Supporting Information for

Antimicrobial Resistance Monitoring of Water Environments: A Framework for Standardized Methods and Quality Control

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Summary of contents:
25 pages, 18 figures, 4 tables

1. Supplemental Methods

a. Systematic Literature Reviews

Eight independent systematic literature reviews were conducted at the beginning of this work (September 2020). The targets were: sul1, intI1, tetA, blaCTX-M, vanA, E. coli, Enterococcus, and environmental pathogens. Searches were conducted on Web of Science Core Collection for articles published between 2000 and May 1, 2020 in English, using a tiered literature search strategy. For gene targets, articles were included that met the following criteria: (1) intended to measure antibiotic resistance, (2) focused on surface water, recycled water, and/or wastewater, and (3) employed qPCR to quantify the specific gene target (Keenum et al., 2022). For organism targets, articles were included that met the following criteria: (1) intended to measure or characterize antibiotic resistance, (2) focused on surface water, recycled water, and/or wastewater, and (3) employed culture-based methods, and (4) targeted the organism of interest (Calarco et al., 2022 (in-preparation)).

To identify studies that assessed antibiotic resistance (tier one), a topic search was conducted using the following keywords: “antibiotic resistan*” OR “antimicrobial susceptibility” OR “antimicrobial resistan*” OR “drug resistan*” OR “multi-drug resistan*” OR “resistome” OR "ARG" OR "antibiotic resistan* gene"). Within the publications found in tier one, a second search was conducted to identify studies focusing on the relevant water matrices of interest (tier two) using a topic search with the following keywords: 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") .

For gene targets, within the publications found in tiers one and two, an additional topic search (tier three) was applied to ensure the studies utilized PCR: TS = (“*PCR” OR “polymerase chain reaction” OR “microfluidic PCR” OR “*PCR array”). Finally, in tier four, each gene was searched independently: TS = (“sul1” OR “intI1” OR “vanA” OR “blaCTX-M” OR “tetA”).

For organism targets, within the publications found in tiers one and two, an additional topic search (tier three) was applied to ensure the studies utilized culture methods: 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"). Finally, in tier four, each organism target was searched independently: TS = (“Escherichia coli” OR “E. coli” OR “enterococc*” “Acinetobacter” OR “A. baumannii” OR “Aeromonas” OR “Pseudomonas” OR “P. aeruginosa”).

Literature returned via this search strategy was manually screened by two independent members of the research team to exclude any irrelevant papers. Irrelevant papers included, but were not limited to, those that did not address the specific water environments of interest (ship ballast water, aquaculture operations) or that were designed to detect the presence/absence of a gene or identify a gene after culture enrichment. Any disagreements between the two screeners on relevance were presented to multiple coauthors in order to reach a consensus. Studies retrieved by literature searches and that met eligibility criteria were subjected to extraction of data relating to the parameters outlined in Table S2 (supplementary material). Relevant quantitative data points were extracted from text, figures, and tables manually.

b. Online Expert Survey Design

The survey first captured information about the expert particip ants and the organizations that they represented and assessed their confidence and expertise in environmental AMR monitoring. Participants provided information about any AMR methods currently used, their familiarity with culture, qPCR, and metagenomic 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.

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 a survey with 19 core questions. An additional six 'display' questions, i.e., ones that pop-up only for participants with a relevant answer to the previous question, were incorporated. Institutional Review Board (IRB) evaluation deemed that the study was exempt according to Virginia Tech IRB—20-659.

c. Survey Participants

i. Participant Recruitment

Experts were identified via multiple avenues, with the aim of recruiting individuals who have published research on the topic of antimicrobial resistance as it relates to the water environment or were otherwise familiar with antimicrobial resistance from an environmental perspective and are engaged in relevant governmental or non-governmental initiatives. Particular efforts were made to ensure inclusion of U.S. water industry representatives, e.g., utility representatives that had volunteered to assist with this research sponsored by the U.S. Water Research Foundation. A list was initially compiled of research known by the authors and their professional networks. Attendee lists from relevant professional conferences were compiled, including: The 5th International Meeting on Environmental Dimensions of Antibiotic Resistance (Hong Kong, 2019), the University of North Carolina Water Microbiology Conference (Chapel Hill, NC 2019), and the Gordon Research Conference on Microbiology of the Built Environment (Biddeford, MA 2018), as well as those subscribed to an AMR email listserv of > 100 international experts in environmental aspects of antimicrobial resistance maintained by Dr. Ed Topp (Principal Research Scientist, 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. Postdoctoral researchers were eligible to take the survey, but students were considered to be ineligible. Using this approach, a list of 327 individuals was compiled and emailed the survey.

All individuals received a standardized invitation email and one standardized reminder email afterwards. Survey invitations were sent via email using Qualtrics' embedded Distribution software over a period of 2 months, from October 2020-December 2020. 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.

ii. Participants Completing the Survey

A total of 105 surveys were returned (32% response rate). The majority of participants represented academic institutions or universities (67%, n=70). Thirteen of the participants (12%)

worked for government and/or regulatory organizations, nine participants (8.5%) worked for water or wastewater utilities, seven (6.5%) worked in water engineering and/or consulting, four (4%) worked at research institutes, and one (1%) 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 further assess the participants' relevant fields of expertise, respondents selected from a dropdown list of environments that they specialize in or have worked with, with unlimited selections. Wastewater was the leading environment relevant with the participants' expertise (n=85), 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). Breaking out the "other" by written responses, seawater (n=2), sediments (n=2), groundwater (n=2), wildlife (n=1), coral reef (n=1), stormwater (n=1), and biofilms (n=1) were entered by the experts.

d. Expert Workshop

An expert workshop was hosted virtually on May 18, 20, 25, and 27, 2021, for three-hours each day, using Zoom web-conferencing (Zoom Video Communications, Inc. San Jose, California). Forty nine participants attended at least a portion of the workshop, consisting of 9 representatives of U.S. 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. 43 participants were based in the US, and 6 participants were based in other countries. Key international participants currently involved in standardizing methods for monitoring of antimicrobial resistance in water environments were invited to and participated in the workshop: the co-principal investigators of the Establishing a Monitoring Baseline for Antimicrobial Resistance in Key Environments (EMBARK) project[1], which is funded by The Joint Programming Initiative on Antimicrobial Resistance (JPIAMR), a representative of the WHO Tricycle initiative involved in the standardization of ESBL *E. coli*, and a representative of the Global Water Research Consortium on AMR. Based on a poll conducted on day 1, in which participants were asked to self-rank their familiarity with objectives and methods for AMR surveillance: 22.2% of respondents self-identified as experts, 48.1% as very familiar, 22.2% somewhat familiar, and 7.4% beginner. No participants selected "not at all".

Two weeks before the workshop, participants were provided with the expert survey results, systematic literature reviews of the candidate AMR monitoring targets (Draft systematic review manuscripts based on Web of Science searches spanning 2020-2019), and draft standard operating procedures (SOPs) for proposed standard methods compiled by the co-authors. The targets selected for the literature reviews and draft SOPs were chosen based on the expert survey: *Escherichia coli*, *Enterococcus* spp., *Pseudomonas aeruginosa*, *Aeromonas* spp., *Acinetobacter* spp., *sul*1, *int*I1, *tet*A, *van*A, and *bla*CTX-M. A literature review of studies incorporating metagenomics for environmental AMR monitoring was also provided. The workshop consisted of

presentations by the WRF Project 5052 team members, invited external presentations, interactive break-out sessions, plenary discussions, and a panel discussion.

Break-out groups focused on five topics: (1) What key questions can be answered by surveillance of AMR in water and wastewater systems? (2) What are the advantages and challenges to fecal indicator bacteria (i.e., *E. coli* and *Enterococcus*) as culture-based AMR 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 antibiotic resistance gene (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 sessions were followed up with polls that allowed participants to rank targets and methods that are most relevant and practical for addressing specific AMR monitoring objectives, based on their expertise and experience in the group discussions. End-of-day surveys were carried out each day in which participants were asked to self-rank their familiarity with the topics covered in that session. In general, end-of-day surveys received around a 50% response rate.

2. Supplemental Results- Expert Survey

a. Assessing Survey Participant Background

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 worked with wastewater and surface water. When split out by continent of their organization, similar patterns were observed (**Figure SI 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.

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

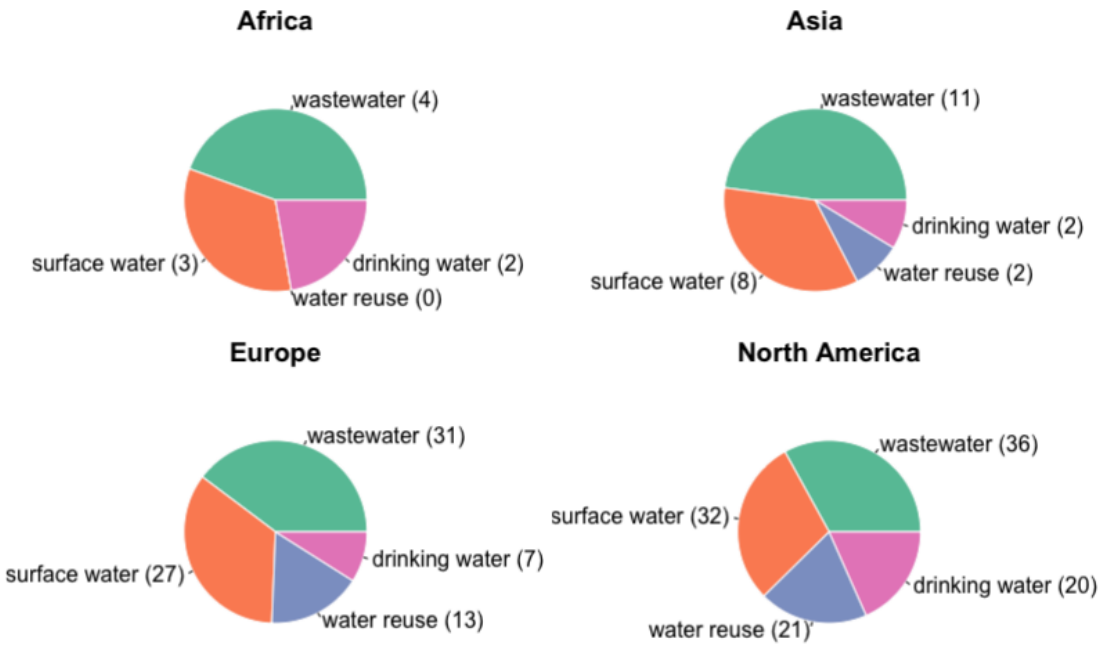

Figure SI 1: *Water environments which participants reported that they were engaged in monitoring, sub-analyzed by continent*

Survey participants were asked to self-rank themselves on familiarity with laboratory methods on AMR monitoring (**Table SI 1**).

Table SI 1: *Self-reported Ranking of Familiarity with AMR monitoring Laboratory Methods, by Organization Type of Respondent*[1]

Organization Type	Familiarity Ranking
Academic/University	4.43
Research Institute	4.75
Government	3.79
Water Utility	3.33
Water Engineering/Consulting	3.14
Other	2.00

[1]*group mean, from individual 1-5 rankings, with 1 being basic awareness of literature and 5 being someone who regularly carries out AMR monitoring/testing*

Survey participants were asked questions specific to eight (8) methodologies relevant to AMR monitoring in water environments **(Table SI 2)**: PCR, qPCR, ddPCR, commercialized rapid MPN-based culture methods (i.e., IDEXX, Westbrook, ME), metagenomics, qPCR array or microfluidic qPCR, membrane filtration, and culturing fecal coliforms.

Table SI 2: *Participants Reporting Current Use of Various Methodologies Relevant to AMR Monitoring of Water Environments*[1]

Method	Which of these are currently implemented by you/your lab/your organization? (counts)	Which of these methods do you outsource (i.e., send to external commercial labs) for analysis? (counts)
PCR	78	4
qPCR	85	10
ddPCR	30	8
IDEXX	32	3
Metagenomics	53	66
qPCR array or microfluidic qPCR	21	22
Membrane Filtration	79	4
Fecal Coliforms	78	4

[1]Multiple choice matrix table question with multiple selections available.

b. Assessing Preferences for Targets for Monitoring AMR in Water Environments

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 in response to the question if they have been tested in their labs within the last 12 months and were also 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 SI 2**).

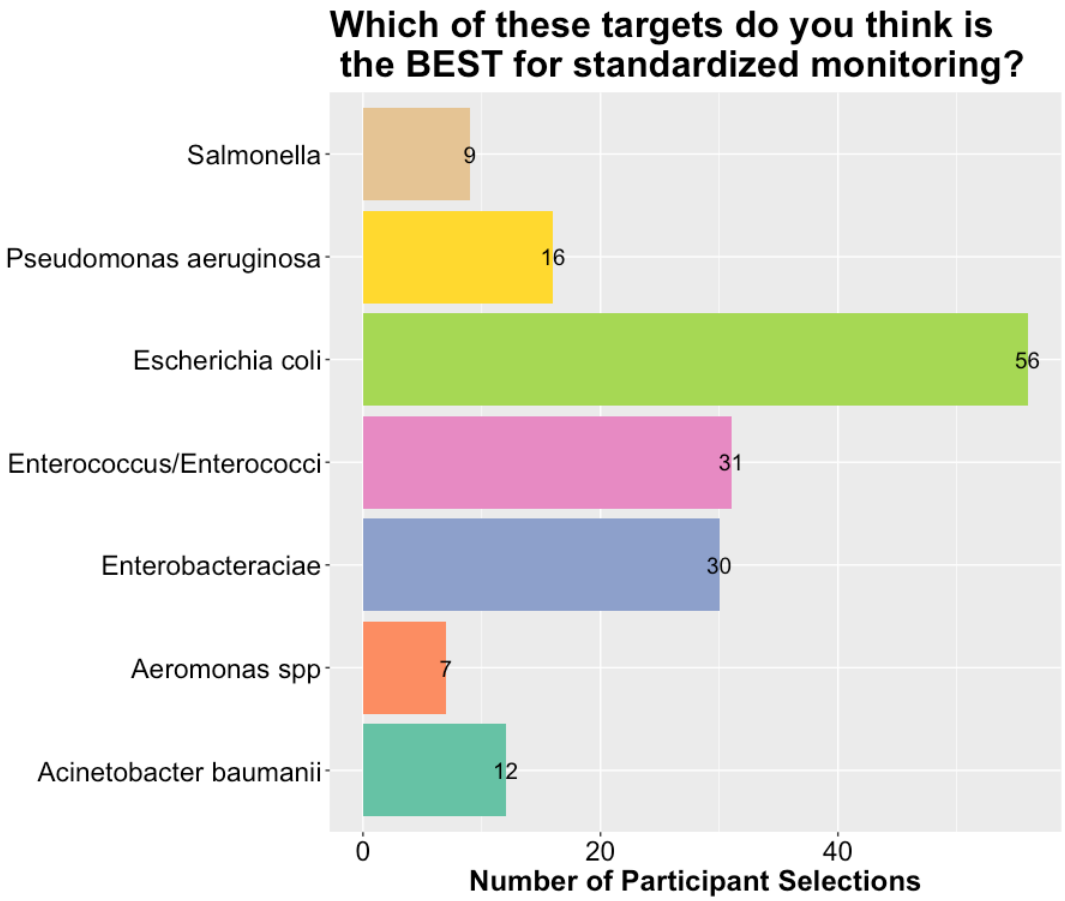

Figure SI 2: *Preferred target bacteria for standardized culture-based monitoring of AMR of water environments. Participants could select up to two targets.*

Eighty-nine survey respondents (85%) reported that they had an understanding of qPCR. The characteristics ranked most important for monitoring by experts were genes reported to occur in human pathogens and ARGs with clinical relevance. Fifty-four respondents (51%) reported that they are 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 SI 3**.

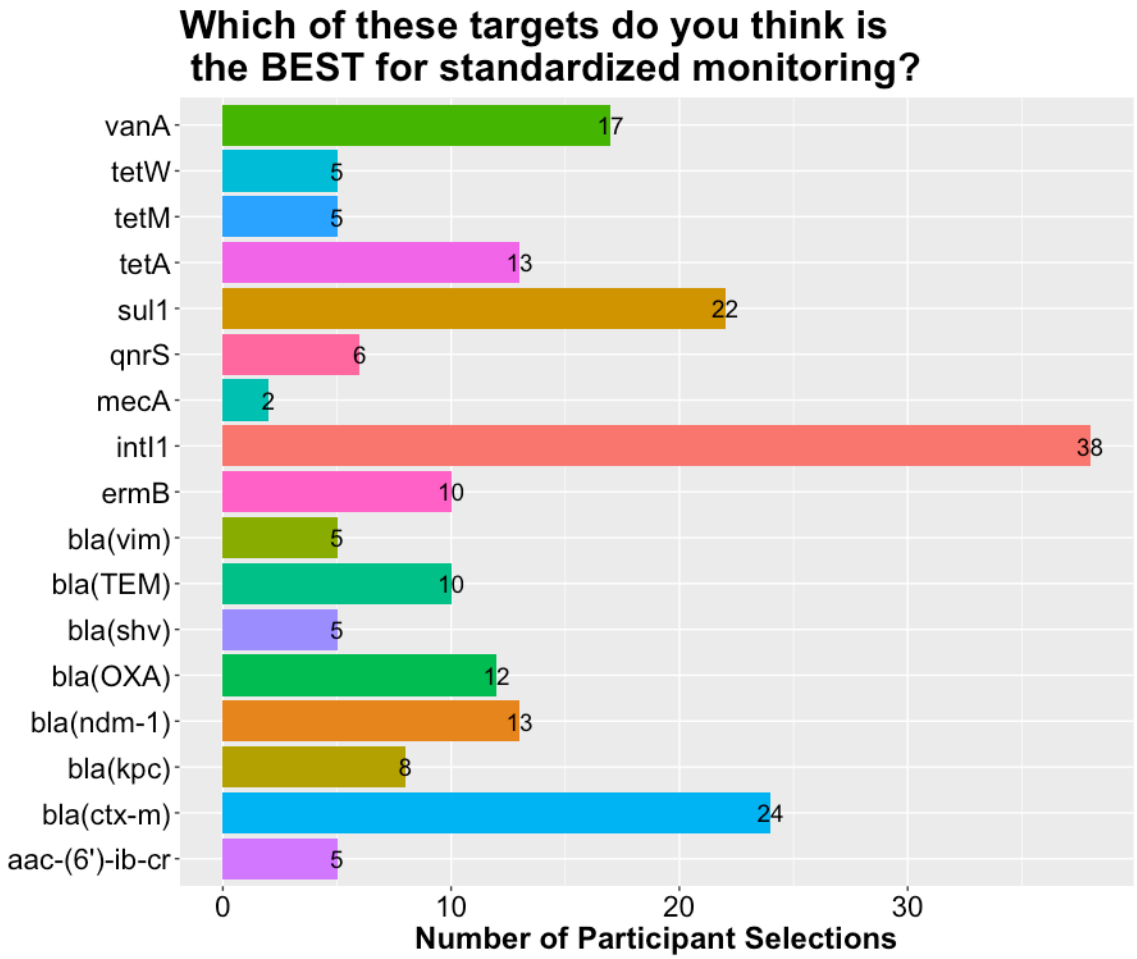

Figure SI 3: *Preferred target genes for qPCR-based monitoring of AMR of water environments. Participants could select up to three targets.*

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

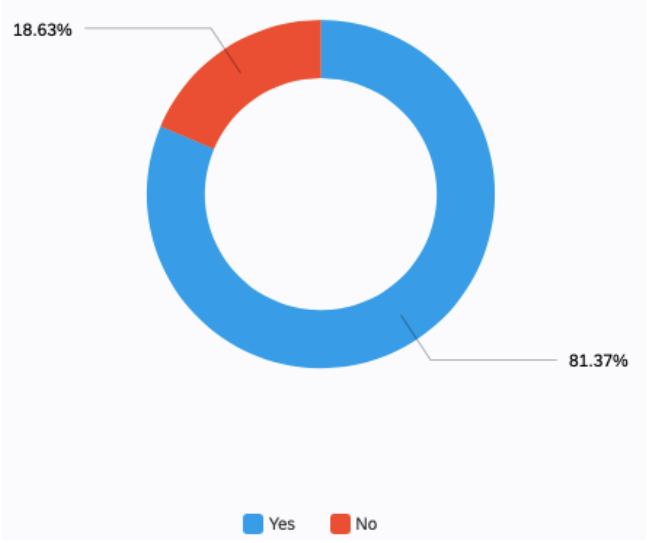

Figure SI 4: *Experts' and/or their organizations familiarity with next-generation sequencing data analysis*

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 SI 3**) 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 SI 3: *Experts' Ranked Preference*

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

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 SI 5**).

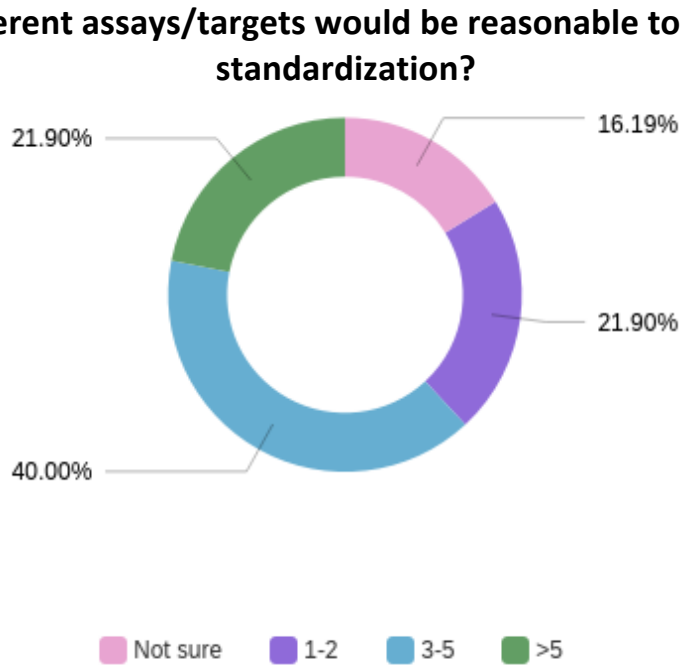

Figure SI 5: *Experts' recommendations regarding the number of targets that is reasonable to recommend for AMR monitoring of water environments. Participants could select from four bins: 1-2, 3-5, >5, or not sure.*

The survey further queried how much turnaround time is acceptable for AMR monitoring methods, and found that many didn't see turnaround time as a barrier (26 selections for "not a concern"), 26 participants thought 3 days was appropriate, and 23 thought that 1-day was appropriate (**Figure SI 6**).

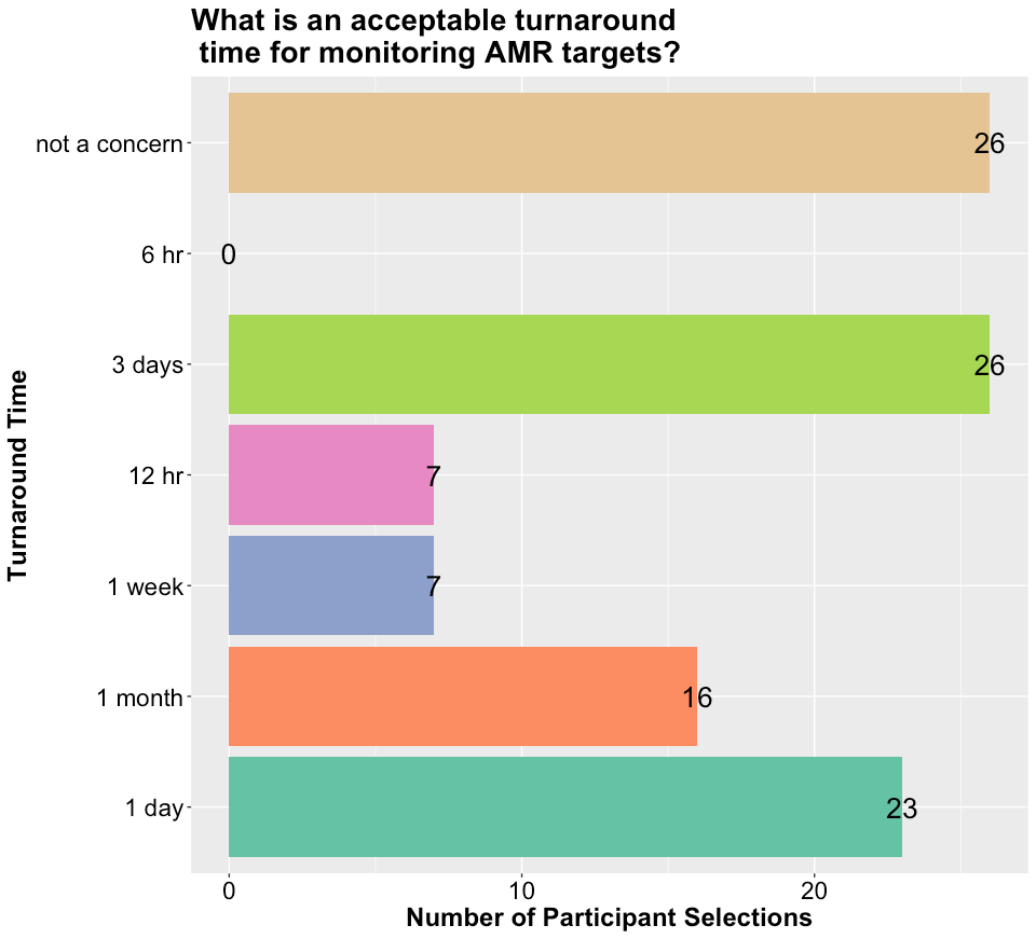

Figure SI 6: *Expert survey results regarding acceptable turnaround times for AMR monitoring methods.*

Participants were asked about reasonable costs per sample for an assay. Nine (9) indicated that $3/sample was reasonable, 27 selected $10/sample, 23 selected $25/sample, 23 selected $50/sample, 17 selected $100/sample, and 3 selected $300/sample or more.

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 SI 7)**. Timeliness of results, low technical skill requirement, and precedence of a standardized method tended to rank lower.

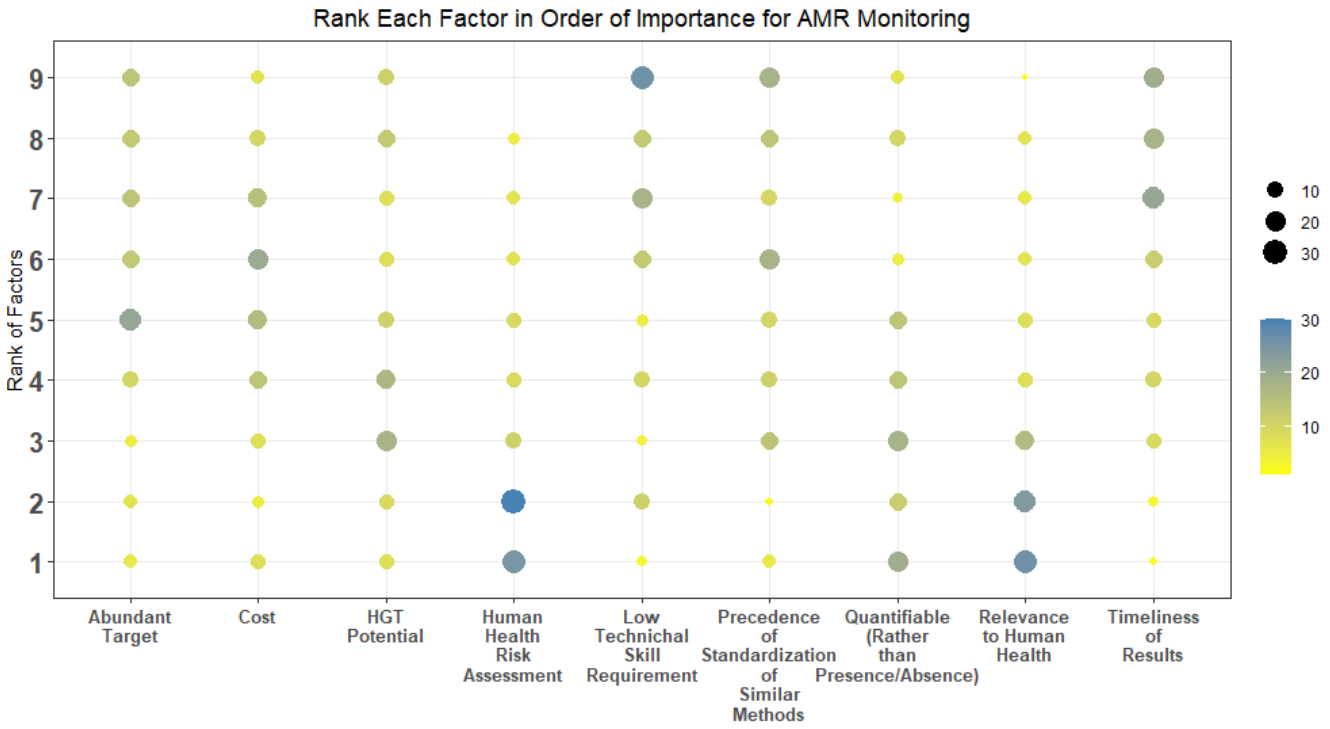

Figure SI 7: *Expert ranking of factors of importance for AMR monitoring of water environments, with 1 being most important and 9 being least important. The color scale indicates number of respondents selecting the indicated ranking (y-axis) for each factor (x-axis).*

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, difficulty selecting meaningful targets, matrix interference, inability to identify host of gene, general lack of information, legislation, and a lack of standardization.

3. Supplemental Results- Expert Workshop

a. Goals of Monitoring

On day 1, following sessions on the purpose of monitoring, a poll was conducted asking participants (n=45) for their initial thoughts on which monitoring practices were most feasible, most informative, and which were the best combination of feasible and informative **(Figure SI 8)**. The participants indicated that qPCR was the likely the most feasible for monitoring by U.S. water utilities, followed by culture, metagenomics, and "other". On which is the most informative, the majority of participants selected metagenomics, followed by culture, then qPCR, then "other". When asked to select one method that balances both feasibility and information yielded, qPCR was selected by a majority, followed by culture, then other, then metagenomics.

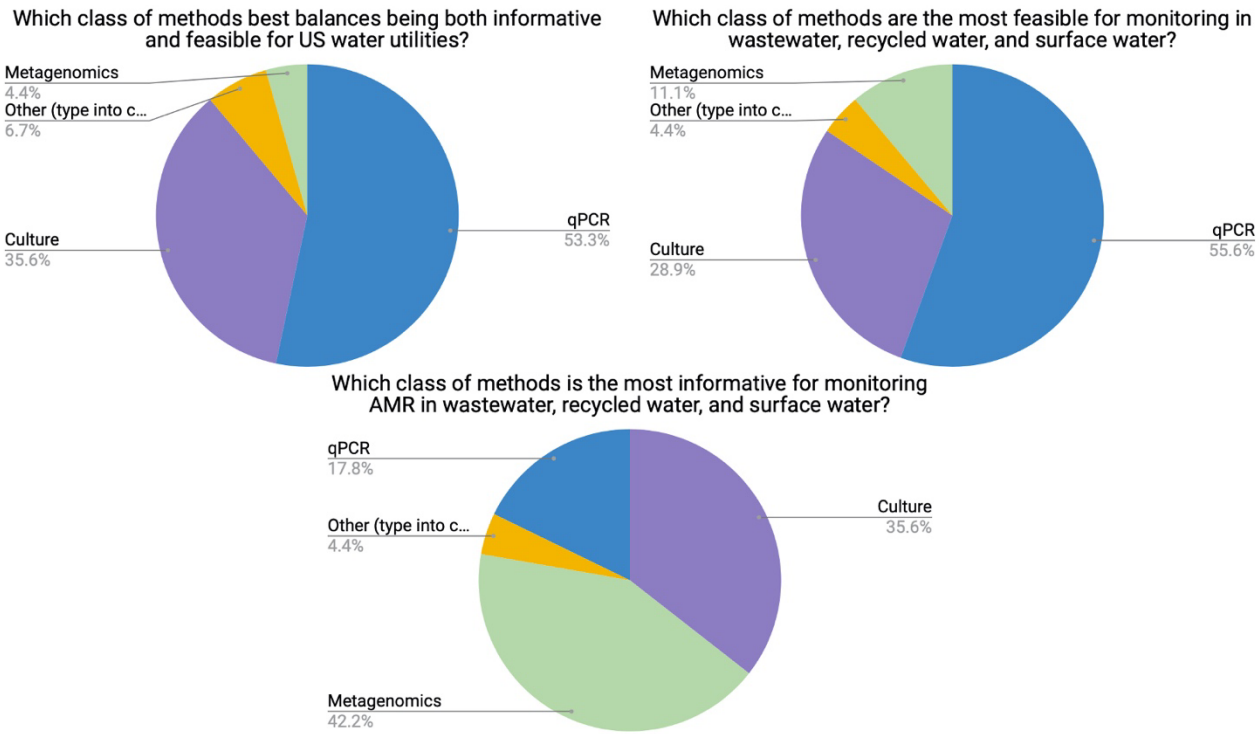

Figure SI 8: *Workshop participant poll results on feasibility and informative nature of method categories for AMR monitoring of water environments.*

b. Workshop Sessions on Culture-Based Methods

During the end-of-day survey, participants (n=27) were asked to self-rank their familiarity with *E. coli* as a monitoring target, *Enterococcus* as a monitoring target, and environmentally-relevant culture bacteria as targets (**Table SI 4**).

Table SI 4: *Participant (n=24) self-reported familiarity with the culture-based methods discussed that day at workshop*

The topic of "_" as an AMR Monitoring Target:	Expert	Very Familiar	Somewhat Familiar	Beginner	Not at all familiar
E. coli	3	11	11	11	0
Enterococcus spp.	0	11	9	9	0
environmentally-relevant organisms	0	9	12	12	0

Breakout sessions were productive for discussing each draft SOP (*E. coli*, *Enterococcus spp.*, environmentally-relevant organisms). Participants observed that utilities are very comfortable running culturable *E. coli* assays, but not PCR or qPCR for confirmation or ARG detection. However, it is becoming more common for water utilities to be equipped with qPCR, and some participants viewed that recommendation of culture for monitoring would be a step

backwards. Feasibility of in-house versus out-sourced analysis was discussed. For example, water utilities are widely capable of culturing fecal indictors, but not subsequent testing of isolates, which could be sent to certified labs for susceptibility testing, genotypic testing, or whole genome sequencing. DNA extraction is also fairly low tech, and it is conceivable that utilities could extract DNA in house and send elsewhere for metagenomic sequencing or qPCR analysis.

Breakout groups discussed the question of which culture target with strong niches for regrowth in the environment is the best candidate. Many participants preferred *Pseudomonas* as the easiest to grow and with high enough occurring abundance to capture, as well as its clinical-relevance. It was suggested that a baseline will need to be developed, whether for pseudomonas or another environmental organism, to act as a comparison point. This was suggested via development of a naturally occurring environmental baseline, or via the use of upstream and downstream sampling in each surface water region.

In terms of participant assessments of the proposed SOPs, details were requested to be added, such as the temperature and media used for storage, and how long samples can be stored at given temperatures. It was also suggested that while the mEI agar works well for wastewater, it may require additional steps for success with environmental water samples. Some participants had concerns about how specific any PCR confirmation assays could be, given the specificity of available gene targets for *Enterococcus*. Similarly, for *E. coli* there are questions around which gene targets would be most applicable as monitoring targets, as well as how geographically specific these genes are. In addition, feedback suggested that the existing draft method may have too much potential for false positives, and setting a Cq value criterion might be key to reduce these. On the sampling end of the protocol, participants expressed concern about when and where to sample in order to get samples that are both representative of the water environment and data that is comparable to other sites. Pipe type, temperature, time of day, season, and other factors may impact resulting data measurements and should be considered and factored into a decision tree.

For the optional disk diffusion section of the proposed SOP, participants asked for a ranking of antibiotics in order of importance. With a ranked system, users could select the top antibiotics to test for, with the number tested selected according to budgetary and labor restrictions at their site, as opposed to suggesting a list of nine antibiotics which requires about twice the amount of plates and labor, and is likely excessive for most purposes.

A poll was conducted (n= 35) following sessions on culture-based techniques 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, *Enterococcus* as the second choice, and *P. aeruginosa* as the third choice (**Figure SI 9**).

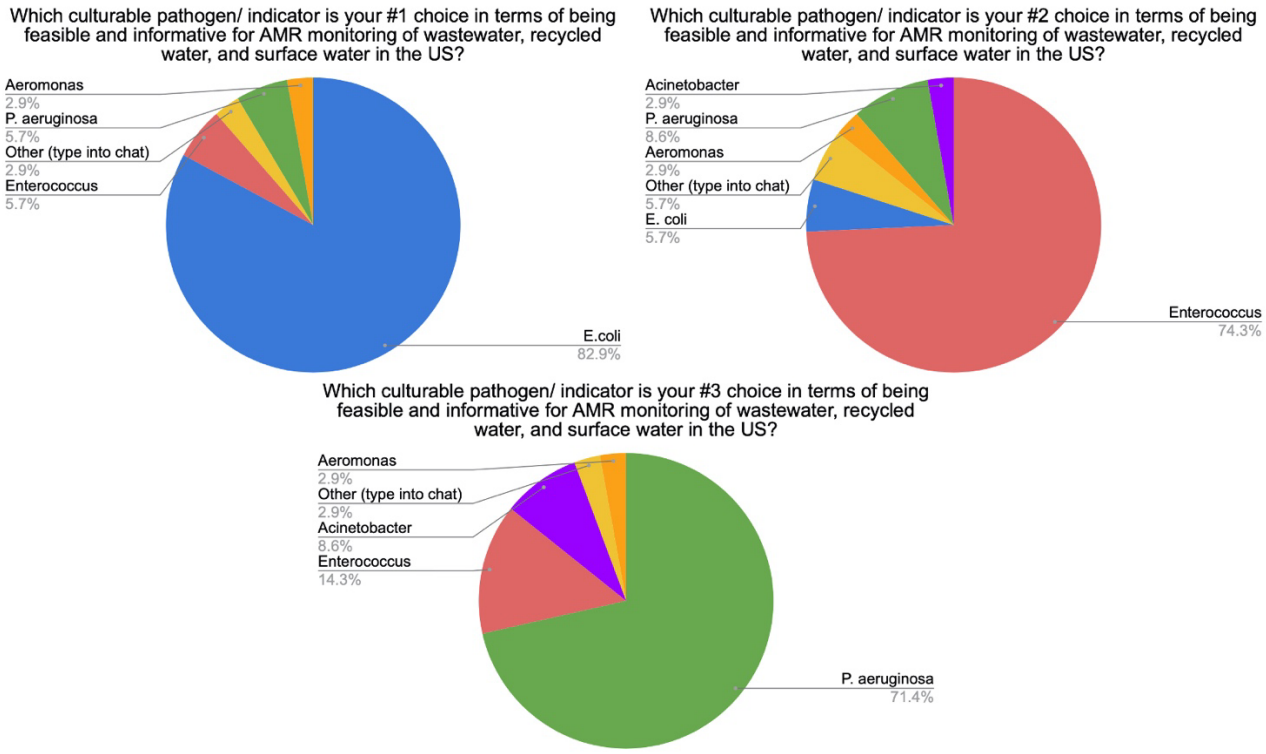

Figure SI 9: *Ranking of culture targets for AMR monitoring of water environments based on poll conducted following sessions focused on culture-based methods*

c. <u>qPCR Session</u>

During the end-of-day survey, participants (n=24) were asked to self-rank their familiarity with qPCR-based AMR monitoring. 12.5% of participants identified themselves as an expert, a majority chose very familiar (54.2%), followed by somewhat familiar (25%), and finally beginner (8.3%). Participants (n= 11) were also asked to self-rank their familiarity with high throughput qPCR (HTqPCR) and digital droplet qPCR (ddPCR). Only 9.2% identified as expert, 27.3% chose very familiar, 36.4% chose somewhat familiar, followed by beginner (18.2%), and not at all, at (9.1%).

Participants commented that calculations surrounding the recovery efficiency, limit of detection, equivalent sample volume, and relative abundance are important to include and report. Though, it was suggested not to correct for recovery efficiency. Normalization approaches vary amongst experts, as some normalize to 16S rRNA (as proposed in the original draft SOP), others normalize rpoB, and some would prefer no normalization. In terms of calculating the limit of detection (LOD) and limit of quantification (LOQ), some would prefer a more statistical approach. It was noted that the measurement of LOD/LOQ will require an entirely different method for ddPCR as opposed to qPCR. This information, as well as discussion on the differences between ddPCR and qPCR, syber and probe assays, types of standards, and more were requested to be components of the final guidance document.

Throughout breakout session groups, *bla*CTX-m, *sul*1, and *van*A were the highest ranked choices for a monitoring priority. Participants singled out *bla*CTX-m as the most significant for indicating health risks, and *sul*1 and *intI*1 as the most significant for measuring treatment removal rates. Many agreed that aligning efforts with CDC, FDA, and NARMs would be beneficial.

Inhibition is a key consideration for a method intended for national uptake, as water chemistry, target occurrence levels, and equipment are highly variable across the U.S. Participants agreed that an inhibition assessment should be included in the standard method developed. Many recommend using the salmon testes DNA assay in EPA Method 1611[2]. Though, there is some concern with the salmon DNA interacting with future metagenomics analysis, which may favor the use of a NIST eukaryotic DNA standard[3].

On the third workshop day, a poll was conducted to follow up discussion on qPCR targets for AMR monitoring. The poll asked participants (n=33) to rank their top 3 qPCR monitoring targets based on feasibility and how informative they are for wastewater, recycled water, and surface water in the US. A strong majority ranked *intI*1 as first choice, then the rankings became more divided for second and third choice (**Figure SI 10**). 36.4% of ranked *sul*1 as the second choice, followed closely by *van*A (27.3%), *bla*CTX-M (15.2%), *tet*A (12.1%), *intI*1 (6.1%), and "Other" (3%). For third choice qPCR target, *bla*CTX-M received a clearer majority at 36.4%, followed by *van*A (18.2%), *sul*1 (15.2%), *intI*1 (12.1%), *tet*A (9.1%), and "Other" (9.1%).

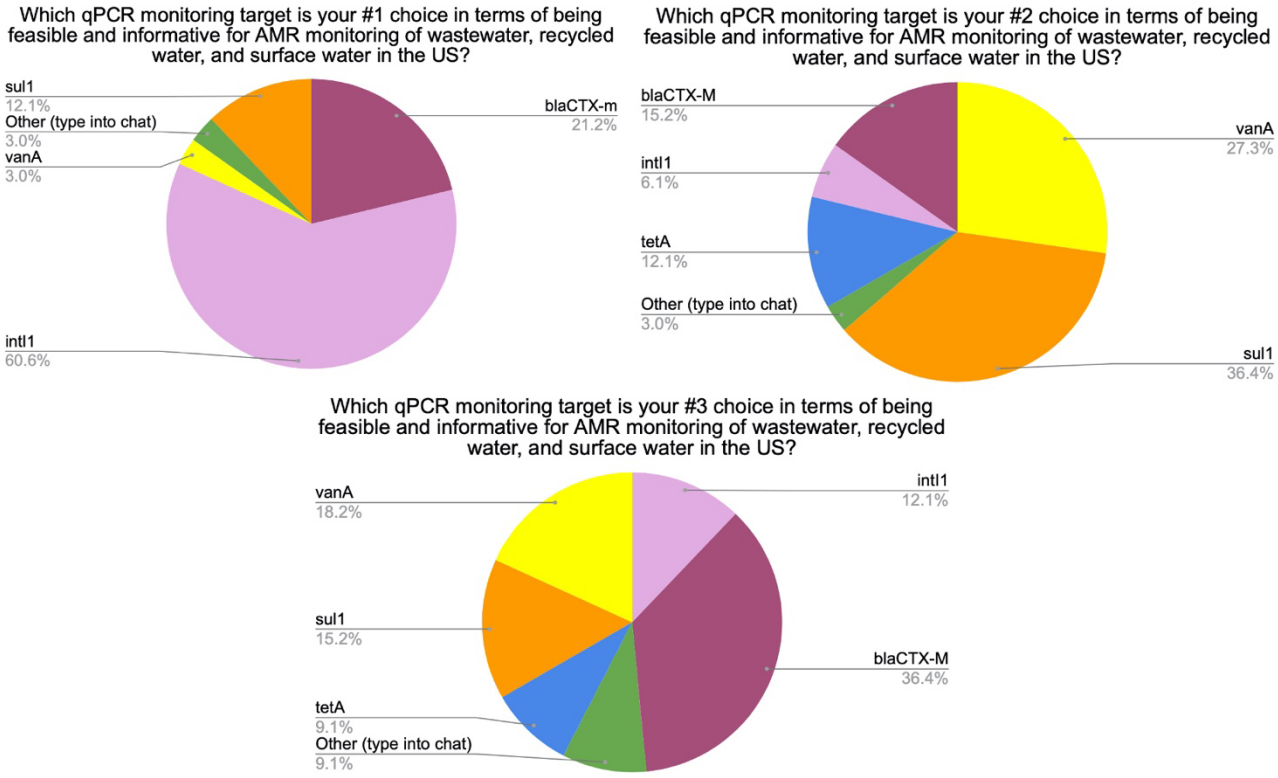

Figure SI 10: *Ranking of qPCR targets for AMR monitoring of water environments based on poll conducted following sessions focused on q-PCR-based methods*

d. Metagenomics Session

During the end-of-day survey, participants (n=11) were asked to self-rank their familiarity with metagenomics. 18.2% self-identified as an expert, 27.3% very familiar, 45.5% somewhat familiar, and 9.1% beginner.

Breakout session feedback indicated that participants were hopeful and optimistic about future uses for metagenomics in the context of AMR monitoring and standardized methods. Many participants judged themselves or their organizations to be under-prepared at the moment to

conduct comparable and meaningful metagenomics work. The need for further research and standardization was widely recognized. However, despite impressions that metagenomics is the least developed/standardized method, participants remained strong in the opinion that it is the most promising for AMR research and should be a focus of standardization efforts. Feedback indicates that metagenomics is viewed as the best tool for surveying water samples for numerous targets, instead of narrowing down on select targets. This could be an asset in risk assessments, helping to find indicators for ARG abundance/diversity or predicting where resistance may emerge in the future. In addition to the challenge of standardization and comparability of data, participants expressed concern about the price point of metagenomics sequencing currently and the inaccessibility that results for many communities.

At the end of the metagenomics sessions, a poll was conducted on attitudes, preferences, and perceptions in regards to metagenomics for AMR monitoring. First, participants (n = 27) were asked to select their preferred normalization method (**Figure SI 11**). 40.7% of respondents chose "don't know," 22.2% 16S rRNA, 14.8% "a collection of single copy genes," 7.4% FPKM, and the remaining options each received 3.7% of selections: TPM, *rpoB* gene, None, and Other.

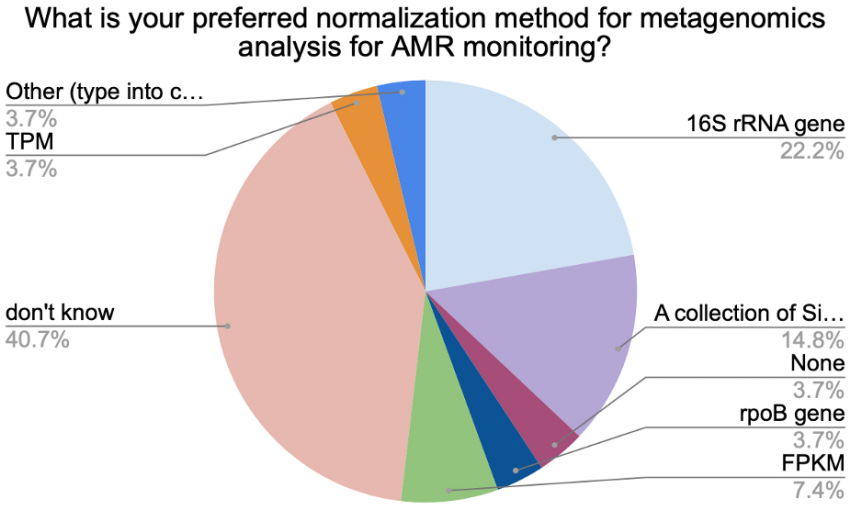

Figure SI 11: *Participant poll of recommended normalization methods for metagenomic-based monitoring of AMR*

Next, participants (n=27) were asked to estimate when they think the US water sector will be ready to implement standard methods for AMR monitoring. 40.7% of respondents chose 10 years, followed by 5 years (33.3%), 3 years (14.8%), now (7.4%), and never (3.7%).

Participants (n=27) were asked which metagenomics metric is the most informative for AMR monitoring in water environments. The top selection was a three-way tie (22.2% each) between comparative index of co-occurrence of ARG/MGE/pathogen markers on same contig, total clinically-relevant ARGs, and total ARGs. "Other" was selected by 18.5% of respondents, followed by emerging/newly evolved ARGs (7.4%) and don't know (7.4%).

Lastly, participants were asked if their organization currently conducts metagenomics and if their organization has any plans to conduct metagenomics in the next 3 years. To the first, 74.1% responded Yes, their organization/group is conducting metagenomic analysis (25.9% no). To the latter question, a slight increase of 77.8% responded that their organization/group is planning to

implement metagenomics in the near future, representing just one response indicating that their organization intends to begin metagenomics.

e. US Water Utility Perspectives

During the final survey, administered following the session on water utilities, participants (n= 28) were asked to self-rank their familiarity with water utility perspectives on AMR monitoring. 50% responded very familiar, 35.7% somewhat familiar, 10.7% beginner, and 3.6% expert.

Discussion with respect to how to best involve water utilities in AMR monitoring in the future tended to either focus on barriers to AMR monitoring or general things to consider. Some barriers discussed include lack of standard methods, lack of responsible party for interpretation of results, lack of regulatory drivers, lack of trained staff and available equipment, costs, lack of buy-in from upper management, lack of political will, and the need for strong justification for such a monitoring program. It was recommended that these methods begin with a focus on larger WWTPs and plants who are engaged in water reuse. Among discussion topics categorized as things to consider, were: resolution, reporting, feasibility, how it fits into the public health system, public messaging (in absence of public messaging: FOIA), synergy with existing activities, and comparability of data across utilities.

Questions, concerns, ideas, and comments regarding how utilities may or may not fit into this system were common throughout all breakout groups and sessions. For example, the need to demonstrate a value provided by AMR monitoring was a recurring concern for participants. 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, there was recognition of the need to demonstrate that gene removal relates to human health impacts. One participant suggested that a kit be developed; allowing utilities to easily and conveniently follow the standard method with instructions and materials built in.

Participants (n=27) were asked which objective would be most informative and useful for US water utilities **(Figure SI 12**); 37% chose wastewater-based epidemiology, followed by quantifying removal of AMR through wastewater/recycled water (29.6%), identifying types of AMR of clinical concern that might escape treatment (25.9%), and “other). For Other, answers were written in: “determining if conditions during treatment or in receiving environments are conducive to evolution of new forms of AMR” and “1, 2, 3 are all relevant.”

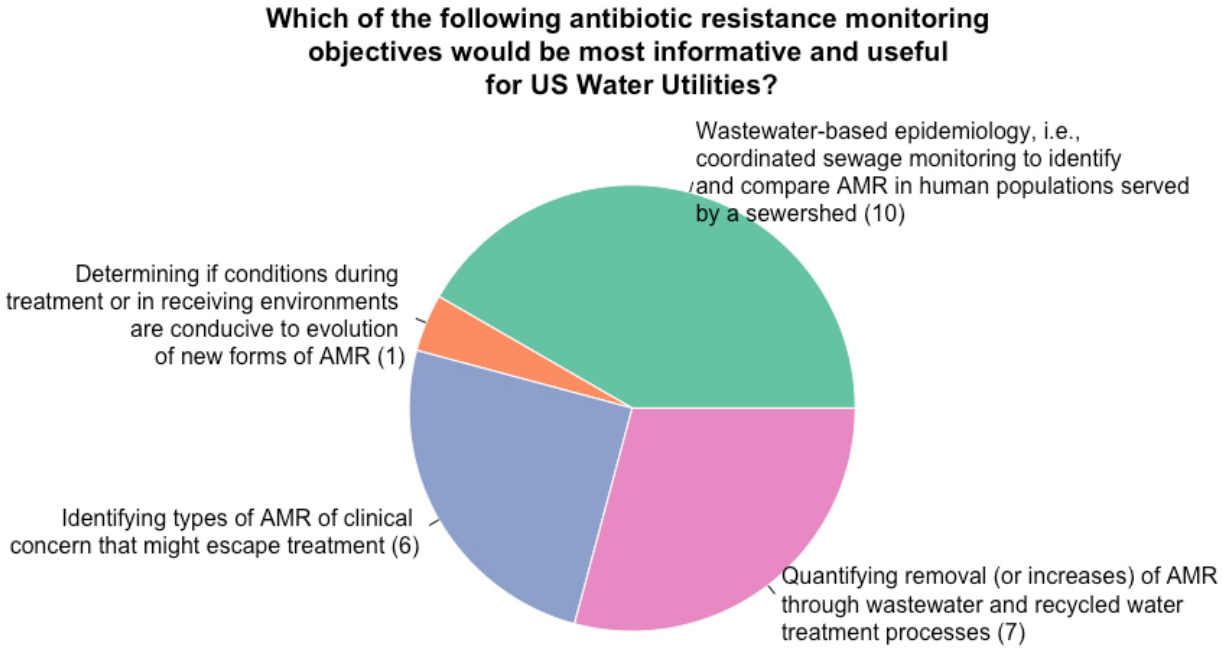

Figure SI 12: *Participant survey results following session focused on US water utility perspectives, Day 4*

The survey asked (n=26) if any methods should be categorically 'ruled out' for use in US water utilities for the monitoring of AMR. The majority responded "None" (65.4%), followed by metagenomics (26.9%), whole genome sequencing (19.2%), HTqPCR (19.2%), ddPCR (7.7%), and culturing resistant fecal indicators (3.8%).

f. Overall themes, knowledge gaps that need addressed to move forwards

Overall, there is and has been immense agreement amongst experts that standardization is necessary; the lack of standard methods are holding back the water environment from making further leaps in understanding the occurrence, adaptation, spread, and exposure risks associated with ARGs and antibiotic resistant organisms. One aim of the Water Research Foundation Project (Project 5052) sponsoring the survey and workshop is to establish an open-access database for researchers across the globe, to upload and download data from different regions, water types, and contamination levels. These efforts will facilitate public health assessments ensuring that data are comparable across databases due to the use of standard methods. With these data, exposure pathways can be assessed, risks associated with wastewater exposure evaluated, and any links between wastewater occurrence and community disease incidence (as done with COVID-19) can be determined. In addition to this, more basic-science research would benefit the field. The mechanism behind hypothesized and observed health risks are a key part of human health risk assessments, and more effort should be spent on determining detrimental health effects in controlled laboratory experiments.

Not only are human health risks fundamental, but ecological risks and risk for emergence of new resistance types are crucial to AMR research. What is the environmental impact of not only the selection pressures, but the resulting changes in microbiomes and organisms alike? Are there impacts of these resistome changes on the food chain, and if so, how will these impacts affect species in the future as we anticipate rapid growth in antibiotic resistance in the environment? In

order to look ahead, we must understand the risk factors that drive emergence of new resistance genes, bacteria, and drug-resistant pathogens. The risks of emergent drug-resistant pathogens should be accounted for in all risk assessments that consider AMR in the environment.

At the end of the workshop, the final survey polled participants for their opinions and preferences on various topics and questions, with the option for open-ended feedback as well. One question asked (**Figure SI 13**), if you/your organization were to conduct AMR monitoring in the environment, what would the primary goal be? Results (n=27) indicate that 63% would aim to inform human health risk, followed by public health monitoring (51.9%), assess potential for treatment processes to remove AMR (44.4%), assess potential for evolution and spread of AMR (29.6%), basic research (29.6%), informing policy (22.2%), and "other" (3.7%).

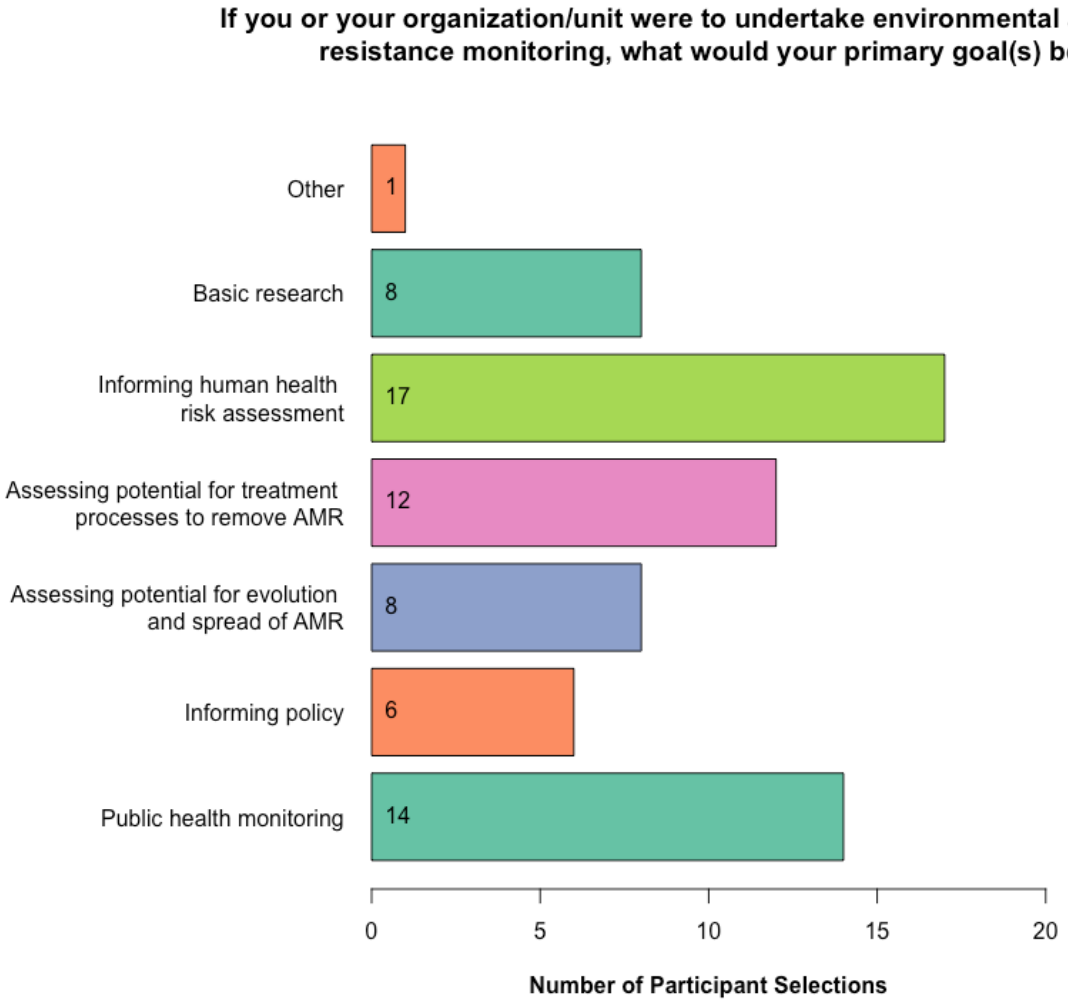

Figure SI 13: *Final survey results from Expert Workshop, Day 4*

Another asked, 'If you/your organization were to initiate AMR monitoring next year, what methods would you propose?' (**Figure SI 14**). Results indicated that 56% would propose qPCR methods, metagenomics at 52%, then ddPCR (48%), culture resistant fecal indicators (48%), culture resistant bacteria (32%), whole genome sequencings (20%), HTPCR (12%), and Other (8%).

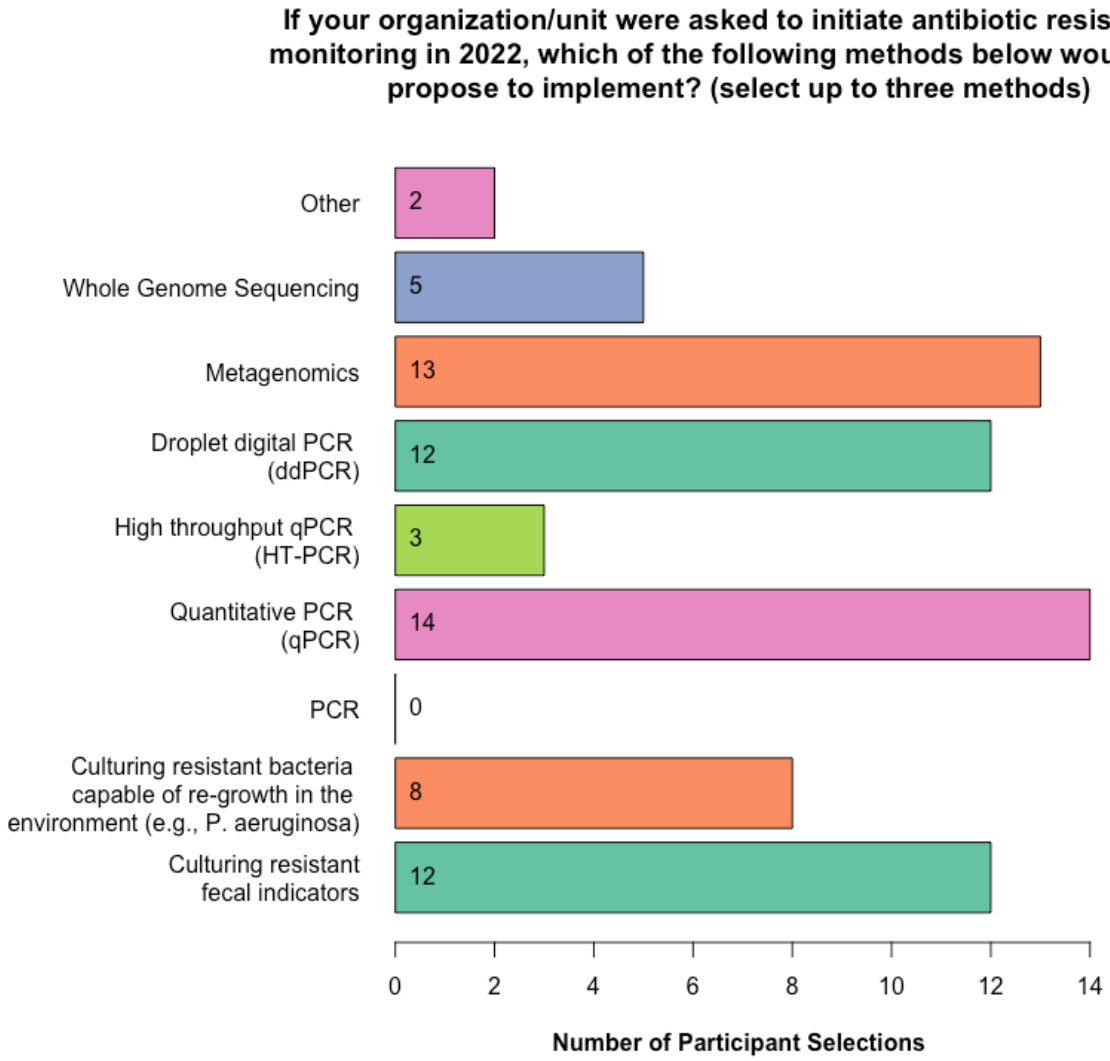

Figure SI 14: *Final survey results from Expert Workshop, Day 4*

Final rankings of AMR monitoring targets were captured from participants on the last day (n=26). First, participants were asked to rank the targets for the best option to monitor AMR in wastewater-based epidemiology. The top three were metagenomics (38.5%), resistant *E. coli* (26.9%), and *intI*1 (11.5%) (**Figure SI 15**).

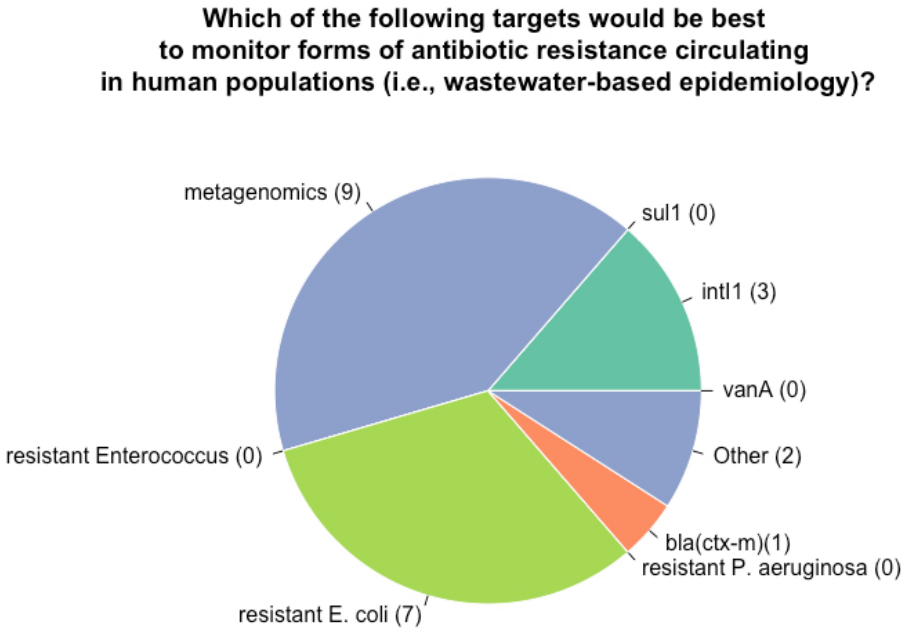

Figure SI 15: *Final workshop poll results reflecting rankings of monitoring targets for the purpose of wastewater-based epidemiology.*

Next, participants were asked to rank the targets for the best option for the reduction of public health risks associated with wastewater exposures. Resistant *E. coli* received a clear consensus ranking at 50% of selections, followed by *intI*1 (16.7%) and metagenomics (8.3%) (**Figure SI 16**).

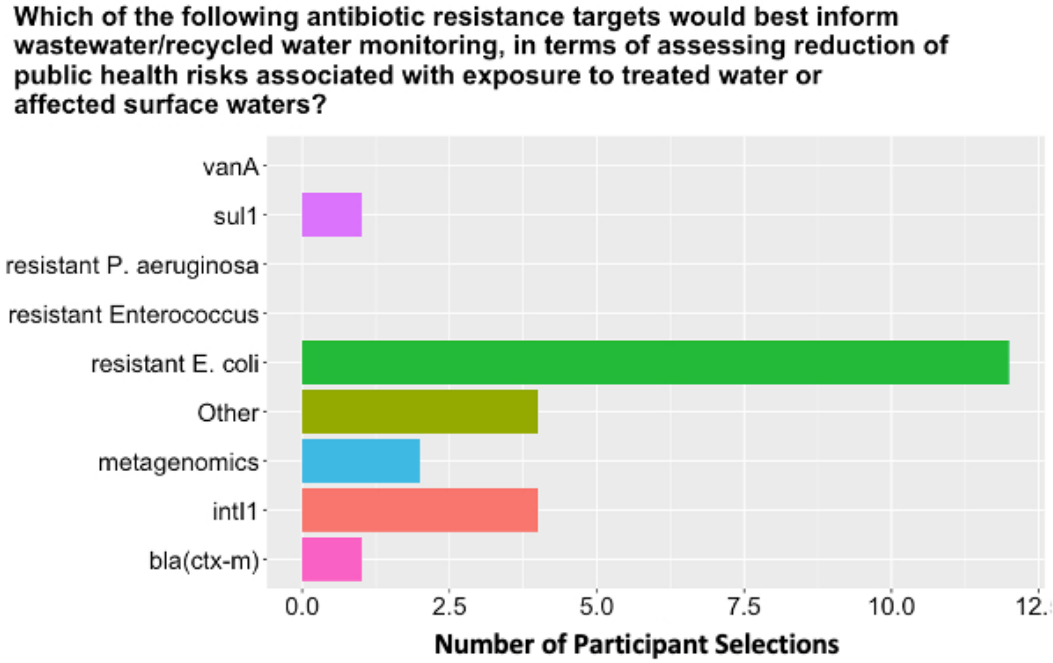

Figure SI 16: *Final workshop poll results reflecting selections of monitoring targets for the purpose of demonstrating that wastewater/recycled water treatment processes reduce public health risks*

Finally, participants were asked which target best informs water monitoring in terms of reducing potential for evolution and spread of AMR among bacteria (**Figure SI 17**). This question received slightly less consensus, with 30.4% selecting metagenomics and another 30.4% selecting *intI*1. Next, resistant *E. coli* received 13% of selections and resistant enterococcus received 8.7%.

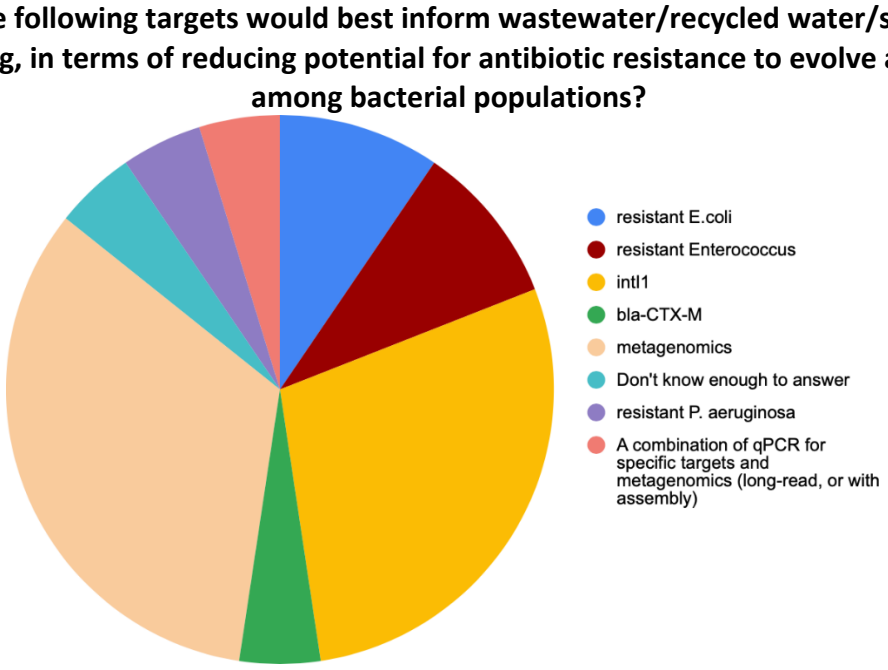

Figure SI 17: *Final workshop survey results reflecting rankings of monitoring targets for the purpose of demonstrating that wastewater/recycled water treatment processes reduce public health risks*

g. Usefulness of this Workshop

As the final question in the final survey, participants were asked to rank from 1 to 5 (with 1 being not productive and 5 being most productive) how productive the WRF 5052 Expert Workshop on AMR Monitoring in Water was towards identifying suitable antibiotic resistance monitoring targets for the US water industry. The results were left skewed, with 50% of respondents ranking a 5 out of 5, 33.3% ranking a 4 out of 5, and 16.7% ranking a 3 out of 5 (**Figure SI 18**).

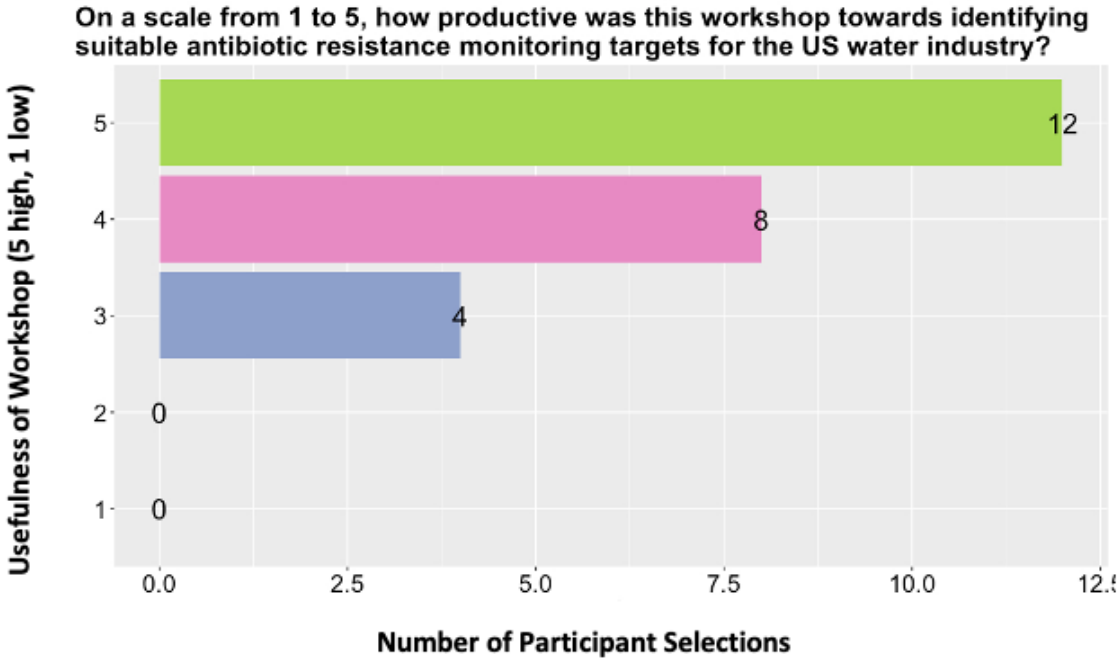

Figure SI 18: *Poll results on the usefulness of the workshop for identifying suitable AMR targets. 5 was the most productive and 1 was the least productive.*

4. References

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