 2018 human health risk assessment framework in the context of the draft version of the Figure 1-2 decision tree, which was provided in the workshop packet. In particular, she emphasized the need to harmonize the recommendations of Project 5052 with measurements that will be informative for risk assessment.

It was apparent in subsequent group and break-out discussions, as well as polling (Figure 4-1), that the participants were in strong agreement regarding the importance of prioritizing targets for AMR that are informative of human health risk. Based on this input, culture and qPCR were highlighted in the final decision tree as most amenable to informing human health risk assessment (Figure 1-2). Participants also noted the importance of "risk for emergence of new resistance types" and emphasized that this should also be broadly factored into risk assessment. "Ecological risks" were also a stated concern. For example some participants emphasized the need not only to consider environmental impacts in terms of selection pressures imposed by antibiotics and other antimicrobials, but also the resulting shifts in microbiomes and resistomes. How might such shifts in water microbiomes and resistomes, including corresponding influences in food production affect humans, livestock, and agricultural production in the future?

4.4.3 Coordinating with National and International Efforts to Develop Standard Methods for Antibiotic Resistance Monitoring in the Environment: NARMS, The WHO Tricycle Protocol, and EMBARK

The workshop provided a valuable opportunity to compare and coordinate with parallel national and international efforts aimed at standardizing environmental monitoring of AMR. This provided valuable context for workshop participants and rich contribution to the group discussions and break out groups. The following paragraphs summarize high points of key activities and recommendations of the US NARMS, WHO Tricycle, and EMBARK efforts, as they were presented at the workshop.

Several key individuals employed by federal agencies and who are involved in leading the expansion of the current US NARMS to include environmental monitoring were able to participate. These individuals included Alison Franklin, Amy Kirby, Andrea Ottesen, Daniel Tadesse, Jay Garland, Kim Cook, Lisa Durso, Mark Ibekwe, and Scott Keely. Dr. Jay Garland from the USEPA gave a presentation to inform the workshop participants about the NARMS effort. In terms of monitoring targets, the NARMS team has similarly decided to pilot test a suite of culture-, qPCR, and metagenomic-based methods of monitoring antibiotic resistance. Further,

the group has decided to focus on monitoring surface water as a starting place, because it is an integrated recipient of multiple sources of AMR, including farm runoff and WWTP overflows and discharges. This specific branch of NARMS is referred to as the Surface Water Antimicrobial Resistance Monitoring (SWAM) program. Dr. Garland emphasized two interrelated sampling campaigns that are underway to advance the SWAM effort. The first is coordinating with NRSA, which is a comprehensive national survey that occurs every five years and includes the collection of multiple water quality criteria. The second is a shorter-term, more focused, effort where the candidate AMR monitoring methods will be tested on the Chattahoochee and East Fork Little Miami Rivers. These watersheds are already test beds for CDC and USEPA monitoring, respectively. Members of the NARMS team were highly supportive of exchange of SOPs and samples with Project 5052 as these efforts got underway.

Key international efforts aimed at standardizing methods for monitoring antibiotic resistance in the environment are also underway and were represented at the workshop. On the first day, Dr. Jorge Matheu from the WHO delivered a highly relevant presentation about the development of the WHO Tricycle Protocol for ESBL *E. coli*. This presentation was particularly beneficial as a means of briefing the audience regarding lessons learned in the development of the Tricycle Protocol, and how they might inform development of standard methods as part of Project 5052. Dr. Mattheu spoke of how the Tricycle Protocol was born out of the 2015 WHO Global Action Plan on AMR, which emphasized the need to "strengthen the knowledge through surveillance and research." The WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) accordingly acted to develop a protocol suitable for One Health (People, Animals, Environment) monitoring. A strong priority for WHO is broad accessibility of methods among member states, especially low-income countries that have suffered the brunt effects of the spread of antibiotic resistance. Because of this, only culture-based methods were considered. Specifically, *Salmonella*, *Campylobacter* spp., *Vibrio*, *Clostridium*, and *Staphylococcus aureus* were selected as targets containing pathogenic members that are highly relevant to antibiotic resistance, while *Escherichia coli* and *Enterococcus* spp. were selected as relevant fecal indicators. After testing at 16 pilot sites in Africa, Southeast Asia, South America, and the Middle East, *E. coli* was identified as a robust indicator that is easily implemented and widely detected across One Health matrices. By contrast, organisms such as *Salmonella*, which has > 2500 Serovars, was deemed unrealistic for broad-scale deployment. The Tricycle Protocol was recently published by WHO and is beginning to gain wide scale adoption (WHO, 2021; Matheu, 2021). Challenges encountered in the development and launching of the TriCycle Protocol include: Lack of existing standard methodologies; Lack of existing infrastructure, reagents, and supplies for bacterial culture and identification; Lack of in-field laboratory QA/QC; lack of human resources with the appropriate skill sets; and cost and financing (e.g., the method is triple the cost of standard monitoring). Recommendations included: identifying and working with existing capacity, offering training on site, providing supplies, and providing vetted SOPs.

Finally, the co-principal investigators of the EMBARK project, Dr. Johan Bengtsoon-Palme (Sweden) and Dr. Thomas Berendonk (Germany) also participated in the workshop and presented their perspectives to the group. EMBARK is funded by The Joint Programming Initiative on Antimicrobial Resistance (JPIAMR), which is based in the EU and has the objective

of coordinating AMR research (JPIAMR, 2022). Currently 29 countries are contributing members of the JPIAMR and correspondingly have access to financial resources to support research and monitoring in this domain. While Canada is currently a JPIAMR member, the US is not, but it is hoped that the US will take the opportunity to join in the near future. EMBARK shares similar goals as Project 5052. EMBARK was inspired by the need for baseline monitoring data in order to address the following key knowledge gaps and research needs related to environmental dimensions of antibiotic resistance:

- Determine important sources of resistance
- Determine important human exposure settings
- Identify changes over time
- Allow temporary interventions
- Early warning for emerging resistance threats

Accordingly, the specific objectives of EMBARK are to:

- Establish a baseline for resistance in different environments
- Standardize and compare difference methods for environmental monitoring
- Develop methods to detect emerging resistance threats
- Develop a modular monitoring framework allowing comparison of data between agencies and countries

Similar to Project 5052, EMBARK has identified culture-based, qPCR-based, and metagenomics-based methods to all be of value for monitoring of antibiotic resistance in the environment. They additionally have emphasized value in whole genome sequencing (WGS) characterization and tracking of isolates and functional metagenomics as a means of detecting new ARGs before they become widely disseminated and problematic in the clinic. The EMBARK group is also widely recognized as being at the forefront of metagenomic-based monitoring of AMR in the environment. Specific pros and cons of metagenomics identified by the EMBARK team are discussed further below in Section IV.4.6.

4.4.4 Pros and Cons of Culture-Based Fecal Targets for Monitoring

Dr. Harwood, WRF 5052 Co-PI, provided an overview of the advantages and disadvantages of culture-based fecal indicators, *E. coli* and *Enterococcus* spp., as targets for antibiotic resistance monitoring of water environments. First, she emphasized that culture-based techniques are the tried-and-true way to ensure that the target is viable in the sample, whereas it can be assumed that a substantial portion of DNA emanates from dead cells. Viable organisms are obviously much more meaningful to epidemiological studies and risk assessment, while DNA-based methods can be particularly misleading when applied after engineering controls that kill cells, such as disinfection. She also emphasized that further analysis of culturable cells is possible, through phenotypic and genotypic profiling, including WGS analysis. This approach eliminates ambiguity of DNA-based techniques in terms of linking host bacterial cells with genetic and physiological properties, such as carriage of ARGs, MGEs, and virulence genes. With respect to *E. coli* and *Enterococcus* spp. in particular, she emphasized that they have a major advantage in terms of having stood the test of time in terms of their wide application across the US and throughout the globe for regulatory monitoring of wastewater and regulatory monitoring.

These methods are extremely well validated and standard methods are available that can be adapted for antibiotic resistance monitoring. Both targets are also clinically-relevant, e.g., ESBL *E. coli*, carbapenem-resistant *Enterobacteriaceae*, and vancomycin-resistant *Enterococcus*. Finally, she emphasized that both *E. coli* and *Enterococcus* strains are known to harbor an array of plasmids and other MGEs that carry multidrug resistance and thus are relevant to studying mobile resistance. When comparing the two, *E. coli* has the advantage in the U.S. of more commonly being monitored for regulatory purposes and also that the WHO Tricycle Protocol is already available for adaptation. On the other hand, including *Enterococcus* spp. as well will capture important forms of resistance relevant to Gram positive bacteria that will be missed if monitoring *E. coli* alone.

Dr. Harwood's presentation was followed by Project 5052 team member presentations providing overviews of the draft SOPs for *E. coli* and *Enterococcus* spp. In the break-out discussions, there was broad agreement with the advantages of targeting these organisms, as laid out by Dr. Harwood. It was also apparent that water utility staff are very comfortable running *E. coli* culture assays. However, it was noted that participants were generally reluctant to select a "best" culture target because this depends on the water sample type and the monitoring objective. A challenge with any culture target is that it overlooks what is happening across the vast majority of the microbiome. Some workshop members expressed the sentiment that *E. coli* and other fecal indicators are not without their drawbacks (e.g., VBNC, are more easily killed by engineering controls than other pathogens) and provide a limited view of the water quality. For this reason it was urged that, even if *E. coli* and/or *Enterococcus* spp. are determined to be a good starting place, that it is important to push the envelope on taking advantage of more modern technologies, such as metagenomics. Table 4-4 provides a summary of the advantages and disadvantages of the culture-based fecal targets discussed at the workshop.

Table 4-4. Summary of Advantages and Disadvantages of Fecal Indicator Targets for Antibiotic Resistance Monitoring of Water Environments.

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Method	Advantages	Disadvantages
Culture of Fecal Indicators	- Best aligns with current utility infrastructure and regulatory requirements for in-house monitoring - Enumerated target is viable - Targets with direct clinical relevance can be prioritized - Isolates can be further characterized for multi-drug resistance and virulence factors	- Some methods yield high frequency of false positives, requiring validation - Viable but non-culturable organisms will not be detected - Specialized media can be costly - Methods are laborious, particularly when quantitative data are desired - Any one target will not fully capture human health risk or the broader resistome
Escherichia coli	- Aligns with existing regulatory requirements - USEPA and ISO standard methods available - Recreational water regulations in U.S. - Human clinical and One Health relevance - Utilities already have infrastructure in place - Modified mTEC agar well validated for drinking water, surface water, and wastewater - WHO Tricycle protocol already available	- Current regulatory trends are moving towards more precise targets, rather than indicators. - *E. coli* is a genetically complex target - Clinical relevance and forms of resistance are site-specific
Enterococcus spp.	- Provides insight into Gram + AMR to complement Gram – monitoring - USEPA and ISO standard methods available - Recreational water regulations in U.S.; drinking water standards in EU - mEI agar well validated for drinking water, surface water, and wastewater	- Broad range of *Enterococcus* species that vary in clinical relevance - Speciation and genotyping needed for determining clinical relevance

A poll was conducted (n= 35) following sessions on culture-based targets to assess group consensus on which organisms are most feasible and informative for monitoring AMR in wastewater, recycled water, and surface water in the US. A majority of participants selected *E. coli* as the first choice and *Enterococcus* spp. as their second choice (Figure 4-2).

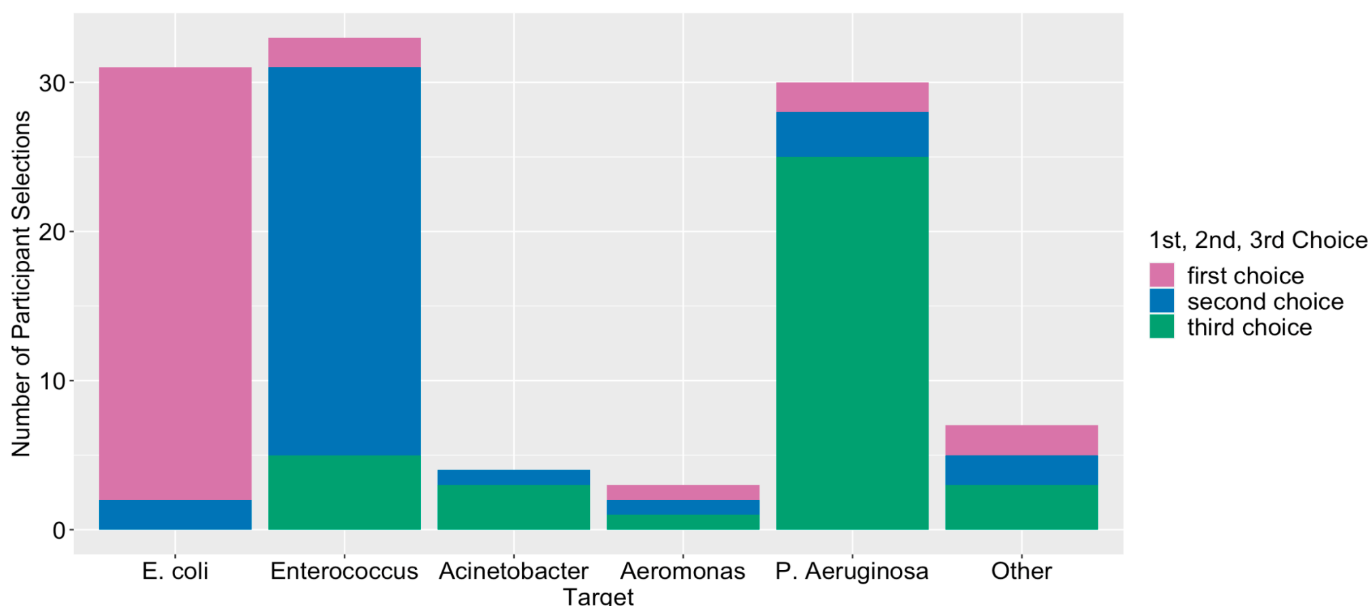

Figure 4-2. Workshop Participants Ranked the Provided Targets by Their First, Second, and Third Choice for a Feasible and Informative Target for AMR Monitoring in Wastewater, Recycled Water, and Surface Water in the United States.

4.4.5 Pros and Cons of Culture-Based Targets with Environmental Niches for Monitoring

Dr. Nick Ashbolt, who is a widely recognized authority on the microbial ecology of opportunistic pathogens, was invited to give a presentation making the case for the value of targeting environmentally relevant pathogens. In his presentation, Dr. Ashbolt emphasized that organisms that are capable of growth in environmental niches (i.e., saprozoic organisms) are more likely to be influenced by factors of interest in the environment. Therefore, such organisms would theoretically be of greater value as targets for monitoring of antibiotic resistance in water environments, because they would be more likely to acquire ARGs or otherwise be enriched by ambient antibiotic/antimicrobial concentrations, other stressors of interest, and interactions with the surrounding microbiome. Two prime examples of such organisms highlighted by Dr. Ashbolt were *Pseudomonas aeruginosa* and *Aeromonas* spp. At the other extreme, strictly enteric organisms, such as Campylobacters, will only provide a measure of die-off during treatment, or potentially viable but non culturable states. Somewhere in between, i.e., capable of persisting in a viable state and sometimes growing, were *E. coli*, *Enterococcus* spp. Dr. Ashbolt additionally shared work suggesting that *Arcobacter*

spp. are also important enteric microbes, but survive and grow somewhat better than *E. coli* and *Enterococcus* spp. in environmental niches.

Following Dr. Ashbolt's presentation, a Project 5052 team member gave an overview of the draft SOPs for *P. aeruginosa*, *Aeromonas* spp., and *A. baumannii*. In the workshop discussions, it was highlighted that a fundamental challenge with these organisms is that they are much more challenging to cultivate than *E. coli* and *Enterococcus*. This was blamed in part on the fact that there are no regulatory requirements for these organisms and thus the methods are primarily geared for clinical testing and have not been well validated for environmental samples. Among the three organisms, *P. aeruginosa* benefits from existing commercial protocols, such as IDEXX Pseudalert™ and *Pseudomonas* Isolation agar, which are geared towards drinking water samples. However, these media are prone to growth of non-target organisms when challenged with wastewater and recycled water samples. *Aeromonas* spp. benefits from an existing USEPA standard method for monitoring biostability of drinking water, but was generally viewed by the workshop participants as inconsequential as a human pathogen. *Acinetobacter baumannii*, on the other hand, was seen as an urgent antibiotic resistant pathogen of concern with suspected wastewater linkages, but among the three organisms, suffers from the least development of validated protocols for environmental monitoring.

Ultimately, workshop participants clearly favored *P. aeruginosa* among the targets with environmental niches, but it consistently was a third choice among culture-based targets overall (Figure 4-2). Table 4-5 summarizes the key strengths and weaknesses of targeting the pathogens with environmental niches for growth discussed at the workshop.

Table 4-5. Summary of Advantages and Disadvantages of Key Pathogens with Environmental Niches for Consideration for Antibiotic Resistance Monitoring.

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Method	Advantages	Disadvantages
Culture of Pathogens with Environmental Niches	- Some targets are commonly monitored in the field and standard protocols are available - Enumerated target is viable - Targets with direct clinical relevance can be prioritized - Isolates can be further characterized for multi-drug resistance and virulence factors	- Some methods yield high frequency of false positives, requiring validation - Viable but non-culturable organisms will not be detected - Specialized media can be costly - Methods are laborious, particularly when quantitative data are desired - Any one target will not fully capture human health risk or the broader resistome - Methods are particularly poorly developed for these targets in water samples
Pseudomonas aeruginosa	- Environmentally-relevant target that can re-grow in water systems and capture potential for evolution of new forms of resistance - Multi-drug resistant forms are highly clinically relevant - Especially relevant to recycled water, where fecal indicators are expected to be diminished	- Standard methods not established for wastewater, recycled water, or surface water - Not currently monitored by water utilities - Sampling protocols will require more complexity (where, when) to capture regrowth
Acinetobacter baumannii	- Clinically relevant with emerging evidence of environmental sources - Carbapenem-resistant *A. baumannii of* particular concern	- No published method to date reliably recovers the target from wastewater, recycled water, or surface water
Aeromonas spp.	- USEPA standard methods available for assessing regrowth in drinking water that could be adapted - Published research indicates that it captures potential for new forms of resistance to evolve during wastewater treatment	- Less human clinical relevance than the other culture-based options - Not widely monitored by utilities, methods would need validation for wastewater, recycled water, and surface water

4.4.6 Pros and Cons of qPCR-Based Monitoring

qPCR-based monitoring was covered in presentations both by Dr. Jay Garland of the USEPA and by Dr. Satoshi Ishii of the University of Minnesota.

Dr. Garland shared that the NARMS SWAM team is currently monitoring *int*I1, *sul*1, *tet*W, and *bla*TEM. This decision was made in part due to limited availability of DNA and the corresponding need to choose targets that are abundant and good quantitative markers of anthropogenic inputs (i.e., *int*I1, *sul*1, and *tet*W), but also include a highly clinically relevant ARG

(*bla*TEM), which is rarely detected but deemed to be important when it is. He also noted that NARMS SWAM is currently moving towards translating these assays to ddPCR as a more sensitive technique that provides absolute quantification. Finally, Dr. Garland summarized the benefits of qPCR/ddPCR in that they provide an integrated measure across the microbial community and quantitative data over a wide range (several logs), which is useful for modeling efforts. They also quantify 16S rRNA genes, for estimations of relative abundance, and fecal indicator targets, for comparison of AMR and water quality trends, in their panels.

Dr. Ishii specifically focused on the benefits of high-throughput qPCR (HT-qPCR), which can target and quantify hundreds of ARGs and other genes of interest in a single assay. This is an improvement with respect to the “one at a time” gene quantification approach typical of qPCR and ddPCR. There are three main commercial platforms for HT-qPCR: Fluidigm Biomark AD, TakaraBio SmartChip (Wafergen), and Thermo Fisher Open Array. One challenge with HT qPCR is the very small reaction volumes (6-100 nL) inherently limits detection. This can be overcome by a pre-amplification step, and he shared literature indicating that this incurs minimal to no bias in the resulting quantification. Dr. Ishii shared in house data demonstrating specific, sensitive, and high throughput quantification of 48 genes across 48 samples in a single step. His group has demonstrated the method on wastewater, drinking water, and stormwater.

Breakout group discussions of qPCR took place following Dr. Garland’s presentation and a presentation by a Project 5052 team member, going over the draft qPCR SOP. A full workshop group discussion then took place following Dr. Ishii’s presentation during the next session. Some workshop participants viewed qPCR as a promising “middle” ground where antibiotic resistance monitoring could be launched across the US. It was noted that US water utilities were finally beginning to buy-in to qPCR for pathogen monitoring and some of the larger utilities had recently purchased qPCR instrumentation. For example, qPCR monitoring of viral markers has proven beneficial for SARS-CoV-2 monitoring and in potable water reuse scenarios. Not everyone shared this opinion, however. There was concern that some utilities would not be open to using qPCR as a measure of gene removal during treatment, due to a mixed history with norovirus gene targets. Additionally, they may argue that gene removal has no indication on health impacts and therefore is not relevant.

Participants commented that calculations surrounding the recovery efficiency, LOD, equivalent sample volume, and relative abundance are important to include and report. Normalization approaches seem to vary amongst experts, as some normalize to 16S rRNA (as proposed in the original draft SOP), others normalize to pepper mild mottle virus, and some would prefer no normalization. In terms of calculating the LOD and LOQ, some would prefer a more statistical approach. It is noted that the measurement of LOD/LOQ will require an entirely different method for ddPCR as opposed to qPCR. Inhibition is a key consideration for a molecular method intended for national uptake, as water chemistry, target occurrence levels, and equipment are highly variable across the US. Participants agreed that an inhibition assessment should be included in the standard method developed. Many recommend using the salmon testes DNA assay in USEPA Method 1611 (USEPA, 2012).

Throughout breakout session groups, *bla*CTX-m, *sul*1, and *van*A were the most popular choices for a monitoring priority. Participants singled out *bla*CTX-m as the most significant for indicating health risks, and *sul*1 and *int*I1 as the most significant for measuring treatment removal rates. Many agreed that aligning efforts with CDC, FDA, and NARMs would be beneficial. Table 4-6 summarizes the advantages and disadvantages of qPCR-based methods and targets for AMR monitoring.

Table 4-6. Summary of Advantages and Disadvantages of the qPCR-Based Methods and Targets for AMR Monitoring of Water Environments Chosen for Validation.

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Method	Advantages	Disadvantages
qPCR	- Captures ARGs carried across a microbial community - Higher throughput than culture, more targets feasible - Instrumentation is increasingly available to water utilities - Quantitative info useful for modeling - Samples can be preserved long-term for retrospective analysis as new targets emerge	- Does not distinguish DNA from viable vs non-viable bacteria - Requires lab infrastructure equipped with quality control measures for molecular work - Requires personnel with expertise in molecular biology - Multiple levels of controls required for false positives/negatives - Assays require lab-specific troubleshooting and validation
*int*I1	- Strong correlation with anthropogenic sources - MGE associated with cassettes carrying multiple ARGs, thus tracking mobile multi-drug resistance - Abundance makes it suitable for assessing treatment removal efficiencies	- MGE, not an ARG - Sometimes gene cassettes do not contain ARGs
*sul*1	- Strong correlation with anthropogenic sources - Tends to be associated with *int*I1 - Abundance makes it suitable for assessing treatment removal efficiencies	- Sulfonamide resistance is already widespread, as these antibiotics have been used for decades - It is likely too late to effectively mitigate spread of sulfonamide resistance
*bla*CTX-M	- Greatest human health threat among candidate targets - Emerging concern about environmental/ waterborne sources/ pathways of spread	- Less likely to detect in environmental waters, resulting in non-detects and therefore not suitable for quantifying removal rates.
*van*A	- High clinical relevance - Relevant to Gram + bacteria, particularly *Enterococcus*	- Less likely to detect in environmental waters, resulting in non-detects and therefore not suitable for quantifying removal rates.
tet(A)	- Frequently detected in the environment - Tetracycline a widely used antibiotic for human and agricultural applications	- Not as strong anthropogenic indicator compared to *int*I1 and *sul*1 - Less widely utilized by researchers, utilities, and regulators compared to other relevant gene targets

4.4.7 Pros and Cons of Metagenomic-Based Monitoring

The topic of metagenomics was introduced first by the EMBARK team. Specifically, they noted the following drawbacks and benefits:

Benefits

- Potential to detect all (known) ARGs in a sample
- Samples can be re-investigated retrospectively
- Costs and waiting times are rapidly declining
- Easy to standardize
- Enables other uses of data – more flexible

Drawbacks

- Only finds the most common ARGs
- Cannot for sure tell if ARGs give a relevant phenotype
- Fairly expensive (today)
- Comparatively long turnaround time (today)
- Requires some type of bioinformatic infrastructure

Subsequently, Dr. Emily Garner, a member of the PAC and faculty member at West Virginia University, provided a more in-depth presentation summarizing the process of metagenomic data generation from water samples and the specific kinds of information that metagenomic sequencing data can provide. In addition to the benefits noted by EMBARK, she additionally noted several benefits of the non-target nature of metagenomic analysis, including: no need to select targets *a priori*, no need for PCR primers and thus elimination of uncertainties regarding their specificity, and the ability to capture numerous targets in a single assay. Additional drawbacks that she noted were that, due to the nature of metagenomic sequencing, the data are reported in relative abundance units, which cannot directly inform risk assessment. Dr. Garner additionally noted that short-read Illumina sequencing fails to provide direct context in terms of whether an ARG is carried on an MGE or within a pathogen host. She noted that long-read DNA sequencing, such as nanopore, is promising to address this shortcoming, but lacks sequencing depth. Dr. Garner provided several examples of the kinds of analyses that can be performed with metagenomic data, such as: relative abundance of total ARGs, relative abundance of ARGs of clinical concern, diversity of ARGs, and discriminatory analysis of ARGs. She further demonstrated MetaCompare as a means to compare the relative "resistome risk" of different samples, i.e., the relative carriage of ARGs on MGEs and in pathogens hosts.

To lead off the discussion, a member of the Project 5052 team presented an overview of the systematic literature review, including independent analysis of data extracted from the identified papers. It was emphasized that standardization is critical if metagenomics is to meet its full potential towards addressing the larger research questions highlighted in Chapter 1. For example, comparing ARG composition in a given sample relative to samples collected from similar environments locally, regionally, or globally? One key issue identified that needs to be addressed was that sequencing depth as currently applied only captures the most dominant ARGs, and misses rare ARGs. Another key issue is that there is wide variation in denominators

used for normalizing metagenomic data (e.g., 16S rRNA genes, *rpo*B, FPKM) and that scientific consensus is needed in order to improve comparability in reporting.

In the workshop discussions, metagenomics was widely viewed by participants as a "promising method of the future." Hope and optimism regarding the future role of metagenomics for antibiotic resistance monitoring in water environments was apparent throughout the discussions and break-out groups. However, participants acknowledged that metagenomics is still in the "wild west" phase as far as its trajectory towards standardization. Thus, there was some level of disagreement with the EMBARK assessment that metagenomic methods are "easy to standardize." Still, there was broad agreement that such standardization is necessary, and steps need to be taken to achieve this. In particular, further research aimed to address quality control concerns was identified as a need. Consensus is also needed in terms of best practices in the generation and reporting of metagenomic data, starting with sample collection. While the culture of sharing metagenomic data within the scientific community was applauded, it was noted that such data are of little value if key metadata are not also provided. Quality control measures and metadata that should be reported include:

- Sampling locations, e.g., key information regarding type of water system and location within the system the sample was collected (e.g., influent sewage versus activated sludge are often both reported as "wastewater)
- Sample volumes and concentration methods. This is particularly critical in moving towards qMeta techniques and reporting units as genes/volume.
- Inclusion of field blanks to address contamination with ambient DNA
- DNA extraction kits and inclusion of blank DNA extractions to address ambient DNA contamination.
- Spiking of internal standards in DNA extracts prior to sequencing for quality control to support qMeta
- Selection of appropriate sequencing platform and reporting it (e.g., Illumina versus Nanopore)
- Selecting sequencing depths that are appropriate for the research question and reporting these depths (guidance is needed on this)
- Normalizing data to a common denominator, or multiple denominators, to facilitate comparison across studies.

Despite impressions that metagenomics is the least developed/standardized method in our arsenal, participants continued to assert that it is the most promising for AMR research and should be a focus of standardization efforts. Feedback indicated that metagenomics is viewed as the best tool for broadly surveying environmental waters and assessing numerous targets, instead of narrowing down on select targets (Figures 4-3, 4-4, 4-5). Although not directly compatible with QMRA, metagenomics was viewed as being a broader asset for risk assessments, helping to find indicators for ARG abundance/diversity or predicting where resistance may emerge in the future. Developing qMeta will help in this regard. In addition to the challenge of standardization and comparability of data, researchers were concerned about the cost of metagenomics sequencing and general accessibility, although both are anticipated to improve in the coming years.

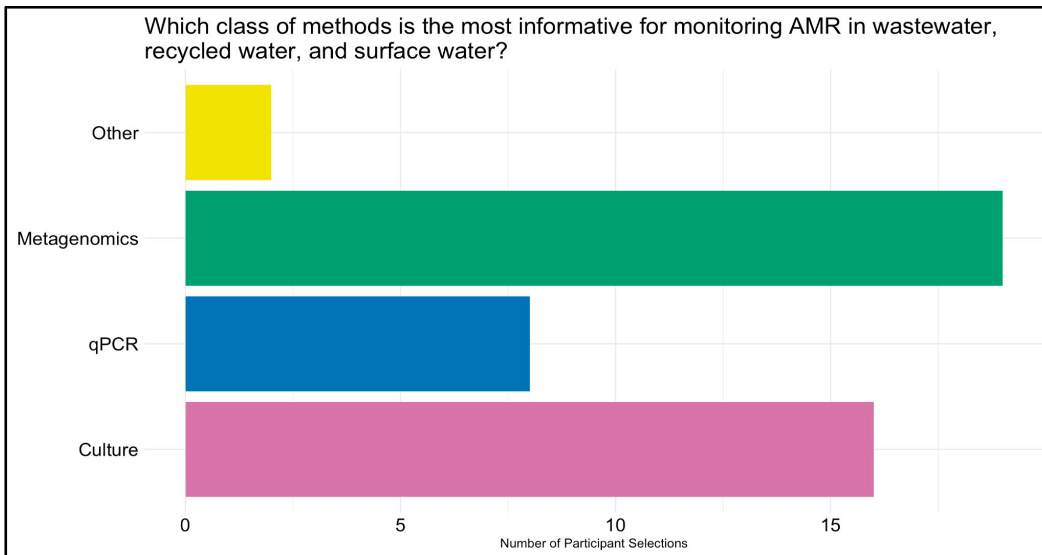

Figure 4-3. Workshop Participants Were Polled in Real-Time to Determine Impressions around Which Methods for Monitoring AMR are the Most Informative.

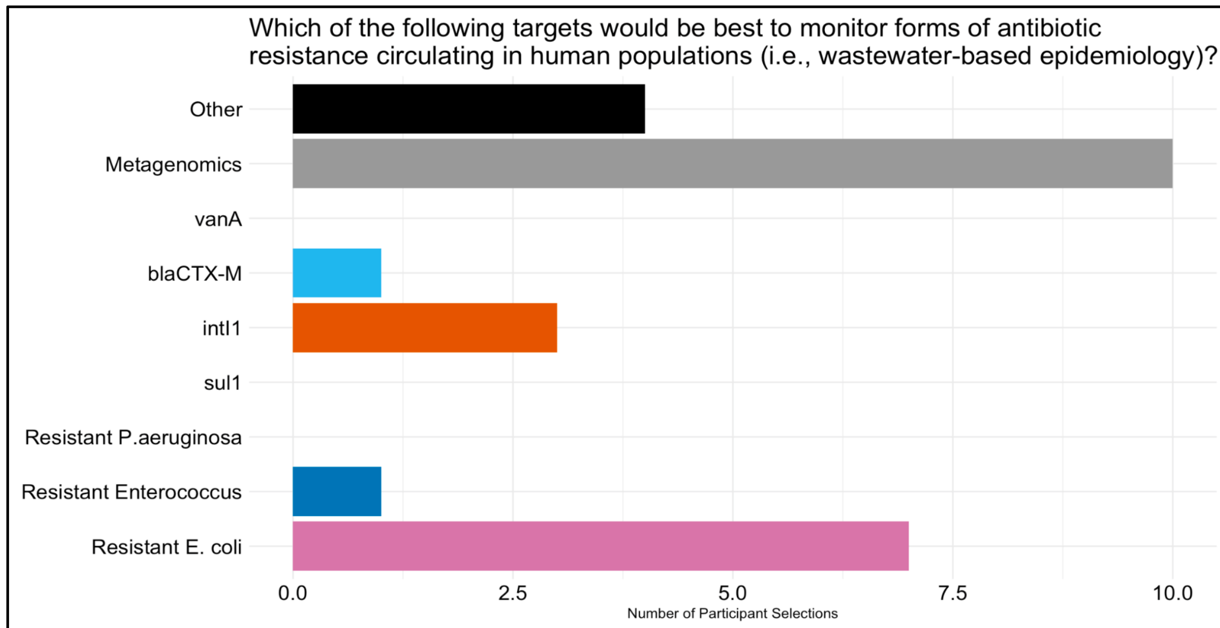

Figure 4-4. Workshop Participants Were Polled in the Final End-of-Day Survey and Selected the Targets Which They Found To Be the "Best" Option to Monitor AMR with the Goal of Conducting Wastewater-Based Epidemiology.

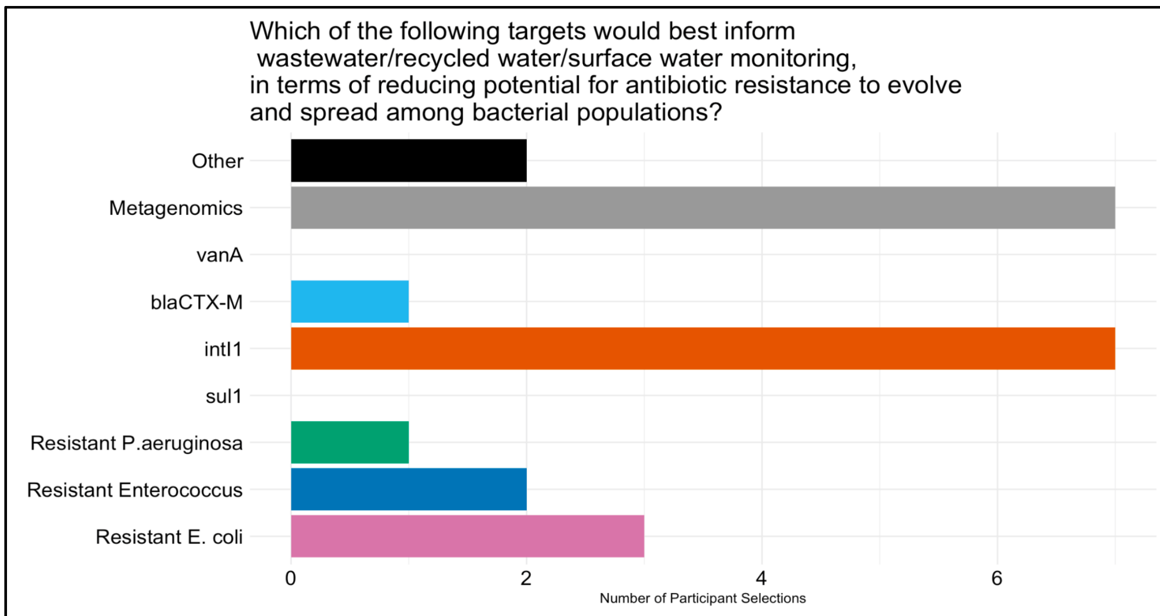

Figure 4-5. Workshop Participants Were Polled in the Final End-of-Day Survey and Selected the Targets Which They Found Best Inform Water Monitoring of AMR with the Goal of Reducing the Evolution and Spread of Resistance.

4.4.8 Feasibility for Adoption of Antibiotic Resistance Monitoring by U.S. Water Utilities

The workshop concluded with a session emphasizing feasibility for US water utilities. First Dr. Raul Gonzalez of the Hampton Roads Sanitation District (HRSD) provided a presentation with his insights as a representative of a "proactive" US water utility engaged in non-regulatory monitoring of public health concerns in wastewater, recycled water, and surface water. This presentation was followed by a panel discussion featuring representatives of water utilities and water engineering and human health risk consultants, who each took turns introducing themselves and sharing their views. Subsequently, there was a full workshop group Q&A focused on feasibility for US water utilities. In order to prime the participants with US water utility concerns in mind, the workshop poll soliciting final recommendations followed this session.

Dr. Gonzalez noted that HRSD employs culture, qPCR, ddPCR, and metagenomic-based analysis of water and wastewater samples. It is recognized, however, that most water utilities will at best have access to culture-based methods, with some recently having acquired qPCR instrumentation (Figure 4-6). Subsequent discussion highlighted that large water utilities with large WWTPs and those engaged in water reuse will be looked to as leaders in any potential antibiotic resistance monitoring efforts that might be adopted by other water utilities.

A major theme of this final session was that most US water utilities will be hesitant to engage in antibiotic resistance monitoring without incentives, regulatory requirements, or general fit within existing public health monitoring systems. The value of AMR monitoring needs to be demonstrated and to induce buy-in from upper management and political will. Identifying synergy with existing initiatives, such as NARMS/SWAM or specific endeavors underway at a

given utility, were also emphasized as a promising way to advance antibiotic resistance monitoring initiatives targeting wastewater, recycled water, and surface water (Figure 4-7).

Key barriers identified included lack of expertise, equipment, supplies, and funding. Additionally, critical needs identified included clear and feasible SOPs, a framework for interpreting the data in terms of human health risk, a responsible party for interpretation of results, and guidance for communicating findings to the public. In the further development of SOPs, participants additionally emphasized the importance of establishing method resolution and including recommendations for reporting that are standardized and comparable across utilities. Development of user-friendly sampling and analysis kits that require minimal expertise and allow utilities to conveniently follow the standard method and generate reproducible data was suggested. Similarly, IDEXX (Westbrook, ME) kits, such as Colilert™, Enterolert™, and Pseudalert™ have greatly expanded capacity for monitoring of pathogens and indicators in water samples. It was also noted that it would be beneficial to have centralized services for analysis of samples. For example, most utilities could be trusted to isolate *E. coli* or extract DNA, but it would be useful to be able to send those isolates and DNA extracts to centralized labs for further analysis such as antimicrobial resistance susceptibility testing, whole genome sequencing, qPCR, and metagenomics.

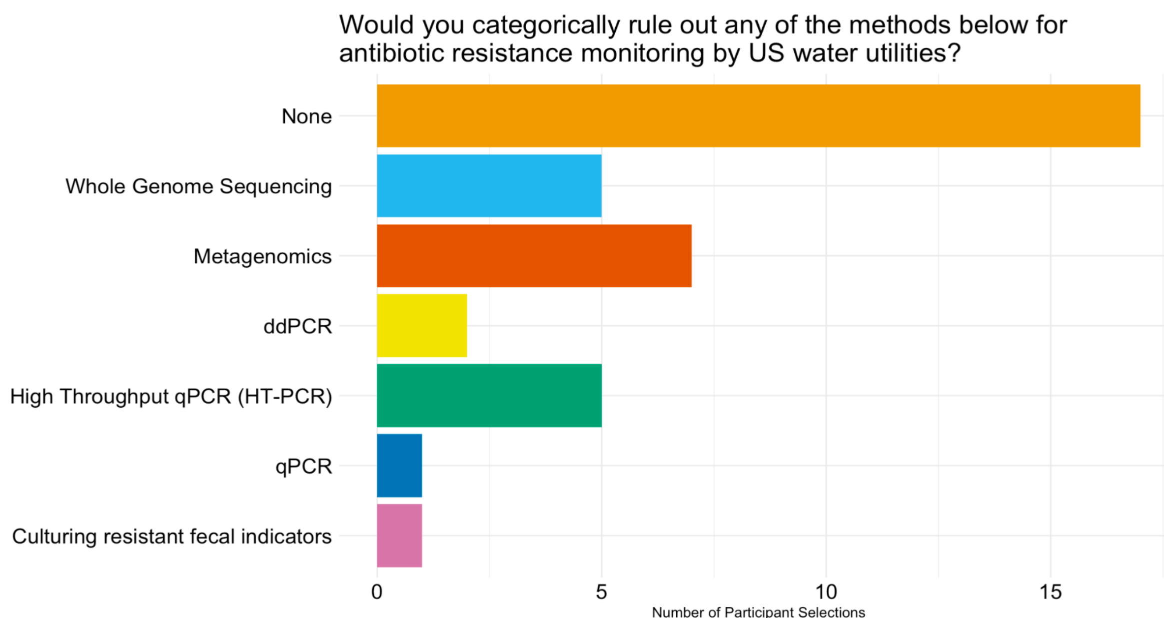

Figure 4-6. Expert Workshop Participant's Perspectives on Which Methods are Feasible and Appropriate for U.S. Water Utilities to Undertake.

Which of the following antibiotic resistance monitoring objective would be most informative and useful for US Water Utilities? (n=24)

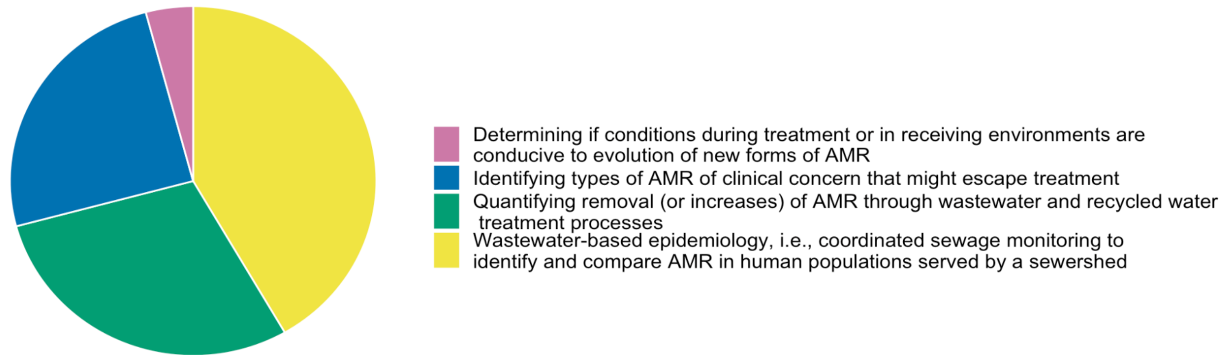

Figure 4-7.Expert Workshop Participants were Polled on Which Methods Best Balanced the Demands of a U.S. Water Utility in Finding a Method That is Both Informative and Feasible.

4.5 Final Rankings and Recommendations

Given the consensus that no one method will adequately address all monitoring objectives, participants suggested that a comprehensive guidance document would be very useful to the research, regulatory, public health, and water environment communities. Such a document could help water utilities, and others, to evaluate options in considering initiating a monitoring program for antibiotic resistance. Several participants expressed that it is important to be forward thinking in recommendations and that it is not necessary to rewind and repeat the development of water quality methods as they evolved in the water and wastewater field. In other words, it is important to look ahead at what technologies, questions, and targets will be key for monitoring 10 years from now. This kind of forward thinking was reflected in a substantial number of participants selecting metagenomics as the method of choice if they were to initiate monitoring in 2022 and as the most informative method for WBE (Figure 4-8; Figure 4-4). At the same time, many participants were practical in recommending culturing resistant fecal indicators as a reasonable starting place that would only require minor adjustment (inclusion of antibiotics in the media) to existing standard methods for meeting regulatory requirements in order for water utilities to implement (Figure 4-8; Figure 4-4).

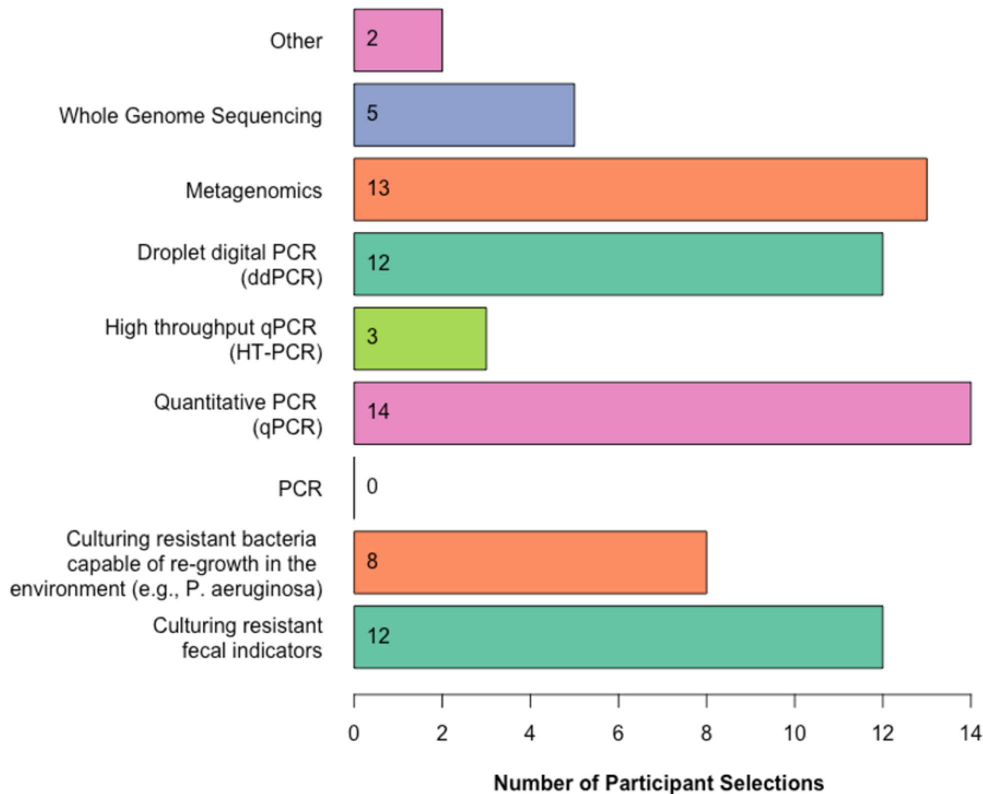

Figure 4-8. Results of Concluding Survey from the Expert Workshop

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Although many participants were enthusiastic about metagenomics, it was universally recognized that there is too much uncertainty at this point to develop and validate an SOP specifically as part of Project 5052. In particular, participants emphasized the importance of including highly specific details in the SOPs; starting with collection, processing, and storage of samples and ending with data analysis, reporting, and sharing. At this point, there is too much divergence in the application of metagenomics that needs to be resolved first before such a detailed SOP could be confidently developed. Given this, based on the workshop input, SOPs for ESBL *E. coli* and qPCR of *sul*1 and *intI*1 were selected for further refinement and validation (Chapter 5). Ideally, the details provided in Chapter 3 could aid interested parties in developing standard methods for the other targets, including metagenomics.

Regardless of the targets and methods selected for monitoring, it was recognized that it is important to be aware and transparent regarding what methods can and cannot tell you, and also what is not being monitored and therefore unknown. Accordingly, assistance with risk communication around the problem of AMR in a water environment context was recognized as a major need. For example, what does a utility communicate to the public, given that interpretation of the data in a risk context is still evolving?

Questions, concerns, ideas, and comments on how utilities may or may not fit into antibiotic resistance monitoring initiatives were common throughout the workshop general and breakout group discussions. In terms of incentivizing water utilities to monitor for antibiotic resistance, it appears that a few proactive utilities are likely to take the lead until there is some type of regulatory requirement, other incentive, or synergy. Including antibiotic resistance on the USEPA voluntary contaminant monitoring list could be helpful. The NARMS expansion and planned initiative to integrate monitoring with the National Rivers and Streams Assessment in 2022 was viewed as a promising avenue to demonstrate the value of environmental antibiotic resistance monitoring. Water utilities engaging in water reuse are also often under pressure from the public to take every possible precaution to ensure that the water is safe and are also in a key position as potential leaders in future antibiotic resistance monitoring.

An interesting twist related to the current COVID-19 pandemic is the rise in popularity of WBE. Monitoring sewage for SARS-CoV-2 was widely popularized and attracted a great deal of positive press for water utilities. WBE infrastructure has correspondingly rapidly been established across the US and throughout the world that could be harnessed for antibiotic resistance monitoring. Interestingly, participants selected WBE as the top choice as far as monitoring objectives that would be "most informative and useful to US Water Utilities (Figure 4-9)." WBE could provide a highly effective on ramp to initiate AMR monitoring by US Water Utilities.

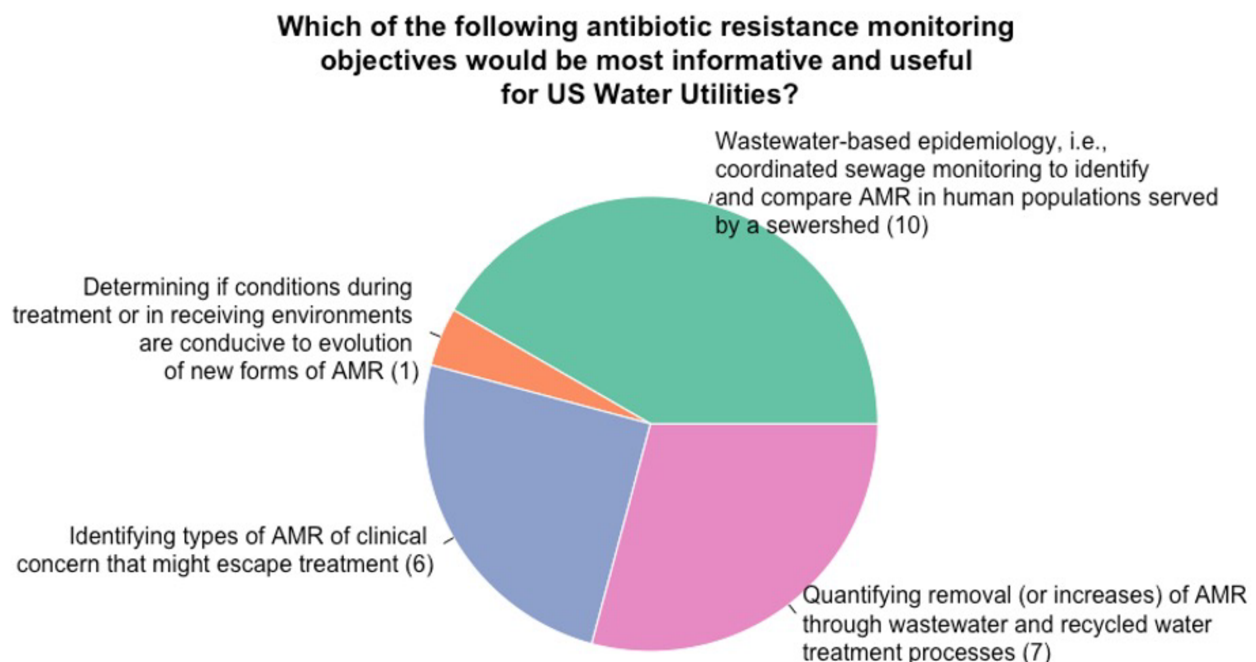

Figure 4-9. Participant Survey Results Following Session Focused on U.S. Water Utility Perspectives, Day 4

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It is hoped that the products of Project 5052 are an important step in meeting the needs raised by the workshop participants. This report, and the derivative works (Liguori et al., 2022;

Keenum et al. 2022; Davis et al., In-Revision; Calarco et al., In-Preparation; Davis et al., In-Review; Milligan et al., In-Review), provide a science-based framework and expert-vetted decision tree for formulating monitoring objectives and selecting appropriate targets and methods. Moving into the future, it is further hoped that the WARD developed through this effort (Chapter 6) will continue to serve as a forum for sharing, discussing and updating protocols. WARD is also configured to share data in a manner that can help water utilities see how their monitoring data compare to other water utilities and ultimately can help to address broader monitoring objectives summarized in Chapter 1.

CHAPTER 5

Multi-Lab Validation of ESBL E. coli, intI1, and sul1 SOPs

SOPs for culture-dependent analysis of ESBL *E. coli* and qPCR of *int*I1 and *sul*1 were selected for pilot-testing and validation. An interlaboratory comparison of these analyses was carried out at VT and USF. The team partnered with five US water utilities to obtain shipments of wastewater, recycled water, and surface water for testing. This afforded the opportunity to further refine the SOPs, assess efficacy of QA/QC measures, and also establish expected ranges of measurements in the target matrices.

5.1 Initial Assay Pilot-Tests

Prior to engaging the utility partners in sample collection, an internal validation program was carried out to test the specificity, LOD, LOQ, quantitative range, and repeatability of the culture and qPCR protocols, as well as the clarity of the instructions. The written SOPs were further refined following initial pilot testing at USF and VT. At VT, multiple new technicians were trained to help carry out the protocols, whereas measurements at USF were carried out by a single operator. Analyzing data from multiple users helped to assess clarity of the SOPs and also potential contribution of individual operator variation.

5.1.1 ESBL *E. coli*

Culture methods were refined during the first few weeks of internal testing at VT and USF. Wastewater influent, recycled water or wastewater effluent (depending on treatment plant), and surface water samples were collected from a local WWTP for this purpose. Clarifications and adjustments to the culture SOP were made during this internal testing phase. During validation studies, one aim was to collect "recycled water influent," essentially meaning the water that enters the recycled water treatment process. The treatment achieved by this point varies across plants due to differences in the separation or inclusion of recycled treatment with wastewater treatment, and treatment trains used. This initial testing helped to predict the dilutions needed for each sample type in order to obtain quantitative counts of cefotaxime-resistant *E. coli* on membranes in subsequent sampling events.

5.1.2 qPCR

Three qPCR assays were initially pilot-tested (Table 5-1): *sul*1 based on the method of Pei et al. (2006); qPCR of *int*I1 based on the method of Stokes et al. (2006); and *int*I1 based on the method of Barraud et al. (2010). The Stokes et al. *intI1* assay and the Pei *et al. sul1* assay rely on DNA-intercalating dyes such as SYBR green for detection of DNA and therefore require only primers in the reaction, while the Barraud et al. *sul1* assay is probe-based (Table 5-1).

Table 5-1. Primers and Probes for Each Assay Tested by qPCR at Virginia Tech

Gene Target	Primer/Probe	Sequence (5' to 3')	Source
*int*I1	Forward Primer	CTGGATTTCGATCACGGCACG	Stokes et al., 2006
*int*I1	Reverse Primer	ACATGCGTGTAAATCATCGTCG	Stokes et al., 2006
*int*I1	Probe	(6-FAM)ATTCCTGGCCGTGGTTCTGGGTTTT(BHQ1)	Barraud et al., 2010
*int*I1	Forward Primer	GCCTTGATGTTACCCGAGAG	Barraud et al., 2010
*int*I1	Reverse Primer	GATCGGTCGAATGCGTGT	Barraud et al., 2010
*sul*1	Forward Primer	CGCACCGGAAACATCGCTGCAC	Pei et al., 2006
*sul*1	Reverse Primer	TGAAGTTCCGCCGCAAGGCTCG	Pei et al., 2006

After completion of the SOP, each qPCR assay was run independently, at least two times, by constructing independent standard curves on two separate days, by two different researchers and using two different thermal cyclers, for a total of 12 runs. The assay efficiency, R^2 value, LOD, and LOQ were tracked over each of these test runs and used to determine which assays were performing the most consistently.

The Pei et al., 2006 assay for *sul*1 and the Barraud et al., 2010 assay for *int*I1 were selected for further testing as the most sensitive and repeatable. Both assays consistently produced an R^2 value > 0.97. The Stokes assay produced highly variable standard curves and therefore was not selected for further testing.

The *intI1* qPCR assay was further tested at USF using a mastermix produced by Thermo Fisher (TaqMan Environmental Master Mix 2.0), with a standard Taq polymerase (AmpliTaq Gold DNA Polymerase) and not the "fast" polymerase (Bio-Rad SsoAdvanced) that was used at VT. This testing revealed that the SsoAdvanced enzyme initially used at VT resulted in a lower assay efficiency. The USF implementation of the qPCR protocols produced high efficiency and high R^2 values in the standard curves (Table 5-2). USF also analyzed the standard curves with varying end points (5 gene copies, 10 gene copies or 100 gene copies) to determine the effect of the lowest standard on the performance of the standard curve. All technical replicates (see V.8.1 for definition) containing 5 gene copies amplified for both assays and standard curve performance remained acceptable, therefore the LOQ and LOD of the assays was designated as 5 gene copies (GC).

Table 5-2. Test Run of 10 Standard Curves and A Test Plate of the Pei Sul1 qPCR Assay Conducted at USF, with Assay Performance Calculated with Lowest Standard at 5 GC, 10 GC or 100 GC.

	10^8 to 5 GC		10^8 to 10 GC		10^8 to 10^2 GC	
Standard Curve #	R^2	Efficiency	R^2	Efficiency	R^2	Efficiency
1	0.988	107.4%	0.995	100.8%	0.999	94.5%
2	0.989	107.3%	0.993	101.8%	0.999	94.6%
3	0.99	107.2%	0.994	101.3%	0.999	94.7%
4	0.981	108.6%	0.995	100.9%	0.997	89.4%
5	0.976	107.3%	0.989	103.1%	0.998	94.2%
6	0.984	106.7%	0.991	98.9%	0.997	84.6%
7	0.987	106.9%	0.996	100.2%	0.998	94.7%
8	0.991	106.2%	0.993	101.9%	0.999	94.6%
9	0.989	107.1%	0.992	101.4%	0.999	94.8%
10	0.982	108.4%	0.99	102.8%	1	95.1%

The performance of *sul*1 (Pei et al., 2006) and *int*I1 (Barraud et al., 2010) assays differed by lab (Table 5-3). These findings emphasize that the reproducibility of qPCR analysis can be imperfect even for skilled labs. One researcher produced the USF data, while several produced the data at Virginia Tech, in order to gain a sense of operator variation. There was also a difference in mastermix used for the *sul1* assay (BioRad SsoFast EvaGreen Supermix (VT); Thermo Fisher Power SYBR Green Master Mix (USF).

Table 5-3. Performance of qPCR Assays in Each Lab.

Lab (n = number of unique dilution curves)	***sul*1** (Pei et al., 2006)		***Int*I1** (Barraud et al., 2010)	
	R^2	Efficiency	R^2	Efficiency
Virginia Tech (n= 12)	0.942 - 0.999	69.9 - 88.1%	0.963 - 0.996	64.4 - 83.3%
University of South Florida (n = 18)	0.995 - 0.999	82.5 - 92.6%	0.995 - 1.000	89.4 - 94.4%

5.2 Partnering Water Utilities and Field Sampling Plan

We secured partnerships with five water utilities across the US who are producing recycled water in order to pilot test the *E. coli* culture and *intI1* and *sul*1 qPCR protocols on an array of wastewater, recycled water, and surface water samples. The partnering utilities were located in Virginia, Florida, Georgia, Nevada, and California, thereby capturing geographical variability from the east coast, south, west and California. Utilities are designated by the state in which they are located, in order to respect data privacy considerations. The ideal sampling plan included: wastewater influent, wastewater effluent, recycled water influent, recycled water effluent, upstream surface water, and downstream surface water (Figure 5-1). However, the precise sampling locations varied by water utility. For example, in some cases the discharge was to a reservoir instead of a river, or there was no discharge at all, so a neighboring river or other surface water body was tested instead. When a surface water body was sampled in place of or in addition to an upstream and/or downstream sample, the sample was labeled as

"ambient surface water". Samples collected at each facility were shipped separately to USF and VT.

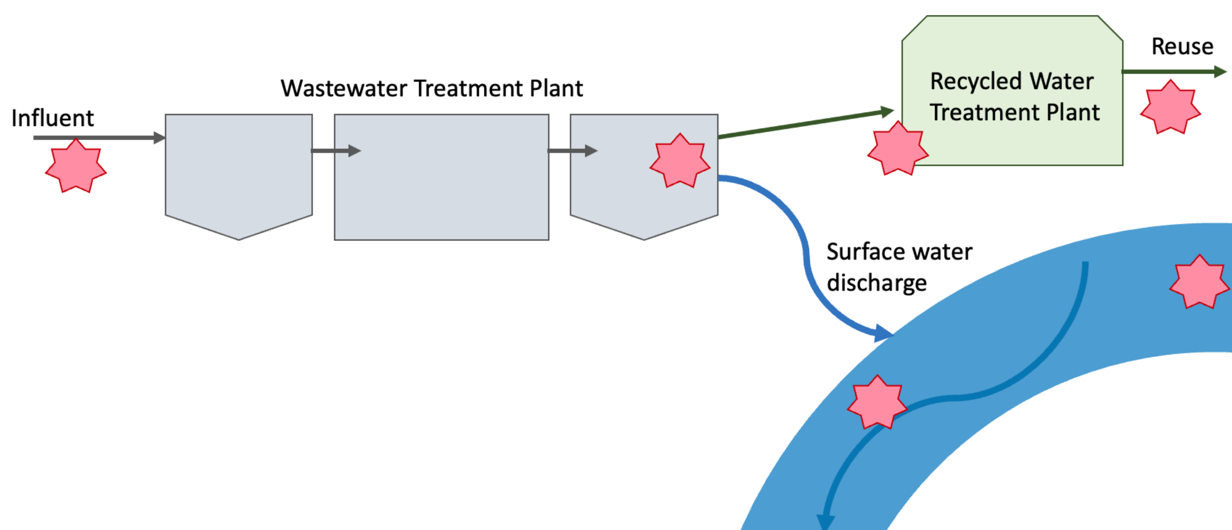


Figure 5-1. Idealized Wastewater Treatment Plant with Recycled Water Treatment and Surface Water Discharge. Red stars indicate sampling locations that are likely to be of interest for monitoring efforts. The aim of the pilot testing of the SOPs was to assess ARB and ARGs at geographically distributed WWTPs that produce recycled water and discharge to surface water. The treatment trains, setups, discharge points, and sampling sites varied at each site due to site-specific circumstances.

5.3 Field Sample Collection, Preservation, Transport, and Controls

Sampling protocols were provided to each utility partner at least two weeks prior to the scheduled sampling event. Sampling kits containing pre-labeled, sterile sampling bottles and coolers with return shipping labels were provided in advance. The following controls were also included in the sampling kits:

- Trip blank (bottle of sterile, autoclaved water that was never opened)
- A field blank (bottle of sterile, autoclaved water that was opened and exposed to the atmosphere during sampling).

The kits were prepared independently by USF and VT staff, and therefore the background water used in these controls and shipped back to each lab differed.

Project 5052 team members communicated with utility staff prior to each sampling event to address any questions related to the protocols. The sampling protocol provided to water utilities is provided in Appendix B-1. For WWTP and recycled water samples, valves were opened and flushed for 30 seconds prior to sampling. Then, all site-labeled bottles were filled, capped, and returned to the cooler. Ammonia, temperature, pH, conductivity, and dissolved oxygen (DO) were also collected from the WWTP and recycled water plant, if applicable. At one WWTP sampling site, the field blank bottle was opened during sample collection, then re-capped and returned to the cooler (Results of this analysis are presented in Chapter V).

Surface water samples were collected upstream and downstream of WWTP effluent discharge. GPS coordinates of sampling sites were collected at time of sampling. Bottles labeled with each site with lid still on were dipped about 1 foot below the water's surface, then the lid was removed to collect the sample and the bottle is re-capped and stored. pH, turbidity, conductivity, and river flow rate or capacity were also collected from the surface water body.

Sampling kits arrived with cooler packs which partners were instructed to place in the freezer upon receipt. Instructions stipulated that samples were to be transferred as soon as possible to the coolers containing frozen cooler packs. The coolers were shipped "overnight-early" by UPS to guarantee arrival as soon as possible, usually within 18-22 hours of shipment. Any samples arriving more than 48 hours after sampling were flagged as late and temperatures of bottles were taken on arrival. More information on the impact of shipping time is discussed in Section V.5. Sample processing began immediately upon receipt.

5.4 ESBL-*E. coli* Culture

5.4.1 ESBL-*E. coli* culture on Modified mTEC (Modified WHO Tricycle Protocol)

The selective antibiotic for isolation of presumptive ESBL *E. coli* was cefotaxime at a final concentration of 4 μg/ml. Modified mTEC agar was the primary isolation medium (USEPA 2014). For brevity, it is simplified here as 'mTEC', though note it is a slightly different agar composition (USEPA 2014; Hach 2022). Culture of *E. coli* on mTEC and mTEC + 4 μg/ml cefotaxime was carried out both at USF and VT, primarily by a single operator at each location. Culture media (BD Difco Chromogenic Dehydrated Culture Media: Modified mTEC) and antibiotics used were confirmed to be from the same manufacturer (Thermo Fisher), although lot numbers might have differed. Therefore, a high level of replication was expected between labs. Most sample sites were processed in parallel (in both VT and USF labs), with the exception of surface water upstream and recycled water influent, which were both processed at VT only.

Biological duplicate samples were provided to each laboratory for culture and processed by membrane filtration in technical duplicate (2 filters processed for each dilution of each biological duplicate). Filter cups and cassettes were sterilized by autoclaving in advance, at minimum one set for each sample type. Samples were filtered using 0.45-um cellulose acetate membrane filters (Fisher: cat no. 09719555) and immediately placed onto mTEC plates (both with and without 4 μg/ml cefotaxime) and incubated for 2 hours at 37°C before being transferred to a water bath preheated to 44.5°C, where plates were incubated for an additional 22 hours (USEPA 2014).

Plates were removed after 22 hours (+/- 2 hrs) in the water bath and colonies were counted and tabulated. Five (5) colonies were selected from each water type and streaked onto mTEC without 4 μg/mL cefotaxime, repeating the above incubation and water bath procedure. When a pure culture was achieved, a loop of each isolate was suspended in molecular grade water and DNA was extracted (placed in a thermal cycler at 95°C for 15 minutes). *E. coli* colonies were then confirmed via the *uidA* gene target using PCR (modified from Chern et al., 2009). Further details on culture methodology are outlined in the full protocol in the Appendix (Appendix B2).

5.4.2 Method Outline for Comparison of mTEC Method with WHO Tricycle Protocol and Effect of Water Bath Versus Incubator

mTEC was selected for the WRF 5052 ESBL *E. coli* SOP because of its high specificity and because of existing USEPA standard methods (USEPA Method 1603). However, a comparison of the WRF 5052 and WHO Tricycle methods for quantifying cefotaxime-resistant *E. coli* was conducted at USF on a select subset of wastewater influent samples from partner facilities and neighboring surface water samples. Bacteria were cultured on tryptone bile X-glucuronide (TBX) agar (Tricycle) or mTEC, with (cefotaxime-resistant *E. coli*) and without (total *E. coli*) 4 μg/ml cefotaxime (cefotaxime sodium salt, Millipore Sigma Product No. 219504; Fisher Sci Cat No. AC454950010). The incubation conditions were also varied. The Tricycle Protocol instructs incubation of plates in a 35° incubator for 22-24 hours (incubator method), whereas the WRF 5052 SOP follows the USEPA method by incubating plates in a 35° incubator for 2 hours and then in a 44.5° water bath for 20-22 hours (water bath method). The comparison of incubation conditions (water bath vs. incubator) was added due to the high level of background growth that occurred on plates incubated using the incubator method compared to the plates that were incubated using the water bath method. The following was the matrix for this comparative study:

- TBX (with and without cefotaxime)- incubator method (WHO Tricycle)
- TBX (with and without cefotaxime)- water bath method
- mTEC (with and without cefotaxime)- incubator method
- mTEC (with and without cefotaxime)- water bath method (Project 5052 SOP)

5.5 Method Outline for qPCR of *int*I1 and *sul*1

qPCR assays targeting *intI*1 or sul1 were carried out independently at VT and USF. A single lab operator processed and analyzed all samples at USF, while several operators assisted with the sample processing and analysis at VT. Also, at USF, the field and trip blanks were filtered first and analyzed in separate qPCR reactions, to minimize any possibility of contamination. At VT, the field and trip blanks were filtered last, after filtering of all the other samples was completed. Those blanks were then processed and analyzed last during the qPCR set-up, in order to capture any potential contamination throughout the process.

Filter cups and cassettes were sterilized by autoclaving, and at minimum one set was used for each sample type (i.e., influent, recycled water, surface water). Samples intended for DNA extraction were filtered (filters volumes are sample-type-specific and outlined in the protocol) using 0.22- µm cellulose acetate filters (EMD Millipore: cat no. GSWG047S6), placed in 5 ml tubes, and topped with 50% ethanol prior to freezing at -20°C.

DNA extraction was performed using FastDNA SpinKit for Soil (MP Biomedicals, Irvine, CA) following manufacturer instructions. The longest duration for each step was selected when a range was provided in the manufacturer's instructions (for example, when directions were: "centrifuge for 5-10 minutes,'' centrifugation was conducted for 10 minutes; 15 ml tubes were used when recommended; and the heat block was included for improved recovery). DNA extracts were stored in 100 µl cryotubes at -20°C.

qPCR was conducted using the protocol described in the Appendix (B-3) with *sul*1 and *int*I1 as the gene targets (Table 5-1). The negative control PCR reaction used was a non-template control, consisting of the same mastermix as samples but with molecular grade water in place of DNA template.

Data were entered into Excel spreadsheets and statistical analyses were conducted in RStudio (RStudio Team, 2020).

5.6 Culture Data and Results

Some samples (30 out of 128) did not arrive on time due to shipping delays. The sample groups which were delayed included wastewater and effluent, blanks, and recycled water influent and effluent samples. Samples that did not meet QA/QC requirements due to delayed sample shipping and processing outside of the 48-hour holding time were removed from the analysis, unless otherwise noted. The concentration of total and cefotaxime-resistant *E. coli* (log_{10} CFU/100mL) for each quantifiable technical replicate across all sampling points for six utilities is summarized in Figure 5-2.

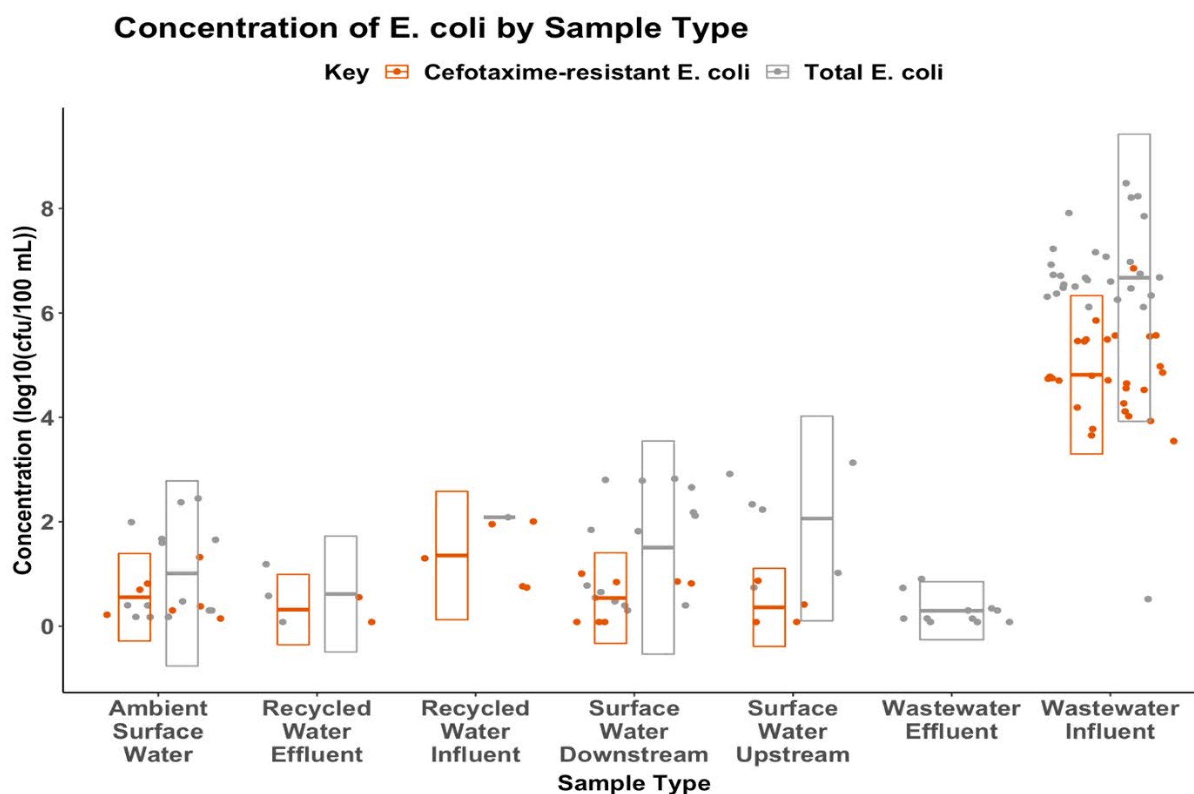


Figure 5-2. Cefotaxime-resistant *E. coli* (orange) and Total *E. coli* (grey) Concentrations (log(CFU/100mL+1)) Measured across All Samples (Including Duplicates).

VT and USF data sets were combined for this analysis. Samples where *E. coli* or cefotaxime-resistant *E. coli* were not detected are not included in the analysis. Crossbars represent the mean +/- standard deviation for each group in each water type.

All samples were analyzed at Virginia Tech; but recycled water influent and surface water upstream samples were not included in the analysis conducted at USF in order to optimize

shipping costs and resource/labor availability. Total *E. coli* concentrations were 1 to 2 logs higher than cefotaxime-resistant *E. coli* at all sites and utilities for each water type (Figure 5-2). Total *E. coli* and cefotaxime-resistant *E. coli* were detected in all wastewater influent samples (Table 5-4). Detection frequency of total *E. coli* (percent of samples with at least one colony) was highest in the influent samples, followed by ambient surface waters, downstream surface waters, upstream surface waters (data from VT only), recycled water influent (data from VT only), wastewater effluent, and recycled water effluent. Detection of cefotaxime-resistant *E. coli* was highest in influent, followed by ambient surface water, recycled water influent (VT only), upstream surface water (VT only), downstream surface water, recycled water effluent, and wastewater effluent.

Table 5-4. Detection Frequency (%) for Total *E. coli* and Cefotaxime-Resistant *E. coli* in Each Sample Type, by Lab.

Sample Type (n, total)	Total *E. coli*		Cefotaxime-resistant *E. coli*	
	USF	VT	USF	VT
Wastewater Influent (55)	100%	100%	100%	100%
Wastewater Effluent (37)	50%	63.6%	0%[b]	0%[b]
Ambient Surface Water (21)	100%	100%	100%	66.7%
Upstream Surface Water (15)	N/A	80%	N/A	50%
Downstream Surface Water (35)	80%	91.7%	50%	25%
Recycled Water Influent (11)	N/A[a]	70%[c]	N/A	60%[c]
Recycled Water Effluent (40)	0%	20%	0%	10%

[a] N/A - USF did not process these samples
[b] No cefotaxime-resistant *E. coli* were detected
[c] Several plates from this sample type were too numerous to count

Less than 10% of *E. coli* were resistant to cefotaxime in all sample types and utilizes, where this value was calculable (Table 5-5). In wastewater influent, cefotaxime-resistant *E. coli* comprised 0.15 to 3.66% of the population. Cefotaxime-resistant *E. coli* were not detected in any wastewater effluent sample (Table 5-4). In surface water upstream, cefotaxime-resistant *E. coli* comprised 0 to 8% of the population. In surface water downstream, cefotaxime-resistant *E. coli* comprised 0 to 1.2% of the population. In ambient surface water, *E. coli* comprised 0 to 8.13% of the population. Resistance percentages could not be calculated in recycled water influent due to issues with dilutions, which produced mTEC plates with too many colonies to count from Virginia, California, and Georgia utility samples. In the two samples where any *E. coli* were detected in recycled water effluent, resistance percentages were either 1.39% or 92.9%. This latter percentage was flagged as an outlier because it was inconsistent with the duplicate.

Table 5-5. Percent of Cefotaxime-Resistant *E. coli* by Lab and Utility.

Sample Type	Utility (% resistance)							
	Lab	Florida 1	Florida 2	Virginia	Nevada 1	Nevada 2	California	Georgia
Wastewater Influent	USF	0.40	0.15	0.22	0.47	0.58	3.41	1.32
	VT	2.83	0.50	0.84	0.19	2.57	3.66	1.21
Wastewater Effluent	USF	0[a]	N/A	N/A	0[a]	0[a]	0[a]	NC
	VT	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	
Surface Water Upstream	USF	N/A[b]	N/A	N/A	NC		N/A	N/A
	VT	0.32	8.0	0[a]			0.10	0[a]
Surface Water Downstream	USF	1.05	0[a]	0[a]	NC		0.15	0[a]
	VT	1.20	0[a]	0[a]			0.07	0[a]
Ambient Surface Water	USF	NC[c]	NC	NC	1.66		NC	NC
	VT				0[a] – 8.13			
Recycled Water Influent	USF	N/A	N/A	N/A	NC		N/A	N/A
	VT	0[a]	0[c]	TNTC[e]			TNTC	TNTC
Recycled Water Effluent	USF	0[a]	0[a]	0[a]	NC		0[a]	0[a]
	VT	0[a]	0[a]	0[a]			92.9[d]	1.39

[a] 0% - total *E. coli* and/or cefotaxime-resistant *E. coli* was not detected.
[b] N/A - samples were not collected from this utility for processing.
[c] NC - Not calculated due to overcrowded plates (TNTC)
[d] Outlier – replicates did not match; high colony count on one plate resulted in high resistance percentage

Concentrations of total *E. coli* and cefotaxime-resistant *E. coli* measured in each sample type at USF and VT were compared by paired t-tests (Table 5-6). All p-values indicated that there was no significant difference between the measurements performed at USF versus VT. A p-value could not be determined for cefotaxime-resistant *E. coli* in wastewater effluent because none were detected in any samples. No comparison could be made for downstream surface water or recycled influent, which were only processed at VT.

Table 5-6. Mean Concentrations (log10 CFU/100 ml) of *E. coli* and Cefotaxime-Resistant *E. coli* at USF vs. VT.

Sample Type	Total *E. coli* (log_{10} CFU/100 ml)			Cefotaxime-resistant *E. coli* (log_{10} CFU/100 ml)		
	USF	VT	P-value	USF	VT	P-value
Wastewater Influent	6.87	6.47	0.4635	4.61	5.02	0.1258
Wastewater Effluent	0.14	0.25	0.4278	ND[b]	ND[b]	N/A[b]
Surface Water[a]	2.07	2.45	0.2121	0.37	0.51	0.2935
Recycled Water Effluent	ND[b]	0.19	N/A[b]	ND[b]	0.08	N/A[b]

[a] Ambient and downstream surface water data were combined for surface water comparisons.
[b] A comparison between USF and VT could not be made. Cefotaxime-resistant *E. coli* was not detected (ND) in samples processed at USF or VT.

5.6.1 Method Comparison for Culturable *E. coli*

The comparison study of mTEC (USEPA, 2014) and TBX (WHO, 2021) culture methods for *E. coli* was carried out at USF. The rationale for this study, which was performed on influent samples from a subset of utilities and surface water samples, was that the performance of mTEC is well-understood in the US, but TBX is much less frequently used. The fact that TBX has been chosen for the WHO Tricycle protocol makes it a possible prospect for a monitoring tool in the US, therefore we determined the performance of both methods, including media and incubation regimes. Total and cefotaxime-resistant *E. coli* were enumerated in influent samples from four water utilities and three surface water sites in the Tampa Bay area. Surface water sites included one brackish estuary (BTD) and two freshwater rivers (BFC, HR). Two different incubation methods with different temperature regimes were applied to each medium. The "incubator method" incubated plates at 35°C for 22-24 hours in an incubator (per WHO Tricycle protocol) whereas the "water bath method" incubated plates at 35°C for 2 hours and then in a 44.5°C water bath for 20-22 hours (per USEPA Method 1603). Each site was sampled once and filtered in duplicate at three volumes. Up to 20 colonies from each medium in each incubation condition were isolated for confirmation of *E. coli* from each site.

The study determined that the incubation conditions did not have a significant effect on the concentration of total *E. coli* or cefotaxime-resistant *E. coli* enumerated on either media (Table 5-7). *E. coli* isolates were, however, more frequently confirmed from influent and surface water samples when the water bath method was used compared to the incubator methods, regardless of the culture medium (Table 5-8).

Table 5-7. Concentrations of Total *E. coli* and Cefotaxime-Resistant *E. coli* (log CFU/100mL) in Wastewater Influent (n=4) and Surface Water (n=3) Processed by Incubator Method (35°C) and Water Bath Method (35°C/44.5°C).

		Total *E. coli* (log_{10} CFU/100 ml)				Cefotaxime-resistant *E. coli* (log_{10} CFU/100 ml)			
		TBX		mTEC		TBX		mTEC	
	Site	Incubator	Water Bath	Incubator	Water Bath	Incubator	Water Bath	Incubator	Water Bath
Wastewater	Georgia	6.71	6.56	6.66	6.60	5.27	4.39	5.16	4.86
	Virginia	6.32	6.23	6.26	6.37	4.69	4.08	5.49	3.65
	California	7.62	7.72	7.67	7.68	5.48	5.72	5.62	5.53
	Nevada	8.1	7.95	8.04	7.85	5.99	5.72	5.90	5.55
Surface Water	BFC	2.59	2.55	2.66	2.68	0.6	0.57	0.62	0.45
	BTD	1.58	1.62	1.88	2.83	0.17	0.11	0.11	0.03
	HR	2.19	2.15	1.90	1.91	ND[a]	ND	ND	0.08

[a]ND, not detected

Comparisons of confirmation frequency (Fisher's exact test) were made by medium, by microbial target (total vs cefotaxime-resistant *E. coli*) and by incubation method. The overall confirmation frequency (combining data for total and cefotaxime-resistant *E. coli* and both media) using the water bath method was significantly higher than the incubator method **for influent**, but not for surface water. Note that fewer colonies were tested for surface water than for influent (267 colonies vs 640).

The confirmation frequency for isolates from mTEC plates (combining both total and cefotaxime-resistant *E. coli* and both incubation methods) was higher than that for isolates from TBX plates **for influent** but not for surface water. No other comparisons produced significant differences.

Table 5-8. Confirmation Rates (%) of Total *E. coli* and Cefotaxime-Resistant *E. coli* at Each Site Sampled for Incubator Method (35°C) and Water Bath Method (35°C/44.5°C).

		Total *E. coli*				Cefotaxime-resistant *E. coli*			
		TBX		mTEC		TBX		mTEC	
	Site	Incubator	Water Bath	Incubator	Water Bath	Incubator	Water Bath	Incubator	Water Bath
Wastewater	Georgia	80	90	90	100	95	100	95	100
	Virginia	85	90	95	100	90	95	100	100
	California	85	95	90	100	100	95	100	100
	Nevada	85	95	90	100	95	100	95	100
Surface Water	BFC	90	95	90	95	66.6	66.6	75	100
	BTD	90	100	95	100	75	100	100	100
	HR	100	100	100	100	n/a[a]	n/a	n/a	100

[a]n/a: no colonies were obtained on plates from this media/condition

The lower confirmation frequency for media processed using the incubator method versus the water bath method can be explained by the high levels of background growth present on the plates using the incubator method (Figure 5-3). In addition to obtaining higher confirmation rates on plates incubated using the water bath method, less time was required to obtain pure cultures of isolates and colony counting was more accurate and rapid. Plates processed by the incubator method required several rounds of streaking for isolation to obtain pure cultures, whereas plates processed by the water bath method required one or two rounds of isolation. Plates that were processed using only the incubator method also required smaller volumes of sample to be filtered to obtain countable plates compared to their water bath method counterparts due to the background growth that occurred on the incubator method plates.

This experiment indicates that mTEC and TBX media produce similar estimates of total and cefotaxime-resistant *E. coli* concentrations. Incubation conditions (at 35°C for the incubator method and 35°C/44.5°C for the water bath methods) influenced the specificity of the assays, as the water bath method produced significantly higher confirmation rates. Confirmation rates were also significantly higher on mTEC agar compared to TBX.

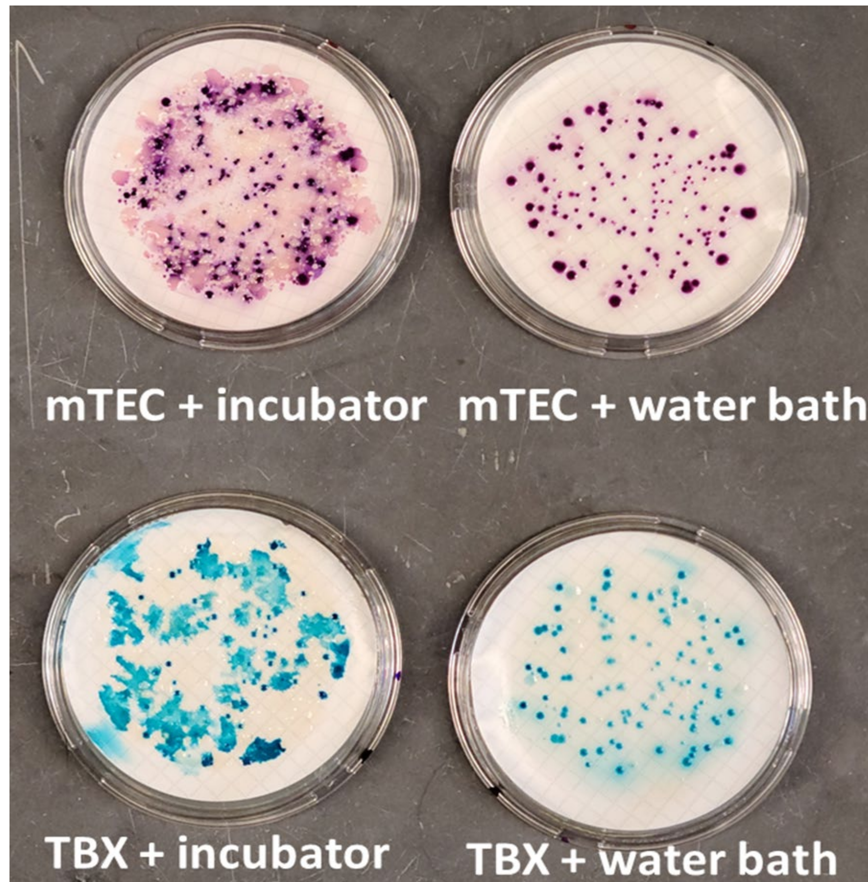


Figure 5-3. Depiction of Differences between Incubator (left) and Water Bath (right) Methods of Incubation for mTEC (top) and TBX (bottom) Agar.

The same volume of sample from the same site is filtered onto each of the four plates.

5.7 qPCR Data and Results

The ranges of *sul1* and *int*I1 for each sample type measured in this study are summarized in Table 5-9. To better understand any differences between qPCR measurements of *sul*1 and *int*I1 made in this study, a comparison was made with values reported in the literature. The expected ranges in concentrations of *sul*1 and *intI*1 in wastewater, recycled water and surface water shown in Table 5-9 were derived from the literature review summarized in Section 3.4. *sul*1 and *int*I1 concentrations were found to be most variable in recycled water. The gene target ranges measured at USF and VT are further illustrated in Figures 5-4 and 5-5. In all samples processed at USF, the concentration of gene target was within 0.05 log GC/mL of the expected range. In samples processed at VT, the range of concentrations of *intI*1 found in wastewater influent and effluent were outside of the high end of the expected range (Table 5-9). All other sample ranges were within the expected values.

Table 5-9. Summary of Gene Target Ranges Measured for *Sul*1 And *Inti*1 in All Sample Types.
Expected ranges were derived from the literature review (Section 3.4). Ranges outside the expected range are highlighted in gray.

Sample Type	Target	Expected Range (estimated[a] log_{10}(GC/mL)) s	USF Range (log_{10} (GC/mL))	VT Range (log_{10} (GC/mL))
Wastewater Influent	*Sul*1	1.5 to 10	5.56 to 9.24	5.34 to 8.07
	*Intl*1	1.5 to 7.5	5.88 to 7.54	5.44 to 9.23
Wastewater Effluent	*Sul*1	1 to 8	2.37 to 5.91	3.26 to 6.08
	*Intl*1	2 to 5	2.94 to 5.04	3.02 to 6.94
Surface Downstream*	*Sul*1	0 to 8	2.41 to 7.45	3.68 to 5.91
	*Intl*1	1 to 8	2.29 to 5.42	3.07 to 6.38
Surface Ambient*	*Sul*1	0 to 8	3.89 to 4.48	4.12 to 4.20
	*Intl*1	1 to 8	3.61 to 5.20	3.17 to 5.61
Recycled Effluent*	*Sul*1	-4 to 12	1.35 to 6.69	3.42 to 5.68
	*Intl*1	-1.5 to 10.5	1.95 to 4.96	2.36 to 8.61

[a]*Based on literature review, which did not distinguish between types of surface water or recycled water*

The ranges for *sul*1 were wider in samples processed at USF compared to VT, particularly at the lower range of concentrations. Recycled water effluent samples had the largest variability in *sul*1 abundance for samples processed at both USF and VT, which is consistent with the literature. The mean concentrations of *sul*1 were within a log difference between samples processed at USF and VT for all sample types (Figure 5-4).

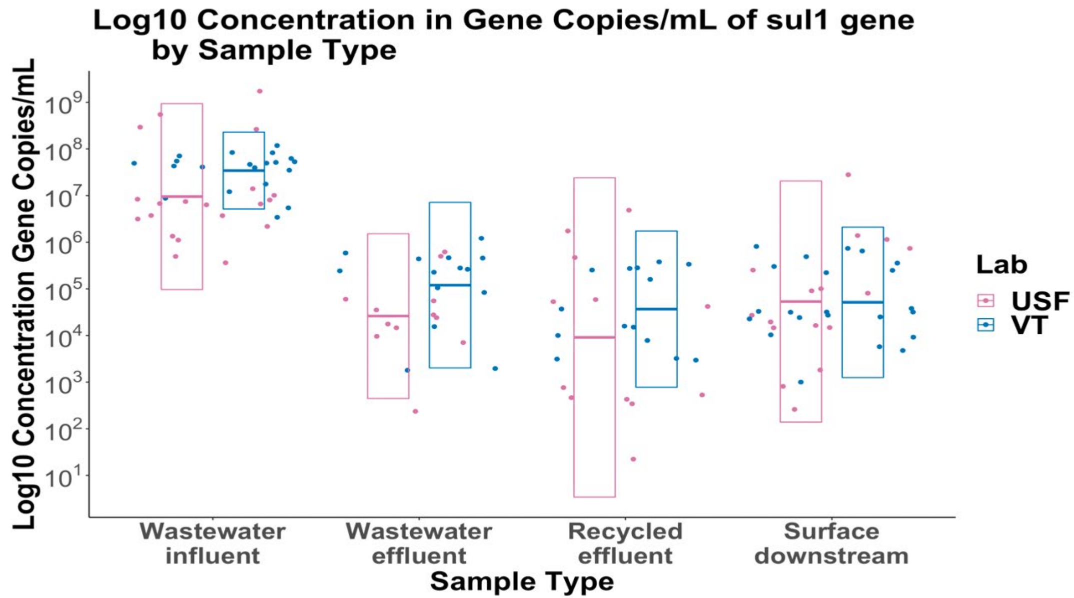


Figure 5-4. *Sul*1 Gene Target Abundance in Each Sample Type.
Samples processed at USF are shown in pink and samples processed at VT are shown in blue. Crossbars represent the mean ± standard deviation.

The ranges for *int*I1 were greater in samples processed at VT compared to USF (Figure 5-5). Recycled water effluent samples had the largest variability in *int*I1 abundance for samples processed at both USF and VT, which is consistent with the literature. The mean concentrations of *int*I1 were within 1 log between samples processed at USF and VT for wastewater and ambient surface water. There was greater than a 1 log difference in means for wastewater effluent, downstream surface water, and recycled water effluent samples. In both wastewater influent and effluent, some samples processed at VT were outside of the expected range observed in the literature.

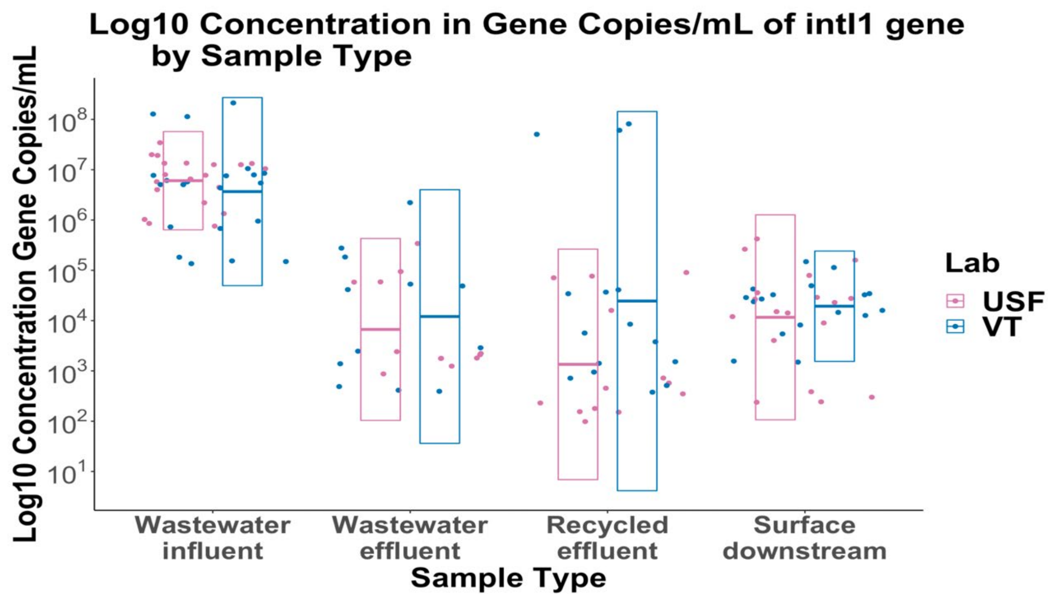


Figure 5-5. *Int*I1 gene Target Abundance in Each Sample Type.
Samples processed at USF are shown in pink and samples processed at VT are shown in blue. Crossbars represent the mean ± standard deviation.

Further analysis included breaking the data out by state and utility. Figure 5-6 shows the concentration of the *int*I1 gene in each sample type, grouped by utility. Figure 5-7 shows the concentration of the *sul1* gene in each sample type, grouped by utility.

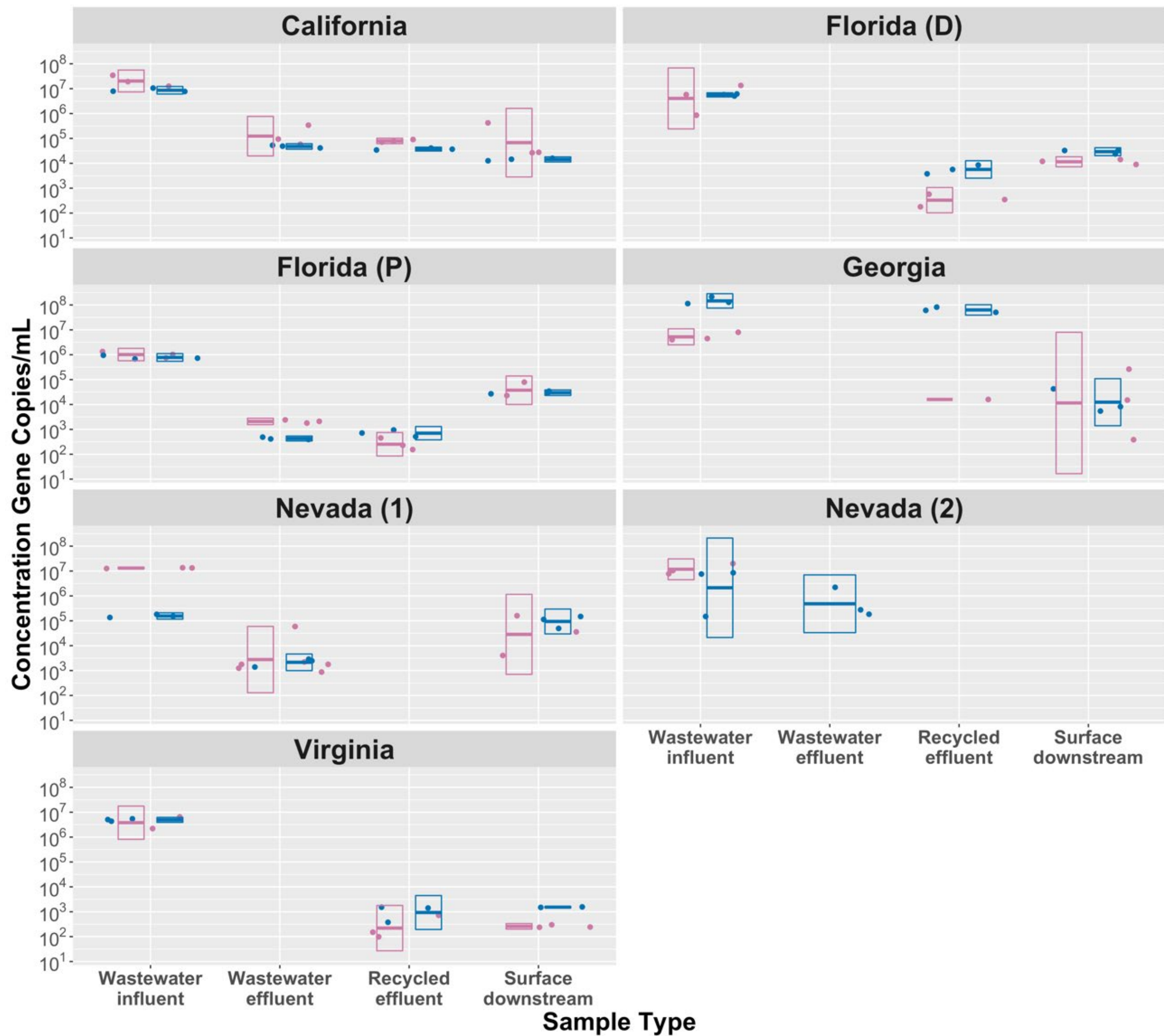


Figure 5-6. intI1 Gene Copy Concentration (Log10 GC/ml) by Sample Type, Utility, and Processing Laboratory.

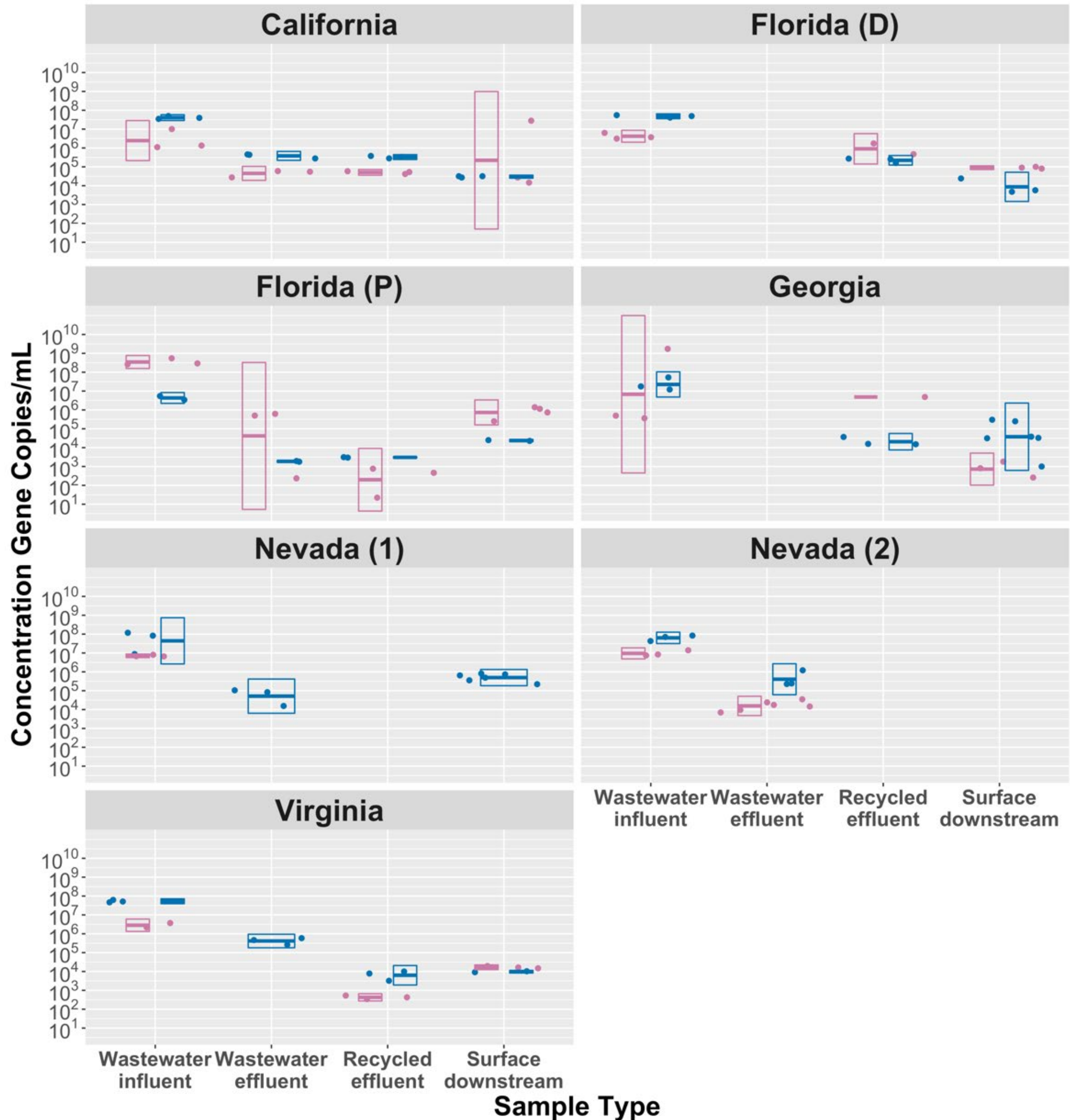


Figure 5-7. Sul1 Gene Copy Concentration (Log10 GC/ml) by Sample Type, Utility, and Processing Laboratory.

A 2-way ANOVA was conducted on the qPCR datasets from both labs to examine if the resulting concentrations were significantly different between processing laboratories. There was no significant difference between labs for the gene target *int*I1 (p=0.06567) nor for the gene target *sul*1 (p=0.1984). There were significant differences as a function of sample type (i.e. water

matrix) for both *sul*1 (p=0.0238) and *int*I1 (p=0.00933) datasets, which would be expected (e.g., sewage influent contains different numbers of the gene targets than treated effluent).

5.8 Evaluation of QA/QC

5.8.1 Replication Implemented in This Study

To account for natural variability in water samples and determine the importance of replicates, both biological and technical replicates were included in all aspects of this study. Biological replicates are samples that were collected and stored independently, and are intended to provide information about the inherent variation of the measured variable in the water environments. Biological replicates help to ensure that the sampling is representative of the target environment. Statistical comparisons are typically applied to biological replicates. Technical replicates are not independent samples and are used to determine the precision of the method. Technical replications help to capture variability in outcome measures across days, researchers, time, or the variation inherent to the technique or instrument used. Examples of technical replicates are duplicate membranes filtered for each sample volume, or three triplicate wells analyzed in qPCR assays. In this study, two biological replicates were analyzed for all culture analysis and three biological replicates were analyzed for all molecular analysis. Three technical replicates were used for all analyses conducted, both for culture and molecular techniques.

5.8.1.1 Culture

A comparison between the *E. coli* concentrations derived by culture from each biological replicate was conducted. The difference in concentration in the culture biological replicates was not significant for total *E. coli* (p=0.34, paired t-test) or cefotaxime-resistant *E. coli* (p=0.29, paired t-test). Such replication is important in culture data to ensure that the results were not obtained by chance or due to things such as collecting a sample that was not representative of the water body. Performing culture analyses at three dilutions in technical duplicate allows for a wider range of concentrations to be targeted, minimizing the chance that the sample was too diluted or too concentrated to obtain countable plates.

5.8.1.2 qPCR

The standard deviation from the mean was calculated for molecular technical replicates (individual qPCR reactions for a given biological replicate). The percent deviation from the mean was chosen for comparison and calculated by subtracting the concentration of the technical replicate from the mean concentration for all three technical replicates, then dividing by the mean of all three technical replicates. Measurements of *int*I1 and *sul*1 at USF and VT produced highly variable percent standard deviation measurements (Table 5-10), particularly at VT. Higher variability in the qPCR measurements can be attributed in part to multiple technicians and also to outlier data points. The outliers skewed the average percent standard deviation from the mean and suggests the need in the SOPs for a protocol on how to identify outliers and address them in any further analyses. Performing molecular analyses in triplicate allows for the elimination of outliers from the data. When two out of three data points are nearly the same and a third data point is more than 2 standard deviations different from the other points, the third data point can be treated as an outlier. Performing molecular analyses in singlet or duplicate hinders the ability to screen for variability and error in the analyses.

Table 5-10. Lowest, highest, and Average Percent Standard Deviation from the Mean for Each Target in Each Sample Type, by Lab, Amongst the Technical Replicates.

Target	Sample	Lab	% Standard Deviation from the Mean		
			Low	High	Average
*Int*I1	Recycled Effluent	USF	0.01%	8.1%	1.7%
		VT	0.2%	1313%	58.7%
	Surface Downstream	USF	0.01%	5.1%	1.8%
		VT	0.4%	845.1%	53.4%
	Wastewater Influent	USF	0.01%	18.2%	1.8%
		VT	0.05%	2188.3%	89.3%
	Wastewater Effluent	USF	0.03%	5.2%	1.8%
		VT	0.08%	2226.9%	119.3%
*Sul*1	Recycled Effluent	USF	0.01%	3.5%	1.2%
		VT	0.02%	15.5%	4.6%
	Surface Downstream	USF	0.01%	4.8%	1.2%
		VT	0.3%	37.4%	9.8%
	Wastewater Influent	USF	0.01%	6.7%	1.3%
		VT	0.2%	29.3%	4.5%
	Wastewater Effluent	USF	0.01%	9.8%	1.4%
		VT	0.3%	147.1%	12.3%

To address the question of variance in qPCR measurements, the percent deviation from the mean was examined for each technical replicate. Graphed below in Figure 5-8 is the percent standard deviation from the mean for each technical replicate by sample type and processing laboratory, on a linear scale.

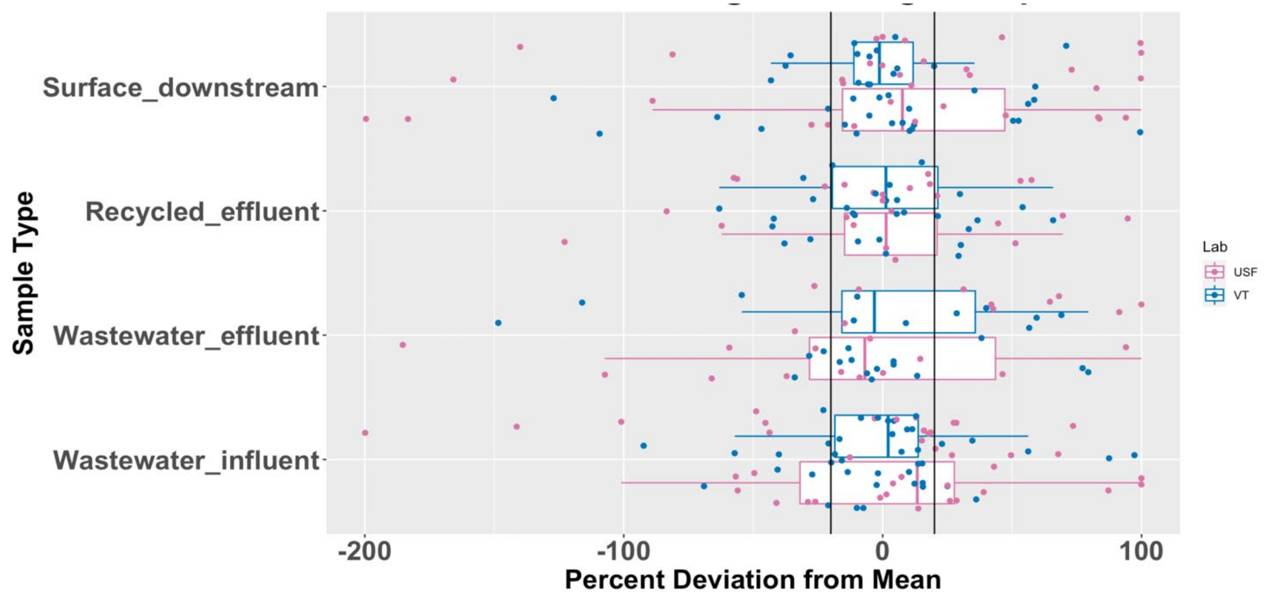


Figure 5-8. The Percent Deviation from Mean for Biological Replicates, where Mean is the Average of Biological Triplicates.

For each main sample type that was tested at both USF and VT, the percent standard deviation from the mean was calculated for each biological replicate, with the mean being that of the three biological replicates. Here, the black vertical lines indicate +/- 20% deviation.

Next, analysis was conducted to determine the variance between the two labs, using the absolute value of the percent deviation from the mean. Variance in technical replicates was found to be statistically significantly different across the two labs (Kruskal-Wallis, p=2.2e-16). Variance in biological replicates was found to be significantly different across the two labs (Kruskal-Wallis, p=0.0001278).

Next, analysis was conducted to determine the variance between the six utilities sampled, using the absolute value of the percent deviation from the mean. The results indicated that variation from the mean was significantly associated with the utility from which samples were taken (Kruskal-Wallis, p=0.0004538).

5.8.2 Blanks and Negative Controls Implemented in This Study

The importance of “blanks,” or negative controls, is well agreed-upon within the community (Borchardt et al., 2021). In this study, the use of multiple types of blanks at each step of the process was examined. Table 5-11 outlines which blanks were used for which methods, qPCR or culture, and how they were treated differently at each processing laboratory.

Table 5-11. The Multiple Types of Negative Controls Used in This Study.

Designation	Used for qPCR	Used for Culture	Virginia Tech	University of South Florida
Trip Blank	X		Autoclaved nanopore water, sealed with tape to prevent disruption in shipping or sampler from opening it.	Autoclaved and UV sterilized deionized water, sealed with tape to prevent disruption in shipping or sampler from opening it.
Field Blank	X		Autoclaved nanopore water; Samplers were instructed to remove the cap prior to any wastewater sampling event, collect wastewater sample, then re-cap this field blank.	Autoclaved and UV sterilized deionized water; samplers were instructed to remove the cap prior to collecting wastewater influent, collect wastewater influent, then re-cap this field blank.
Filter Blank/Extraction Blank	X		50 ml of sterile PBS, carried through extraction	50 ml of sterile PBS, carried through extraction
Non-Template Controls	X		Molecular grade water	Molecular grade water
Negative Control		X	The inoculation loop being used for samples, empty and flame-sterilized	Non-target organism (*Enterococcus* ATCC)

Throughout this study, several blanks were found to amplify at higher-than-expected concentrations. Below, these concentrations have been plotted by utility, processing laboratory, type of blank, and gene target, for clarity (Figure 5-9).

Figure 5-9. Concentration of Gene Targets Detected in Each Blank.

All blanks are plotted here as individual points, by sampled utility, processing laboratory, type of blank, and gene target. The samples are coded on the y-axis here as "processing laboratory: state: utility code". Concentrations are presented in gene copies per mL as quantified using the aforementioned qPCR methods.

In terms of blanks, it is recommended to incorporate as many controls as possible throughout the processing and analysis train in order to assess any potential points of contamination. This is especially important for DNA-based analysis, because there can be many sources of contamination during sample collection and processing. For qPCR, the main blanks recommended include a field blank, a trip blank, and a filter blank. Additional blanks could be added to the DNA extraction step. The field blank is sterile, ideally DNA-free water in a 1 L sampling bottle that is sealed, shipped to the sampling site, opened briefly during sampling, and resealed. The trip blank is a sterile, ideally DNA-free water in a 1 L sampling bottle which is treated the same as the field blank, except it is never opened until all samples are filtered. The filter blank is sterile PBS which is filtered through a sterile filter cup during sample filter concentration. These sampling blanks may be Molecular Grade Water, budget permitting, or an ultrapure water such as Nanopure. In this study, all water implemented for blank was autoclaved prior to shipment. If a new bottle of molecular grade water is used, then autoclaving is not necessary. In this study, no trip or field blanks were plated for culture, but negative and positive controls were used.

Each type of blank serves to isolate potential points of contamination in the sampling and analysis system. First, a DNA extraction blank can indicate any contamination that occurred during the actual extraction of the samples. Second, the filter blank can indicate if the filter cassettes, sterile PBS, or other tools used in filter-concentration were contaminated. Thirdly, the field blank can indicate background levels of contamination in the environment where sampling is taking place; this may include contaminated gloves that open the container, or airborne contamination. And lastly, the trip blank can indicate any fundamental contamination

in the sampling system, such as the water used for laboratory processing or if the sampling bottles were not fully sterilized. Ideally the blanks will yield no qPCR signal. However, in the case that there is contamination, the blanks will help to identify the source of contamination. Based on which blanks give a positive signal, it is possible to quickly isolate and address any contamination problems.

Interpreting qPCR Signals in Blank Samples and Recommended Actions:

- Signal in NTC qPCR reagent(s) are contaminated, re-run with new reagents. Pipettors may be contaminated; initiate thorough cleaning. If signal persists, verify it is true signal (e.g., examine melt curves or sequence product) and not instrument error. NTC products can be sequenced to identify whether it is non-specific product or primer dimer. Examine qPCR practices; are blanks being processed after samples with high target concentrations? See more troubleshooting in SOPs
- Signal in trip blank only (other negative controls are clean)- the water used to prepare the blanks is contaminated, the sampling bottles were not effectively sterilized, or the trip blank was erroneously opened and/or used for a sample. Check water supply and autoclave for future analysis.
- Signal in field blank only (i.e., other negative controls are clean) indicates that the process of collecting the sample in the field is introducing contamination. The field sampling process should be re-visited to minimize opportunity for contamination.
- As a general rule of thumb, if the qPCR signal in the blank is two standard deviations lower than the mean of the measurements in the corresponding samples, then the contamination is considered negligible and analysis can proceed. However, if the signal is within two standard deviations of the mean of the sample measurements, then it is not possible to discern signal from noise and the data collected should be discarded and the sampling and analysis repeated.

5.8.3 Inhibition Assessments and Control Strategies Implemented in This Study

Inhibition was assessed and controlled in this study using the dilution method. A representative subset of all samples from each utility were serial diluted to run a qPCR dilution curve. The final calculated concentration data was then plotted to illustrated which dilution resulted in the best amplification (with melt curve analysis for QA/QC). The selected optimal dilution for each sample type and utility was then applied to the remaining samples for all subsequent qPCR analyses.

5.8.4 Samples that Did Not Meet QA/QC for Culture Due to Late Arrival

During the first sampling event, several challenges arose and resulted in the samples collected not meeting QA/QC guidelines. Notably, the shipping carrier lost a significant portion of the samples for an additional 24 hours, putting them outside of the 48-hour processing window by the time of their arrival. The team initially decided to process and analyze the samples in an attempt to measure the importance, or lack thereof, of the 48-hour processing window.

In addition to the late-arriving cooler, the blanks for the USF samples were lost during sampling and never received by the laboratory. On day two of processing samples at USF, an error was discovered with the water bath which rendered all cultures unusable. USF personnel re-

cultured each sample again on day two, which was again over 48 hours after sampling, and after the water samples had been in refrigeration for about 24 hours. Therefore, those culture results were not usable as a comparison to the late samples which arrived at VT after being lost in transit. QA/QC failures of this sampling trip are outlined below in Table 5-12.

Due to the combination of QA/QC failures discussed above and in the below table, the team opted not to analyze and present the data collected under these conditions due to an inability to assign causation to any effects on the culturing results. Instead, they re-sampled this water utility for a second time and fortunately all samples arrived to the laboratory on time and met the stated QA/QC criteria.

Table 5-12. Detailed Outline of QA/QC Failure Comparison for One Utility
(1st sampling event and re-sample)

Sampling Date	Lab	Sample Type	Arrival	qPCR Processing	Culture Processing
08-23-21	VT	Wastewater	Late[1]	< 8 h of arrival	< 8 h of arrival
		Recycled Water	Late[1]	< 8 h of arrival	< 8 h of arrival
		Surface Water	On time	< 8 h of arrival	< 8 h of arrival
		Blanks	Late[1]	< 8 h of arrival	< 8 h of arrival
	USF	Wastewater	On time	< 8 h of arrival	> 24 h of arrival
		Recycled Water	On time	< 8 h of arrival	> 24 h of arrival
		Surface Water	On time	< 8 h of arrival	> 24 h of arrival
		Blanks	Late[1]	n/a	n/a
02-09-22	VT	Wastewater	On time	< 8 h of arrival	< 8 h of arrival
		Recycled Water	On time	< 8 h of arrival	< 8 h of arrival
		Surface Water	On time	< 8 h of arrival	< 8 h of arrival
		Blanks	On time	< 8 h of arrival	< 8 h of arrival
	USF	Wastewater	On time	< 8 h of arrival	< 8 h of arrival
		Recycled Water	On time	< 8 h of arrival	< 8 h of arrival
		Surface Water	On time	< 8 h of arrival	< 8 h of arrival
		Blanks	On time	< 8 h of arrival	< 8 h of arrival

[1]Late = After 48 hours of sample collection; On time = within 48 hours of sample collection
[2]Culture was conducted outside of the 24 h culture window on this occasion due to a misuse/malfunction of water bath which caused the first set of plates to melt. Therefore, samples were re-plated outside of the 24 h window in order to obtain usable data.

5.8.5 Impacts of Late Sample Arrival on qPCR Results

Plotted in Figure 5-10 are the resulting gene concentrations for the first utility that was sampled and re-sampled due to delays in shipping with the first event (Table 5-12). Samples which arrived late are plotted in pink with on-time samples in blue, allowing comparison of the impact of meeting the QA/QC guidelines. This graph demonstrates the variability observed in the samples that arrived outside of the processing window. Not only did these samples arrive late, but due to the expectation of their arrival within 24 hours, there was insufficient ice within the cooler, which resulted in the samples arriving at or above room temperature (20-22 °C). Some additional uncertainty arose due to all three of the VT blanks for this sampling event arriving in the late cooler, and none of the USF blanks being returned. This eliminated the possibility of a comparison point for the samples that arrived on-time or for inter-lab comparison of blanks,

but ultimately allowed for direct comparison of the late samples to their (late) blanks at Virginia Tech.

A two-sided t-test examined the difference in late and on time samples and found no statistically significant difference in the measured concentrations ($p=0.9803$). The results of this examination indicate that the qPCR data were not significantly impacted by the delayed arrival of the samples, nor the arrival at or above room temperature. This is especially remarkable given that the samples were collected several months apart (August versus February). This suggests that samples intended for molecular analysis remain valid for analysis at least an additional day outside the sample processing window. However, we still recommend staying within the criteria stated in the SOP. Further testing would be necessary to validate that longer holding times are acceptable for qPCR analysis, for example in situations where it is not possible to analyze within the 48-hr window specified for on-time analysis.

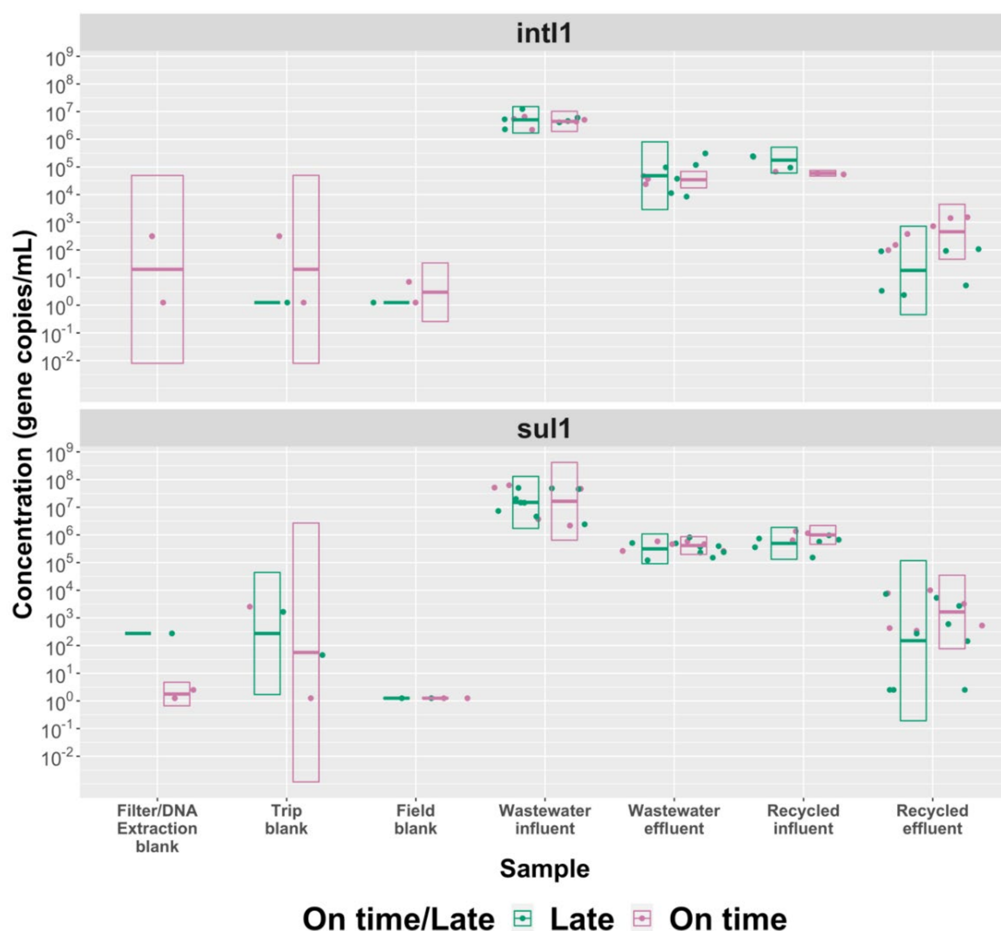


Figure 5-10. Concentration of Gene Targets by qPCR in Late vs. On Time Shipments from the Same Utility
The concentration of each gene target, *int*I1 and *sul*1, measured by qPCR, are plotted by sample type and whether the individual sample arrived on time or late, according to the 48-hour processing window outlined in the QA/QC guidance of this study. All samples here were collected from the same utility, but during two different sampling events due to the samples which arrived outside of the 48-hour window on the first sampling event (Table 5-12).

5.9 Comparison of qPCR and ddPCR

A sub-aliquot of VT DNA extracts was shipped overnight on dry ice to collaborators at USEPA-ORD for independent analysis via ddPCR. This served to provide a comparison to the qPCR measurements and also to independently assess apparent contamination of a number of the field blanks, trip blanks, and filter blanks with *int*I1 and *sul*1.

Both sets of results were converted to concentration in gene copies per mL for comparison, the overall results for the gene target *int*I1 are plotted below by sample type (Figure 5-11).

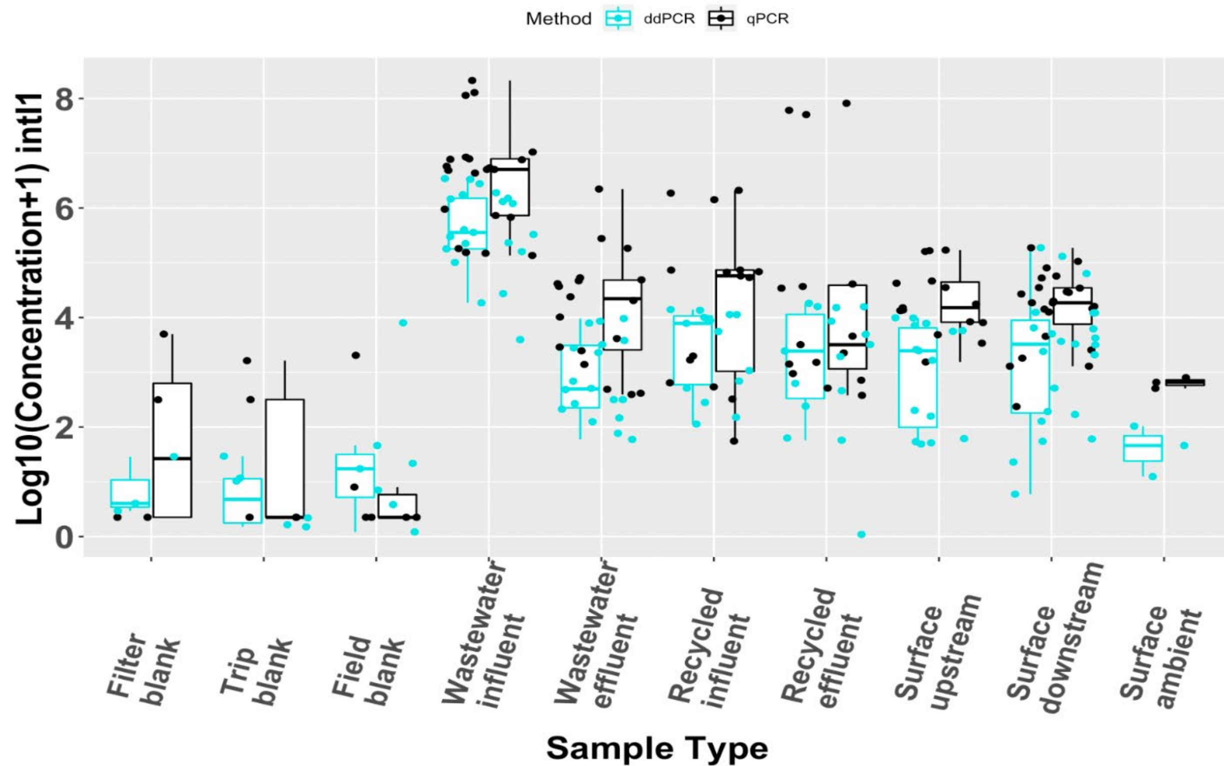


Figure 5-11. IntI1 Concentration by Sample Type, Comparing ddPCR and qPCR Methods

Boxplot comparing concentration data by sample type, for the gene target intI1, with color indicating the methodology used, ddPCR or qPCR, of the same sample DNA extracts. This figure demonstrates that general patterns and concentrations hold across sample types for both methods, although qPCR measurements were systematically higher than ddPCR.

The overall results for the gene target *sul*1 are plotted below by sample type (Figure 5-12), using the same method as used above for *int*I1.

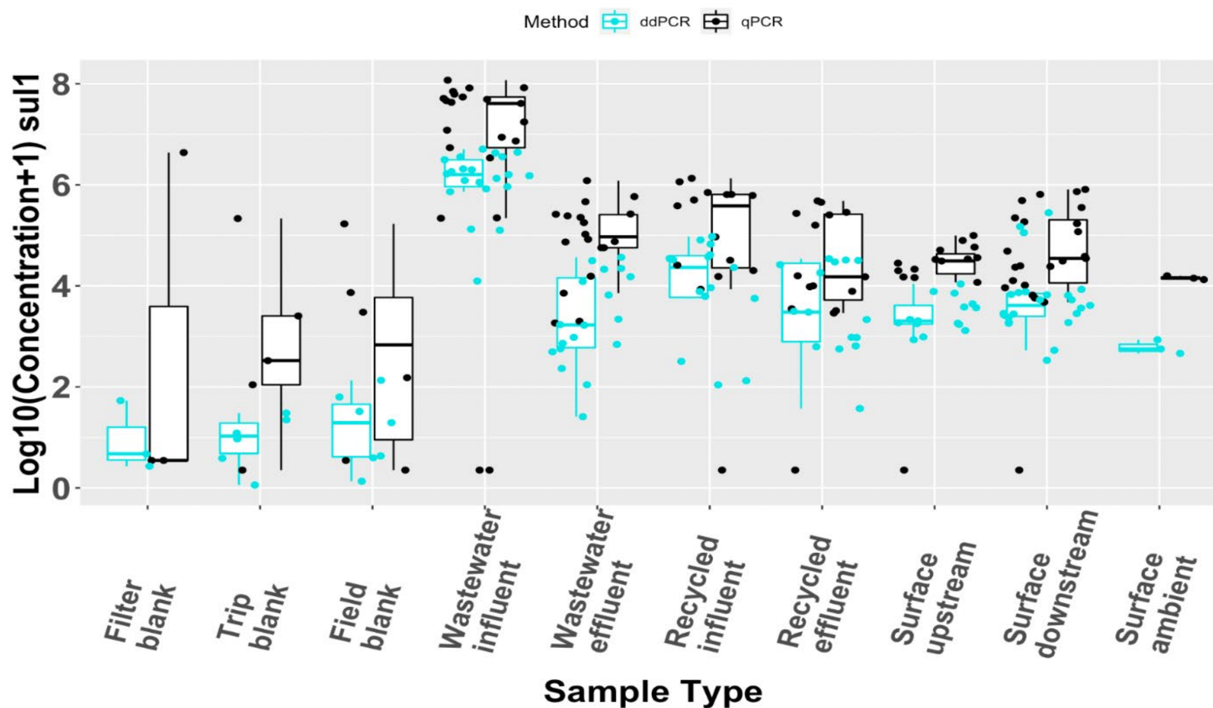


Figure 5-12. sul1 Concentration by Sample Type, Comparing ddPCR and qPCR Methods

Boxplot showing overall spread of concentration data by sample type, for the gene target sul1, with color indicating the methodology used, ddPCR or qPCR, of the same sample extracts. This figure demonstrates that general patterns and concentrations hold across sample types for both methods, though qPCR measurements were systematically higher than ddPCR.

A linear model was fit to the log transformed qPCR and ddPCR data to determine the correlations between *sul*1 and *int*I1 measurements produced from qPCR versus ddPCR. A linear regression indicated significant linear relationship between qPCR and ddPCR measurements (log^{10}) for both *sul*1 (r^2= 0.351; p= 3.17e-13) and *int*I1 (r^2=0.6028; p=2.2e-16).

Comparison of results from the qPCR and ddPCR overall indicates that general patterns were consistent between the methods. The exact numerical quantification by each method varied, but that variation tended to be one-sided, i.e. the resulting concentrations were consistently higher using qPCR compared to ddPCR. This pattern was not impacted by sample type or utility. The higher concentrations detected by qPCR could potentially be a result of the use of Qubit-confirmed G-blocks for standard curves. Because fluorometers measure total DNA in a sample, it is possible that quantification is not precise or that degraded G-block standard is measured during the dilution of the 10^8 G-block standard, resulting in a standard in which the actual concentration is less than what was initially measured. Because standard curves are programmed in qPCR, the use of a standard that in fact has a lower concentration than what it is designated as in the standard curve, would result in a systemic over-estimation of all samples. Another possibility is that the samples themselves experienced an additional freeze-thaw cycle before being shipped to USEPA for analysis. The freeze thaw cycle also could have systematically degraded the DNA targets and resulted in lower estimates of concentrations across samples by ddPCR. One possibility to reduce the potential for DNA degradation with freeze thaw cycles in the future would be to elute in buffer instead of water, whereas all DNA

extracts were eluted into water in this study. This was not examined in this study, however, and would require further testing and verification.

The analysis of the negative controls (Field blanks, Filter blanks, and Trip blanks) using ddPCR allowed for an additional test of the blanks which amplified during qPCR. The ddPCR concentrations of amplifying blanks were notably lower than those measured via qPCR for both *sul*1 and *int*I1 (Figure 5-12). This suggests that contamination observed in the blanks by qPCR was largely a result of contamination during setting up the qPCR reactions, and not as a result of sample collection, processing, or DNA reaction.

5.10 Conclusion from the SOP Validation

Overall, it was demonstrated that the 5052 ESBL *E. coli* SOP is user-friendly and highly repeatable between laboratories. Additionally, a cross-comparison study showed that the mTEC-based protocol provides comparable results to the TBX-based WHO Tricycle Protocol, but provides a higher confirmation rate to species for *E. coli*. The overall findings here are beneficial to the water utility community because it was shown that simple modification to USEPA mTEC-based standard methods results in a robust and globally comparable measure of ESBL *E. coli*.

The *sul*1 qPCR SOP was also repeatable between laboratories. However, the *intI*1 assay was not as repeatable. Comparison between labs helped to improve the efficiency and LOD of both assays by identifying a master mix and corresponding enzyme that results in higher assay efficiency. Assessment of the different handling of sample blanks at USF versus VT illuminated opportunities for introduction of false positives in the sample analysis. A matrix was developed to inform users regarding actions that should be taken when blank samples or qPCR negative controls yield positive signals.

qPCR and ddPCR measurements were also found to yield highly similar trends across samples, although ddPCR yielded a systematically lower signal (about 1-log lower). This could be either because the standard curve implemented in qPCR by Virginia Tech employed standards that were slightly lower in concentration than estimated based on what the supplier and fluorometry measurements indicated. Another possibility is that there was some DNA extract degradation with the extra freeze-thaw cycle that was incurred when the extracts were shipped to EPA for analysis. Notably, mean *sul*1 measurements were highly similar between VT and USF labs, suggesting that the qPCR standard curves were accurate. However, *int*I1 measurements were systematically lower when measured by the USF lab, and more on par with the ddPCR measurements. This suggests that the standard curve for *int*I1 at VT may have slightly overestimated sample concentrations.

CHAPTER 6
Water Antibiotic Resistance Database (WARD) Website

6.1 Overview

The WARD website is an online system providing a data repository and analytical tool to support antibiotic resistance surveillance of water environments. WARD is embedded in the Virginia Tech AgroSeek website (Liang et al. 2021), which provides full service storage and analysis of metagenomic data and metadata derived from a variety of environments, including wastewater, recycled water, and surface water. The WARD website expands this capacity specifically to also support culture, qPCR, and qPCR-array data and metadata relevant to antibiotic resistance monitoring. Additionally, WARD provides user-friendly data upload, analysis, and visualization tools for analysis and comparison within and across projects. Integrated with the AgroSeek website's functionality, WARD provides researchers, regulators, and practitioners a convenient means to share AMR monitoring data from water environments. Thus, WARD can provide a valuable mechanism to address the broader research and monitoring objectives described in Chapter 1, particularly those that require global comparison of data sets.

WARD is also designed to provide a forum for users to discuss and share sampling and analysis protocols as the field progresses. The current SOPs and QA/QC procedures developed through Project 5052 are posted on the website, and these can be updated with time by posting and tracking updated versions. At this time, users are invited to test the WARD service and post comments about their experiences and suggestions. This can help to improve and further fine-tune WARD into the future, and also improve application of the SOPs developed herein to encourage industry-wide adoption. The following sections provide an overview of WARD.

6.2 Methods

The WARD website is built with the WordPress framework and is collocated with the AgroSeek website. qPCR and culture data are organized in PostgreSQL databases. Visualization tasks are handled using Python and R programs.

To make it easy for users to upload and share data, two comprehensive metadata templates were designed (specialized Excel spreadsheets) that are tailored to culture and qPCR data generated from water surveillance projects. Each provides various water type options and collects corresponding essential information, including the treatment stages, the treatment train, and the intended usage. Users can easily upload data to the server via one Excel file using the appropriate template format and perform data management and analysis. Spreadsheets are included in Appendix B4.

6.3 Tutorial

Please visit WARD Tutorial page for details. The following section provides an overview of the WARD workflow (Figure 6-1) and key screenshots from the website (Figure 6-2). The home page includes an introduction to the project (Figure 6-3) and links to the project login page (Figure 6-4). After logging in, users can create a new project or work from an existing one (Figure 6-5). In each project, the user can

upload files or view their existing files (Figure 6-6). If uploading a new file, templates are provided to ensure data is in the appropriate format (Figure 6-7). When uploaded, data can be viewed on the webpage (Figure 6-8) as well as visualized using the visualization function (Figure 6-9; Figure 6-10).

6.4 Workflow and Website Screenshots

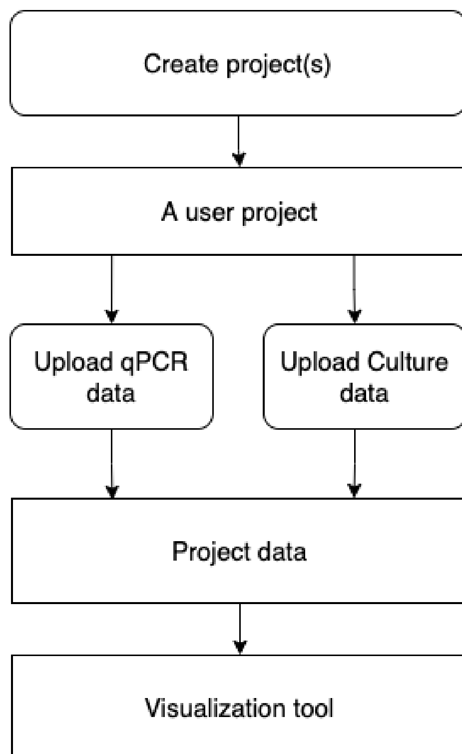


Figure 6-1. WARD Workflow.

Figure 6-2. WARD Welcome Page.

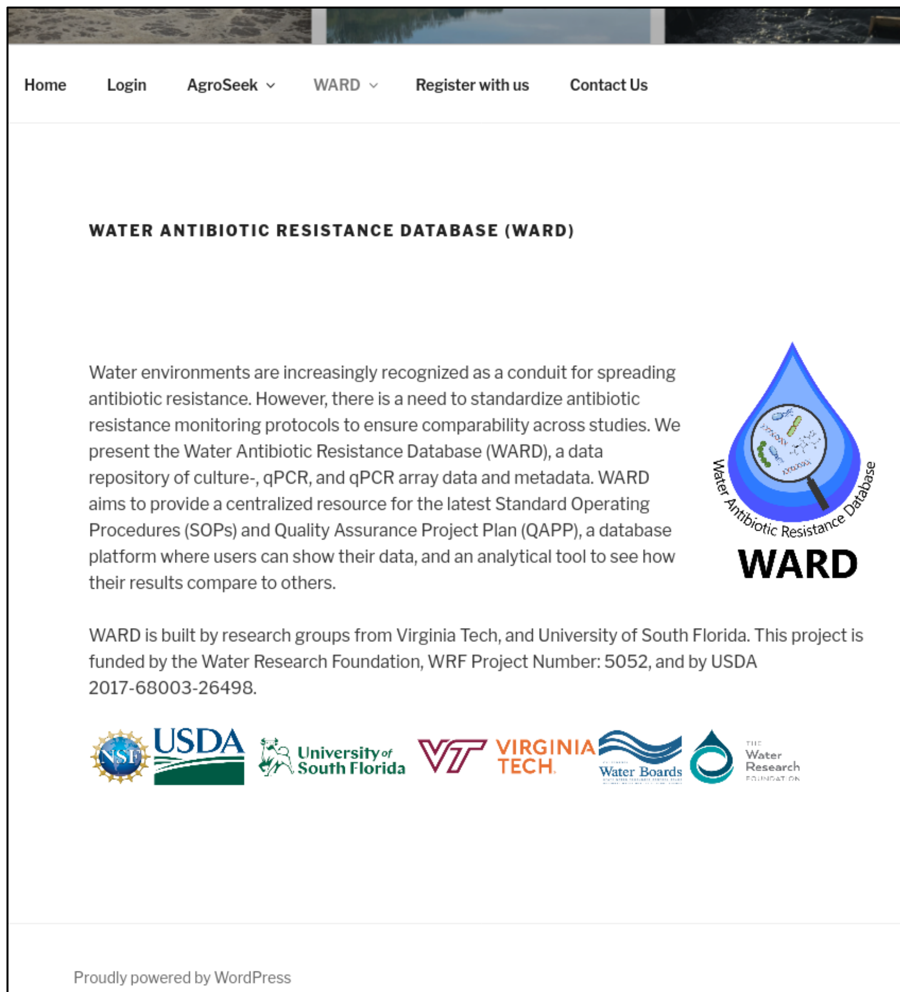


Figure 6-3. WARD Introduction Page.

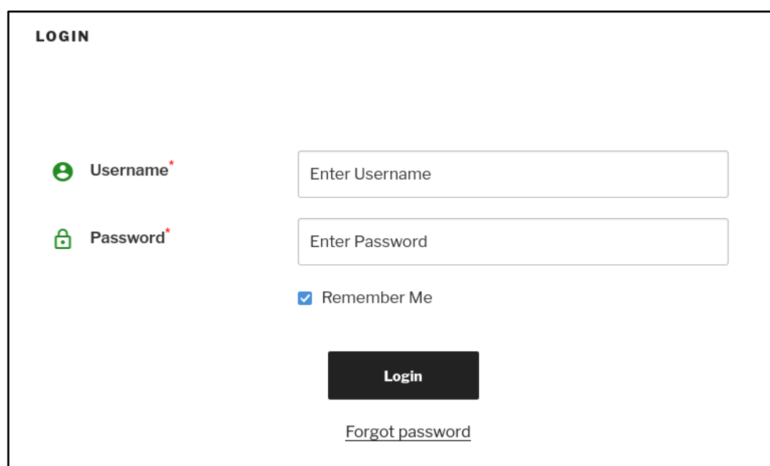


Figure 6-4. WARD User Login Page.

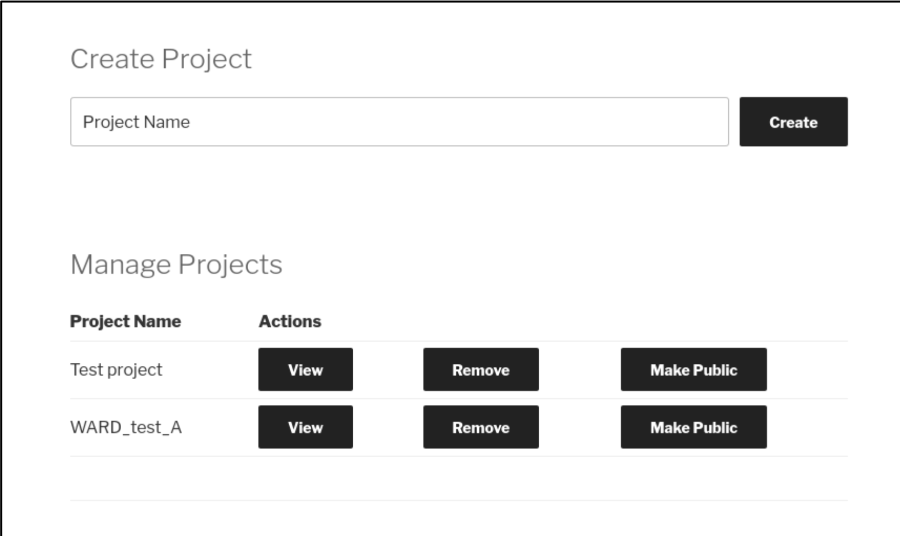


Figure 6-5. WARD Project Management Page.

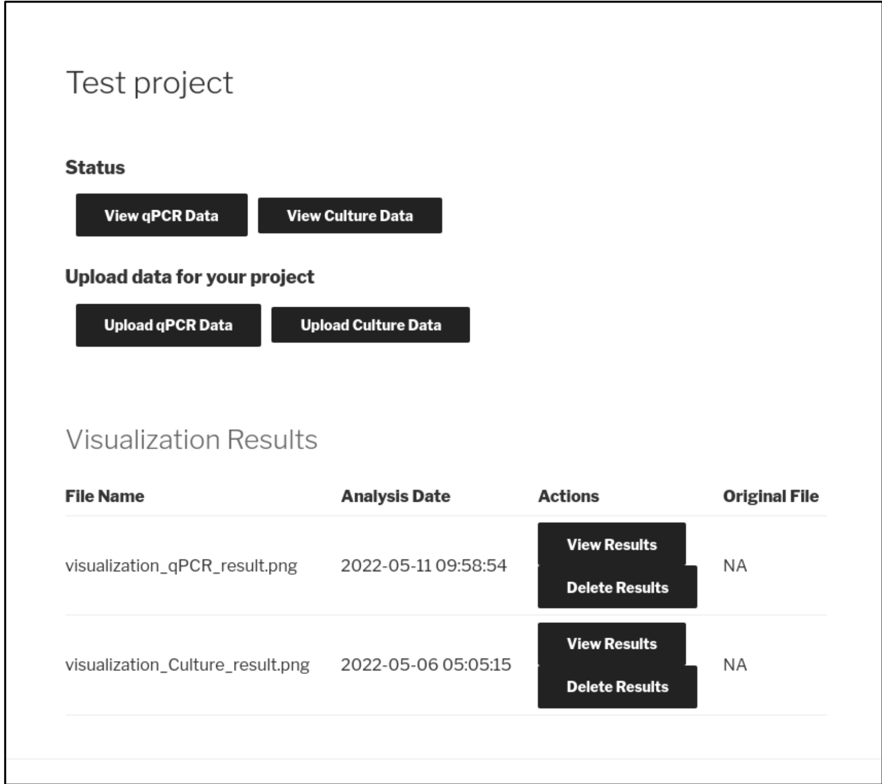


Figure 6-6. WARD Project Page.

Download metadata template:

Template for qPCR data:
Download

Template for culture data:
Download

Please use the recommended units in the metadata templates:

Antibiotic concentration: ug/mL
DNA concentration: ng/μL
qPCR gene measurements: gc/mL
C and N total organic matter concentration: weigtht%

Upload qPCR data file:

Browse... No file selected. Submit qPCR data

Upload Culture data file:

Browse... No file selected. Submit Culture data

Figure 6-7. WARD Data Uploading Page.

View Current data

Sample	water_type	treatment_stage	treatment_plant_type	intended_use	treatment_train	impacted_
GAG-P-RE1	Recycled water effluent	Not applicable	Not applicable	Indirect potable	Other	Not applical
GAG-P-RE2	Recycled water effluent	Not applicable	Not applicable	Indirect potable	Other	Not applical
GAG-P-RI1	Recycled water influent	Not applicable	Not applicable	Not applicable	Other	Not applical
GAG-P-RI2	Recycled water influent	Not applicable	Not applicable	Not applicable	Other	Not applical
GAG-P-WI1	Wastewater influent	Influent	Conventional Biological	Not applicable	Not applicable	Not applical
GAG-P-WI2	Wastewater influent	Influent	Conventional Biological	Not applicable	Not applicable	Not applical

Download Data File
Visualize data
Clear data
Back

Figure 6-8. WARD Data View Page.

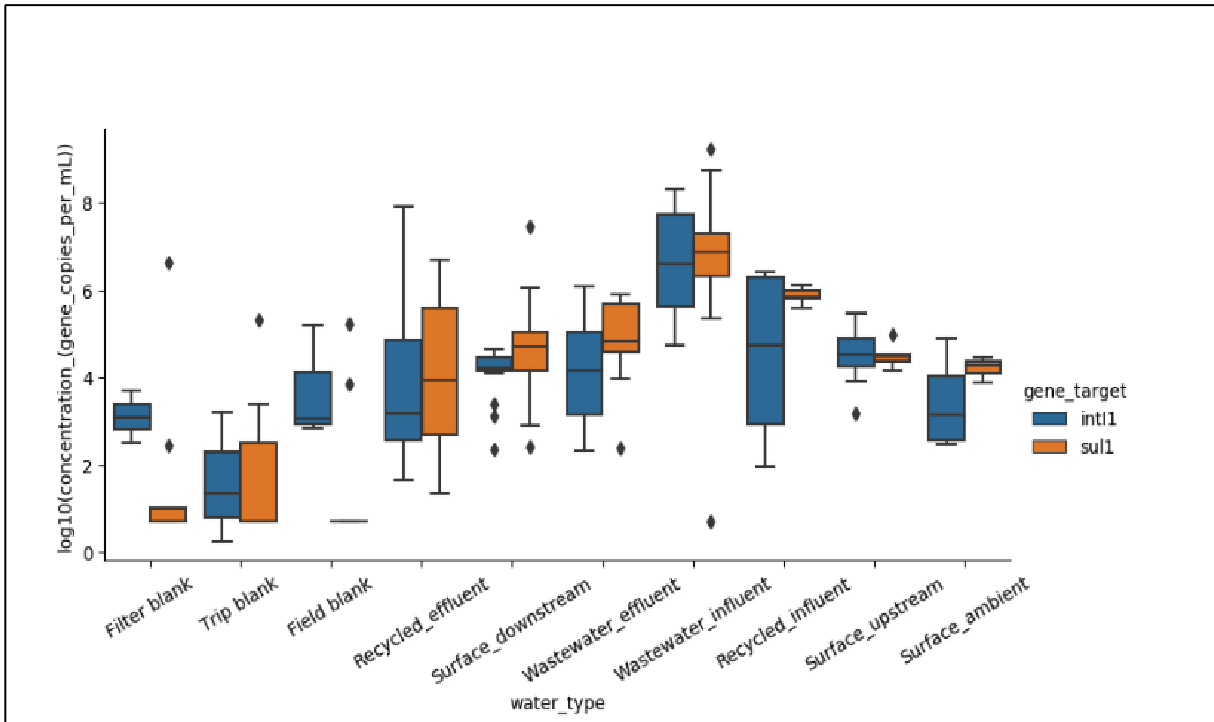


Figure 6-9. WARD qPCR Data Visualization Page.
(Preliminary data used for example only)

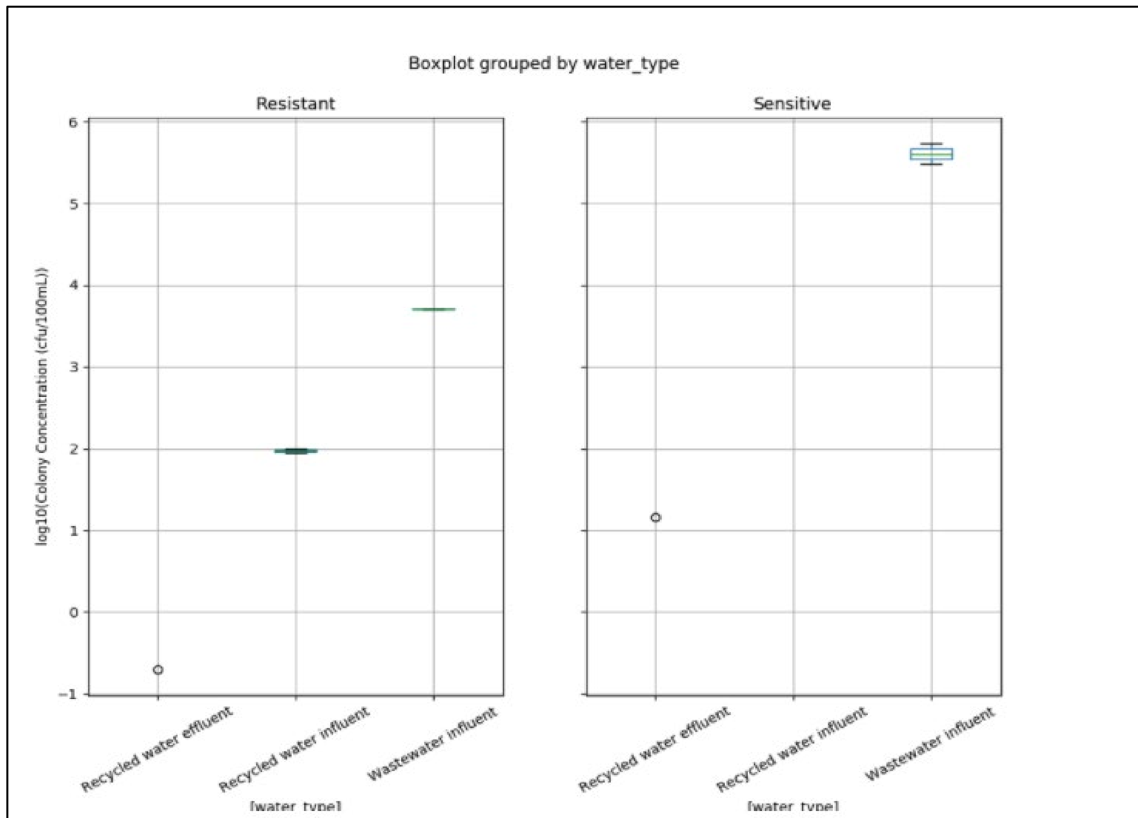


Figure 6-10. WARD Culture Data Visualization Page.
(Preliminary data used for example only)

CHAPTER 7

Conclusion and Next Steps

SOPs for monitoring antibiotic resistance in wastewater, recycled water, and surface water samples were successfully developed and validated. Specifically, the team developed SOPs and QA/QC guidelines for the following targets/methods:

Table 7-1. Overview of the Methods Included in the SOPs and QA/QC Guidelines Developed

Target	Method	Benefits
ESBL *E. coli*	mTEC culture media supplemented with 4 μg/ml cefotaxime (Appendix B2)	-Modified USEPA standard method commonly implemented for regulatory purposes -Comparable to the WHO Tricycle Protocol
*sul*1	SYBR Green qPCR (Pei et al. 2006) (Appendix B3)	-Highly indicative of anthropogenic sources of antibiotic resistance -Readily quantifiable in impacted environments -Commonly associated with multi-antibiotic resistance
*int*I1	TaqMan Probe qPCR (Barraud et al. 2010) (Appendix B3)	-Encodes a mobile genetic element that typically carries multiple ARGs -Indicative of mobile, multi-antibiotic resistance -Highly indicative of anthropogenic sources of antibiotic resistance -Readily quantifiable in impacted environments

These targets and methods were selected through extensive stakeholder input, literature review, and lab validation. Suitability and feasibility for monitoring by US water utilities was a key criterion guiding the selection of the above targets. Capabilities for culturing *E. coli* are nearly universal among U.S. water utilities for regulatory purposes, while qPCR is also becoming a more common tool, especially for viral monitoring. SOPs for wastewater, recycled water, and surface water sampling for the purpose of culture- and qPCR-based monitoring of antibiotic resistance were also developed (Appendix B1) and are expected to be broadly valuable for multiple assays, beyond those described here.

Stakeholders were clear in their recommendation that no one target will capture all dimensions of antibiotic resistance of interest in a given environment. Therefore, it is critical to define the monitoring objectives first before selecting monitoring targets and methods. A key next step of

this research would be to pursue standardization of other targets that were highly recommended based on the literature review, expert survey, and workshop. For example, *Enterococcus* spp. as a Gram-positive target capturing distinct forms of antibiotic resistance of interest. Also opportunistic pathogens with environmental niches for growth, such as *Pseudomonas aeruginosa,* could provide distinct insight into the potential for evolution and spread of antibiotic resistance within environmental matrices.

Experts and stakeholders were especially enthusiastic about the potential of metagenomics as a future monitoring tool, especially for wastewater-based epidemiology purposes. However, they also recognized that there is much work to be done before metagenomics can be standardized as a method for antibiotic resistance monitoring. The literature review and workshop proceedings summarized in this report should provide a very useful step in pursuing the development of SOPs for these other targets.

Going into the future, WARD will be a useful web tool for further discussing, developing, and improving the protocols developed here and those that may follow. WARD will also provide a useful data repository, analysis, and sharing tool to support collaboration in addressing larger research questions related to antibiotic resistance, such as estimated exposure levels and efficacy of water treatment for reducing markers of antibiotic resistance. Users across the community are invited to upload and share their data and metadata via WARD. The more data that is shared, the more robust conclusions will be possible with respect to better understanding antibiotic resistance in water environments. WARD will also be a key forum for feedback and continual improvement of the tool and the SOPs.

APPENDIX A

Expert Survey

A.1 Survey Questions

WRF 5052 Expert Survey on Antimicrobial Monitoring

Survey Flow

Block: Default Question Block (27 Questions)

Page Break

Start of Block: Default Question Block

1 **WRF 5052: Standardizing Methods with QA/QC Standards for Investigating the Occurrence and Removal of Antibiotic Resistant Bacteria/Antibiotic Resistance Genes (ARB/ARGs) in Surface Water, Wastewater, and Recycled Water** PI: Dr. Amy Pruden, Virginia Tech Co-PIs: Dr. Valerie (Jody) Harwood, University of South Florida, Dr. Lenwood Heath, Virginia Tech
The purpose of Water Research Foundation Project 5052 (WRF 5052) is to identify suitable targets for antibiotic resistance monitoring of water, wastewater, and water reuse systems. Targets may include antibiotic resistant bacteria (ARBs) and antibiotic resistance genes (ARGs). The goal of this survey is to poll experts working in academia and in the water industry in terms of current practice and laboratory capacity for ARB/ARG monitoring.

---

2 Name:

____

---

3 Organization:

____

Q26 My organization is located on which continent:

- ○ North America (1)
- ○ South America (2)
- ○ Europe (3)
- ○ Asia (4)
- ○ Africa (5)
- ○ Australia/Oceana (6)

---

4 Title:

____

5 My organization is primarily:

- ○ Academic/University (1)
- ○ Water Utility (2)
- ○ Water Engineering/Consulting (3)
- ○ Commercial Lab (4)
- ○ Other (5) ____

6 My primary role in my organization is:

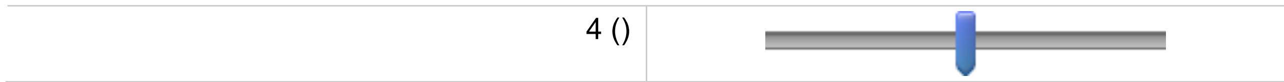
- ○ Academic (PI) (1)
- ○ Post-Doctoral Researcher (2)
- ○ Laboratory Technician (3)
- ○ Manager (4)
- ○ Other (5) ____

Q33 Rank yourself on your level of familiarity with laboratory methods for assessing antibiotic resistance in environmental samples (i.e., non-clinical samples): (rank 1-5 with 1 being low and 5 being high).

For example, basic awareness of literature or generally that environmental sources/reservoirs of antibiotic resistance are a concern would be a "1," while a senior scientist whose primary

expertise is on environmental sources/reservoirs of antibiotic resistance or someone who works regularly in a laboratory using these methods would be a "5".

1 2 3 3 4 5



Q34 Which target environment(s) are the focus of your expertise? (Select as many as relevant, but we anticipate most would select 1-3 environmental matrices **predominantly** studied): Soil, livestock/animals, manure, drinking water, surface water, reuse water, wastewater, human clinical samples

- ▢ Soil (1)
- ▢ Livestock/Animals (2)
- ▢ Manure (3)
- ▢ Drinking water (4)
- ▢ Surface water (5)
- ▢ Reuse/Recycled water (6)
- ▢ Wastewater (7)
- ▢ Human clinical samples (8)
- ▢ Other (9) ____

---

7 Which of the following aquatic environments do you currently monitor, test, or research? (choose all that apply)

- ▢ wastewater (1)
- ▢ surface water (2)
- ▢ drinking water (3)
- ▢ water reuse (4)
- ▢ Other (5) ____

8 Please complete the table below

	Which of these methods are currently implemented by you/your lab/your organization? (4)	Which of these methods do you outsource (i.e., send to external commercial labs) for analysis? (5)	Mark all of the methods for which you judge you have a HIGH level of understanding. (6)	Mark all of the methods for which you judge you have a MODERATE level of understanding. (7)	Mark all of the methods for which you judge you have a LOW level of understanding. (8)	Which of these methods do you judge hold the MOST PROMISE for standardized for antibiotic resistance monitoring for the water industry? (9
PCR (1)	☐	☐	☐	☐	☐	☐
qPCR (2)	☐	☐	☐	☐	☐	☐
ddPCR (3)	☐	☐	☐	☐	☐	☐
IDEXX (4)	☐	☐	☐	☐	☐	☐
metagenomics (Illumina / Nanopore / PacBio sequencing) (5)	☐	☐	☐	☐	☐	☐
qPCR array or microfluidic qPCR (6)	☐	☐	☐	☐	☐	☐
Membrane Filtration (7)	☐	☐	☐	☐	☐	☐
Fecal Coliforms (9)	☐	☐	☐	☐	☐	☐

other (8)	☐	☐	☐	☐	☐	☐

9 **Click and drag** each of the following into order the order of importance for standardizing AMR monitoring in water, with 1 being the most important for AMR testing
_______ Cost (1)
_______ Relevance to Human Health (e.g., assay targets human pathogens or forms of resistance to clinically-important antibiotics) (2)
_______ Meaningful Target for Human Health Risk Assessment (3)
_______ Timeliness of Results (4)
_______ Precedence of Standardization of Similar Methods (5)
_______ Ability to Broadly Capture Potential for Antibiotic Resistance to Spread within a Microbial Community (9)
_______ Abundant Target (i.e., one that is not rare and is likely to be detected and quantifiable) (8)
_______ Quantifiable Target (i.e., rather than a presence/absence-based assay) (6)
_______ Low Technical Skill Requirements (7)

10 How many different assays/targets do you think would be reasonable to recommend for standardization?

◯ Not sure (1)

◯ 1-2 (2)

◯ 3-5 (3)

◯ >5 (4)

Q28 What is an acceptable turnaround time for monitoring AMR targets?

- 6h (2)
- 12 hr (3)
- 1 day (4)
- 3 days (10)
- 1 week (5)
- 1 month (6)
- Turn around time is not a concern (9)

Q32 What cost per sample is reasonable for AMR monitoring in water systems (select the highest applicable value in your opinion)?

- $3 USD/sample (1)
- $10 USD/sample (2)
- $25 USD/sample (3)
- $50 USD/sample (4)
- $100 USD/ sample (5)
- $300 USD/ sample or higher (6)

---

11 Have you ever utilized or are you familiar with culture-based methods for detecting ARBs in water systems?

- ◯ Yes (4)
- ◯ No (5)

Display This Question:

If 11 = Yes

12 Please complete the table below for bacterial groups to target with AMR monitoring

	For which of these targets do you currently have an assay in your lab? (1)	Have you tested for this target in the past 12 months? (2)	If a standard method were available, would you consider testing for this target? (3)	Which of these targets do you think is the BEST for standardized monitoring of antibiotic resistance in water systems (choose 2)? (4)
Acinetobacter baumanii (44)	☐	☐	☐	☐
Enterobacteraciae (45)	☐	☐	☐	☐
Enterococcus/Enterococci (46)	☐	☐	☐	☐
Escherichia coli (47)	☐	☐	☐	☐
Pseudomonas aeruginosa (48)	☐	☐	☐	☐
Aeromonas spp. (49)	☐	☐	☐	☐
Salmonella (50)	☐	☐	☐	☐

13 Have you ever utilized/are you familiar with qPCR-based methods for detecting ARGs in water systems?

○ Yes (1)

○ No (2)

Display This Question:

If 13 = Yes

14 Please complete the table below for genetic determinants to target with AMR monitoring

	For which of these targets do you currently have an assay in your lab? (1)	Have you tested for this target in the past 12 months? (4)	If a standard method were available, would you consider testing for this target? (2)	Which of these targets do you think are the BEST for standardized monitoring of antibiotic resistance in water systems (choose up to 3)? (3)
intI1 (1169)	☐	☐	☐	☐
sulfonamide*sul1 (1170)*	☐	☐	☐	☐
multidrug*aac-(6')-ib-cr* (1171)	☐	☐	☐	☐
beta-lactam*bla(ctx-m) (1172)*	☐	☐	☐	☐
beta-lactam*bla(kpc) (1173)*	☐	☐	☐	☐
beta-lactam*bla(ndm-1) (1174)*	☐	☐	☐	☐
beta-lactam*bla(OXA) (1175)*	☐	☐	☐	☐
beta-lactam*bla(shv) (1176)*	☐	☐	☐	☐
beta-lactam*bla(tem) (1177)*	☐	☐	☐	☐
beta-lactam*bla(vim) (1178)*	☐	☐	☐	☐

beta-lactam*mecA (1179)*	☐	☐	☐	☐
MLS *ermB (1180)*	☐	☐	☐	☐
quinolone*qnrS (1181)*	☐	☐	☐	☐
tetracycline*tetA (1182)*	☐	☐	☐	☐
tetracycline*tetM (1183)*	☐	☐	☐	☐
tetracycline*tetW (1184)*	☐	☐	☐	☐
glycopeptide*vanA (1185)*	☐	☐	☐	☐

Q27 Does your organization have capacity for or are you familiar with qPCR array or microfluidics qPCR techniques?

○ Yes (1)

○ No (2)

Display This Question:

If Q27 = Yes

Q29 Please rank the following attributes with respect to importance for tailoring qPCR array/microfluidics PCR for AMR monitoring:
_______ Total ARG Relative Abundance (e.g., normalized to 16S rRNA rRNA or other housekeeping genes) (1)
_______ Total ARG Absolute Abundance (e.g., ARGs/mL) (2)
_______ Clinically-Relevant ARGs (3)
_______ Total ARG Diversity (e.g., Shannon or Chao Index) (4)
_______ ARGs reported to occur on Mobile Genetic Elements (8)
_______ ARGs reported to occur in Human Pathogens of significant concern (CDC's list) (11)
_______ ARGs reported to occur frequently in water systems (12)
_______ Mobile Genetic Elements (e.g., plasmids, transposons, and integrons) (13)
_______ Other: (10)

Display This Question:

If Q27 = Yes

Q31 Please rank your ideal features of a well-designed qPCR array/microfluidic qPCR assay?"
_______ Rapid (1)
_______ Quantitative (2)
_______ Sensitive (3)
_______ Specific (4)
_______ User-Friendly (5)
_______ Sample Throughput (6)
_______ Reports absolute abundance and not just relative abundance (8)

Display This Question:

If Q27 = Yes

Q30 What do you see as the biggest barrier to adoption of qPCR array/microfluidic qPCR as a standard method for AMR monitoring of water systems?

____

Q21 Does your organization have capacity for or are you familiar with bioinformatic analysis of next generation DNA sequencing data?

◯ Yes (4)

◯ No (5)

Display This Question:

If Q21 = Yes

Q25 Please rank the following with respect to next generation DNA sequencing-derived metrics for AMR monitoring:Rank the following metrics in terms of their suitability for AMR monitoring:
_______ Total ARG Relative Abundance (e.g., normalized to 16S rRNA genes or RPKM) (1)
_______ Total ARG Absolute Abundance (e.g., ARGs/mL) (2)
_______ Clinically-Relevant ARGs (3)
_______ Total ARG Diversity (e.g., Shannon or Chao Index) (4)
_______ ARGs Occurring on Contiguous DNA Strand with Mobile Genetic Elements (5)
_______ ARGs Occurring on Contiguous DNA Strand that is Taxonomically-Classified as Pertaining to a Genus Known to Contain Human Pathogens (6)
_______ Mobile ARGs (e.g., databases specifically tailored to ARGs that are known to be mobile and exclude intrinsic ARGs) (7)
_______ ARGs reported to occur frequently in water systems (11)
_______ Total Mobile Genetic Elements (e.g., plasmids, transposons, and integrons) (8)
_______ Emerging ARGs (i.e., bioinformatically- or functionally-predicted ARGs that have not yet been reported in the clinic) (9)
_______ Other: (10)

---

15 If applicable, please share your thoughts on how you/your organization are/is currently working to expand/improve methods for antibiotic resistance monitoring of water systems.

____

____

____

____

____

16 Any additional comments/ targets that you feel were missed in this survey and should be considered?

____

____

____

____

____

End of Block: Default Question Block

APPENDIX B
Protocols

B.1 Standard Operating Procedure for Water Sample Collection and Shipping Provided to Water Utilities Participating in the Validation Study (Chapter 5)

B.1.1 Sampling Materials List and Protocol, by Water Type

Overview

Table B-1. Schedule Overview for Sampling

Upon Receipt	Day 1 Tuesday	Day 2 Wednesday
(1) Put ice packs in freezer (2) Check materials list against items packaged- notify Krista Liguori (KristaLiguori@vt.edu or 484-744-7044) of missing items.	(1) Conduct sampling and complete protocol (2) Ship packages for overnight delivery to VA Tech and University of South Florida	(1) Samples arrive at Virginia Tech/ University of South Florida

Upon Arrival

- Put ice packs in the freezer.
- Check that all materials are present.

Materials Included in Shipment from VA Tech to WWTPs

- Gloves
- Coolers **(3)**
- Ice Packs **(6-9)**
- Sampling bottles
    - 50 ml falcon tubes **(2)**
    - 250 ml bottles **(8)**
    - 1 L bottles **(25)**
    - 2 L bottles **(15)**
- Paper towels
- Sharpie marker
- Packing tape
- Control bottles (4 H_2O filled)
- Forms:
    - Sample/Bottle Inventory Sheet
    - Metadata Form

Table B-2. Sample Volumes and Number of Replicates to be Collected

Water Type	Sample	50 mL	250 mL	500 mL	1 L	2 L
Wastewater	Influent	2	8	-	-	-
	Effluent	-	-	-	10	-
Reuse/ Recycled	Influent	-	-	-	5	-
	Effluent	-	-	-	4	6
Surface Water	Upstream	-	-	-	2	3
	Downstream	-	-	-	4	6
Controls	Trip Blank	-	-	1	1	-
	Field Blank	-	-	-	2	-

Definitions

PPE Personal protective equipment
L Liter
MGD Million gallons per day

B.1.2 Wastewater

Protocol

- At the sample collection site, put on gloves and any other necessary PPE.

Wastewater Treatment Plant Sampling: Untreated Influent

- Open valve/sample port and allow wastewater to run for 30 seconds to flush.
- First 50 ml bottle: remove the bottle cap- do not touch the inside of the cap or the inside of the bottle. Place the open bottle such that the appropriate sampling port is just inside the bottle, BUT NOT TOUCHING THE INSIDE OF THE BOTTLE. Fill until sample water reaches the top of the square part of the bottle. Do not fill the stem of the bottle. Replace the cap on the bottle. Repeat for remaining 3-50 ml bottles and 6-250 ml bottles.
- Collect wastewater for additional measurements
  - Take measurements for the following parameters using in house equipment. Document the measurements on the provided log sheet.
    - Ammonia
    - Temperature
    - pH
    - Conductivity

        - DO
        - *any additional standard measurements taken at the facility *

Wastewater Treatment Plant Sampling: Final Effluent (after disinfection)

- Open valve/sample port and allow water to run for 30 seconds to flush effluent.
- Remove lid from ***Field* Blank** and leave in proximity while sampling continues. Do not open Trip Blank bottle at all, it should remain sealed until arrival back at the university.
- Take one 1-L bottle: remove the bottle cap- do not touch the inside of the cap or the inside of the bottle. Place the open bottle such that the appropriate sampling port is just inside the bottle, BUT NOT TOUCHING THE INSIDE OF THE BOTTLE. Fill until sample water reaches the top of the square part of the bottle. Do not fill the stem of the bottle. Replace the cap on the bottle. Repeat for remaining 3-1 L bottles and 12-2 L bottles.
- Replace lid on the Field Blank.
- Collect water for additional measurements
    - Take measurements for the following parameters using in house equipment. Document the measurements on the provided log sheet.
        - Chlorine (Total and free)
        - Ammonia
        - Temperature
        - pH
        - turbidity
        - Conductivity

Questionnaire - Metadata Collection

- Separate sheet attached

B.1.3 Reuse/Recycled Water

Protocol

- At the sample collection site, put on gloves and any other necessary PPE.

Recycled Water Sampling: Influent to Tertiary Treatment

- Open valve/sample port and allow water to run for 30 seconds to flush recycled water.
- First 1 L bottle: remove the bottle cap- do not touch the inside of the cap or the inside of the bottle. Place the open bottle such that the appropriate sampling port is just inside the bottle, BUT NOT TOUCHING THE INSIDE OF THE BOTTLE. Fill until sample water reaches the top of the square part of the bottle. Do not fill the stem of the bottle. Replace the cap on the bottle. Repeat for remaining 7-1 L bottles and 24-2 L bottles.
- Collect water for additional measurements
    - Take measurements for the following parameters using in house equipment. Document the measurements on the provided log sheet.
        - Chlorine (Total and free)
        - Ammonia

        - Temperature
        - pH
        - turbidity
        - Conductivity

Recycled Water Sampling: Distributed Water

- Open valve/sample port and allow water to run for 30 seconds to flush water.
- First 2 L bottle: remove the bottle cap- do not touch the inside of the cap or the inside of the bottle. Place the open bottle such that the appropriate sampling port is just inside the bottle, BUT NOT TOUCHING THE INSIDE OF THE BOTTLE. Fill until sample water reaches the top of the square part of the bottle. Do not fill the stem of the bottle. Replace the cap on the bottle.
- Collect water for additional measurements
    - Take measurements for the following parameters using in house equipment. Document the measurements on the provided log sheet.
        - Chlorine (Total and free)
        - Ammonia
        - Temperature
        - pH
        - turbidity
        - Conductivity

Questionnaire - Metadata Collection

- Separate sheet attached

B.1.4 Surface Water

Protocol

- At the sample collection site, put on gloves and any other necessary PPE.

Surface Water Sampling: Upstream and Downstream of Wastewater Effluent Discharge

- Take a sample upstream and downstream of wastewater effluent discharge site
    - The upstream sample can be taken anywhere within a reasonable distance allowing a proper comparison to the downstream sample
    - The downstream sample should be taken as close as reasonably possible after the effluent has mixed with the receiving water (do not sample the effluent directly). Collect GPS coordinates of site locations.
- Collect 1 L from center of flow, ~1ft below surface, making sure to wait for stream/river to clear of resuspended sediment (hip waders may be necessary)
- Keep bottle capped, place under the surface of the flow upstream of your body (to avoid sampling your own flora), then open bottle to take sample and recap
    - Repeat for remaining 1 L bottles and 2 L bottles.
- If river too large to reach center of flow, wade out as far as possible to sample, making sure to sample flowing water

- Place samples on ice for transport back to lab to be shipped as soon as possible
- Collect water for additional measurements from each site
  - Take measurements for the following parameters. Document the measurements on the provided log sheet.
    - GPS coordinates
      - The Compass app on an iPhone will work for this (coordinates are displayed at the bottom of the screen) - If no coordinates are displayed, you will need to go into Settings -> Compass -> allow Compass to access location -> While Using the App. Your current GPS coordinates will then display upon re-opening.
    - Temperature
      - If you have the capacity, take measurements for the following additional parameters and log them.
        - pH
        - turbidity
        - Conductivity
        - River flow rate/capacity

Questionnaire - Metadata Collection

- Separate sheet attached

Shipping

Immediately upon completion of sampling, prepare to ship samples to Virginia Tech and University of South Florida. Ship samples with all included ice packs and ensure all bottles are in cooler. Tape the cooler closed with duct tape or packing tape. Use enclosed shipping label.

- Ship Coolers (2) labeled “VT” OVERNIGHT to Virginia Tech:

  Krista Liguori
  Virginia Tech
  1075 Life Science Circle
  ICTAS II
  Blacksburg VA 24061

- Ship Cooler (1) labeled “USF” OVERNIGHT to University of South Florida:

  Dr. Valerie Harwood
  Univ of South Florida
  4202 East Fowler Avenue
  Dept Integrative Biology
  NES 107
  Tampa, FL 33620

Table B3. Inventory for Each Cooler, Red

Bottle Label	Size	Cooler
WW-Inf-A	250 ml	VT-Red
WW-Inf-B	250 ml	VT-Red
WW-Inf-1	250 ml	VT-Red
WW-Inf-2	250 ml	VT-Red
WW-Inf-3	250 ml	VT-Red
WW-Eff-A	1 L	VT-Red
WW-Eff-B	1 L	VT-Red
WW-Eff-1	1 L	VT-Red
WW-Eff-2	1 L	VT-Red
WW-Eff-3	1 L	VT-Red
R-Inf-1	1 L	VT-Red
R-Inf-2	1 L	VT-Red
R-Inf-3	1 L	VT-Red
R-Eff-1	2 L	VT-Red
R-Eff-2	2 L	VT-Red
R-Eff-3	2 L	VT-Red
Field Blank	1 L	VT-Red
Trip Blank	1 L	VT-Red

Table B4. Inventory for Each Cooler, Blue

Bottle Label	Size	Cooler
R-Inf-A	1 L	VT-Blue
R-Inf-B	1 L	VT-Blue
R-Eff-A	1 L	VT-Blue
R-Eff-B	1 L	VT-Blue
S-Up-A	1 L	VT-Blue
S-Up-B	1 L	VT-Blue
S-Up-1	2 L	VT-Blue
S-Up-2	2 L	VT-Blue
S-Up-3	2 L	VT-Blue
S-Down-A	1 L	VT-Blue
S-Down-B	1 L	VT-Blue
S-Down-1	2 L	VT-Blue
S-Down-2	2 L	VT-Blue
S-Down-3	2 L	VT-Blue

Table B5. Inventory for Each Cooler, Green

Bottle Label	Size	Cooler
WW-Inf-A	50 ml	USF - Green
WW-Inf-B	50 ml	USF - Green
WW-Inf-1	250 ml	USF - Green
WW-Inf-2	250 ml	USF - Green
WW-Inf-3	250 ml	USF - Green
WW-Eff-A	1 L	USF - Green
WW-Eff-B	1 L	USF - Green
WW-Eff-1	1 L	USF - Green
WW-Eff-2	1 L	USF - Green
WW-Efff-3	1 L	USF - Green
R-Eff-A	1 L	USF - Green
R-Eff-B	1 L	USF - Green
R-Eff-1	2 L	USF - Green
R-Eff-2	2 L	USF - Green
R-Eff-3	2 L	USF - Green
S-Down-A	1 L	USF - Green
S-Down-B	1 L	USF - Green
S-Down-1	2 L	USF - Green
S-Down-2	2 L	USF - Green
S-Down-3	2 L	USF - Green
Trip Blank	500 mL	USF - Green
Field Blank	1 L	USF - Green

B.2 Standard Operating Procedure for Culture of Cefotaxime-Resistant *Escherichia coli* from Water Samples

Standard Operating Procedure for the Enumeration of Culturable Antibiotic-Resistant Escherichia coli in Surface Water, Recycled Water, and Wastewater

Overview

This protocol is a comprehensive description of sample collection and processing methods for culture-dependent enumeration of total and cefotaxime-resistant *Escherichia coli* from surface water, recycled water and wastewater by membrane filtration. It includes calculations of *E. coli* concentrations, the proportion of cefotaxime-resistant *E. coli,* and confirmation of *E. coli* to species from isolated colonies. The protocol is adapted from USEPA Method 1603 for *E. coli* (USEPA, 2014) and the WHO Global Tricycle Surveillance (World Health Organization, 2021) of extended spectrum beta lactam (ESBL) resistant *E. coli*. Consistent with the WHO Global Tricycle Surveillance method, the antibiotic selected for this protocol is cefotaxime. The Clinical and Laboratory Standards Institute (CLSI) Performance Standards for Antimicrobial Susceptibility Testing (CLSI, 2017) classifies cefotaxime resistance in *E. coli* as resistance to ≥4 µg/mL cefotaxime. This concentration of cefotaxime is also stipulated in the WHO Global Tricycle Surveillance of ESBL *E. coli*.

Contents

1. Media and Reagent Preparation
2. Sample Collection
    a. Field Sampling Data Sheet
3. Membrane Filtration and Incubation
4. Plate Counting and Data Analysis
    a. Plate Count Data Sheet
5. Confirmation of Species
6. Confirmation of Antimicrobial Resistance
7. Multi-Antibiotic Resistance Profiling (Optional)

B.2.1 Media and Reagent Preparation

For quality control, use commercial media whenever possible. Check expiration date, and do not use media that has hardened.

B.2.1.1 Materials, Equipment, Reagents, Microbiological Agents, and Media

Materials

- Gloves
- 0.2 mL PCR tubes or 2 mL microcentrifuge tubes
- 15 mL Falcon tubes
- 50 mm pre-sterilized polystyrene petri dishes (Hach Product No. 1485299)
- Tape
- Sharpie
- Autoclave tape
- Foil
- pH probe or pH paper (Fisher Scientific Cat No. 50-278-26)

Equipment

- Erlenmeyer flask (150 mL; 250 mL or larger)
- 1 L autoclavable bottles

- Bunsen burner
- Hot plate
- Scale
- Stir bar
- 4°C refrigerator
- -20°C freezer
- Optional: laminar flow workspace

Reagents

- Cefotaxime sodium salt (Millipore Sigma Product No. 219504; Fisher Sci Cat No. AC454950010)
- Reagent grade water
- 1N HCl
- 1N NaOH
- Sodium dihydrogen phosphate (NaH_2PO_4)
- Disodium hydrogen phosphate (Na_2HPO_4)
- 70% ethanol

Control microorganisms

(can be purchased from the American Type Culture Collection (ATCC, 10801 University Blvd, Manassas, VA, 20110-2209; http://www.atcc.org)

- Positive antibiotic-resistant control: *E. coli* NC11 (ESBL-producing) - growth and characteristic colonies on mTEC + cefotaxime
- Antibiotic-sensitive control: *E. coli* ATCC 25922 (non ESBL-producing) - growth and characteristic colonies on mTEC; no growth in presence of cefotaxime
- Negative control: *Enterococcus faecalis* ATCC 19433 - no growth on mTEC

Media

- Modified mTEC agar
- Optional for secondary isolation: Eosin methylene blue (EMB)-Levine agar (Oxoid Cat No. CM0069) or formulated as follows (per 1 L media):
    - 10 g pancreatic digest of gelatin
    - 10 g lactose
    - 2 g dipotassium phosphate
    - 0.4 g eosin Y
    - 65 mg methylene blue
    - 15 g agar
- Optional for secondary isolation: MacConkey agar (Oxoid Cat No. CM0115) or formulated as follows (per 1 L media):
    - 20 g peptone
    - 10 g lacrosse
    - 5 g bile salts

  - 5 g sodium chloride
  - 75 mg neutral red
  - 12 g agar
- Tryptic Soy Agar (TSA) (Oxoid Cat No. CM0131) or formulated as follows (per 1 L media):
  - 15 g casein peptone (pancreatic)
  - 5 g soya peptone (papainic)
  - 5 g sodium chloride
  - 15 g agar
- Mueller-Hinton Agar (Oxoid Cat No. CM0337) or formulated as follows (per 1 L media):
  - 2 g beef infusion solids
  - 17.5 g casein hydrolysate
  - 1.5 g starch
  - 17 g agar

B.2.1.2 Procedures

Preparation

❑ Wipe down workspace with 70% ethanol

Preparing Cefotaxime Antibiotic Stock Solution (50 mg/mL)

1. Add 250 mg of cefotaxime sodium salt to 5 mL of reagent grade water in a 15 mL Falcon tube.
2. Vortex to dissolve.
3. Add antibiotic solution to a syringe and attach 0.22 µm syringe filter.
4. Press antibiotic solution through the syringe filter.
5. Aliquot in 50 µL amounts in PCR tubes.
6. Store at -20°C for up to 1 year. Do not subject to multiple freeze-thaw cycles.

Modified mTEC Agar Preparation (for 20 to 25 plates, 50 mm each)

1. Add 4.56 g of modified mTEC powdered agar to 100 mL of reagent grade water in a 250 mL or larger labeled Erlenmeyer flask. Cover loosely with foil.

a. A flask at least twice the volume of the amount of media being prepared is recommended to avoid loss due to boiling over during heating and autoclaving.

2. Heat on a hot plate with a stir bar until media begins to boil, agar has fully dissolved, and solution is clear.
3. Autoclave at 121°C for 15 minutes.
4. Cool to 45-50°C, preferably by tempering in a water bath.
5. Check pH by using sterile pipet to deliver a drop of media onto pH paper.
6. Adjust pH to 7.3 ± 0.2 with HCl or NaOH if necessary.
7. For control plates without antibiotics:
   a. In a laminar-flow workspace or near a lit Bunsen burner flame, carefully pour ~4 mL of media into each 50 mm plate.
8. For 4 µg/mL cefotaxime antibiotic plates:

   a. In a laminar-flow workspace or near a lit Bunsen burner flame, add 8 µL of antibiotic stock solution to a sterile 150 mL flask with a mark at precisely 100 mL.
   b. Fill flask to 100 mL line with agar tempered to 45-50°C.
      i. If media is too hot, the antibiotic activity will be destroyed.
   c. Gently swirl to mix antibiotic into agar.
   d. Add ~4 mL of media to each 50 mm plate
9. Allow plates to cool to room temperature, sheltered from light to avoid degradation of antibiotic due to light sensitivity, until condensation evaporates.
10. Test media by streaking controls:
    a. Positive antibiotic-resistant control: *E. coli* NC11 (ESBL-producing)
       i. Streak on mTEC + cefotaxime (observe characteristic colony morphology and growth); incubate as detailed below
    b. Antibiotic-sensitive control: *E. coli* 25922 (non ESBL-producing)
       i. Streak on mTEC (observe characteristic colony morphology and growth) and mTEC + cefotaxime (should see no growth); incubate as detailed below
    c. Negative control: *Enterococcus faecalis* ATCC 19433
       i. Streak on mTEC (should see no growth); incubate as detailed below
11. Transfer cooled, uninoculated plates to the package sleeve they were packed in and refrigerate
12. Store plates no longer than one week before use

Phosphate Buffered Saline (PBS)

1. Add 0.58 g sodium dihydrogen phosphate (NaH2PO4), 2.5 g disodium hydrogen phosphate (NA2HPO4), and 8.5g sodium chloride (NaCl) for every 1 L of reagent grade water.
2. Add to 1 L autoclavable bottles.
3. Autoclave after preparation at 121°C for 15 minutes.
4. Final pH should be 7.4 ± 0.2.

Following primary isolation on mTEC, subsequent isolation streaks may be made on a selective-differential media that is less expensive, such as the optional media Eosin-Methylene Blue (EMB)-Levine or MacConkey agar.

EMB-Levine Agar Preparation (for 20 to 25 plates, 50 mm each)

1. Add 3.74 g of EMB-Levine powdered agar to 100 mL of reagent grade water in a 250 mL or larger labeled Erlenmeyer flask. Cover loosely with foil.
   a. A flask at least twice the volume of the amount of media being prepared is recommended to avoid loss due to boiling over during heating and autoclaving.
2. Heat on a hot plate with a stir bar until media begins to boil, agar has fully dissolved, and solution is clear.
3. Autoclave at 121°C for 15 minutes.
4. Cool to 45-50°C, preferably by tempering in a water bath.
5. Check pH by using sterile pipet to deliver a drop of media onto pH paper.
6. Adjust pH to 7.1 ± 0.2 with HCl or NaOH if necessary.

7. For 4 µg/mL cefotaxime antibiotic plates:
   a. In a laminar-flow workspace or near a lit Bunsen burner flame, add 8 µL of antibiotic stock solution to a sterile 150 mL flask with a mark at precisely 100 mL.
   b. Fill flask to 100 mL line with agar tempered to 45-50°C.
      i. If media is too hot, the antibiotic activity will be destroyed.
   c. Gently swirl to mix antibiotic into agar.
   d. Add ~4 mL of media to a 50 mm plate
8. Allow plates to cool to room temperature, sheltered from light to avoid degradation of antibiotic due to light sensitivity, until condensation evaporates.

MacConkey Agar Preparation (for 20 to 25 plates, 50 mm each)

1. Add 5.2 g of MacConkey powdered agar to 100 mL of reagent grade water in a 250 mL or larger labeled Erlenmeyer flask. Cover loosely with foil.
   b. A flask at least twice the volume of the amount of media being prepared is recommended to avoid loss due to boiling over during heating and autoclaving.
2. Heat on a hot plate with a stir bar until media begins to boil, agar has fully dissolved, and solution is clear.
3. Autoclave at 121°C for 15 minutes.
4. Cool to 45-50°C, preferably by tempering in a water bath.
5. Check pH by using sterile pipet to deliver a drop of media onto pH paper.
6. Adjust pH to 7.4 ± 0.2 with HCl or NaOH if necessary.
7. For 4 µg/mL cefotaxime antibiotic plates:
   a. In a laminar-flow workspace or near a lit Bunsen burner flame, add 8 µL of antibiotic stock solution to a sterile 150 mL flask with a mark at precisely 100 mL.
   b. Fill flask to 100 mL line with agar tempered to 45-50°C.
      i. If media is too hot, the antibiotic activity will be destroyed.
   c. Gently swirl to mix antibiotic into agar.
   d. Add ~4 mL of media to a 50 mm plate
8. Allow plates to cool to room temperature, sheltered from light to avoid degradation of antibiotic due to light sensitivity, until condensation evaporates.

Before confirmation of antibiotic resistance, isolates must be streaked onto a general growth media, such as Tryptic Soy Agar (TSA) that also contains cefotaxime. Colonies are then selected from the TSA agar plates and used for Kirby-Bauer disk diffusion (see section 5) on Mueller-Hinton agar plates.

Tryptic Soy Agar (TSA) Preparation (for 20 to 25 plates, 50 mm each)

1. Add 4 g of TSA powdered agar to 100 mL of reagent grade water in a 250 mL or larger labeled Erlenmeyer flask. Cover loosely with foil.
   a. A flask at least twice the volume of the amount of media being prepared is recommended to avoid loss due to boiling over during heating and autoclaving.
2. Heat on a hot plate with a stir bar until media begins to boil, agar has fully dissolved, and solution is clear.

3. Autoclave at 121°C for 15 minutes.
4. Cool to 45-50°C, preferably by tempering in a water bath.
5. Check pH by using sterile pipet to deliver a drop of media onto pH paper.
6. Adjust pH to 7.3 ± 0.2 with HCl or NaOH if necessary.
7. For 4 µg/mL cefotaxime antibiotic plates:
    a. In a laminar-flow workspace or near a lit Bunsen burner flame, add 8 µL of antibiotic stock solution to a sterile 150 mL flask with a mark at precisely 100 mL.
    b. Fill flask to 100 mL line with agar tempered to 45-50°C.
        i. If media is too hot, the antibiotic activity will be destroyed.
    c. Gently swirl to mix antibiotic into agar.
    d. Add ~4 mL of media to a 50 mm plate

Mueller-Hinton Agar Preparation (for 20 to 25 plates, 50 mm each or 4 plates, 100 mm each)

1. Add 3.8 g of Mueller-Hinton powdered agar to 100 mL of reagent grade water in a 250 mL or larger labeled Erlenmeyer flask. Cover loosely with foil.
    a. A flask at least twice the volume of the amount of media being prepared is recommended to avoid loss due to boiling over during heating and autoclaving.
2. Heat on a hot plate with a stir bar until media begins to boil, agar has fully dissolved, and solution is clear.
3. Autoclave at 121°C for 15 minutes.
4. Cool to 45-50°C, preferably by tempering in a water bath.
5. Check pH by using sterile pipet to deliver a drop of media onto pH paper.
6. Adjust pH to 7.3 ± 0.2 with HCl or NaOH if necessary.
7. Add ~4 mL of media to a 50 mm plate or ~25 mL of media to a 100 mm plate.

B.2.2 Sample Collection

B.2.2.1 Materials, Equipment, and Reagents

Field Blank Control Samples

- Autoclavable carboys (3) (ThermoFisher Cat No. 2250-0050)
- Tape/labels and sharpie
- Gloves
- Molecular grade DNA-free water (if accessible, otherwise ultrapure water can be autoclaved)

Wastewater Influent

- 50 mL Falcon tubes (1 per sample)
- Tape/labels and sharpie
- Gloves
- Biohazard bag
- Hand sanitizer
- Sample pole (optional)
- 5-gallon bucket (optional)
- Cooler with ice packs (large enough to fit all sampling containers)
- Multiparameter water quality meter (optional)
- 70% ethanol

Wastewater Effluent and Recycled Water – Grab samples

- Autoclavable carboy (ThermoFisher Cat No. 2250-0050)
- Tape/labels and sharpie
- Gloves
- Biohazard bag
- Hand sanitizer
- Sampling pole (optional)
- 5-gallon bucket (optional)
- Cooler with ice packs (large enough to fit all sampling containers)
- Multiparameter water quality meter (optional)
- 70% ethanol
- 0.5 mg/L sodium thiosulfate

Surface Water

- 2 L collection bottles (1 per sample)
- Tape/labels and sharpie
- Gloves
- Biohazard bag
- Hand sanitizer
- Sample pole or waders (optional)
- 5-gallon bucket (optional)

- Cooler with ice packs (large enough to fit all sampling containers)
- Multiparameter water quality meter (optional)
- 70% ethanol

Wastewater Effluent and Recycled Water – Hollow Fiber Filtration

- Autoclavable carboy (ThermoFisher Cat No. 2250-0050)
- Tape/labels and sharpie
- Gloves
- Biohazard bag
- Hand sanitizer
- Rexeed 25-S hollow fiber filters (1 per sample)
- Innovaprep high volume elution fluid can – Tris (1 per sample)
- Innovaprep LVC can interface
- Sampling pole (optional)
- 5-gallon bucket (optional)
- Cooler with ice packs (large enough to fit all sampling containers)
- Multiparameter water quality meter (optional)
- 70% ethanol
- 0.5 mg/L Sodium thiosulfate

B.2.2.2 Preparations

❑ Place labels on each sampling bottle with location/date/initials
❑ Sterilize sampling bottles by autoclaving
❑ Prepare mTEC agar with and without cefotaxime (see section B.2.1); label plates with sampling site, volume filtered, and dilution

B.2.2.3 Procedures

Water samples should be processed within 24-30 hours of sampling to avoid decay of culturable bacteria. The appropriate number of personnel should be available in the laboratory to process samples quickly. Optimally, filters should be placed on modified mTEC agar within 2-3 hours of receipt in the lab.
Go to sampling procedure 1-4 depending on sample types to be collected (wastewater, recycled water, or surface water):

Sampling Procedure

1. Wastewater influent and effluent sampling
    a. Put on gloves before beginning.
    b. Collect appropriate volumes from each wastewater treatment stage. In general, less sample can be filtered from the less processed stages (e.g. influent), and less is needed to obtain countable plates (see Table B-6).
        i. Use a sampling cup attached to a long pole to pour wastewater into collection vessels, or dip the vessels directly into the wastewater.

ii. If sampling multiple sites using the same sampling cup, disinfect between sites using 70% ethanol and rinse the sampling cup with water from the new site before collection.

c. For sites that follow chlorine/chloramine disinfection, quench using 480 µL of 0.5 mg/L sodium thiosulfate per 10L of sample.

d. Take physiochemical measurements on site with multi-parameter water quality meter and bucket.

   i. If unable to collect measurements on site, collect a sample of water into an appropriate container size to complete analyses in lab.

e. Cap bottles, check labels are correct, disinfect the outside of the bottle with ethanol, and store in cooler on ice.

f. Remove used gloves and discard in biohazard bag.

g. Fill out sampling metadata sheet with metadata and sample-specific measurements (see Table B-7 field sampling metadata sheet).

2. Recycled water sampling – grab sampling
   a. Put on gloves before beginning.
   b. Collect the target volume of treated wastewater intended for reuse in sterile bottles or autoclavable carboys (see Table B-6).
      i. Use a sampling cup attached to a long pole to pour recycled water into collection vessels, or dip the vessels directly into the recycled water.
      ii. If sampling multiple sites using the same sampling cup, disinfect between sites using 70% ethanol and rinse the sampling cup with water from the new site before collection.
   c. For sites that follow chlorine/chloramine disinfection, quench using 480 µL of 0.5 mg/L sodium thiosulfate per 10L of sample.
   d. Take physiochemical measurements on site with multi-parameter water quality meter and bucket.
      i. If unable to collect measurements on site, collect a sample of water into an appropriate container size to complete analyses in lab.
   e. Cap bottles, check that labels are correct, disinfect the outside of the bottle with ethanol, and store in cooler on ice.
   f. Remove used gloves and discard in biohazard bag.
   g. Fill out sampling metadata sheet with metadata and sample-specific measurements (see section 4 for sampling metadata sheet).
3. Surface water sampling
   a. Put on gloves before beginning.
   b. Collect appropriate volume depending on type of surface water being sampled (see Table B-6).
      i. If sampling multiple sites, work from downstream to upstream, to avoid disruption of downstream sampling sites.

ii. Either by wading or using a pole, collect from as close to center of water body as feasible from beneath the surface (10-30 cm depth).
iii. Be careful not to disrupt sediment and sample the water upstream of your standing position to avoid sampling your own or sediment flora.
iv. If sampling multiple sites using the same sampling cup, disinfect between sites using 70% ethanol and rinse the sampling cup with water from the new site before collection.

c. Take physiochemical measurements on site with multi-parameter water quality meter and bucket.
i. If sampling multiple sites using the same bucket, rinse bucket with water from the new site before collection.
ii. If unable to collect measurements on site, collect a sample of water into an appropriate container size to complete analyses in lab.

d. Cap bottles tightly, check labels are correct, and store in cooler on ice.
e. Remove used gloves and discard in biohazard bag.
f. Fill out sampling metadata sheet with metadata and sample-specific measurements (see section 4 for sampling metadata sheet).

4. Treated effluent and recycled water – hollow fiber filtration.
a. Put on gloves before beginning.
b. Collect 50L of treated wastewater effluent or recycled water in carboys
i. Use a sampling cup attached to a long pole to pour recycled water into collection vessels, or dip the vessels directly into the recycled water.

c. For sites following chlorine disinfection, quench using 480 µL of 0.5 mg/L sodium thiosulfate per 10L of sample.
d. Use a peristaltic pump and attach sterile tubing to the hollow fiber filter.
i. The blue port should be connected to the tubing running through the peristaltic pump.

e. Remove to port on the side of the hollow fiber filter near the orange end (but not located on the orange color).
f. Screw the port on the orange end securely closed.
g. Place the tubing into the carboy containing the sample and turn on the peristaltic pump.
h. Filter 50L of sample through the hollow fiber filter.
i. Once filtered, remove the tubing and place the hollow fiber filter back into its packaging and store in a cooler on ice until elution.
j. For multiple samples, fill the tubes with 70% ethanol between samples and hold the ethanol in the tubes for a minimum of 60 seconds.
k. Repeat the process for additional samples.
l. To elute samples from the hollow fiber filter, remove the cap on the orange and blue ports of the hollow fiber filter.
m. Attach the LVC can interface to the orange port.
n. Place a labeled 500mL collection container underneath the blue port.

o. Push the elution canister into the LVC can interface and hold firmly until sample is no longer eluting from the blue port.
p. Cap the collection container, disinfect the outside with ethanol, and store in a cooler until processing.
q. Remove used gloves and discard in biohazard bag.
r. Fill out sampling metadata sheet with metadata and sample-specific measurements (see section 4 for sampling metadata sheet).

Table B-6. Suggested Grab Sample Volumes and Filtration Amounts for *E. Coli* and Cefotaxime-Resistant *E. Coli* Based on Water Matrix Being Sampled.

Water Matrix	Sample Volume to Collect	Target	Filtration Amounts/Dilutions
Raw Sewage (Influent)	50 mL	*E. coli*	1 mL at 10^{-2}, 10^{-3}, 10^{-4}
		Cefotaxime-resistant *E. coli*	1 mL at 10^{-1}, 10^{-2}, 10^{-3}
Treated Sewage (Effluent) & Recycled Water	20 L	*E. coli*	2 L or more undiluted
		Cefotaxime-resistant *E. coli*	2 L or more undiluted
Surface Water Polluted	2L	*E. coli*	1, 10, 100 mL
		Cefotaxime-resistant *E. coli*	10, 100, 250 mL
Surface Water Clean	2L	*E. coli*	1, 10, 100, 250 mL
		Cefotaxime-resistant *E. coli*	500 mL
Marine Water	2L	*E. coli*	1, 10, 100 mL
		Cefotaxime-resistant *E. coli*	500 mL

Table B-7. Field Sampling Metadata Sheet

Project Name:
Sampling Date:
Personnel:

Sampling Site	Sample Type	Sample Volume	Chlorine Quench? (Y/N)	Time	Temp (°C)	Turbidity	Salinity (ppt)	Dissolved Oxygen (mg/L)	pH	Comments (wet weather, dry weather, tides, etc.)

Time Received in Lab:
Received By: Processing Time End:

B.2.3 Membrane Filtration and Incubation

B.2.3.1 Materials, Equipment, and Reagents

Materials

- Filter membranes, 47 mm, 0.45 µm. Either filter material below is permitted.
  - Nitrocellulose: Fisher Cat No. 09-719-555
  - Mixed-cellulose ester: Fisher Cat No. HAWP04700

Equipment

- Incubator
- Water bath
- Vacuum pump or other vacuum source
- Filter cups/holders (sterile) – 1 per site preferred
  - VWR Cat No. 28199-440
  - Pall Product ID 4238
- Large Erlenmeyer flask (e.g. 2 L) with desiccant
- Bunsen burner/ethanol flame
- Sterile graduated tubes (for serial dilutions)
- Vortex mixer
- Autoclavable squirt bottle
- Gallon sealable plastic bags
- Large clear bag(s) designated for water immersion

Reagents

- PBS
- 70% ethanol

B.2.3.2 Procedures

Ensure Samples are Valid for Analysis

Upon receipt of samples, QA/QC procedures shall be enforced before analysis of samples. Coolers should arrive within 48 hours of the time of sampling, sample bottles should be intact (without holes, cracks, or leaks), coolers should arrive below room temperature (20°C). If any of the above criteria are not met, the sample has failed QA/QC and should be removed from processing.

Replication

Samples are processed in two technical replicates for each dilution of each unique biological sample. For example, two different sample bottles are used to collect wastewater influent, designated 'wastewater influent A' and 'wastewater influent B' (biological duplicates). In each biological duplicate, three different target dilutions are filtered onto membranes in technical replicate. For example, 'wastewater influent A' would have two filters at dilution 1, two filters at dilution 2, and two filters at dilution 3 for each media.

Serial Dilutions

It is recommended that a minimum of three dilutions be analyzed per sample to ensure countable plates (10-100 colonies per membrane). See Table B-6 for recommendations. At least

two, and preferably three technical replicate plates per dilution should be prepared. An example of serial dilution procedure is shown in Figure 1.

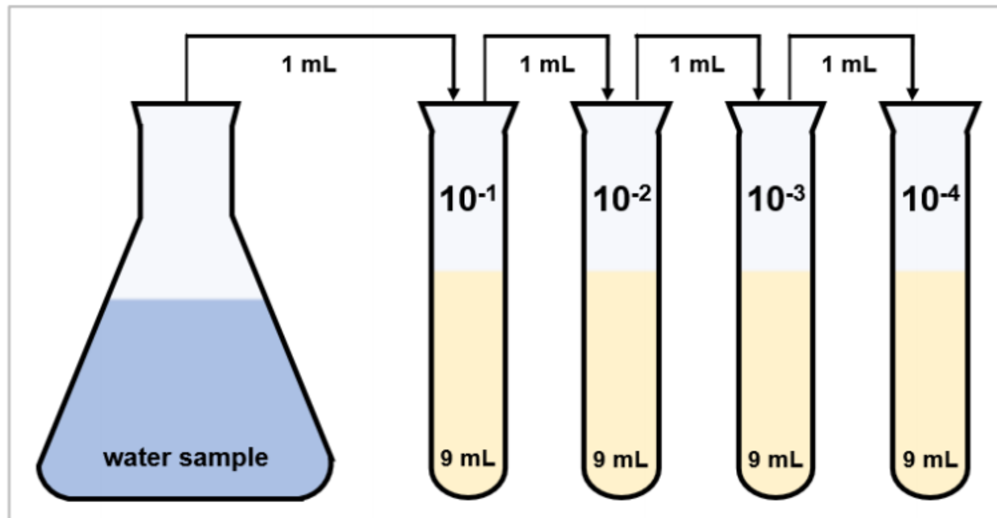


Figure B-1. Example Procedure for Serial Dilutions.
Yellowish volume represents sterile PBS.

Before Starting

- At least 24 hours ahead of time, set incubator to 35°C and equilibrate
- Wipe down workspace with 70% ethanol
- Assemble sterile filter cups/holders and attach them to flask or vacuum manifold (Figure B-2)
- Prepare a small beaker of 95% ethanol for sterilizing forceps (fill ~2 cm depth)
- Light Bunsen burner or ethanol flame
- Have all reagents prepared and at room temperature
- If filter funnels are to be used for more than one sample, filter the samples that are likely to contain the least bacteria first (e.g. filter effluent first, influent last)
- Make serial dilutions in PBS appropriate for the sample matrix (Table B-6)
- Set water bath to 44.5°C

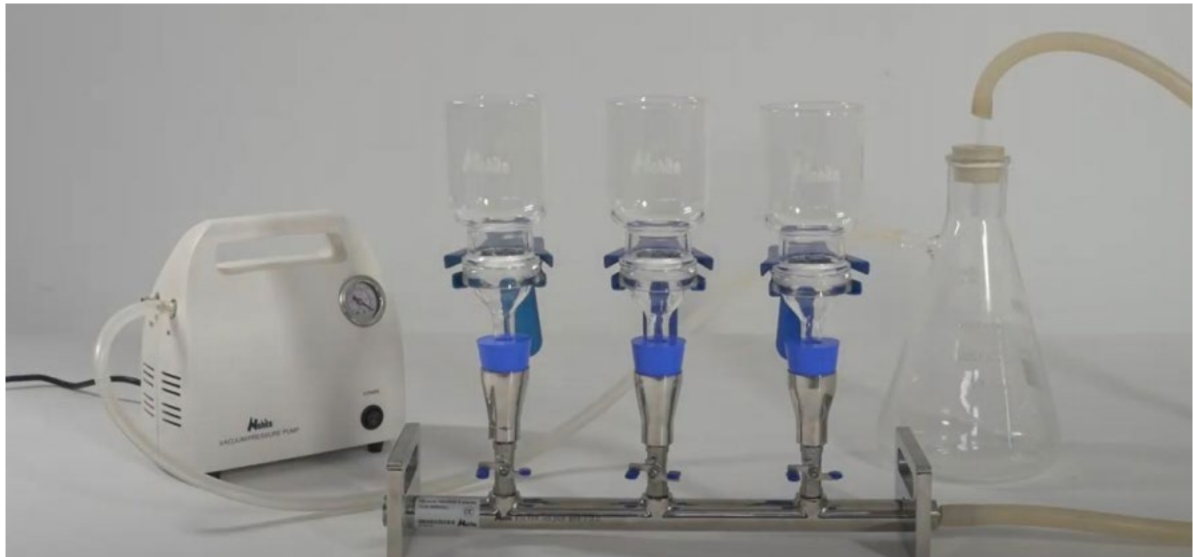


Figure B-2. Example Vacuum Manifold and Filtration Setup with Vacuum Pump.

Membrane Filtration Procedure

1. Flame sterilize forceps (wait a few seconds after the flame goes out) and place a single 0.45 µm membrane in each filter cup, grid side up, and secure.
2. With the vacuum pump off, pour water sample into filter cup, then turn on vacuum filter
   a. For samples with debris (e.g. sand) or visible turbidity, allow the sample to settle for 2 minutes and decant sample off the top for use.
   b. When filtering a small sample volume (e.g. 1 mL or less), with the vacuum off, add 30 mL of PBS and then the sample before applying a vacuum. This will ensure that cells are evenly distributed on the membrane.
3. Rinse sides of filter cup with PBS in autoclavable squirt bottle, then turn vacuum filter off.
4. Re-sterilize forceps, remove membrane from holder cups, and place membrane onto agar plate, being careful not to touch any other surface (side facing up while filtering is placed facing up on agar plate).
   a. Avoid bubbles between membrane and agar surface.
   b. Reset membrane if bubbles occur.
   c. Use the forceps to press along edges of membrane to ensure the membrane is properly seated.
5. Close Petri dish, invert.
6. If filtering more than one sample per filter cup, after all dilutions from a given sample have been plated, rinse the filter cup with PBS (volume equivalent to the highest volume of sample filtered). Then process the next sample.
7. After the last sample has been processed, rinse the cup with PBS as above. Then, filter 100 mL of PBS through a fresh membrane and place it on a mTEC plate. This is the METHOD BLANK and will be incubated alongside samples.
8. Wrap sample, control, and blank plates together in saran wrap, place in a gallon sealable plastic bag, then in a larger clear plastic bag designed for immersion (e.g. Ziploc). Ensure that the bag is securely closed.

9. After 2 hours at 35°C ± 0.5°C, transfer sample, control, and blank plates to a water bath set at 44.5°C ± 0.2°C for 22 ± 2 hours. Incubate all plates upside down
   a. Tape the opening edge of the large clear plastic bag against the outside of the water bath to prevent any water from getting inside. The opening edge should be above the water line, but all samples must be fully submerged.
   b. Weigh down the bag to ensure full submersion in the water bath ("donuts" for flasks work well for this job).

B.2.4 Plate Counting and Data Analysis

After incubation, determine the dilution of each sample that will provide appropriate colony forming units (CFUs) in a countable range (aim for 10 – 200 colonies per plate). Count only one dilution. Plates with less than 10 colonies can be counted if no other plates present a countable number. Plates with colonies above the countable limit are labeled as 'too numerous to count' (TNTC). Count and record the number of purple/magenta colonies on each membrane. Count colonies on mTEC agar plates with and without cefotaxime and mark the colonies on the outside of the plate with a black or blue pen or fine-tipped sharpie. Take pictures of the plates with countable colonies for future reference. An example of typical colony morphology is illustrated in Figure B-3.

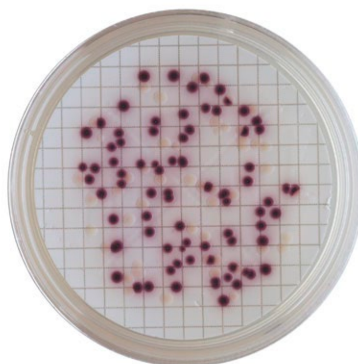


Figure B-3. An mTEC plate with typical *E. coli* morphologies, in magenta

$$\frac{E.coli}{100\ mL} = \frac{Average\ E.coli\ colonies\ at\ countable\ dilution}{Volume\ of\ sample\ filtered\ (mL)} x\ 100$$

Calculation of CFU/100 ml: Make sure to account for the number of dilutions represented by each individual plate when calculating the representative volume. For example, if 1 mL of a 1:10 dilution was filtered, the volume of the sample filtered is 0.1mL. Add up all raw colony counts for each dilution for a single sample and divide that number by the total undiluted sample volumes they represent per counted plate (including plates with zeros). For example, if a sample filtered at 10-2 has 10, 13, and 16 colonies, the average number of *E. coli* colonies at the countable dilution would be 13. The volume of the sample filtered in mL would be 0.1 mL, giving a calculated CFU/100mL of 13,000 or 1.3x104. Record data as CFU/100mL in the incubation and plate count data sheet in Section B.2.7.

To calculate the proportion of *E. coli* that are resistant to cefotaxime, divide the total number of cefotaxime-resistant *E. coli* for each sample by the total number of presumptive generic *E. coli* for each sample. Do not use logarithmic values for this calculation. Record % cefotaxime-resistant *E. coli* data in the incubation and plate count data sheet (Table B-8).

$$\%\ Cefotaxime - resistant\ E.coli = \frac{\frac{CFU\ of\ Cefotaxime-resistant\ E.coli}{100mL}}{\frac{CFU\ of\ total\ E.coli}{100\ mL}}\ x\ 100.$$

Table B-8. Incubation and Plate Count Data Sheet (Excel)

Date and time processing began:

Date and time into incubator at 35°C ± 0.5°C:

Date and time into water bath at 44.5°C ± 0.2°C:

Date and time plates removed from water bath for counting:

Site	Rep	mTEC without antibiotics				mTEC with antibiotics				Cefotaxime-resistant (%)
		Dilution 1:	Dilution 2:	Dilution 3:	CFU/100mL	Dilution 1:	Dilution 2:	Dilution 3:	CFU/100mL	
	1									
	2									
	1									
	2									
	1									
	2									
	1									
	2									
	1									
	2									
	1									
	2									

For dilution columns, enter the dilution at the top of the row and number of colonies observed at each dilution for each replicate and circle or highlight the selected group used for CFU/100mL calculations. It is recommended that a different plate count data sheet be used when dilution volumes are changing between sites. Plates with colonies above the countable limit are labeled as 'too numerous to count' (TNTC); Plates with no colonies are below detection limit.

B.2.5 Confirmation of *E. coli* Species

Confirmation to species is necessary when culturing from environmental samples. Many media are optimized for a clinical setting, where the bacterial population is less diverse and results in fewer non-target bacteria phenotypically resembling the target species on selective-differential media. Prior to conducting sampling events, it is recommended that the mTEC method be tested within the facility. Several isolates should be confirmed on mTEC and mTEC + cefotaxime per sample type to be tested and at least 90% confirmation should be consistently achieved. Data that are collected with the intention for publication should also be confirmed throughout the project. Five colonies should be confirmed from each media (mTEC and mTEC + cefotaxime) per sample type for each sampling event. This SOP details a method using real-time PCR of the *uidA* gene. Note that this method can also be carried out using conventional PCR on agarose gels.

B.2.5.1 Materials, Equipment, and Reagents

Materials

- 2 mL microcentrifuge tubes
- 0.2 µL PCR tubes

Equipment

- 250 mL Erlenmeyer flask
- Real-time thermocycler
- 4°C refrigerator
- -20°C freezer
- Gel electrophoresis chamber
- UV lamp or UV imager
- Centrifuge
- PCR hood
- Template-addition hood
- Microwave or hot plate

Reagents

- Agarose (ThermoFisher Cat. No. 17850)
- 50x TAE buffer (ThermoFisher Cat. No. B49)
- Go Taq Green Environmental Master Mix (Promega Cat. No. M7122 or M7123) – or comparable alternative
- Nuclease-free water (IDT Cat. No. 11-05-01-14)
- Ethidium bromide (concentration 10 mg/mL) – or comparable alternative
- 50 bp DNA ladder (ThermoFisher Cat. No. SM0371)
- uidA405 forward primer
- uidA405 reverse primer

B.2.5.2 Procedures

Ordering and Stock Preparation

1. Order primer and probes from https://www.idtdna.com (Table 2).
2. Upon arrival, hydrate at aliquot primer and probes.
   a. Primers should be hydrated according to the DNA concentration of the lyophilized pellet to create 100 µM master stocks and aliquoted 1:10 in nuclease free water to create 200 µL working stocks at 10 µM concentration.
3. Store master stock and working stocks at -20°C. Do not subject to multiple freeze-thaw cycles.

Table B-9. Primers for *E. coli* confirmation PCR assay.

Assay Name	Target Species and Gene	Sequences (5' to 3')	Reference
uidA405	*Escherichia coli uid*A gene (β-glucuronidase)	uidA405 Forward primer: CAACGAACTGAACTGGCAGA	(modified from Chern et al., 2009)*
		uidA405 Reverse primer: CATTACGCTGCGATGGAT	

*The probe from the Chern et al., 2009 assay was omitted to create a more versatile assay that can be run as both a SYBR green assay on a real time thermocycler or using a conventional thermocycler and agarose gel. The specificity of the assay was checked in silico and determined to be adequate for confirmation of colonies that already resembled *E. coli* on the selective-differential media. This modification is NOT recommended for quantification of *E. coli* in environmental samples.

Isolation of Colonies

1. Using a sharpie, draw quadrants onto an mTEC, MacConkey, or EMB plate.
2. Using a flame-sterilized loop, needle, or sterile pipette tip, touch a single colony on the membrane filter of the correct morphology.
3. Streak for isolation on a mTEC, MacConkey, or EMB plate amended with cefotaxime unless for total *E. coli*).
   a. Begin by zig-zagging with the loop, needle, or pipette tip in quadrant one.
   b. Re-sterilize the loop or needle or discard the pipette tip and get a new pipette tip.
   c. Drag the loop, needle or pipette tip once through the first quadrant and begin zig-zagging through quadrant two.
   d. Repeat steps b and c, dragging through quadrant two into quadrant three and from quadrant three into quadrant four.
4. Repeat for each colony to be isolated for confirmation testing.
5. Incubate plates at 35°C for 22-24 hours.

DNA Extraction Procedure

1. Add 50 µL of nuclease-free water to PCR tubes, one per sample to be extracted.
2. Using a sterile toothpick, pipette tip, or metal loop, pick one isolated colony from a pure culture and add to a tube of 50 µL nuclease-free water.
3. Vortex at high speed for one minute.

4. Repeat for all samples to be extracted (up to 90 at a time) and the positive *E. coli* control.
5. Add tubes to a thermocycler and run at 95°C for 15 minutes.
6. Freeze DNA template at -20°C for up to 24 hours or use immediately in qPCR reaction.

PCR Procedure

1. Turn on UV lamp in PCR and template-loading hoods and allow exposure for at least 15 minutes.
2. Put on gloves.
3. Place PCR strip tubes in support base. Place plate and 2 mL microcentrifuge tube in hood under UV to prevent contamination.
4. Remove PCR reagents (and samples if necessary) from -20°C freezer and allow to thaw
   a. SYBR Green qPCR Master Mix should be stored at -20°C until first use. After one freeze-thaw cycle, store in refrigerator. If already using thawed Go Taq Green Master Mix, do not remove from refrigerator at this point.
5. As reagents thaw, move to cold block or on ice. Once samples are thawed, place in refrigerator. If samples will be used for repeated runs within five days, keep in refrigerator.
6. Prepare a balance plate for centrifuge by adding 25 µL of water to each well being used on a blank plate (do not cover, may be reused).
7. Calculate master mix for all reactions (include two extra reactions to account for pipette error)
   a. 12.5 µL SYBR Green qPCR Master Mix
   b. 1 µL forward primer
   c. 1 µL reverse primer
   d. 5.5 µL nuclease-free water
8. Change gloves.
9. Move cold block with reagents and SYBR Green qPCR Master Mix into PCR hood after turning of UV.
10. Assemble master mix in 2 mL microcentrifuge tube. Vortex briefly to mix.
11. Load 20 µL in each well being used.
12. Transfer to qPCR plate to template-loading hood. Move template DNA extracted from samples and positive control (see DNA extraction procedure) from refrigerator to template-loading hood.
13. Add 5 µL of template to appropriate wells.
    a. In three tubes, add 5 µL of nuclease-free water in place of the sample template. This is your no template control.
    b. In three tubes, add 5 µL of positive control DNA extract in place of the sample template. This is your positive control.
14. Seal the plate and transfer to centrifuge. Centrifuge at 1000 RPM for 1 minute.
15. Place plate in qPCR thermocycler and begin run:
    a. 50°C for 2 min
    b. Forty PCR Cycles of:
       i. 95°C for 15s

    ii. 60°C for 1 min
    iii. 95°C for 10 min
  c. Melt curve: 60°C to 95°C in 0.5°C increments

16. For each sample, check amplification and melt temperature against the positive control to determine whether the sample is positive or negative for *E. coli* species confirmation.

B.2.6 Confirmation of Antibiotic Resistance

Colonies whose phylogeny is confirmed should also be confirmed for antibiotic resistance. This can be accomplished by re-streaking on Mueller-Hinton agar containing the selection antibiotic, by Kirby-Bauer disk diffusion, or by assessing the minimum inhibitory concentration of antibiotic (CLSI, 2017; Hudzicki, 2012; Matuschek et al., 2014). The diameter of the zone of inhibition should be recorded and intermediate versus full resistance denoted for both CLSI and EUCAST standards. Additional antibiotics to be used in multi-antibiotic profiling (optional) can be found in Section B.2.7.

B.2.6.1 Materials, Equipment, and Reagents

Materials

- 30 µg cefotaxime antibiotic disks (1 per sample)
- Mueller-Hinton agar plates (see section 1)
- TSA agar plates (see section 1)
- 2 mL microcentrifuge tubes (1 per sample)
- Sterile swab (1 per sample)

Equipment

- 0.5 MacFarland standard or means of checking absorbance (e.g. Nanodrop)
- Sterilized loop, needle, or toothpick
- Incubator set at 35°C

Reagents

- Sterile saline

B.2.6.2 Procedures

Kirby-Bauer Disk Diffusion Procedure

1. The day before performing a Kirby Bauer test, streak the isolate out onto a TSA plate amended with the selection antibiotic, if applicable.
2. Warm Mueller-Hinton plates (1 per isolate tested) to room temperature.
3. Fill a 2 mL microcentrifuge tube with ~1mL of sterile saline.
4. From the TSA plate, touch four or five isolated colonies with a loop, needle, or toothpick and inoculate the sterile saline.
5. Vortex to create a smooth suspension, around 0.5 MacFarland standard.
6. The suspension absorbance can be checked by Nanodrop or other means until you feel comfortable with the amount you should add – the absorbance at 625 nm should be 0.08 to 0.13; dilute or add more bacteria to reach this range.

7. Use this suspension within 15 minutes of preparation.
8. Dip a sterile swab into the inoculum tube.
9. Rotate the swab against the side of the tube (above the fluid level) using firm pressure, to remove excess fluid.
10. Inoculate the dried surface of a Mueller-Hinton plate by streaking the swab three times over the entire agar surface; rotate the plate approximately 60 degrees each time to ensure an even distribution of the inoculum.
11. Rim the plate with the swab to pick up any excess liquid.
12. Leaving the lid slightly ajar, allow the plate to sit at room temperature for 3 to 5 minutes to dry.
13. After drying, place the cefotaxime antibiotic disk (and other disks if testing for multi-drug resistance, see Section B.2.7) on the surface of the agar, using forceps to place the disk in the center of the plate; apply gentle pressure to ensure complete contact.
    a. Between plates, flame sterilize the forceps by dipping them in ethanol and running it through a flame.
    b. If testing multiple antimicrobials (see Section B.2.7), a disk dispenser can be used to place the antibiotic disks. Use forceps to apply gently pressure to each disk to ensure complete contact.
14. Once the disk is in place, replace the lid, invert the plates, and incubate them at 35°C for 24 hours.
15. Following incubation, measure the zone of inhibition diameter for each disk to the nearest millimeter (round up).
16. Record diameter and compare to both CLSI and EUCAST guidelines, report susceptible, full resistance or intermediate resistance for both guidelines.

B.2.7 Multi-Antibiotic Resistance Profiling (Optional)

If profiling *E. coli* isolates for resistance to multiple antibiotics, a 100 mm Mueller-Hinton agar plate should be used. It is not recommended to use a 50 mm plate for more than one antibiotic disk. A disk dispenser can be used to simultaneously deliver multiple antibiotic disks to each agar plate. The diameter of the zone of inhibition should be recorded and intermediate versus full resistance denoted for both CLSI and EUCAST standards.

B.2.7.1 Materials, Equipment, and Reagents

Materials

- 10 µg gentamicin antibiotic disks (1 per sample)
- 200 µg fosfomycin antibiotic disks (1 per sample)
- 20/10 µg amoxicillin-clavulanate antibiotic disks (1 per sample)
- 10 µg imipenem disks (1 per sample)
- 1.25/23.75 µg trimethoprim-sulfamethoxazole disks (1 per sample)
- 30 µg nalidixic acid disks (1 per sample)
- 30 µg tetracycline antibiotic disks (1 per sample)
- Mueller-Hinton agar plates (see section 1)

- TSA agar plates (see section 1)
- 2 mL microcentrifuge tubes (1 per sample)
- Sterile swab (1 per sample)

Equipment
- 0.5 MacFarland standard or means of checking absorbance (e.g. Nanodrop)
- Sterilized loop, needle, or toothpick
- Incubator set at 35°C

Reagents
- Sterile saline

Procedures
Refer to section B.2.6.2 for Kirby-Bauer disk diffusion procedure.

B.3 qPCR Standard Operating Procedure

Standard Operating Procedures for The Detection and Quantification of Antibiotic Resistance Genes (ARGs) from Water Using Quantitative Polymerase Chain Reaction (qPCR)

Table of Contents

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  B. qPCR Template Example
  C. qPCR Thermocycler Protocol and Plate Setup Examples
  D. Troubleshooting qPCR using ROX Passive Reference Dye
  E. gBlock Creation

B.3.1 Definitions

Biological replicate: Replicate to ensure the natural variability of the sample matrix is captures (i.e., three 1L samples were collected from site A = 3 biological replicates)

Technical replicate: Replicate to ensure the method variability is captured (i.e., DNA from filter A was used in wells C1, C2, and C3 of a qPCR assay = 3 technical replicates)

B.3.2 Sample Collection

Materials
1. Gloves (2 per sample collector per sampling point)
2. Sterile sampling bottles – 1 per biological replicate at each sampling point

a. We recommend triplicate biological replicates for qPCR (see Table B-10 for volume suggested for each water environment)

3. Tape/labels and permanent marker
4. Cooler with ice packs (large enough to fit all sampling bottles)
5. Biohazard bag
6. 50% ethanol (0.5L per sampling site)
7. Hand sanitizer
8. Paper Towels

Preparation Before Field Deployment

- ❑ Autoclave sampling bottles and Field and Trip Blanks to sterilize
- ❑ Place labels on each sampling bottle with location/date/initials (including the Field and Trip Blank)

Table B-10. Sample Volume per Water Type and Section with Detailed Protocol

Environment	Section	Minimum (L)	Maximum (L)
Recycled Water – At Treatment Facility	1	1	4
Recycled Water – Distribution/Point of Use	2	1	4
Surface Water	3	1	4
Wastewater – Influent	4	0.05	0.25
Wastewater – Primary Clarification	4	0.1	0.5
Wastewater – Activated Sludge	4	0.1	0.5
Wastewater – Secondary Clarification	4	0.5	1
Wastewater – Post Secondary	4	2	4

<u>Go to Section 1-4 depending on your target water environment:</u>

1. Recycled Water at Treatment Facility
   a. Put on gloves and other relevant PPE before beginning
   b. Sampling apparatus
      i. If using a sampling port at the plant, flush to remove stagnant liquid from pipes
      ii. If using a sampling apparatus, sterilize with ethanol and paper towels
         1. Inoculate sampling apparatus by repeatedly filling and emptying in recycled water
   c. Collect water in sterile bottle (see Table B-10 for suggested volume)
   d. Cap bottle, check label is correct, and store in cooler
   e. Collect any biological replicates by repeating step c and d
   f. Take any additional necessary sample-specific measurements (i.e.. pH, time, GPS coordinates, weather observations, dissolved oxygen (DO), biodegradable dissolved organic carbon, metals, turbidity, free & total chlorine, etc.)
   g. Remove used gloves and discard in biohazard bag
   h. The Field and Trip Blanks are not utilized at this step and should remain sealed.

2. Recycled Water Distribution System or Point-of-Use Sampling
   a. Put on gloves and other relevant PPE before beginning

b. Depending on research question, flush or do not flush sampling tap to remove stagnant liquid
c. Collect water in sterile bottle (see Table B-10 for suggested volume)
d. Cap bottle, check label is correct, and store in cooler
e. Collect any biological replicates by repeating step c and d
f. Take any additional necessary sample-specific measurements (i.e.. pH, time, GPS coordinates, weather observations, dissolved oxygen (DO), biodegradable dissolved organic carbon, metals, turbidity, free & total chlorine, etc.)
g. Remove used gloves and discard in biohazard bag
h. The Field and Trip Blanks are not utilized at this step and should remain sealed.

3. Surface Water Sampling (for multiple sampling locations, work from downstream to upstream, repeat for each sampling point)
   a. Put on gloves and other relevant PPE before beginning
   b. Collect water in sterile bottle (see Table B-10 for suggested volume)
      i. Either by wading or using a pole, collect sample as close to center of water body as feasible
      ii. Avoid skimming superficial water
      iii. Be careful not to disrupt sediment
   c. Cap bottle, check label is correct, and store in cooler
   d. Collect any biological replicates by repeating step b and c
   e. Take any additional necessary sample-specific measurements (i.e.. pH, time, GPS coordinates, weather observations, dissolved oxygen (DO), metals, turbidity, etc.)
   f. Remove used gloves and discard in biohazard bag
   g. The Field and Trip Blanks are not utilized at this step and should remain sealed.

4. Wastewater Sampling (repeat for each sampling point)
   a. Put on gloves and other relevant PPE before beginning
   b. During wastewater sampling, the Field Blank should be removed from the cooler, opened carefully, and left open in a clear, clean space nearby for the duration of sampling. No water should be removed nor added.
   c. Sampling apparatus
      i. If using a sampling port at the plant, flush to remove stagnant liquid from pipes
      ii. If using a sampling apparatus, sterilize with ethanol and paper towels
         1. Inoculate sampling apparatus by repeatedly filling and emptying in wastewater from the sampling point
   d. Collect water in sterile bottle (see Table B-10 for suggested volume)
   e. Cap bottle, check label is correct, and store in cooler
   f. Collect biological replicates by repeating step c and d
   g. Cap the Field Blank bottle and returned it to the cooler.

h. Take any additional necessary sample-specific measurements (i.e.. pH, time, GPS coordinates, weather observations, dissolved oxygen (DO), biodegradable dissolved organic carbon, metals, turbidity, free & total chlorine, etc.)
i. Remove used gloves and discard in biohazard bag
j. The Trip Blank is not utilized at this step and should remain sealed.

B.3.3 Sample Processing and Concentration

Materials

1. Gloves (2 per researcher)
2. Filters (47mm, 0.22-micron filters: mixed cellulose, nitrocellulose, or polycarbonate)
3. Filter holder (one sterile holder per sampling point)
4. Vacuum manifold
5. Forceps
6. Small beaker
7. Sterilizing agent (70% ethanol or bleach)
8. Bunsen burner or ethanol flame
9. 50% ethanol (~2 mL/sample)
10. 2 mL microcentrifuge tube (1 per sample, per technical replicate)

Steps (repeat for each sample)

1. Add forceps to small beaker, then pour enough sterilizing agent in to cover the ends of the forceps
2. Place filter assembly (base and funnel of filter holder) in a port of the vacuum manifold (see Figure 1 for example setup)
3. Flame-sterilize forceps and use to aseptically place a sterile filter on the base of the filter holder
4. Place the funnel of the filter holder onto the base. Be sure that the filter has no wrinkles
5. Pour water volume into the funnel of the filter holder, continue to add as needed
a. Do not allow the filter to dry out
b. It is recommended to filter samples in order from most clean to most dirty, thereby reducing risk of contamination
6. Turn on the vacuum pump, open the vacuum manifold valve for the manifold ports in use (close valve before adding additional water to the funnel)
7. After all water has been filtered, or to refusal (no flow through in 15 minutes), close the valve
8. Using flame-sterilized forceps, fold or roll the filter small enough to fit within a 2 mL microcentrifuge tube
9. Transfer the folded of rolled filter into a pre-labeled 2 mL microcentrifuge tube
10. Label the microcentrifuge tube with the site, replicate number, date, and volume filtered
    a. Volume filtered will be required when calculating gene copies per mL of qPCR runs
11. If DNA will be extracted immediately, proceed to section 3. Otherwise, fill the 2 mL microcentrifuge tube with 50% ethanol and store at -20C until extraction

12. Repeat steps 3-10 for each technical replicate

B.3.4 DNA Extraction from Filter-Concentrated Water Samples

Materials

1. Gloves (2 per researcher)
2. Biosafety cabinet
3. Forceps
4. Small beaker
5. Ethanol (70%)
6. DNA Extraction Kit
   a. FastDNA Spin Kit for Soil (example protocol given in appendix)
      i. MP Bio Cat# 6560200
   b. DNeasy PowerSoil Kit
   c. DNeasy PowerWater Kit
7. DNA Extraction Kit Protocol (keep track of the version used and ensure it is the same throughout the project)
8. 0.5 mL O-ring microcentrifuge tubes (1 per DNA extract)
9. Optional: Razor Blades and Weigh Boats

Steps

1. Add forceps to a small beaker, then pour just enough ethanol to cover the ends of the forceps
2. Fold filters (from sample processing) into quarters, then using flame-sterilized forceps, rip the filters into small, uniform pieces
   a. Alternatively, dip a razor blade into 70% ethanol using forceps and flame-sterilize the blade
   b. Allow the blade to cool enough to handle
   c. Cut the filters into small, uniform pieces in a sterile weight boat
   d. Wipe the weigh boat with ethanol between technical replicates and replace the weigh boat with a new, sterile weigh boat between samples
3. If samples were stored in 50% ethanol before extraction, centrifuge the ethanol at 5000 x g for 10 minutes, decant the supernatant, and resuspend the pellet in the first buffer used in the DNA extraction kit (see FastDNA Spin Kit for Soil protocol in the appendix for an example)
4. Follow manufacturer's instructions for DNA extraction kit
   a. See below for an example protocol for FastDNA Spin Kit for Soil
5. Qubit or Nanodrop extracts to assess the DNA extraction yield
   a. Follow manufacturer's instructions, using 1 µL of each DNA extract
6. Store DNA extracts in 0.5 mL O-ring microcentrifuge tube at -80C

B.3.5 qPCR Preparation

a. Sample Preservation and Validity for Analysis

After extraction, the sample DNA can be stored at 4°C in a water solution at -20°C for several months. For long-term storage or archiving of sample DNA, we recommend storing at -80°C. Some groups are moving towards storing DNA in buffer (Kralj et al., 2021), however that was not examined in the development of this protocol. While extracted DNA is stable, repeated freeze-thaw cycles can cause significant damage and result in compromised quantitative data. Therefore, we recommend tracking the number of freeze-thaw cycles (e.g. mark the tube each time it is thawed) and/or aliquoting the sample DNA into smaller volume tubes designed for only 1-2 uses each.

b. Laboratory Specifications

Any PCR-based reaction should be conducted in a clean lab and workspace, ideally meeting the criteria described in the USEPA Guidance Document: Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples (USEPA 2004). This is meant to minimize the potential for cross-contaminating samples from airborne contamination and due to pipetting or other sources.

c. Negative Controls

- **No Template qPCR Control:** Nuclease-free water added to a "blank" qPCR reaction
- **Trip Blank:** Molecular-grade water sealed in a sampling bottle, transported in the field, and kept unopen during sampling
- **Field Blank:** Molecular-grade water sealed in a sampling bottle, transported in the field, and kept open with the lid off over the duration of the sampling
- **Filter/DNA Extraction Blank:** Filter used for sample concentration that is also subject to the same DNA extraction steps

Materials

1. Gloves (2 per researcher)
2. Biosafety cabinet (preferably in a 'clean room' or designated PCR/qPCR space)
3. Tube rack
4. 2 mL microcentrifuge tubes
5. qPCR plates
6. Pipettes
7. Molecular grade water (i.e., Nuclease free water)
8. Master mix
   a. Recommended: PowerSYBR Green for SYBR assays and TaqMan Environmental Master Mix 2.0 for TaqMan Assays
9. Standards (see appendix for gBlock)
10. Primers/probe (see table B-9)
11. Sharpie
12. Aluminum foil
13. Adhesive PCR plate seal

14. Adhesive film applicator
15. Vortexer
16. Thermocycler
17. Computer with appropriate software connected to thermocycler
    a. This protocol uses a BioRad CFX96 thermocycler with software BioRad CFX manager to generate examples shown in Section B.3.7 Appendix.

Steps

1. Clean bench area using DNA Away then 70% ethanol
    a. Tip: make sure you are near a vortex
2. Set up gloves, all empty tubes, tube rack, pipettes, primers, pipette tips, sharpie, and water in biosafety cabinet. Turn on UV light and set timer for 30 minutes
3. Thaw samples and standards on ice blocks
4. Input plate information and run conditions on the thermocycler (see Section B.3.7 Appendix for an example)
    a. Adding the ROX fluorophore as a passive reference dye is suggested
5. Label a qPCR template with standard curve and samples (see Section B.3.7 Appendix for example) and input plate information on the thermocycler
6. Once UV step is complete, bring primers, probe (if applicable), standard, and mastermix into the hood and add to the ice block
7. Put on the gloves that were UV-ed
8. Gently vortex each reagent before adding ingredients to a 2 mL tube based on recipe (see Table B-11)
    a. Tip: add ingredients in order of increasing cost in case mistakes are made (water, then primers/probe, then master mix)
    b. Note: repeated freeze-and-thaw is not recommended for most master mixes, check the manufacturer's instructions on storage after opening
9. Vortex the qPCR master mix matrix
10. Add 20 µL of qPCR master mix matrix to each well to be used
11. Create a standard curve by diluting the gBlock 1 to 10 in 2 mL microcentrifuge tubes
    a. 50 µL of 10^9 standard into 450 µL of nuclease free water is recommended, to reduce discrepancies caused by low volume serial dilutions
    b. A standard curve of at least 10^7 to 10^1 is suggested
12. Dilute sample DNA in nuclease free water if needed to achieve quantifiable results
13. Add either 5 µL of gBlock standard or template to each corresponding well of the qPCR plate
    a. Each sample/standard/negative control should be analyzed in triplicate
    b. Depending on the DNA concentration, a 1:10 dilution of template should be used
14. Remove the adhesive backing from the plate seal and place it on the qPCR plate, touching only the side (which are regions to be torn off and discarded later)
15. Use the applicator to press down the edges to form a firm seal
    a. Make sure there are no wrinkles in plastic seal

16. Add plate to thermocycler and run protocol

Table B-11. qPCR Assay Target Information

Target	Sequences (5' to 3')	Amplicon length (bp)	Cycling Conditions	Reference
*sul*1	F: CGCACCGGAAACATCGCTGCAC	163	95C for 15m; 50 cycles of 95C for 15s, 65C for 30s; 72C for 30s + melt curve	Pei et al. 2006
	R: TGAAGTTCCGCCGCAAGGCTCG			
*intI*1	F: GCCTTGATGTTACCCGAGAG	196	95C for 10m; 45 cycles of 95C for 30s, 60C for 1m	Barraud et al. 2010
	R: GATCGGTCGAATGCGTGT			
	P: (6-FAM) ATTCCTGGCCGTGGTTCTGGGTTTT (BHQ1)			

*initial denaturation time and temperature not given in reference

Table B-12. qPCR Master Mix Matrix Recipe to Create a 25 µL Total Reaction Volume

		***sul*1 (Pei et al. 2006) - SYBR**		***intI*1 (Barraud et al. 2010) – TaqMan**	
Reagent	Working Stock Concentration	Final Concentration	Volume per reaction (µL)	Final Concentration	Volume per reaction (µL)
Master mix	2x	1x	12.5	1x	12.5
Forward primer	10 µM	200 nM	0.5	400 nM	1
Reverse primer	10 µM	200 nM	0.5	400 nM	1
Probe	10 µM	N/A	N/A	200 nM	0.5
Water			6.5		5
Sample Template			5		5

B.3.6 Interpreting qPCR Results

Materials

1. Thermocycler
2. Computer with appropriate software connected to thermocycler
3. Excel or Google Sheets

Steps

1. When the thermocycle run ends, qPCR plate can be removed and disposed of with biohazard waste
2. Check the standard curve
   a. Using the same baseline threshold, keep track of the 10^8 or 10^7 standard curve Cq value on every run to catch any drift in your standards. If you see this value drift over time, a fresh aliquot of the standard should be used. If the problem persists, a new aliquot of the gBlock standard should be made of another gBlock ordered.
   b. The standard curve should not plateau. If it does, this is indicative of contamination or background noise. You should establish the lowest standard value which does not exhibit plateauing as your limit of quantification. Plateauing can be evidence of degraded standards.
   c. Only samples which amplify in the linear portion of your standard curve can be reliably quantified. A minimum of five standard curve dilutions amplifying is required to establish a standard curve.
   d. R^2 values should be above 0.98 to be acceptable.
   e. Efficiency should ideally be close to 100%, though lack of ideal primer sets for a target gene can cause this number to vary. Monitor efficiency values and rerun plates with unacceptable efficiency values. You should expect values between 80-120%.
   f. Monitor slope and y-intercept of your standard curves to prevent drift of your standards as described in item (a). Delta Cq should be ~3.3
   g. Only standards that fall in the linear portion of your standard curve are quantifiable. Samples that amply before any standards should be rerun using an adjusted standard curve to target higher values and any samples that amplify after should be rerun with lower standards. If you have attempted to amplify lower standard for a given assay and find that you can consistently expect to amplify, consider values amplifying below your standards to be "below quantification" (more details provided in item #7)
   h. The lowest standard that amplified in at least duplicate (on the linear portion of the curve, without plateauing) should be used as the limit of quantification
3. Check melt curve (for SYBR green assays)
   a. Melt curves illustrate the dissociation of DNA by temperature, allowing comparison between samples and standards as a QA for specificity and to reduce concerns of non-target amplification
   b. Do all samples peak at the same temperature as your standards? If not, exclude samples with different melt peak temperatures. These are non-target amplification

    i. If this is happening in a small portion of your samples and only occurs in samples that amplified very late in the run, this is probably an acceptable level of non-target amplification
    ii. If this is a persistent problem or is happening very early in the run, you may need to optimize the assay for your sample type (non-target amplification is likely competing with your target gene amplification). Start by running your thermocycler program on a representative set of samples with a temperature gradient to identify the best annealing temperature.
    iii. Note: some assays that use genes that contain natural variation, such as 16S rRNA genes, may exhibit 1-2C of variation in melt peak temperature

4. Review samples for positive hits
    a. Determine if the batch of samples analyzed is valid:
        i. No template negative control should be below the detection limit
        ii. Filter/DNA extraction blank should be below the detection limit. If it is not, the data should not be analyzed further and sources of contamination in the filters or DNA extraction kits should be identified and eliminated for future analysis.
        iii. Trip Blank and Field Blank should below the detection limit, or otherwise measure at least 2-logs less than the lowest sample measurement. If they do not, the data should not be analyzed further and sources of contamination in lab water or during the sample collection process should be identified and eliminated for future analysis.
    b. To consider a sample “positive” for the target gene, at least 2 of the 3 triplicates must be quantifiable.
    c. Determine if a sample is non-detectable, detectable but below the limit of quantification (LOQ), or quantifiable:
        i. If all three triplicates are above the LOQ, average these values and consider the sample quantifiable
        ii. If all three triplicates do not amplify or are below the limit of detection (LOD) established in #6, the sample is non-detectable
        iii. If two out of the three triplicates are above the LOQ, average these two values together and consider the sample quantifiable
        iv. If two out of the three triplicates are below the LOQ, consider the sample detectable but below the limit of quantification (did not quantify, DNQ)
5. Data processing
    a. Download SQ values from thermocycler
    b. Average the hits as described in item 4b
    c. Convert to gene copies per mL by multiplying the average SQ by the filter volume (from section III)
6. Calculating limit of detection (LOD) and limit of quantification (LOQ)
    a. LOD: at least two out of three technical replicates amplify at a standard curve concentration
    b. LOQ: all three technical replicates amplify at a standard curve concentration

7. Designating samples that are detected but not quantifiable (DNQ) and below the detection limit (BDL)
    a. Samples that are DNQ are given a value equal to the LOD for statistical analyses
    b. Samples that are BDL are given a value equal to half the LOD for statistical analyses

B.3.7 Appendix

B.3.7.1 FastDNA Spin Kit for Soil Example Protocol (MP Bio Cat# 6560200)

1. Use a flame-sterilized razor blade to cut the filter into small, uniform pieces in a weigh boat or flame-sterilized tweezers to tear the filter into small, uniform pieces and add them to Lysing Matrix E tube
2. If the filter was stored in 50% ethanol, centrifuge 5000 x g for 10 minutes to pellet the contents. Decant the supernatant. Resuspend the pellet in 978 µL Sodium Phosphate Buffer. Transfer all contents to Lysing Matrix E tube
3. Add 978 µL of Sodium Phosphate buffer to Lysing Matrix E tube (if not already added to the pellet)
4. Add 122 µL of MT buffer to Lysing Matrix E tube
5. Load tubes into FastPrep instrument and process 40s at a speed setting of 6.0 m/s
6. Centrifuge tubes at 14000 x g for 10 min
7. Transfer supernatant to a clean 2 mL microcentrifuge tube
8. Add 250 µL PPS to each tube and mix by inverting 10 times
9. Centrifuge tubes at 14000 x g for 5 minutes
10. Transfer supernatant to a clean 5 mL tube
11. Add 1 mL of Binding Matrix Solution
12. Repeatedly invert the tubes for 2 minutes
13. Allow tubes to settle in a rack for 3 minutes
14. Discard 500 µL of supernatant
15. Resuspend the Binding Matrix in each tube by gently shaking or vortexing
16. Transfer 600 µL of the DNA solution to a SPIN filter tube
17. The tubes will likely need to be shaken or vortexed again before transferring
18. Centrifuge at 1400g for 1 min
19. Empty the catch tube
20. Repeat steps 15-17 until the entire volume is processed
21. Add 500 µL prepared SEWS-M Solution to the SPIN filter tube
22. Mix the SEWS-M Solution with the matrix on the filter by pipetting 5-10 times
23. Centrifuge at 14000 x g for 1 min
24. Empty catch tube
25. Centrifuge at 14000 x g for 2 min
26. Air dry SPIN filter for 5 minutes at room temperature
27. Transfer SPIN filters to a new catch tube
28. Add 100 µL DES elution solution
29. Mix the DES elution solution with the matrix on the filter by gently stirring

30. Incubate the tubes at 55C for 5 min in a heat block
31. Centrifuge at 14000 x g for 1 min
32. The DNA in the catch tube is ready to use

B.3.7.2 qPCR Template Example

Assay Information
Target name: **sul1**
Assay name: **sul1**
Name of qPCR run:
Direction of file:
Amplicon length (bp): **163**
Annealing temp (C): **65**

Citation
Pei, R., Kim, S.-C., Carlson, K.H., Pruden, A., 2006. Effect of river landscape on the sediment
5324 concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water
5325 Res. 40, 2427–2435

Sample Information
Type:
Date collected:
Location collected:
Process collected:
Kit for DNA extraction:

Concentration of Standard Curve
1x10^8 - 1x10^2 dilute 50uL of gblock into 450uL of water

qPCR Materials
Power SYBR Green MasterMix 2X - Cat. # 4367806
Forward Primer sul1 F: CGCACCGGAAACATCG
Reverse primer sul1 R: TGAAGTTCCGCCGCAA
Nuclease-free water - Cat. # FERR0582

Reagent	[Working Stock]	[Final]	1x	Reaction Mix	
				26	Number of Samples plus 10% extra
PowerSYBR Green MasterMix 2X		1x	12.50	**325.0**	
Forward primer	10 µM	200 nM	0.50	**13.0**	
Reverse primer	10 µM	200 nM	0.50	**13.0**	
		0	0.00	**0.0**	
Water			6.50	**169.0**	
Sample Template			5.00	**130.00**	
Total Reaction Volume			25	25	

Thermocycling conditions
95C for 15min; 50 cycles of: (95c for 15s, 65C for 30s); 72C for 30s; 60-95C melt curve stage
Instrument
Passive reference dye: ROX
PLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^8	10^8	10^8	10^7	10^7	10^7	10^6	10^6	10^6	10^5	10^5	10^5
B	10^4	10^4	10^4	10^3	10^3	10^3	10^2	10^2	10^2	NTC	NTC	NTC
C												
D												
E												
F												
G												
H												

B.3.7.3 qPCR Thermocycler Protocol and Plate Setup Examples for BioRad CFX96

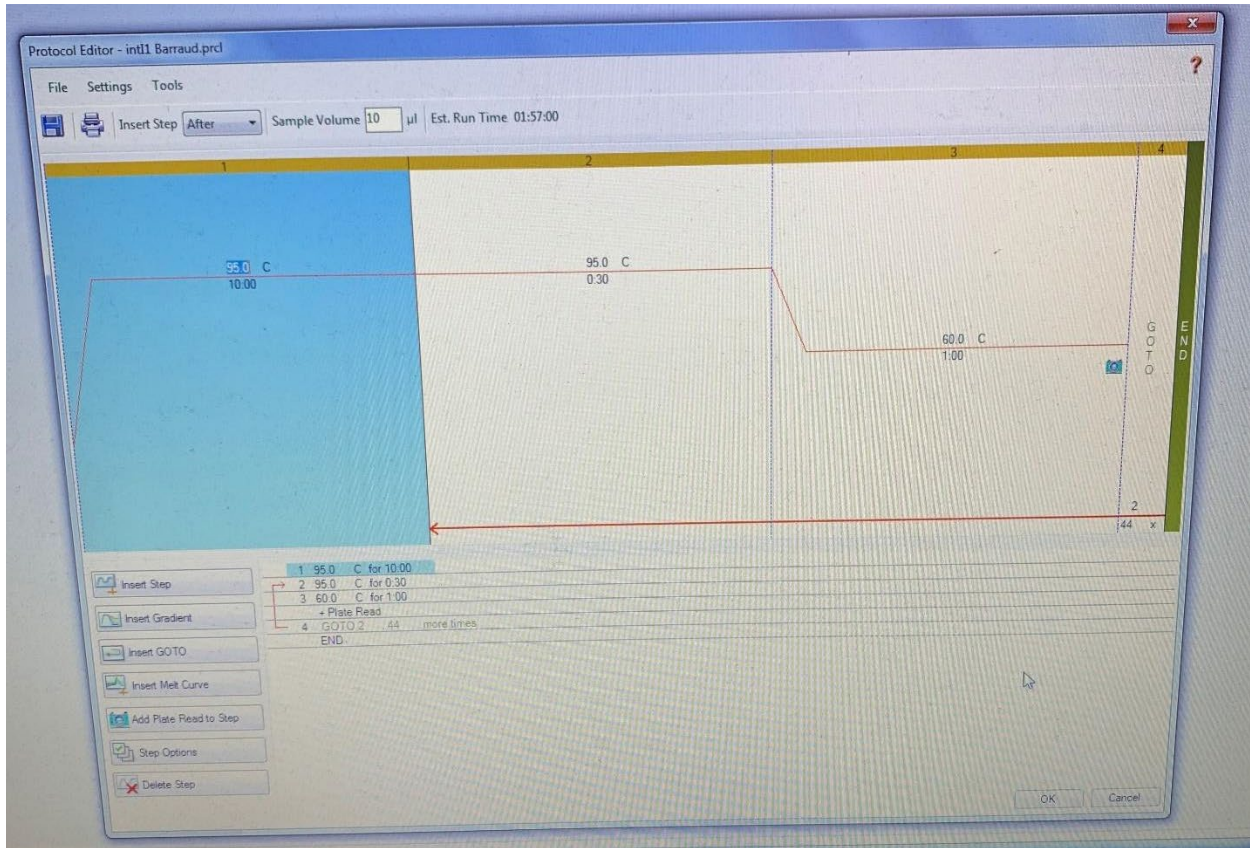

Figure B-4. Example of qPCR Protocol Setup for *IntI*1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std-1 SYBR	Std-1 SYBR	Std-1 SYBR	Unk-1 SYBR FL-2229	Unk-1 SYBR FL-2229	Unk-1 SYBR FL-2229	Unk-9 SYBR FL-2240	Unk-9 SYBR FL-2240	Unk-9 SYBR FL-2240	Unk-17 SYBR FL-2347	Unk-17 SYBR FL-2347	Unk-17 SYBR FL-2347
B	Std-2 SYBR	Std-2 SYBR	Std-2 SYBR	Unk-2 SYBR FL-2230	Unk-2 SYBR FL-2230	Unk-2 SYBR FL-2230	Unk-10 SYBR FL-2242	Unk-10 SYBR FL-2242	Unk-10 SYBR FL-2242	Unk-18 SYBR FL-2348	Unk-18 SYBR FL-2348	Unk-18 SYBR FL-2348
C	Std-3 SYBR	Std-3 SYBR	Std-3 SYBR	Unk-3 SYBR FL-2231	Unk-3 SYBR FL-2231	Unk-3 SYBR FL-2231	Unk-11 SYBR FL-2244	Unk-11 SYBR FL-2244	Unk-11 SYBR FL-2244	Unk-19 SYBR FL-2349	Unk-19 SYBR FL-2349	Unk-19 SYBR FL-2349
D	Std-4 SYBR	Std-4 SYBR	Std-4 SYBR	Unk-4 SYBR FL-2232	Unk-4 SYBR FL-2232	Unk-4 SYBR FL-2232	Unk-12 SYBR FL-2245	Unk-12 SYBR FL-2245	Unk-12 SYBR FL-2245	Unk-20 SYBR FL-2350	Unk-20 SYBR FL-2350	Unk-20 SYBR FL-2350
E	Std-5 SYBR	Std-5 SYBR	Std-5 SYBR	Unk-5 SYBR FL-2233	Unk-5 SYBR FL-2233	Unk-5 SYBR FL-2233	Unk-13 SYBR FL-2246	Unk-13 SYBR FL-2246	Unk-13 SYBR FL-2246	Unk-21 SYBR FL-2352	Unk-21 SYBR FL-2352	Unk-21 SYBR FL-2352
F	Std-6 SYBR	Std-6 SYBR	Std-6 SYBR	Unk-6 SYBR FL-2234	Unk-6 SYBR FL-2234	Unk-6 SYBR FL-2234	Unk-14 SYBR FL-2247	Unk-14 SYBR FL-2247	Unk-14 SYBR FL-2247	Unk-22 SYBR FL-2353	Unk-22 SYBR FL-2353	Unk-22 SYBR FL-2353
G	Std-7 SYBR	Std-7 SYBR	Std-7 SYBR	Unk-7 SYBR FL-2237	Unk-7 SYBR FL-2237	Unk-7 SYBR FL-2237	Unk-15 SYBR FL-2248	Unk-15 SYBR FL-2248	Unk-15 SYBR FL-2248	Unk-23 SYBR FL-2354	Unk-23 SYBR FL-2354	Unk-23 SYBR FL-2354
H	NTC-1 SYBR	NTC-1 SYBR	NTC-1 SYBR	Unk-8 SYBR FL-2239	Unk-8 SYBR FL-2239	Unk-8 SYBR FL-2239	Unk-16 SYBR FL-2253	Unk-16 SYBR FL-2253	Unk-16 SYBR FL-2253	Unk-24 SYBR FL-2355	Unk-24 SYBR FL-2355	Unk-24 SYBR FL-2355

Figure B-5. Example of qPCR Plate Information Input

B.3.7.4 Troubleshooting qPCR using ROX Passive Reference Dye

Using ROX reference dye allows for normalization that would fix skewed amplification readings. To calculate normalized values (Rn), the reported dye signal is divided by the ROX reference dye signal.

ROX reference dye also can be used for troubleshooting.

- If ROX dye reading remains flat, amplification is as expected
- If ROX dye reading increases throughout the run, evaporation may be occurring
- If ROX dye readings momentarily spike or drop, and air bubble or electrical surge may have occurred

B.3.7.5 gBlock Creation

The following gBlock can be used for both the *sul*1 and *intI*1 targets (5' to 3'):

tgcatgatctacgtgcgtcacatgcagtacCGCACCGGAAACATCGCTGCACGTGCTGTCGAACCTTCAAAGCTG
AAGTCGGCGTTGGGGCTTCCGCTATTGGTCTCGGTGTCGCGGAAATCCTTCTTGGGCGCCACCGTTGGC
CTTCCTGTAAAGGATCTGGGTCCAGCGAGCCTTGCGGCGGAACTTCAActgtgcggactctaCTGGATTTCGA
TCACGGCACGATCATCGTGCGGGAGGGCAAGGGCTCCAAGGATCGGGCCTTGATGTTACCCGAGAGCT
TGGCACCCAGCCTGCGCGAGCAGCTGTCGCGTGCACGGGCATGGTGGCTGAAGGACCAGGCCGAGGG
CCGCAGCGGCGTTGCGCTTCCCGACGCCCTTGAGCGGAAGTATCCGCGCGCCGGGCATTCCTGGCCGTG
GTTCTGGGTTTTTGCGCAGCACACGCATTCGACCGATCCACGGAGCGGTGTCGTGCGTCGCCATCACAT
GTATGACCAGACCTTTCAGCGCGCCTTCAAACGTGCCGTAGAACAAGCAGGCATCACGAAGCCCGCCAC
ACCGCACACCCTCCGCCACTCGTTCGCGACGGCCTTGCTCCGCAGCGGTTACGACATTCGAACCGTGCAG
GATCTGCTCGGCCATTCCGACGTCTCTACGACGATGATTTACACGCATGTcactagctcagattcagtagaccgct
gttg

Key:
Spacers
*Sul*1 Forward primer
*Sul*1 Reverse Primer Complement
*IntI*1 Forward Primer
*IntI*1 Probe
*IntI*1 Reverse Primer Complement

Protocol for gBlock Hydration, Working Stock Preparation, and Aliquoting

1. Calculate the required volume of IDTE buffer to be added to the gBlock to reach a concentration of 10 ng/µL. For example, if you have a gBlock that was delivered at 500 ng, then 50 µL of IDTE buffer is needed to reach a final concentration of 10 ng/µL.

2. Calculate copies per µL using the following formula:

Copies per µL = $10\frac{ng}{\mu L} * X\frac{fmol}{ng} * \frac{1x10^{-15}mol}{fmol} * 6.022\ x\ 10^{23}$, where fmol/ng is in gBlock specifications.

3. Calculate dilutions needed to obtain a working stock concentration of 10^9 gene copies per 5µL using the formula:

$$\frac{1460\ \mu L * \frac{1x10^{9}\ gene\ copies}{5\ \mu L}}{Copies\ per\ \mu L}$$

- This results in the volume (µL) needed of hydrated manufacturer gBlock stock.
- 1460 minus [this number] = the volume of IDTE buffer needed to create a 10^9 gene copies/5µL working stock.

4. Before opening the tube, spin it down in a microcentrifuge to ensure the DNA is in the bottom of the tube.

5. Add the volume of IDTE buffer calculated in step 1 to achieve a concentration of 10 ng/µL.

6. Vortex briefly.

7. Incubate at 50°C for 20 minutes. This ensures the solvent comes in contact with the tiny pellet, even if it is stuck to the side of the tube.

8. Briefly vortex and centrifuge.

9. Create working stocks by adding the volume of hydrated manufacturer gBlock and volume of IDTE buffer calculated in step 3 to a 2 mL microcentrifuge tube. Multiple working stocks may be prepared if enough manufacturer stock is available.

9. Aliquot working stock into labeled 0.2 mL PCR tubes in 55 µL volumes. When creating standard curve dilutions for qPCR assays, serial dilutions of 50 µL of 10^9 gBlock stock should be added to 450 µL of molecular grade or nuclease-free water.

10. Store at -20°C or use immediately.

APPENDIX C

WARD Website Data Entry Templates

C.1 WARD Website Culture Spreadsheet Template

Figure C-1: Screenshot of spreadsheet columns A through K

	A	B	C	D	E	F	G	H	I	J	K	L
1	sample_id	water_type	treatment_stage	treatment_plant_type	intended_use	treatment_train	impacted_or_unimpacted	water_age	type_of_blanks	Organism_target	Antibiotic_tested	Antibiotic_concentration_(micrograms_per_mL)
2												
3		Surface water upstre	Influent									
4												
5												
6												
7												

Figure C-2: Screenshot of spreadsheet columns K through T

L	M	N	O	P	Q	R	S	T	U	V
Antibiotic_concentration_(micrograms_per_mL)	Media	Sensitive_colony_concentration_(cfu_per_100mL)	Resistant_colony_concentration_(cfu_per_100mL)	Detection_limit_sensitive	Detection_limit_resistant	Volume_filtered_(mL)	location	date_collected	pH	temperature_C

C.2 WARD Website qPCR Spreadsheet Template

Figure C-3: Screenshots of qPCR spreadsheet:

Columns A through K

	A	B	C	D	E	F	G	H	I	J	K
1	sample_id	water_type	gene_target	assay_citation	concentration_(gene_copies_per_mL)	Limit_of_Detection_(gc_per_mL)	Limit_of_Quantification_(gc_per_mL)	Non-Template_Control_(gc_per_mL)	relative_abundance	(gene_copies)_per_(microliter_PCR_reaction)	(microliter_PCR_reaction)_per_(microliter_diluted_DNA_ex
2											
3											
4											
5											
6											
7											

Columns K through T

K	L	M	N	O	P	Q	R	S	T
(microliter_PCR_reaction)_per_(microliter_diluted_DNA_extract)	Dilution_Factor	(microliter_DNA_extract)_per_(millilitre_sampled_water)	R_squared	Efficiency	location	date_collected	pH	temperature_C	total_chlorine_(milligram_per_liter)

APPENDIX D

Quality Assurance Project Plan (QAPP)

Standardizing Methods with QA/QC Standards for Investigating the Occurrence and Removal of Antibiotic Resistant Bacteria/Antibiotic Resistance Genes (ARB/ARGs) in Surface Water, Wastewater, and Recycled Water
Quality Assurance Project Plan

Water Research Foundation
Contract #5052

Prepared for:
The Water Research Foundation

Prepared by:
Principal Investigators Dr. Amy Pruden and Dr. Lenwood S. Heath (Virginia Tech) and Dr. Valerie J. Harwood (University of South Florida)

Virginia Tech
1075 Life Science Circle, Blacksburg, VA 24060
Email: apruden@vt.edu (Dr. Pruden)
Email: heath@cs.vt.edu (Dr. Heath)

University of South Florida
4202 East Fowler Ave, Tampa, FL 33620
Email: vharwood@usf.edu (Dr. Harwood)

Abstract: This Quality Assurance Project Plan (QAPP) provides recommended policies and procedures for measuring antibiotic resistance in aquatic matrices. Specifically, the QAPP is focused on wastewater, recycled water, and surface water collection and analysis, including planning, implementing, and assessing the Quality Assurance (QA) and Quality Control (QC) objectives to ensure integrity and comparability of the resulting measurements. This QAPP was developed specifically for one culture-based target, cefotaxime-resistant *Escherichia coli*, and two quantitative polymerase chain reaction (qPCR) based targets, *sul*1 antibiotic resistance genes (ARGs) and the *intI*1 Class 1 integron. Many of the practices and procedures are applicable to general QA/QC recommended for ensuring the integrity of culture-based and molecular-based testing of aquatic samples. The program started in Summer 2020 and continued through Spring 2022 The development of this QAPP was supported through WRF Project 5052, "Standardizing Methods with QA/QC Standards for Investigating the Occurrence and Removal of Antibiotic Resistant Bacteria/Antibiotic Resistance Genes (ARB/ARGs) in Surface Water, Wastewater, and Recycled Water."

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D.1 PROJECT MANAGEMENT

Project Title:
Contract Laboratory: Pruden Lab at Virginia Tech
Primary Contact: Dr. Amy Pruden
Phone Number: 540-204-5569
Email Address: apruden@vt.edu

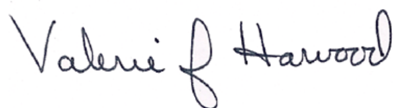
Co-Principal Investigators
Laboratory: Harwood Lab at University of South Florida
Primary Contact: Dr. Valerie J. Harwood
Phone Number: (813) 468-7461
Email Address: vharwood@usf.edu

Laboratory: Heath Lab at Virginia Tech
Primary Contact: Dr. Lenwood S. Heath
Phone Number:
Email Address: heath@cs.vt.edu

D.1.1 Approval Sheet

____ _____________________________

Amy Pruden, PhD. Date
Virginia Tech
Principal Investigator



____ _____________________________

Valerie J. Harwood, PhD. Date
University of South Florida
Co-Principal Investigator

____ _____________________________

Lenwood S. Heath, PhD. Date
Virginia Tech
Co-Principal Investigator

____ _____________________________

Lyndsey Bloxom Date
Research Program Manager
Project Officer

D.1.2 Distribution List

This Quality Assurance Project Plan (QAPP) documents the recommended approach for planning, implementing, and assessing the Quality Assurance (QA) and Quality Control (QC) procedures for measuring antibiotic resistance in aquatic samples. The criteria were developed and vetted through independent testing of wastewater, recycled water, and surface water samples by the Pruden Lab at Virginia Tech and the Harwood Lab at the University of South Florida.

Individuals listed on the approval sheet (subsection A1) and project implementation personnel identified under Project/Task organization (subsection A4, Figure D-1) will receive a copy of this QAPP and its subsequent revisions and/or amendments.

D.1.3 Project/Task Organization

Personnel involved in project implementation are shown in Figure D-1, and responsibilities of each laboratory are summarized below. A brief description of the principal investigator responsibilities are provided in this section.

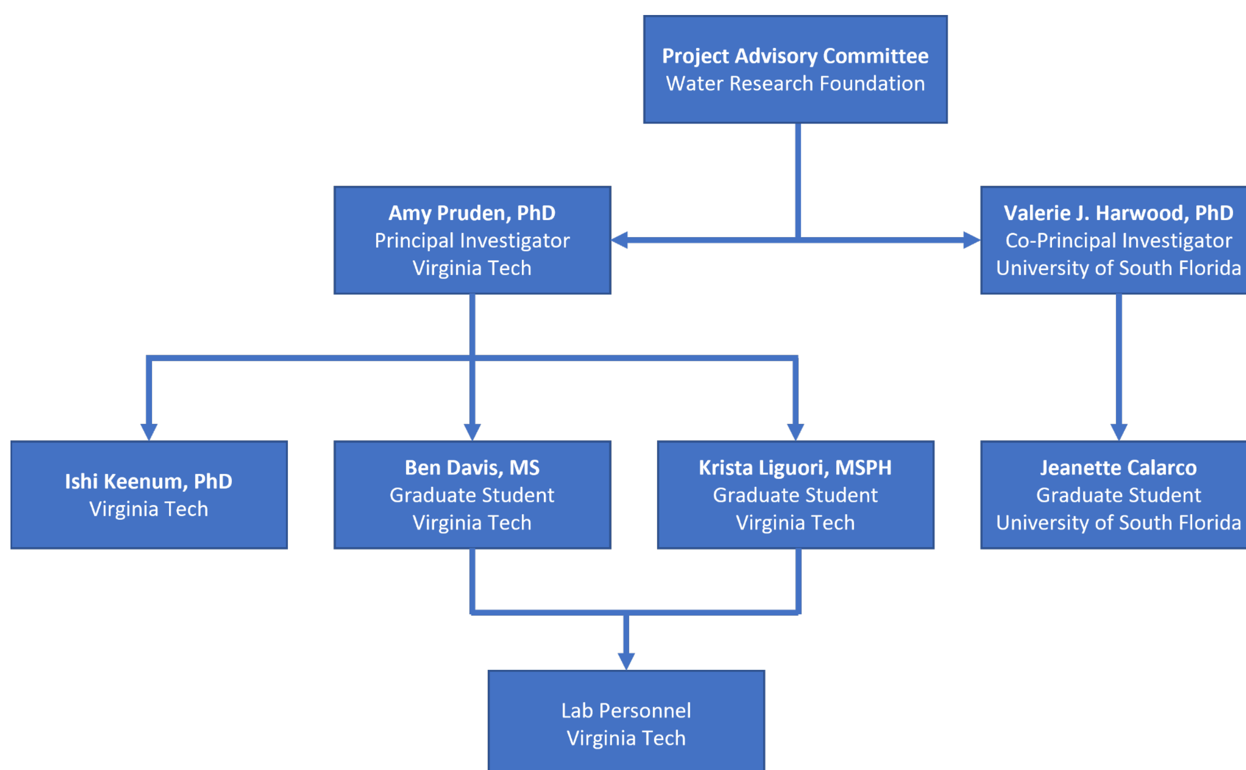


Figure D-1. Project personnel organization

Laboratory Responsibilities

Virginia Tech

- Test and optimize qPCR SOP prior to validation studies
- Receive and process samples from collaborating wastewater treatment facilities for validation studies
- Complete quarterly and final reports to be submitted to project advisory committee

University of South Florida

- Test and optimize culture SOP prior to validation studies
- Receive and process samples from collaborating wastewater treatment facilities for validation studies
- Assist in completion of quarterly and final reports to be submitted to project advisory committee

Project Management Responsibilities

Amy Pruden, PhD will serve as principal investigator and oversee the Virginia Tech laboratory conducting culture and qPCR validation.

As the principal investigator, she will be responsible for the following activities:

- Hiring undergraduate and graduate research assistants at VT
- Laboratory management, equipment, and supplies at VT
- Shipping logistics for all sampling events
- Overseeing validation at VT of all methods

Valerie J. Harwood, PhD will serve as co-principal investigator and oversee the University of South Florida laboratory conducting culture and qPCR validation.

As the co-principal investigator, she will be responsible for the following activities:

- Hiring undergraduate and graduate research assistants at USF
- Laboratory management, equipment, and supplies at USF
- Overseeing validation at USF of all methods

Lenwood S. Heath, PhD will serve as co-principal investigator and oversee the Virginia Tech computer science division conducting website development for an online database.

- Hiring undergraduate and graduate research assistants at VT
- Computer science division management for website/database development
- QA/QC of data management and website development
- Oversee formatting of data for online database

D.1.4 Problem Definition/Background

Antibiotic resistance is a major human health challenge of the 21st century and its spread is limiting options to prevent and treat bacterial infections. A growing body of research has demonstrated that the water environment is a key recipient, pathway, and source of **antibiotic resistant bacteria (ARBs)** and **antibiotic resistance genes (ARGs)**. While several national and international agencies and organizations have recognized that environmental monitoring of ARBs/ARGs is needed in order to better quantify environmental contributions to the overall spread of antibiotic resistance and human (& animal) health burden, such efforts are hampered by a lack of standardized monitoring methods. Methods reported in the literature for detecting and enumerating ARBs and ARGs in water environments are highly varied, making meaningful comparisons across

studies very challenging.

The primary objectives of WRF 5052 "Standardizing Methods with QA/QC Standards for Investigating the Occurrence and Removal of Antibiotic Resistant Bacteria/Antibiotic Resistance Genes (ARB/ARGs) in Surface Water, Wastewater, and Recycled Water" were to:

- Incorporate input from literature review, expert surveys, and an expert workshop in developing a framework for antibiotic resistance monitoring of surface water, wastewater, and recycled water that aligns methods and targets with corresponding monitoring objectives.
- Seek expert workshop input in the development of SOPs for the identified priority targets and validate the SOPs through an interlaboratory comparison of samples collected by representative water utilities.
- Develop a web-based forum for sharing the SOPs and any associated updates, data sharing, data analysis, and discussion of user experiences.

D.1.5 Project/Task Description

Project Overview

The **overarching objective** of this project is to identify, develop, and validate standardized methods for monitoring ARBs and ARGs in water environments, including wastewater, recycled water, and surface water. This objective will be achieved through a systematic review of the literature to identify the most promising methods, followed by an expert workshop to reach consensus on methods to recommend to the water industry and to develop **standard operating procedures (SOPs)**, **quality assurance/quality control (QA/QC)** procedures, and data quality objectives for these methods. Selected methods will be subject to bench-scale pilot-testing and validation in the PI/Co-PI's laboratories in partnership with water utilities. A public website and database will be constructed to facilitate implementation of the methods and to serve as a repository and tool to assess representative ARB/ARG levels. Results will be broadly disseminated via the website and database, conference presentations, journal publications, and a webinar.

D.1.5.1 Constituents to be Monitored and Measurement Techniques

Table D-1. Analytical Microbiology Methods

Analytical Method	Microbe or Target
EPA Method 1603 (modified)	Bacteria: 1) *Escherichia coli* 2) Cefotaxime-resistant *Escherichia coli*
qPCR	Antibiotic Resistance Genes: 1) *sul1* (sulfonamide resistance gene) 2) *intI1* (class 1 integrase genes)

EPA Method 1603 was modified to also include media amended with antibiotics, as detailed in the SOP.

D.1.5.2 Project Timetable

The project's deliverables under the statement of work are summarized in Table D-2.

Table D-2. Project Timetable

	September 1, 2020 – November 30, 2020	December 1, 2020 – February 28, 2021	March 1, 2021 – May 31, 2021	June 1, 2021 – August 31, 2021	September 1, 2021 – November 30, 2021	December 1, 2021 – February 28, 2022	March 1, 2022 – May 31, 2022	June 1, 2022 – August 31, 2022	September 1, 2022 – November 30, 2022
TASK	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	**Q6**	**Q7**	**Q8**	**Q9**
Comprehensive Literature Review									
SOP Protocol Generation and Optimization									
Expert Survey/Workshop			X						
Sample Collection and Processing									
Sample Analysis									
Periodic Report	X	X	X	X	X	X			
Final Report							draft	X	

Shaded squares = processes; **X** = deliverable

D.1.5.3 Geographic Focus

The geographic areas under this WRF study are located in Florida, Georgia, Virginia, Nevada, and California. Six wastewater treatment plants (WWTPs) were selected for this study. The agency and sampling month are provided in Table D-3 where permitted by the collaborating agency.

Table D-3. WWTPs and Sampling Month

#	State	WW Plant Capacity (MGD)	Recycled Water Plant Capacity (MGD)	Sampling Month
1	Florida	33	5	September
2	Georgia	60	37	October
3	Nevada	100	5	November
4	California	25	6	January
5	Virginia	20	30	February
6	Florida	9	64 (pond)	March

D.1.6 Quality Objectives and Criteria

Measurement quality objectives (outlined below) and good laboratory practices will be employed to ensure that the data collected (see section A6.1) comply with USEPA quality objectives. The specific steps taken to achieve measurement quality objectives for the various laboratory methods are detailed in section B4 under Method Validation.

Precision is a value that reflects how closely replicate measures from one sample agree. Precision can be optimized through rigorously controlled laboratory practices and is ensured through replication of measurements and standardized qPCR procedures (e.g., method blanks, positive and negative qPCR controls) for analysis of microbiological and molecular parameters. A method blank consists of a clean, sterile sample containing no DNA that is processed through the entire method, including filtration and incubation if applicable.

Accuracy generally reflects that agreement between an observed value and an accepted reference value.

Bias is a systematic or a persistent distortion of a measurement process that deprives the result of representativeness; i.e., the expected sample measurement is different from the sample's true value expressed in terms of recovery. For the purpose of this study, bias could be encountered if qPCR reactions are inhibited by compounds in the sample matrix, or if a particular assay is prone to nonspecific results (qPCR product generated when the target is not present). Inhibition is detected through the use of 10-fold dilutions. The difference in cT value between the non-diluted (or less diluted) sample and the 10-fold diluted sample will be calculated and a difference of more than 3.3 ∓ 1 cT will be used to indicate inhibition.

Representativeness of data generated in the laboratory to real-work environmental processes is a primary goal of the project. To ensure that results are *representative*, multiple WWTPs, sites, and water matrices across the US (see section A6.3) will be assessed to ensure that laboratory findings represent field conditions.

Comparability will be established by (1) technician analysis in two different labs and (2) by making our laboratory findings and SOPs available to the scientific community to allow replication in other laboratories and ultimately in field environments. The supporting analytical methods (e.g., any standard microbial enumeration and physical/chemical measurements) will be determined by standard methods; and assessment of supporting molecular analyses will likewise be performed by accepted methods.

Completeness will be ensured by paying particular attention to the project's primary outputs: 1) scientific knowledge, publishable in reviewed professional journals; 2) practical, useable and valid SOPs for culture and qPCR; 3) an expert workshop conducted to receive a consensus on microbiological and molecular targets; and 4) a website for sharing the results of the study and a database for depositing antibiotic resistance data among the scientific community. The application of rigorous criteria for complete data sets that adequately support the theses of these publications will be absolutely essential.

D.1.7 Special Training/Certification

Virginia Tech (VT)

All VT laboratory personnel are required to complete training in standard lab safety practices outlined in the University Chemical Hygiene Plan for Virginia Tech. Additional certifications are required for BSL2 lab access which involves general biosafety training and the proper handling of bloodborne pathogens. All personnel in the Pruden lab are required to review and sign the Environmental and Water Resources Laboratory Procedures document administered by the

Civil and Environmental Engineering department's Environmental Health and Safety officers, annually, verifying the completion or renewal of these certifications.

Personnel are trained on sterile lab techniques, pipetting accuracy, and proper use of equipment and implementation of all SOPs. Personnel first demonstrated proficiency in pipetting accuracy and serial dilution by creating a series of dye dots on parafilm, and dyed serial dilution tubes, which were compared to the trainer's series for color-matching. They then demonstrated their abilities to combine sterile technique, pipetting, dilution, and lab equipment and SOP processes by carrying out the intended SOP on one (or as many as required to achieve passing results) set of standards, samples, and negative controls, which were compared to the trainers and other trainees.

University of South Florida (USF)
All USF laboratory personnel are trained in standard health and safety practices through the University of South Florida's Environmental Health and Safety training program for laboratory and research safety, and in USF's Institutional Biosafety Committee training program for biosafety. All lab personnel are required to repeat both training programs annually.

All personnel will be trained on the proper use of equipment and implementation of SOPs. Before carrying out sample analyses using a given method, each worker must demonstrate their capability by providing an experimental plan and performing analyses of positive controls, negative controls, and blind spiked samples. These proficiency tests are recorded in the QA/QC logbook under "Personnel Qualification."

D.1.8 Documentation and Records

Quarterly progress reports will be submitted routinely throughout the project. A final project report will be submitted as a draft to the Water Research Foundation Project Advisory Committee for review and comment prior to final report submission.

VT
Documentation and records include experimental design, daily lab notebook tracking of lab work and results, raw data, and statistical analysis. All lab notebooks are kept in the laboratory at all times and maintained there permanently. Data spreadsheets and analysis notes are kept on the laboratory Google drive and maintained for at least five years after the conclusion of the study.

USF
Records include experimental design and details, raw data from environmental samples (e.g. CFU counts, confirmation rates, qPCR results) and calculations. Quality control data (including instrument and pipette calibration) will be maintained in hard copy in a dedicated QA/QC logbook. Experimental design and raw data are to be recorded in hard copy in a laboratory notebook that is dedicated to the research project. These will be held a minimum of 5 years after the project ends. Records are also kept in electronic form and are maintained on the USF College of Arts and Sciences server and are also backed up by external electronic storage devices to be held a minimum of 5 years after the project ends. Data recorded will be checked for correctness and completeness, as well as transcription accuracy.

D.2 DATA GENERATION AND ACQUISITION

D.2.1 Sampling Process Design (Experimental Design)

A key initial task in this project is the pre-campaign evaluation/optimization and laboratory proficiency tests to generate data for demonstration of capability and describe the project's performance and quality control criteria (see section B6). The Pruden lab at VT evaluated and optimized the molecular assays to be used in this project. The Harwood lab at USF evaluated and optimized the microbiological and membrane filtration protocols.

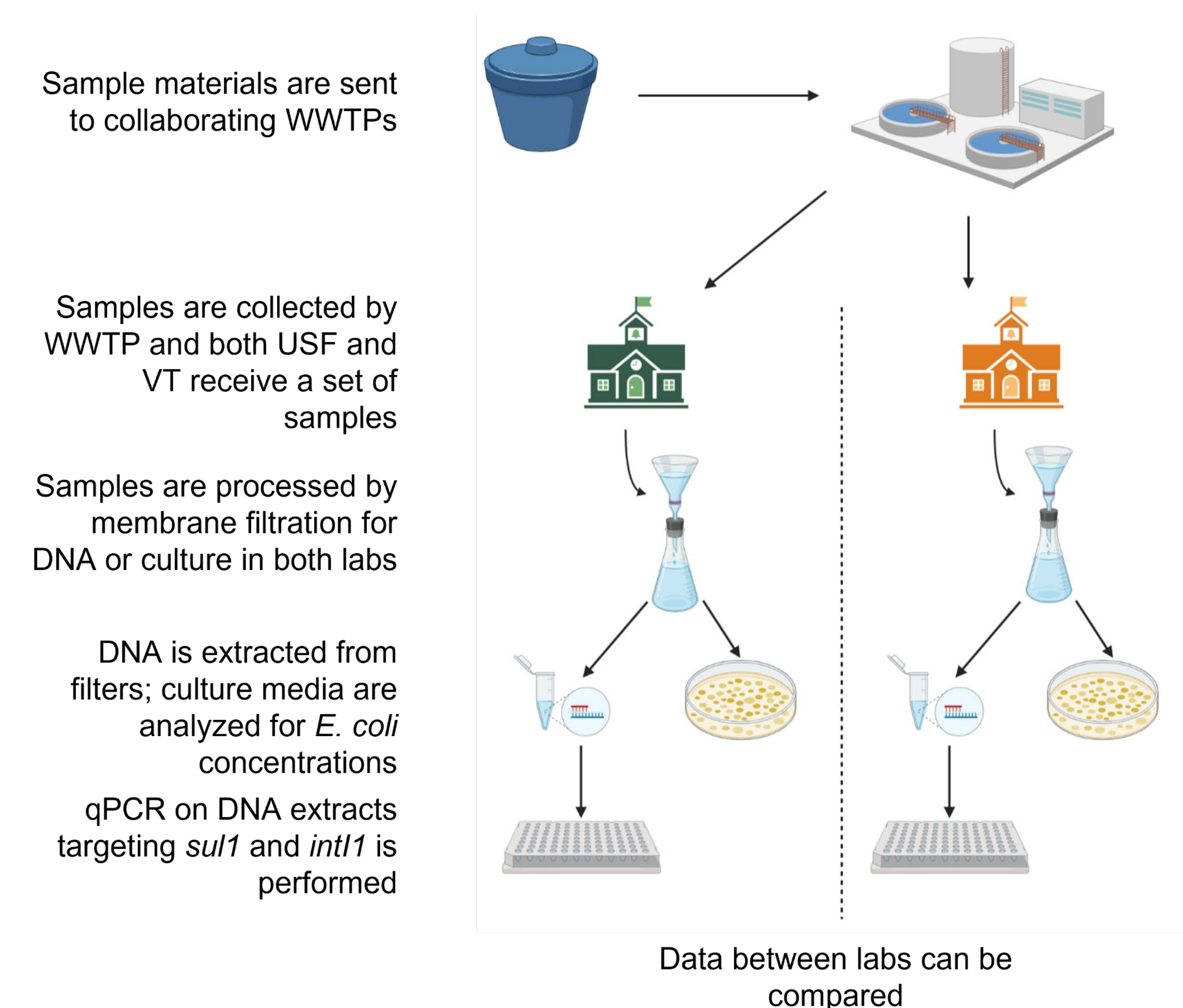


Figure D-2. Overview of sample collection and processing workflow
(Figure Created in BioRender).

D.2.2 Sampling Methods

Grab samples will be collected at the individual sites by WWTP staff personnel. The laboratories will not be directly involved in sample collection but will be responsible for supplying sampling containers and shipping materials. A sampling protocol has been provided to the WWTPs.

Sample Method Requirements

Procedures for environmental sample collection are described in the USEPA Microbiological Methods for Monitoring the Environmental Water and Wastes, EPA-600/8-78-017, Dec 1978. Briefly, samples are collected in sterile plastic bottles by personnel wearing disposable gloves, transported in insulated containers on ice to be kept at 1-4°C, and processed within 48 hours for *E. coli* counts (modified USEPA method 1603). Filters for DNA extraction will be stored until analyzed in 50% ethanol at -20°C.

D.2.3 Sample Handling and Custody

Sample kits will be shipped to WWTPs prior to the agreed-upon sampling date. The laboratories will provide adequate logistical support to WWTPs to ensure all materials are present and ensure the integrity of the samples upon arrival to the laboratory.

Hard copy sample log sheets will be filled out as samples are collected in the field (See Section B.2.2. for Field Sampling Metadata Sheet) and will be stored in a logbook. All samples will be logged into a computer database spreadsheet dedicated to the project upon delivery to the laboratory. The sample collection, date, and time will be recorded, as well as field notes. The analyses performed will be tracked and recorded as they are completed. Supervisors will note the completion of quality control procedures appropriate to each sample. Sample handling will only be performed by personnel who have demonstrated their abilities by prior proficiency testing described in section A8.

A uniform sample identification scheme is to be used in the project. Each sample will be designated first by utility, underscore, then by lab processing, underscore, then site. When distinguished by biological replicate, the replicate number is added onto the end of the sample name. An example is FLD_P_WWI representing the utility Florida (Dunn)_Pruden Lab Wastewater Influent, and the first biological replicate is FLD_P_WWI-1.

Sample Custody

The integrity of samples will be verified using the following sample acceptance criteria routinely employed at the laboratory and specifications identified in the contract:

- Samples arrive at destination laboratory within 48 hour window
- Samples arrive below room temperature (remained refrigerated during transport)
- Broken or leaking bottles will be removed from processing

Responsible Individuals

Each individual laboratory will have ultimate responsibility for ensuring samples are properly handled and transferred.

Krista Liguori, M.S.P.H.
Lab Personnel
Virginia Tech
Phone: 484-744-7044
Email: KristaLiguori@vt.edu

Jeanette Calarco
Lab Personnel
University of South Florida
Phone: (716) 361-7189
Email: JCalarco@usf.edu

D.2.4 Method Validation

Each culture target and qPCR target considered in this study will first go through a method testing and optimization process as well as pilot testing prior to the beginning of validation studies. Culture methods will be tested on wastewater influent and effluent samples collected from a local wastewater treatment plant in each laboratory. Culture samples in validation studies will be processed in biological duplicate, with two pseudo-replicates at up to three dilutions for each biological duplicate. The qPCR target assays will be performed using gBlock standards to generate standard curves within acceptable efficiency (80-120%) and R2 values (>0.98) before analyzing samples from validation studies. Each sample site will be collected with three biological replicates for qPCR and each biological replicate will be filtered onto its own filter for DNA. For each filter, DNA will be extracted and analyzed in technical triplicate by qPCR. The biological replicates will ensure the natural variability of the sample matrix is captured and the technical replicates will ensure the method variability is captured. Any samples of qPCR assays that fall outside of the QA/QC range specified for efficiency and/or R2 will be re-analyzed.

D.2.5 Analytical Methods

The culture and qPCR methodology described is current as of the date of this QAPP (April 2022).

D.2.5.1 Membrane Filtration and Culturing for Enumeration of *Escherichia coli*

EPA Method 1603 – Membrane Filtration Method for the Enumeration of *Escherichia coli*.

Summary of Method:

Total *E. coli* is analyzed by membrane filtration using modified mTEC agar plates (Difco). The medium is prepared by mixing 45.6g of dehydrated modified mTEC agar per liter of deionized water. The suspension is dissolved by boiling for 1 minute while stirring with a magnetic stir bar, and sterilized by autoclaving by 15 minutes at 121°C. Final pH is 7.3 ± 0.2. Cefotaxime-resistant *E. coli* is isolated on mTEC agar tempered to 50°C and amended with 4 µg/mL cefotaxime prior to dispensing, and incubated by the same protocol as total *E. coli*. Media is dispensed into 47 mm petri dishes, allowed to solidify, and then stored at 4°C. Plates are marked as antibiotic-amended if they contain cefotaxime. Samples are membrane filtered and filters are aseptically placed on mTEC plates and incubated at 2 ± 0.5 hours at 35 ± 0.2°C, followed by 22 ± 0.5 hours at 44.5 ± 0.2°C, submerged in a water bath in a sealed, water-tight bag. After incubation, purple or magenta colonies are counted as *E. coli*.

Quality Control:

All media is tested for integrity before use. *E. coli* (ATCC 13706) is used as a positive control and *Enterococcus faecalis* (ATCC 19433) as a negative control. Each control is streaked onto a separate plate and incubated at 2 ± 0.5 hours at 35 ± 0.2°C, followed by 22 ± 0.5 hours at 44.5 ± 0.2°C, submerged in a water bath in a sealed, water-tight bag. For each batch of samples processed by membrane filtration, n

D.2.5.2 Membrane Filtration and DNA Extraction Prior to qPCR

Summary of Method:

Before filtration, each closed sample bottle is dried, and the weight is recorded. Samples are then concentrated by vacuum membrane filtration onto a 0.22-µm filter until clogging. The sample is then reweighed (including the cap) and the difference in mass used to determine the volume of sample filtered. The volume filtered for the first biological replicate of each matrix is used for the subsequent replicates to ensure equally representative filter volumes. Filters are placed in 2 mL centrifuge tubes and 1 mL of 50% ethanol is added for sample preservation. Filters are stored at -20 °C until DNA extraction. To minimize variation in the recovery and quality of the DNA extracted from environmental samples, we will use the commercially available FastDNA Spin Kit for Soil (MPbio).

Quality Control:

All sample batches processed for method validation will include the following controls: method blanks, field blanks, and positive and negative controls. Method blanks will be 100 mL of autoclaved PBS filtered through a sterile filter. In between the handling of blank and soiled filters, tweezers will be flame-sterilized.

D.2.5.3 Quantitative PCR (qPCR)

Summary of Method:

All qPCR protocols for ARGs to be used in the project are based on published methods. ARGs we are presently utilizing include: sulfonamide resistance gene *sul1* (Pei et al. 2006) and class-one integrase gene *intI1* (Barraud et al. 2010). SOPs developed during this project are included as Appendix B.

Quality Control:

To monitor the quality of our qPCR experiments, both positive and negative controls will be used. To verify that no contaminating nucleic acid is present in the assay, each batch of reactions will include a negative control in which template DNA is omitted from the amplification reaction. Method blanks and DNA extraction blanks from processing of samples are also carried through qPCR in order to verify that no contamination has been introduced. A qPCR positive will be added to each batch of reactions to verify that reactions were mixed correctly to produce amplification of the target. Positive controls used in sample processing prior to qPCR will be carried through qPCR to determine the method's capability to amplify target DNA and to assess any inhibitory effects of matrices.

D.2.6 Quality Control Requirements

D.2.6.1 Field QC Checks

Temperature and condition of containers transporting samples will be checked and samples logged into an electronic spreadsheet dedicated to the project. A field blank will also be included for environmental water samples and processed. Field data sheets will be checked and filed into a logbook. If sample conditions do not meet QA/QC requirements, they will not be processed.

D.2.6.2 Laboratory QC Checks

All work surfaces, equipment surfaces, and tube-holding racks are cleaned with a 10% bleach solution, or DNA Away (commercial product designed to remove nucleic acids). All containers and filter funnels used to process samples are washed with a 10% bleach solution, rinsed three times, and then autoclaved at 121°C for 15 minutes. Forceps used to remove filters from funnels are cleaned with DNA Away before and between samples processed, and then diffed into 90% ethanol and flamed before touching the filter.

Microcentrifuge rotors are cleaned with a 10% bleach solution. Microcentrifuges are designated for pre- and post-PCR procedures. Sterile microcentrifuge tubes, PCR tubes, and barrier pipette tips are used for DNA extraction and qPCR. A designated pipette set for PCR is stored in the PCR-UV hood and not used for any other procedure. It is exposed to UV before each performed qPCR assay. A designated pipette set for DNA extraction is cleaned with DNA Away before each batch of samples processed. All qPCR reagents are commercially purchased and stored in a separate freezer, away from any cultures, stored extracted DNA, or DNA amplicons.

Disposable gloves are worn during all sample processing. During DNA extraction, gloves are changed frequently and fresh gloves are always used in the PCR-UV hood.

All equipment calibrations and inspections will be logged in a QA/QC logbook. Failures will be reported to the lab manager and corrective action taken.

D.2.6.3 Data Analysis QC Checks

Experimental design and raw data are to be recorded in a hard copy in a laboratory notebook that is dedicated to the research project. Recorded data will be reviewed by the lab manager or principal investigator. Data analysis will be reviewed by the principal investigators.

D.2.7 Instrument/Equipment Testing, Inspection, and Maintenance Requirements

Table D-4. Equipment Inspection and Maintenance

Equipment Type	Inspection Frequency	Type of Inspection
Autoclaves	Daily	Check for error codes (under service contract)
	Monthly	Spore ampules tested
Dishwasher	Monthly	Check for error codes, refill detergents
Incubators	Daily	Temperature recorded
Refrigerators	Daily	Temperature recorded
Freezers (-20°C)	Daily	Temperature recorded
Freezers (-80°C)	Daily	Temperature recorded
Water baths	Daily	Temperature recorded
PCR-UV hoods	Weekly	Dust wiped from UV light, Replace bulbs after 1,000 uses or as needed
Thermocyclers	Twice a year	Block temperature measured
Biosafety Cabinets	Once per year	Air flow, gas, vacuum

The laboratory manager is responsible for ensuring maintenance and calibration of equipment.

D.2.8 Inspection/Acceptance Requirements

All supplies and consumables in this project will be inspected and accepted by the principal investigator or laboratory manager if QA/QC requirements are met.

D.2.9 Data Acquisition Requirements

All data will be reviewed by the principal investigators before it is included in reports or publications. Data will be graphed periodically to review trends and spot-check for accuracy.

D.2.10 Data Management

Experimental design and raw data are to be recorded in hard copy in a laboratory notebook that is dedicated to the research project. Recorded data will be reviewed by the laboratory manager and/or principal investigator. Hard copies will be help a minimum of 5 years after the project ends. Records, reports, and data analysis are kept in electronic form and are maintained on the institution's server and are backed up by external electronic storage devices to be held a minimum of 5 years after the project ends. Data recorded will be checked for correctness and completeness, as well as transcription accuracy. Data analysis will be reviewed by the principal investigators.

D.3 ASSESSMENT/OVERSIGHT

D.3.1 Assessment and Response Actions

Assessment of experimental design, raw data, and data analysis will take place as data is collected. Progress reports on data collection and analysis will be made on a bi-weekly basis to the principal investigators. The lab manager or technician will stop progress and report to the principal investigator if deficiencies are found. Final decision on any corrective action will be made by the principal investigators and implemented by the laboratory personnel. The co-PIs hold a bi-weekly teleconference where progress is assessed, and problems or challenges are discussed.

D.3.2 Reports to Management

Quarterly reports will be submitted routinely throughout the project duration. A final project report will be submitted in draft form to the Water Research Foundation Project Advisory Committee for review and comment prior to final report submission

D.4 DATA REVIEW AND EVALUATION

Data Review, Verification, and Validation

The U.S. Environmental Protection Agency (USEPA) has developed guidance on the planning process and on criteria for data acceptability in environmental studies in a document entitled Guidance on Systematic Planning Using the Data Quality Objectives Process (http://www.epa.gov/QUALITY/qs-docs/g4-final.pdf). An important goal of study planning is to ensure that available resources are used to collect the data that will be most useful to answering the questions at hand, and the study design is such that meaningful information will be obtained. Guidelines in the USEPA document will be filled wherever applicable. Data recorded will be checked for correctness and completeness, as well as transcription accuracy. Any data that is questionable due to failure of QA/QC practices will be rejected, and experiments will be repeated. Frequent communication from the level of technician to QA/QC officer to PI will ensure timely review of data and correction of any problems.

Culture guidelines were developed based on two existing standardized protocols: USEPA method 1603 (USEPA, 2002) and WHO Tricycle Protocol (WHO 2021). The selection of the culture media, mTEC, was derived from the USEPA method 1603. Rigorous validation on the media in multiple environmental samples was conducted prior to publishing the standardized method. The media used in this method is also used in US monitoring agencies and is widely accepted by US government agencies. The antibiotic and concentration chosen to supplement the media was derived from the WHO Tricycle Protocol. The antibiotic cefotaxime was added at 4 µg/mL to select for ESBL-producing *E. coli*. This meets the antibiotic resistance criteria presented in both the Clinical and Laboratory Standards Institute (CLSI 2017) and European Committee on Antimicrobial Susceptibility Testing Guidelines (EUCAST 2021).

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (Bustin et al., 2009) as well as the Environmental Microbiology Minimum Information Guidelines (Borchardt et al., 2021) both provide guidance for study planning, quantitative (or digital)PCR result reporting and appropriate controls. An important goal of study planning is to ensure that available resources are used to collect the data that will be most useful to answering the questions at hand, and that the study design is such that meaningful information will be obtained. Work by Liguori et al (2022) can aid in determining the best sampling strategy for AMR monitoring. Data recorded will be checked for correctness and completeness, as well as transcription accuracy. Any data that is questionable due to failure of QA/QC practices will be rejected, and experiments will be repeated. Frequent communication from the level of technician to QA/QC officer to PI will ensure timely review of data and correction of any problems.

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