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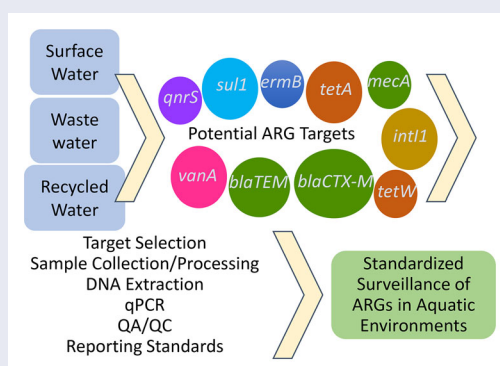
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ABSTRACT

Water environments are increasingly recognized as a conduit for the spread of antibiotic resistance, but there is need to standardize antibiotic resistance monitoring protocols to ensure comparability across studies. Quantitative polymerase chain reaction (qPCR) is attractive as a sensitive means of quantifying antibiotic resistance genes (ARGs) and has been applied broadly over the past two decades to various water matrices. QPCR avoids challenges and biases associated with culture-based methods, providing a reproducible and highly sensitive measure of ARGs carried across a bacterial community. However, there are numerous quality assurance and other aspects of protocols that need to be addressed to ensure that measurements are representative and comparable across studies. Here we conducted a critical review to identify gene targets that are most commonly measured by qPCR to quantify antibiotic resistance in surface water, recycled water, and wastewater and to assess corresponding protocols. Identified targets monitored in water samples included *sul1*, *tetA*, and *int11*, given their abundance and tendency to correlate with anthropogenic inputs, and *vanA* and *blaCTX-M*, as more rarely detected, but highly clinically-relevant targets. We identified 117 peer-reviewed studies meeting search criteria for application of these assays to water matrices of interest and systematically assessed the corresponding protocols, including sample collection and concentration, DNA extraction, primer/probe specificity, amplification conditions, amplicon length, PCR inhibition evaluation, and limit of detection/quantification. Gene copy numbers reported across studies were further compared by assay and water matrix. Based on this comprehensive evaluation, we recommend assays, standardized workflows, and reporting for the five target genes.



KEYWORDS Quantitative polymerase chain reaction; standardization; antibiotic resistance genes; environment

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Introduction

Antibiotic resistance is one of the greatest human health threats of our time, crippling the efficacy of antibiotics for treating deadly bacterial infections. The U.S. Centers for Disease Control and Prevention (CDC) indicate that antibiotic-resistant bacteria (ARB) cause more than 2.8 million infections and 35,000 deaths in the U.S. each year, up from 2 million infections and 23,000 deaths estimated in 2013 (CDC, 2013, 2019a). Although the threat of increasing antibiotic resistance has until recently been primarily associated with hospitals and clinics, community-acquired infections have also been on the rise (CDC, 2019a). 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 environmental sources and pathways can play in the evolution of antibiotic resistance and dissemination to human pathogens (European Commission, 2017; The White House, 2015; WHO, 2015).

The World Health Organization (WHO) and EU Global Action Plans (European Commission, 2017; WHO, 2015, 2019a) call for a One Health (i.e., humans-animals-environment) framework for addressing antibiotic resistance, identifying linkages with water sanitation and a need for corresponding surveillance programs. In the U.S., the recently updated National Action Plan has similarly recognized the need for environmental monitoring (Federal Task Force on Combating Antibiotic-Resistant Bacteria, 2020) and recently the CDC launched a multi-year initiative to fund research on antibiotic resistance, including topic areas focused on water and wastewater-related sources (CDC, 2019b). Building off a national monitoring study of antibiotic resistance genes (ARGs) across the U.S. rivers and streams, the U.S. Environmental Protection Agency (EPA) (Boczek, 2016; Hill et al., 2018), the U.S. Food & Drug Administration, the U.S. Department of Agriculture, and the CDC have teamed together to advance environmental monitoring of antimicrobial resistance to complement ongoing clinical and foodborne monitoring and more holistically advance a One Health framework for understanding and addressing antibiotic resistance. Concurrently, the State of California and others have taken a proactive approach by exploring the possibility of monitoring ARB and ARGs in recycled water, along with other contaminants of emerging concern (California State Water Resources Control Board, 2016; Hong et al., 2018; Olivieri et al., 2016). Harmonizing such efforts through method standardization would be highly valuable, enabling synthesis of the results across studies, locally and globally, so that the trends in antibiotic resistance can be accurately compared for various purposes, including quantifying anthropogenic inputs, assessing ARGs that escape wastewater treatment systems, identifying clinically-relevant ARGs of concern, and informing human health risk assessment (Berendonk et al., 2015; Huijbers et al., 2019). QPCR has grown in popularity for environmental antibiotic resistance monitoring and has now been widely applied for quantifying ARGs in studies of surface water (Lu et al., 2018; Reynolds et al., 2020), recycled water (Garner et al., 2018; Wang et al., 2014), and wastewater (Harnisz et al., 2020; Mao et al., 2015; Pazda et al., 2019). ARGs are the genetic elements (i.e., DNA sequences) carried by ARB that encode proteins that allow them to survive and grow in the presence of antibiotics (D'Costa et al., 2011). The ability of ARGs to be shared among microbial populations via horizontal gene transfer (HGT), leading to evolution of new resistant strains, marks ARGs as unique contaminants of emerging concern (Pruden et al., 2006; Zhang et al., 2009). Further, ARGs can be amplified under selection pressure of antibiotics and other antimicrobials in the environment, serving as indicators of “hot spots” where mitigation efforts might be most effectively directed (Karkman et al., 2018; Pruden et al., 2018). QPCR can be applied to the DNA extracted from an entire microbial community representing a water matrix, providing a highly sensitive and quantitative measure of antibiotic resistance. QPCR also circumvents key biases to culture-based techniques, e.g., the necessity to choose a single species or genera of bacteria to investigate, as well as the limited ability of selective media to isolate and

quantify targets against a complex background of non-target environmental bacteria. Further, culture-based methods overlook viable but non-culturable organisms (Khan & Yadav, 2004).

A key challenge for qPCR-based monitoring of antibiotic resistance is identifying optimal target ARG(s), given that there are thousands of genes and gene variants to choose from, as is apparent from a survey of the current inventory curated in the Comprehensive Antibiotic Resistance Database (<https://card.mcmaster.ca/>) (Alcock et al., 2020; Jia et al., 2017). Individual ARGs will vary in their immediate clinical relevance, as well as their relative mobility and sensitivity to anthropogenic inputs and various selective and co-selective agents (antibiotics, metals, etc.) (Ashbolt et al., 2013; Manaia et al., 2018; Pruden et al., 2018). Mobile genetic elements (MGEs) are also of interest for qPCR monitoring because they are typically the vehicle by which ARGs disseminate. It is also important to consider inherent limitations of qPCR, e.g., that it cannot directly distinguish DNA originating from viable versus non-viable bacteria, or DNA that exists extracellularly. QPCR also relies on the use of primers designed based on previously described ARG variants and thus may not universally capture all versions of these variants. QPCR quantification of ARGs has been applied across various domains of environmental research in a fairly *ad hoc* fashion, implementing different targets and protocols and often with limited detail regarding key quality assurance aspects, such as limit of detection (LOD), limit of quantification (LOQ), positive and negative controls, and accounting for PCR inhibition, issues that have been identified to be problematic for qPCR of environmental samples in general (Borchardt et al., 2021).

The objective of this critical review was to advance a framework for standardized qPCR-based monitoring of antibiotic resistance in surface water, recycled water, and wastewater. Based on an evaluation of prior reviews on environmental antibiotic resistance monitoring (Table 1) and input from an expert survey (Liguori et al., n.d.), we identified five qPCR targets that are commonly applied for monitoring antibiotic resistance in water environments. *sul1* and *tetA* are widely detected ARGs in aquatic environments and tend to be elevated in response to anthropogenic inputs and stressors (Berglund, 2015; Pruden et al., 2012). Similarly, *int1*, the class 1 integron-integrase gene, has also been found to be highly indicative of anthropogenic inputs (Gillings et al., 2015), while further providing an integrative measure of both multi-antibiotic resistance (i.e., through carriage of cassettes harboring multiple ARGs) and mobility (Gillings, 2014). *Bla*CTX-M and *vanA*, on the other hand, are often monitored as highly clinically-relevant ARGs encoding resistance to last-resort antibiotics. Following a systematic review with defined search criteria, various protocol features were evaluated, including: primer/probe specificity, amplicon length, amplification conditions, and LOD/LOQ. Further, reported gene abundances were compared across water matrices. Based on the findings, a path forward for standardization is recommended. This review will help to improve representativeness and comparability of studies employing qPCR-based monitoring to support broader understanding of the drivers of antibiotic resistance in water environments.

Selecting suitable ARG monitoring targets for aquatic environments

To identify ARGs of interest for this critical review, we first evaluated recent literature reviews relevant to ARG monitoring in aquatic environments (Table 1). Notably, Ashbolt et al. (2018) recently compiled a comprehensive summary of occurrence and trends for 50 ARGs monitored across various wastewater treatment plants (WWTPs), as part of the Global Water Pathogens Project. 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 extended

Table 1. Candidate ARGs for monitoring surface water, recycled water, and wastewater examined in the literature.

Antibiotic/function	Target gene(s)	Rationale
Amino-glycoside	<i>aadA1, aadA2, aadA13, aadB, arm, aph(6)-Id, aphA, aphA-3, aphA-13, aphA-6, aph2, npm, rmt</i>	WHO "Access" antibiotic resistance class; ARGs often detected in wastewater (Szczepanowski et al., 2009)
Extended spectrum beta lactamase (ESBL)	<i>ampC, blaCMY, blaCTX-M, blaOXA, blaSHV, blaTEM</i>	WHO resistance class with designations ranging from "Access" to "Reserve" as they are correlated with increased multidrug resistances (Dehshiri et al., 2018) with genes found in waste stabilization ponds (Neudorf et al., 2017), aerated lagoons (Rafraf et al., 2016) and wastewater treatment plants (Chandran et al., 2014; Karlowsky et al., 2017; Perilli et al., 2013; Wen et al., 2016)
Carbapenem-resistant beta lactamase	<i>blaKPC, blaSIM</i>	
Metallo-beta lactamase	<i>blaGES, blaIMP, blaNDM, blaNPS, blaPER, blaSPM, blaTLA, blaVEB, blaVIM</i>	
Class 1 integrase	<i>intl1</i>	Strong indicator of anthropogenic sources of ARGs, mobility, and multi-antibiotic resistance (M. R. Gillings et al., 2015; M. Gillings et al., 2008; Kotlarska et al., 2015; Aubertheau et al., 2017)
Colistin	<i>mcr-1, pmrAB</i>	WHO "Reserve" antibiotic noted for extremely fast global spread. Genes are relatively rarely detected in the environment, but increasing (Liakopoulos et al., 2016)
Efflux pump-multidrug	<i>acrB, acrD, mexB, mexD, mexF, mexI, mexY</i>	Effective against multiple antibiotics by generalized transport of antibiotics and other toxins out of the cell (Yılmaz & Özcengiz, 2017). Often detected in wastewater (Szczepanowski et al., 2009)
Glycopeptide	<i>vanA, vanB, vanC</i>	WHO "Reserve" antibiotic; genes are found in WWTPs (Alexander et al., 2016)
Macrolide	<i>ereA, ereB, ermA, ermB, ermC, ermF, mefA, mefE, mphA, mphB, msr</i>	WHO "Watch" antibiotic class; poorly removed by WWTPs and ARGs tend to be mobile (Bengtsson-Palme et al., 2019; Szczepanowski et al., 2009)
Methicillin	<i>mecA</i>	WHO "Watch" antibiotic, where resistance may result from transfer from <i>S. aureus</i> from non-human sources (WHO, 2019b)
Quinolone	<i>AAC-(6')-Ib-cr, gyr, qnrA3, qnrB1, qnrB2, qnrB4, qnrB5, qnrS, qnrS2, qnrVC</i>	WHO "Watch" antibiotic; genes are found in drinking and wastewater treatment (Figueira et al., 2011; Szczepanowski et al., 2009; Xia et al., 2010)
Sulfonamide	<i>sul1, sul2, sul3</i>	WHO "Access" antibiotic. Sulfonamides on market for longest and resistance genes correspondingly tend to occur in high abundance. Also a strong anthropogenic indicator and tends to co-occur with MGEs (Jacobs & Chenia, 2007; Bergeron et al., 2016; Makowska et al., 2016; Vaz-Moreira et al., 2016)
Tetracycline	<i>tetA, tetB, tetC, tetD, tetE, tetG, tetH, tetL, tetM, tetO, tetQ, tetR, tetS, tetT, tetU, tetW, tetX, tetY, tet31, tet34, tet35, tet36, tet39</i>	WHO "Access" antibiotic, ARGs are widespread, readily detectable, and several assays available (Börjesson et al., 2009b; Jacobs & Chenia, 2007; Storteboom et al., 2010)
Transposase	<i>Tn916/Tn1545</i>	Transposase with recombination module (Ciric et al., 2013) positively correlated in hospital wastewater (Wang et al., 2018)
Trimethoprim	<i>dfr2, dfr5, dfr7, dfr12, dfr13, dfr16, dfr17, dfr18, dfrA1, dfrA12, dfrA19, dfrB2, dfr D</i>	WHO "Access" antibiotic, widely administered antibiotic, genes frequently occur in surface waters and wastewater effluents (Phuong Hoa et al., 2008; Szczepanowski et al., 2009; Xu, 2014)

spectrum beta-lactamases (ESBLs): *bla*CTX-M, *bla*KPC, *bla*OXA-48, *bla*NDM, and *bla*VIM (CDC, 2019a). Further, we took into consideration the targets under investigation in the National Aquatic Resource Survey (NARS) recently launched by the U.S. EPA: *int*I1, *sul*1, *tet*W, *bla*TEM, *bla*KPC, *van*A, and MCR-1 (Keely, 2019). As suggested by Ashbolt et al. (2018), Berendonk et al. (2015), Pruden et al. (2018) and others, we 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.

The sulfonamide class of antibiotics was the first to be introduced in human medicine and has been on the market for the longest (Domagk, 1935). Correspondingly, bacterial resistance to sulfonamides is the most broadly disseminated (Aminov, 2010). Sulfonamides however, remain classified as “Access” antibiotics, indicating that they should be a first or second choice for relevant infections, and are widely used in both human and agricultural sectors (Huber, 1971; Perreten & Boerlin, 2003; WHO, 2019a). *Sul*1 and *sul*2 are the most prevalent sulfonamide ARGs in clinical isolates, with *sul*1 being almost universally incorporated into the class 1 integron (Moura et al., 2012).

Understanding the frequency and prevalence of ARG transfer between bacteria can inform mitigation practices to reduce ARG mobility. The class 1 integron-integrase gene, *int*I1, is a MGE that captures and mobilizes multiple gene cassettes that are known to contain ARGs (Gillings, 2014). In addition to containing ARGs, the *int*I1 gene is frequently linked with genes conferring resistance to disinfectants and heavy metals, making it a target that can be used to track stress responses in addition to antibiotic resistance dissemination (Liebert et al., 1999; Partridge et al., 2009). *Sul*1 and *int*I1 are the two most frequently assessed qPCR targets for evaluating antibiotic resistance in human, animal, and environmental samples (Gillings et al., 2015; Rådström et al., 1991; Stokes & Gillings, 2011). Additionally the *sul*1 and *int*I1 genes reliably exhibit strong correlations with anthropogenic inputs to water environments (Berendonk et al., 2015; Czekalski et al., 2014; Gillings et al., 2015; LaPara et al., 2015; Pruden et al., 2012; Pruden et al., 2019).

Similar to sulfonamides, tetracyclines are also classified as “Access” antibiotics by the WHO because they have activity against a wide range of commonly encountered susceptible pathogens while also showing lower resistance potential than antibiotics classified as “Watch” and “Reserve”. (WHO, 2019a, 2019b). Reliance on tetracyclines in agriculture remains particularly strong (Aminov et al., 2001). Tetracycline resistance has become widespread across Gram-negative and Gram-positive bacteria, including human pathogens, since the 1980s (Chopra & Roberts, 2001). Forty plus tetracycline ARGs have been described to date (Aminov et al., 2001; Jacobs & Chenia, 2007), with many early studies examining *tet*(O) and *tet*(W), which encode ribosomal protection proteins. However, more recently, it has been suggested that *tet*A, a tetracycline efflux pump, may be a more prevalent and relevant target for antibiotic resistance monitoring of anthropogenic and agricultural sources of antibiotic resistance in the environment due to its nearly universal prevalence in the *tet* operon (Nnadozie & Odume, 2019; Rizzo et al., 2013; Yang et al., 2018).

ESBL-producing *Enterobacteriaceae* have been classified as a “serious” health threat by the CDC, causing an estimated 197,400 hospitalizations per year and 9,100 deaths in 2017 in the United States (CDC, 2019a). *bla*CTX-M is the most common genetic variant of ESBL and has been identified as a gene that likely made its way into pathogenic bacterial genomes due to HGT from a nonpathogenic bacterial species (Humeniuk et al., 2002). While *bla*CTX-M is a clinically-relevant target, it is not readily detected in most environmental settings. Thus, detection of such a target could alone raise a flag of concern even in the absence of quantitation or expectation of a quantitative relationship with other inputs.

Vancomycin-resistant *Enterococcus* is also classified as a “serious” health threat by the CDC, causing an estimated 54,500 hospitalizations per year and 5,400 deaths in 2017 in the United States (CDC, 2019a). Until the late 1980s, the glycopeptide antibiotic vancomycin was considered to be a drug of last resort against Gram-positive infections (Cunha, 1995). However, the emergence and widespread dissemination of vancomycin-resistant *Enterococcus* highlight the need to identify corresponding targets for environmental monitoring (Tacconelli & Cataldo, 2008). The core of the vancomycin resistance mechanism relies on *vanH*, *vanA*, and *vanX*, which is thought to have originated in the soil bacterium *Paenibacillus* (Aminov & Mackie, 2007). Among these, the *vanA* operon gene has been identified as a suitable clinically-relevant ARG target (Ashbolt et al., 2018; Berendonk et al., 2015; Keely, 2019; Rizzo et al., 2013).

Literature review protocol

A tiered literature search strategy (conducted in September 2020) supported identification of peer-reviewed articles in the Web of Science Core Collection for each gene target (*sul1*, *int11*, *tetA*, *blaCTX-M*, and *vanA*) that: (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. Notably, papers were considered relevant if qPCR was applied to DNA from a bulk water environment as opposed to isolates derived from the environment of interest.

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”) . 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 “*int11*” OR “*vanA*” OR “*blaCTX-M*” OR “*tetA*”). 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. The number of studies obtained at each tier and for each application area was recorded and in total 117 studies was assessed in this review (Table S1). 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). Additionally, primers that were most commonly used in the literature were assessed for their *in silico* specificity.

Data extraction and analysis

Gene copy data points were extracted from text, figures, and tables manually. Data analysis was conducted in R(v3.5.0) using dplyr and figures were generated using ggplot2 (R Core Team, 2013; Wickham, 2016; Wickham et al., 2015). Gene copy numbers were considered

nonparametric and the Kruskal-Wallis and Wilcoxon rank sum tests were used to evaluate statistical significance. Statistical significance was set at $\alpha = 0.05$.

Primer alignment analyses were conducted in order to assess *in silico* primer specificity by searching and downloading all NCBI entries with matching “Gene Names” for each examined gene. These sequences were then aligned to the relevant primer and probe sets using Geneious Prime (version 2021.2.2, BioMatters Ltd.). Further, recommendations were compared with primer assays contained within the Literature-based, manually-Curated Primer pairs Database (LCPDb) to compare recommendations based on efficacy and specificity scores (Gorecki et al., 2019).

Evaluating commonly used assays for anthropogenically-sensitive targets (*sul1*, *int11*, *tetA*)

In total, 77 records were identified that examined the prevalence of *sul1* in surface water, wastewater, and recycled water (Figure 1(a)). One dominant primer set was found for *sul1*, which originated from Pei et al. (2006) and has an amplicon length of 163 base pairs (bp) (Table 2). The Pei et al. (2006) primers were highly specific (91%), which aligns with the LCPDb score of 1 for both efficacy and specificity (Gorecki et al., 2019). Additionally, this assay was very sensitive, with LOQs ranging from 2 to 250 gene copies (gc). The reported efficiency for the Pei et al. (2006) assay ranged from 74 to 106%. Evaluating the overall prevalence in aquatic environments, *sul1* was most prevalent in wastewater influent and least prevalent in surface waters (Figure 1(b), Kruskal-Wallis, $p < 0.001$). The near ubiquitous use of the *sul1* primer set as developed by Pei et al. (2006) suggests that it is an effective assay for short amplicons. No *sul1* primers were found targeting long amplicons (i.e., >400bp) that had previously been applied for qPCR, although the (Heuer & Smalla, 2007) assay targets a 965 bp amplicon and is widely applied for PCR and is worth considering optimizing for this purpose.

int11 was identified in 65 studies focused on the water matrices of interest. Four dominant primer sets were identified, originating from the works of Barraud et al. (2010), Goldstein et al. (2001), Stokes et al. (2006), and Luo et al. (2010). The Barraud et al. (2010) primers were the most widely used and were relatively specific (82%), but the Goldstein et al. primers were reported to be more specific (92%) (Table 2). The Stokes et al. primers produce the longest amplicon among the four assays. Reported LODs and LOQs were similar across these four assays and ranged from 5 to 50 gc per microliter of DNA extract and for the 14 to 300 gc per microliter of DNA extract, respectively. The efficiencies ranged from 87 to 111%. Comparing studies that reported gc per milliliter sample volume (Figure 2) revealed significant differences in concentrations in recycled water and wastewater as a function of assay used, but this was not the case for surface waters (Recycled: $p < 0.001$, Wastewater: $p = 0.02$, Surface: $p = 0.09$, Kruskal Wallis). In recycled waters and wastewaters, the Goldstein et al. assay yielded higher abundances than the other assays (wastewater $p = 0.001$, recycled $p = 0.004$, Wilcoxon rank sum test). Finally, the Stokes assay appears to carry the advantage both of high sensitivity and exclusion of damaged DNA by the long amplicon (Ayala-Torres et al., 2000; Dodd, 2012; McKinney & Pruden, 2012).

TetA was targeted in 44 of the identified studies, with 21 assessing surface water, 4 assessing recycled water, and 24 assessing wastewater. One primer set developed by Ng et al. (2001) was used in over 60% of articles, while a second primer set, developed by Börjesson et al. (2009a), was utilized in 14% of studies. One long amplicon assay (950 bp) was developed by Guardabassi et al. (2000), but this was only used in one study. This assay was assessed in the LCPDb and found to have both high specificity and efficacy (Gorecki et al., 2019). With respective amplicon lengths of 210 bp and 91 bp, both the Ng et al. and Börjesson et al. (2009a) primers aligned to the same 58% (105 of 181) of the *tetA* sequences (Table 2). The Guardabassi et al. assay aligned to 40% (70 of 177 *tetA* sequences over 1100 bp). The LODs and LOQs across short amplicon assays were consistent and LODs ranged from 1 to 30 gc per microliter of DNA extract and 5 to 330 gc

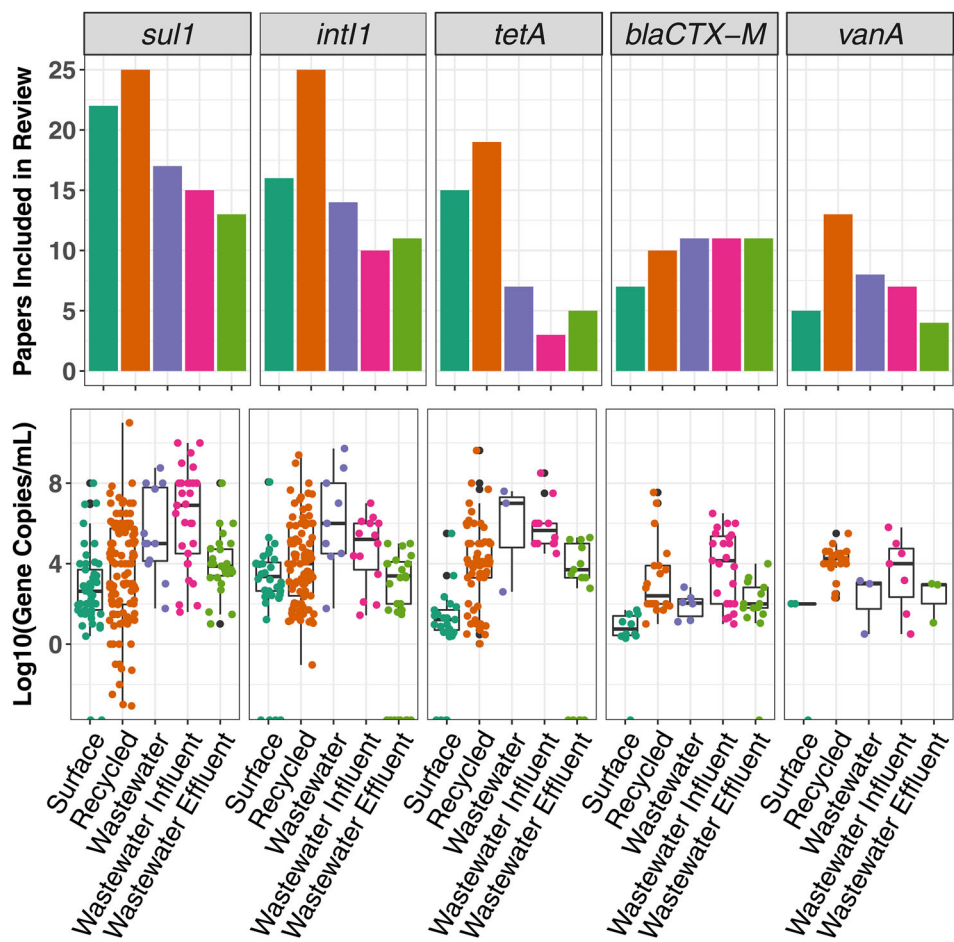


Figure 1. (A) Number of articles that met the search criteria for each gene by water matrix (surface, recycled water, and wastewater). Articles that examined more than one water matrix are double counted. (B) Target gene concentrations measured in each water matrix based on data extracted across the 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.

per microliter of DNA extract, respectively. The efficiencies for all assays ranged from 90 to 105%. Both short amplicon target assays were applied in all target environments, with the Ng et al. (2001) assay yielding significantly higher gene abundances across environments, especially recycled water samples (all water matrices: $p = 0.03$, recycled $p < 0.001$, Wilcoxon). Across aquatic environments, both recycled water and wastewater contained higher abundances of *tetA* than surface waters (Kruskal Wallis, $p < 0.001$).

Evaluating commonly used assays for clinically-relevant indicators of antibiotic resistance

Forty-one (41) studies targeting *blaCTX-M* using qPCR were identified, with application of fourteen to surface water, three to recycled water, and thirty to wastewater. The dominant assays for monitoring *blaCTX-M*-1 were developed by Colomer-Lluch et al. (2011) and Kim et al. (2005), with respective amplicon lengths of 101 and 780 bp. The dominant assays for *blaCTX-M*-32 were developed by Szczepanowski et al. (2009), with an amplicon length of 156 bp; however the

Table 2. Recommended qPCR assays for *sul1*, *int1*, *tetA*, *blaCTX-M*, and *vanA* quantification in water samples, depending on monitoring objective.

Target	Mon-itoring Objective ^a	Amplicon Length, bp	#of Citations (%) ^b	Specificity (%) ^c	Primers (F; R; Probe) 5'-3'	Annealing Temp. Range, °C
<i>sul1</i>			77			
Pei et al. (2006)	S	163	63 (82%)	91	CGCACCAGAAACATCGTGCAC; TGAAGTTCCGCCGCAAGGCTCG;	55-69
Heuer and Smalla, (2007) ^d	S,D	965	0	23	TTCATGGGCAAAAGCTTGATG; GGCCGGAAGGTGAATGCTA;	60
<i>int1</i>			65			
Luo et al. (2010)	S,M	146	6 (9%)	79	GGCTTCGTGATGCTCTCT; CATTCCTGGCCGTGGTTCT;	55-60
Barraud et al. (2010)	S,M	196	34 (52%)	82	GCCTTGATGTTACCCGAGAG; GATCGGTGCAATGCGTGT; ATTCTGGCCGTGGTTCTGGGTTT	59-60.2
Goldstein et al. (2001)	S,M	280	16 (25%)	92	CCTCCCGCACGATGATC; TCCACGCATCGTCAGGC;	50-63
Stokes et al. (2006)	S,M,D	473	9 (14%)	78	CTGGATTTCGATCAGGCACG; ACATGCGTGAATCATCGTCG;	60
<i>tetA</i>			44			
Börjesson et al. (2009a)	S	91	6 (14%)	58	TCAATTTCTGACGGGCTG; GAAGCGAGCGGGTTGAGAG;	60
Ng et al. (2001)	S	210	28 (64%)	58	GCTACATCCTGCTTGCTTC; CATAGATCGCCGTGAAGAGG;	50-64
Guardabassi et al. (2000)	S,D	950	1 (2%)	40	GTAATTCTGAGCACTGTCG; CTGCCTGGACAACATTGCTT;	57.2
<i>blaCTX-M-1</i>			41			
Colomer-Lluch et al. (2011)	C,I	101	8 (20%)	55	ACCAACGATATCGCGGTGAT; ACATCGCGACGGCTTCT; TCGTGCGCCGCTG	62
Kim et al. (2005)	C,D,I	780	15 (38%)	55	CGTCACGCTGTTGTTAGGAA; ACGGCTTTCTGCCTTAGGTT;	60-61
<i>blaCTX-M-32</i>			9			
Szczepanowski et al. (2009)	C,I	156	9 (23%)	55	CGTCACGCTGTTGTTAGGAA; CGTCATCAGCACGATAAAG;	58-64
<i>vanA</i>			32			
Volkman et al. (2004)	C,I	64	10 (31%)	60	CTGTGAGGTCGGTTGTGCG; TTTGGTCCACCTCGCCA; CAACTAACGCGGCACTGTTCCCAAT	60-62
Klare et al. (1995)	C,I	376	6 (19%)	60	TCTGCAATAGAGATAGCCGC; GGAGTAGCTATCCAGCATT;	58-60
Dutka-Malen et al. (1995)	C,D,I	732	5 (16%)	19	GGGAAAACGACAAATTGC; GTACAATGCGGCCGCTTA;	54

^aC- Monitor clinically-relevant ARGs circulating in the community, S-Quantify antibiotic resistance in surface water from anthropogenic sources, M-Assess multi-antibiotic resistance and mobility, D-Quantify degradation and removal by treatment processes, I- Inform human health risk assessment models.

^bTotal number of included references that utilized this assay, percentage was calculated from the total number of gene specific references denoted in the gene identifier row.

^cAssay specificity was defined as the percentage of sequences that aligned to the primer set from NCBI Genbank entries.

^dThe Heuer and Smalla assay has not previously been reported for qPCR and would require optimization as a long amplicon assay for gene quantification.

sequence targeted was found to overlap 100% with that targeted by the Kim et al. primers. This indicates that the two primer sets likely do not truly capture different sequences or variants. Most studies (56%) did not report which *blaCTX-M* gene variant was targeted. This is problematic as the CTX-M enzyme group is not homogenous. Variants are known to differ in terms of mobility and their resistance spectrum (Canton et al., 2012). In terms of specificity, the Kim et al. primers, Colomer-Lluch primers and probe, and Szczepanowski et al. primers all aligned to 55% (71 to 72 of 130) of the *blaCTX-M* (all variant) sequences listed within NCBI Genbank. The LODs were not reported for any of the included assays, while the LOQs ranged from 5 to 100 gc per microliter of DNA extract. The efficiencies of all assays ranged from 87 to 111%. The reported concentrations of *blaCTX-M* did not vary as a function of assay applied. Recycled

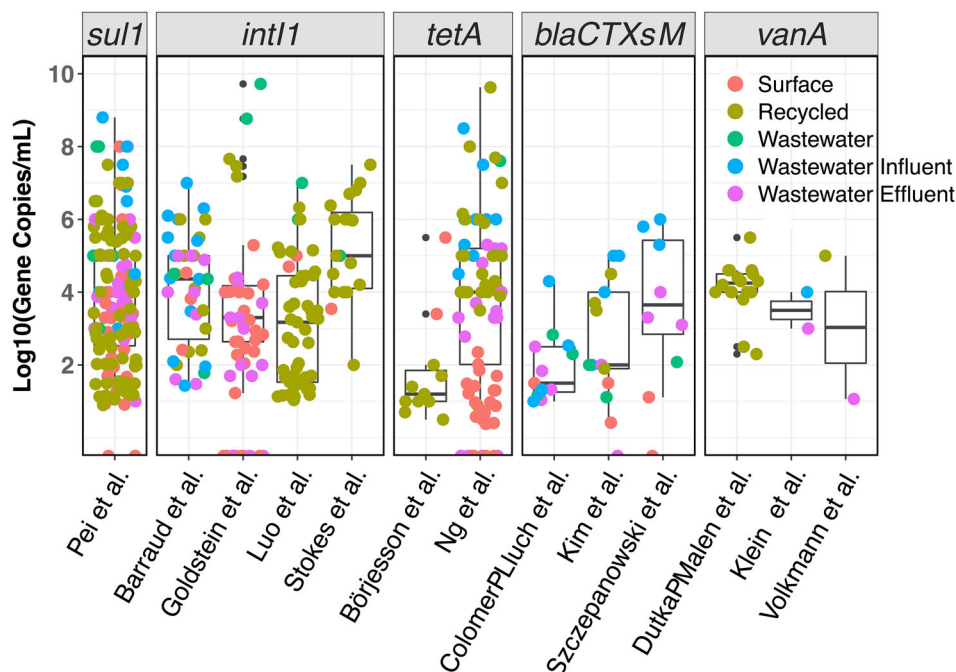


Figure 2. Target gene copies/mL reported across the studies for each water environment as determined by the most frequently utilized assays (x-axis). Box plots indicate the median, first, and third quartiles and whiskers extend no more than 1.5 times the inter-quartile range. Data points are colored by water matrix.

water and wastewater contained higher abundances of *bla*CTX-M than surface waters (Kruskal Wallis, $p < 0.001$). *vanA* was the target of 31 studies, with 10 applied to surface water, 3 to recycled water, and 19 to wastewater. Three assays were utilized most frequently: Volkmann et al., 2007, Klare et al. (1995), and Dutka-Malen et al., 1995, with amplicon lengths of 64 bp, 376 bp, and 732 bp, respectively (Table 2). In terms of specificity, the three primer sets were found to align to the same 60% (3 of 5) of the *vanA* sequences listed in NCBI Genbank. The Klare et al. (1995) assay was included in the LCPDb and found to be highly specific and with high efficacy. The LOQs ranging from 14 to 140 gc per microliter of DNA extract across all three assays. The efficiencies of all assays ranged from 76 to 98%. The levels of *vanA* reported did not vary based on assay applied. When combining data collected across assays, there were no significant differences across studies in levels of *vanA* reported in surface water, recycled water, or wastewater (Kruskal-Wallis, $p = 0.47$).

Proposed standardized qPCR workflow for the study of *sul1*, *int11*, *tetA*, *bla*CTX-M, and *vanA*

Key quality assurance/quality control considerations for qPCR

Table 3 summarizes key quality assurance/quality control and reporting considerations for qPCR-based monitoring of antibiotic resistance in water environments. There are numerous specific aspects of qPCR that must be considered to ensure that the data produced are representative and comparable across studies. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) provides a helpful starting place. MIQE establishes common reporting guidelines for qPCR, including assay efficiencies, LOD, LOQ, R^2 for calibration curves, PCR inhibition, and inclusion of negative controls.

Table 3. Key quality assurance/quality control measures for qPCR for antibiotic resistance monitoring of water environments.

	QA/QC Measure	Rationale
Sample Collection and Processing	Field blank and trip blanks	Differentiate measures true to samples from contamination introduced during sampling
	Storage on Ice	Prevent changes in ARG measurements due to biological activity
	Concentrate within 24 hours	Maximize capture of intact cells and minimize effects of biological activity
	Consistent and efficient DNA extraction procedure	Ensure comparability across samples and representative capture of bacteria
qPCR	Determine LOQ and LOD	Differentiate quantifiable ARGs from qualitative detection and non-detection
	Triplicates in every run	Quantify technical replicate error
	Inhibition control	Minimize false negatives
	R ² , Efficiency	Assess run quality across samples and accuracy of quantitation
	Blank reactions in every run	Verify that contamination has not been introduced in the lab
Reporting	For each new environment, verify specificity by sequencing products	Some environmental samples may be more prone to nonspecific amplification
	Volumes collected/filtered/diluted	Support quantitative comparison across studies and input for risk assessment
	qPCR assay and run conditions	Ensure replicable workflows across studies
	LOQ, LOD	Support accuracy of statistical comparisons of low concentration samples across studies
	Absolute and relative abundance	Provide information about net removal or exposure concentrations for risk assessment or identify conditions that may be imposing selective pressure for ARGs

However, of the 117 studies assessed in this work only 69 (59%) reported any of these parameters. This is consistent with the recent findings of Borchardt et al. (2021), who recently further adapted MIQE guidelines for environmental microbiology samples, noting that only 33% of the evaluated studies implementing digital and qPCR complied with MIQE guidelines. The lack of reporting in regards to LOD and LOQ is especially problematic, as a non-detect in one sample medium may not be equivalent to a non-detect for another medium, due to factors such as differences in sample volumes, concentration factors, template volume in assays, and dilution of DNA extracts (U.S. Environmental Protection Agency 2012). In many studies, it also is not always clear if the LOD/LOQ values provided are in the units of gc/ μ L of DNA extract or original sample volume, with sample volume being more informative and relevant for comparison to other studies.

Sample collection and processing

Across the included studies, sampling methodology was strikingly similar, highlighting that many ARGs could be targeted with a unified sampling method. Primarily, water was collected in sterile containers (mainly autoclavable HDPE plastic or glass) with sample volume varying across studies, but largely depending on the kind of water matrix. For instance, within “wastewater” the sample volume is also a function of the location along treatment train and the associated cell density. As a result, larger volumes are needed from final treated effluent (10 to 2,000 mL) than from activated sludge (50 to 200 mL) (Table S3, supplementary material). Many studies also reported “filtering until clogging,” which in high turbidity samples would mean less volume is actually filtered than is collected. The volume of water passing through the filter before clogging can range widely, although two liters are typically a good goal and may be achievable for less

turbid samples, such as surface water. Across the 117 assessed studies, the average (median) volume sampled was 1860 (500) mL of surface water, 960 (500) mL of recycled water, and 1670 (250) mL of wastewater. A recent study comparing qPCR for ARG quantification by water sample types (surface, recycled water, and wastewater) identified key factors contributing to lab-to-lab variation (Rocha et al., 2019). These included sample volume, concentration method, DNA extraction procedure, storage temperature and duration, thermal cycle program, positive and negative controls, detection limits, and matrix interferences/inhibitors.

Inclusion of replicate samples is essential to assessing variability in the analysis (i.e., technical variability) versus variability in the system under study (i.e., biological variability) and to facilitate statistical comparisons. Commonly, three biological replicates are set as the standard, but in reality, more may be needed in order to accurately account for natural variability in the system or condition of interest in subsequent analyses. Roughly half (52%; 61) of the 117 included studies denoted including technical or biological replicates in their study design. Statistical power analysis tests are recommended before commencing a sampling campaign. Volume of samples is also important because larger volumes increase probability of detecting a low abundance gene, but are also more difficult to concentrate.

Because of the high sensitivity of qPCR, extra care is warranted to ensure that contamination is minimized and quantified throughout the analysis workflow. Inclusion of field blanks (sterile water transported to the field site and transferred to a new sterile bottle in the field) during the sample collection step is a comprehensive means of achieving this, capturing contamination that may be introduced from sampling and transport. It was found that few studies ($n = 4$) mentioned the inclusion of field blanks, although these are recommended in the MIQE general guidelines for qPCR (Bustin et al., 2009). To minimize further microbial growth and alteration of the microbial community profile prior to analysis, it is also important to transport samples at $< 10^{\circ}\text{C}$ from the field to the lab, ideally processing them through to the concentration step in less than 6 hours (US Environmental Protection Agency, 2012). Sometimes, this is not realistic and studies have shown measurements may be consistent for up to 48 hours for samples kept on ice (Pope et al., 2003).

Upon reaching the lab, samples should be immediately concentrated for downstream DNA extraction and analysis. Freezing samples is not recommended because this will damage the cells and diminish the efficacy of subsequent capture of DNA by filter concentration (US Environmental Protection Agency, 2013). One study indicated that water samples stored in a freezer more than 14 days before the concentration step resulted in a reduction of DNA recovery (Hinlo et al., 2017). There was similarity across the 117 studies in terms of sample concentration methodology, with 70% of studies employing vacuum filter concentration onto a 0.22-micron (polycarbonate, mixed cellulose, or nitrocellulose) filter. Filter blanks, i.e., filtering sterile water through identical filters in parallel with the sample processing, are recommended at this step in order to capture contamination introduced by laboratory processing. Filter blanks were also rarely reported across the studies ($n = 19$). “Filtering until clogging” is an effective way to maximize both water volume processed and DNA recovery, though it is critical that all liquid in the filtration manifold passes through the filter. It is also critical that this volume is recorded and reported accordingly to provide a denominator for subsequent analysis. Filters can then be immediately subject to DNA extraction, or stored in 50% ethanol at -20°C until DNA extraction (Li et al., 2018).

DNA extraction

DNA extraction is the next potential source of bias and, as differing downstream applications emerge for extracted DNA, it is critical that extraction methods are chosen with care for the subsequent analyses because multiple analyses (sequencing and molecular work) are often performed

on the same DNA extract. Across the 117 evaluated studies, the most prevalent of the reported DNA extraction kits used were the FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, California) (17%), the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) (16%), and the DNeasy Power Water Kit (QIAGEN, Hilden, Germany) (17%). Across studies, for each targeted gene, there was no correlation between DNA extraction kit and the subsequently quantified gene concentration ([Figure S1, supplementary material](#)), suggesting differing kits are not systematically biasing ARG enumeration when applied to surface water, recycled water, or wastewater. However, it is noteworthy that the most prevalent DNA extraction kits (top 75%) all implemented bead-beating. This is a particularly useful step in environmental samples because it helps to ensure lysis of both Gram-positive and negative bacteria (Guo & Zhang, 2013).

qPCR workflow

Some aspects of the qPCR workflow are consistent regardless of gene target, such as the instruments used in a given laboratory and the laboratory layout. Differences in qPCR instruments should be addressed by determining and reporting the quality assurance/quality control measures as recommended by Borchardt et al. (2021), including the slope (equivalent to the PCR efficiency), goodness of fit, number of standards, and the lowest standard concentration measured. The U.S. EPA provides further guidance on precautions that should be taken to prevent contamination in PCR-based studies, including laboratory design (e.g., positive pressure) and work-flow measures (e.g., separate work spaces for manipulating PCR reagents and DNA extracts) (U.S. Environmental Protection Agency 2004).

To reduce variability and improve precision in qPCR measurements, it is recommended that each qPCR run includes samples run in triplicate as well as triplicate standard curves. Another key decision in the qPCR workflow is determining what will be used as a positive control. Commercially-available and externally validated synthetic DNA standards are recommended to improve accuracy and support uniformity across labs. Finally, it is standard practice to include no template “blank” controls, i.e., background lab-grade water used in the master mix, with every run. No template controls can serve to identify PCR reagent or laboratory airborne contamination. To ensure that an assay is capturing the specific target of interest, which is especially challenging in complex environmental samples, it is also advised that, at the outset of applying a qPCR assay to a new matrix, a subset of resulting qPCR amplicons be cloned and subject to DNA sequencing. A melt curve analysis should further be implemented with every run to verify specificity for all intercalating dye assays (performed in 35 of the included assays). Inclusion of a probe also helps to improve specificity. qPCR inhibitors; e.g., humic/fulvic acids, phenolic compounds, heavy metals, and chelators, can be especially challenging for environmental samples (Wilson, 1997). Addressing inhibition is key to avoid reduced quantitation and false negatives. In order to assess the presence of inhibitors in a target matrix, internal or external amplification controls or dilution curves can be performed. A commonly utilized exogenous DNA control recommended by the EPA is salmon testes DNA, which entails spiking a known concentration of exogenous DNA target (one not anticipated to be present in the sample) and subsequently quantifying the abundance (US Environmental Protection Agency, 2012). This enables the identification of the extent to which inhibition is influencing detection and quantification and allows the researcher to correct values by an appropriate factor for a given set of samples collected from the same water environment. However, Cao et al. (2012) found when evaluating the effectiveness of dilution and internal controls at controlling inhibition in qPCR for the enumeration of *Enterococcus* in environmental waters that this method did not accurately quantify inhibition thus causing inaccurate reporting of the target. Depending on the expected variability and abundance of target gene copies in the DNA extract, a dilution curve on either a subset or all samples can be implemented. If inhibition is at play, an optimal dilution (i.e., that which maximizes signal)

can be selected. Supplements can also be added to the qPCR reaction to enhance amplification, such as proteinase inhibitors, formamide, or bovine serum albumin (Borchardt et al., 2021). Across the included studies, dilution was the most common form of inhibition management, implemented in 60% of studies that noted an inhibition mitigation strategy ($n = 14$), while no studies incorporated salmon testes DNA.

Selecting among available assays for *sul1*, *intI1*, *tetA*, *blaCTX-M*, and *vanA*

Table 2 summarizes key information for selecting targets and assays for qPCR monitoring of antibiotic resistance. Notably, it is critical to consider the monitoring objectives. For example, when the purpose is to gain insight into forms of antibiotic resistance that could pose an immediate health threat in a community served by a wastewater treatment plant, then it would be logical to select a clinically-relevant ARG, such as *blaCTX-M* or *vanA*, and monitor the influent sewage. Further, sensitivity should be prioritized in assay selection when the intention is to maximize potential for detection of such a target that poses an immediate human health concern, but may be rare. For this purpose, shorter amplicons tend to maximize qPCR efficiency and thus sensitivity to detecting rare targets. Such an approach would make sense in a surface water study aiming to quantify risks associated with recreational use. However, if the goal is to demonstrate whether a wastewater or recycled water treatment processes is effectively degrading or inactivating an ARG, then more abundant targets, such as *sul1*, *tetA*, or *intI1*, may be appropriate to be able to generate removal curves. Longer amplicons are also desirable for this purpose because they are more sensitive to DNA damage. Süß et al. (2009) conducted a study of bacteria isolated from clinical wastewater subjected to UV irradiation and demonstrated that DNA damage detection improves as amplicon size is increased (100 bp versus 900 bp amplicon). Another study also found that, after UV disinfection, ARGs with thymine dimers were more effectively excluded by longer amplicons of near 1000 bp (McKinney & Pruden, 2012). Thus, primers capturing longer amplicons are recommended to more accurately differentiate the effects of disinfection processes on ARG functionality in a host cell (Yoon et al., 2017). As evaluating the efficacy of disinfection for inactivating ARGs is a common water quality monitoring goal, as long as adequate standard curves and detection limits are achieved, in most cases it is preferable to select assays targeting longer amplicons. Because of the differential goals of antibiotic resistance monitoring, both short and long amplicon assays are recommended based on this review.

The level of validation undertaken in each original study was evaluated, including the degree to which assays have been adopted in the field (Table 2). Positive control sequences for each synthetic DNA sequence are reported in the supplemental information. Selecting the Pei et al. (2006) assay for the assessment of *sul1* in the environment was clear, due to the overwhelming consensus of the field and specificity of the assay. No long amplicon was found for *sul1* quantification in the environment. However the Heuer and Smalla (2007) primer pair is promising for such a purpose, although adaptation of the long amplicon assay to qPCR would require optimization. The Stokes et al. (2006) assay was selected for the assessment of *intI1* in the environment, given that it provides a longer amplicon. Of potential interest for future application; however are *intI1* primers that were introduced subsequent to the timeframe of this systematic review and are reported to be capable of distinguishing class 1 integrons that carry ARGs versus those that carry empty cassettes (Quintela-Baluja et al., 2021). The Ng et al. (2001) assay was selected for the detection of *tetA* due to its widespread adoption, amplicon length, and extent of validation. When evaluating optimal primers for the *blaCTX-M* gene, it was observed that the primer sequences most used for targeting both *blaCTX-M-1* and *blaCTX-M-32* targeted identical regions of the *blaCTX-M-1* gene and that the Kim et al. (2005) amplicon target was both longer and captured the other target sequences in its amplicon. For *vanA*, the Dutka-Malen et al. (1995) assay is recommended

because it targets a longer amplicon, was subject to substantial validation in the original work, and has been successfully applied in relevant water matrices.

Establishing LOQ and LOD and ensuring specificity

Standard analytical and reporting measures are also critical to ensuring the utility and comparability of qPCR-based ARG measurements. Consistent with the MIQE and environmental microbiology minimum information guidelines, researchers should report all thresholds, controls, calibration curves, LODs, LOQs, the slope and y intercept of standard curves as well as the threshold cycle for the lowest concentration standard to ensure that any drift within standard amplification is minimized (Borchardt et al., 2021; Bustin et al., 2009). If drift appears to be occurring, then a fresh standard aliquot should be used and the run repeated. R^2 values should be >0.98 to be acceptable with a target efficiency of 100%, although lower efficiencies are common with environmental assays because of conditions imposed to ensure specificity within a mixed template background can also lower the efficiency. Plates with unacceptable efficiency values (outside of 80 to 120%) should be re-analyzed.

Importantly, only samples that fall within the linear portion of the standard curve are quantifiable, thus it may be necessary to re-run samples with the high range or low range end of the standard curve extended. The lowest standard that amplifies in at least duplicate (on the linear portion of the curve, without plateauing) should be set as the LOQ for that batch of samples, and any sample measured below that is reported as $<LOQ$. The MIQE guidelines state that the inherent LOD for qPCR is 3 copies per reaction, assuming a 1 microliter template addition to a 25- μ L reaction. To extend this further, a larger volume qPCR reaction can be run with increased DNA extract addition. Samples that amplify in duplicate below the LOQ and above duplicate no template controls can be considered as $>LOD$. Any sample measuring below this threshold is reported as $<LOD$. Due to the stochastic nature of qPCR, it is not uncommon that some portion of analytical replicates fails near the LOD. QPCR can be conducted in various formats to verify specificity (e.g., amplification of the “correct” target), typically via a TaqMan or other “probe” that binds between the primers or an intercalating dye that gives a stronger and stronger signal as the DNA amplifies. Here we focused on intercalating dye assays, specifically EvaGreen (Biotium, Fremont, CA) or SybrGreen (Thermo Fisher Scientific., Waltham, MA) as these were applied across 97% of studies evaluated. A melt-curve should be run upon completion of all runs incorporating intercalating dyes, i.e., to verify that the melting temperature is consistent with the expectation for target amplification products. If the peak melting temperature of the sample is not equivalent to that of the standard, then the sample should be excluded from further analysis. However, it may be of interest to further explore variation in melt curve, as it could indicate variants of the ARG of interest, but this would have to be verified through cloning and sequencing the product. If melt curve variation is widespread among samples, further trouble shooting is recommended, such as verifying the optimal annealing temperature through gradient PCR and sequencing amplicons to verify specificity.

Reporting standards for qPCR-based measurements of ARGs in aquatic environments

Recent guidelines have highlighted the need for standardized reporting of sample collection and analysis procedures for measuring antibiotic resistance in environmental samples (Hassoun-Kheir et al., 2021). In particular, standardizing qPCR assays for ARGs across aquatic environments are critical to support evaluation of trends across studies. Utilizing an array of ARGs that represent differential targets can help capture broader trends and hone in on specific ARGs of concern. Across all studies, there was a dichotomy in reporting of the absolute abundance (i.e., gc per mL of sample water) or of the relative abundance (i.e., ARG gene copies normalized to 16S rRNA

gene copies). Absolute abundances are useful for assessing net flux or numbers of ARGs, which is typically more valuable for informing risk assessment models. Relative abundance, on the other hand, can act to normalize variation in DNA extraction by comparing the ratio of two targets rather than their absolute prevalence and also provide an estimate of the relative proportion of bacterial populations carrying ARGs. Thus, comparison of relative abundances can aid in identifying conditions that may be inducing selection pressure or HGT and thus help to direct mitigation efforts. A major limitation of cross-study comparisons, however, is that calculations for absolute or relative abundance determinations are rarely included. Across all studies, there were over 28 different 16S rRNA assays utilized with the Suzuki et al. (2000) primers employed in 35% of the studies and 3 times more frequently than the next most abundantly used assay (Nadkarni et al. (2002)(10%); Koike et al. (2007)(8%). When calculating absolute abundance, it is critical to take into account all dilution or concentration steps during sample processing, including any dilutions of DNA extracts to address inhibition, the DNA elution volume, and the initial concentrated sample volume. To support comparability of data across studies, it is recommended to report data in both absolute and relative abundance, in table format, making use of the supporting information if necessary.

Here we propose Equation 1 for the determination of absolute abundance of ARGs in a sample and Equation 2 for the determination of relative abundance to the 16S rRNA gene. In Eq.1 the PCR reaction term refers to the total volume of reagents in a given PCR reaction, the Dilution Factor term refers to any dilution performed to limit inhibition and the Diluted DNA Extract term refers to the volume of diluted DNA used for the PCR reaction.

$$\text{Absolute Abundance} \left(\frac{\text{GC}}{\text{mL}} \right) = \frac{\text{Gene Copies(GC)}}{\mu\text{L PCR Reaction}} * \frac{\mu\text{L PCR Reaction}}{\mu\text{L Diluted DNA Extract}} * \text{Dilution Factor} * \frac{\mu\text{L DNA Extract}}{\text{mL Sampled water}} \quad (1)$$

$$\text{Relative Abundance} \left(\frac{\text{GC ARG}}{16\text{S rRNA GC}} \right) = \frac{\text{Absolute Abundance (ARG)}}{\text{Absolute Abundance (16S rRNA gene)}} \quad (2)$$

Finally, it is also critical to verify the normality of the distribution of ARG copies across samples before performing any parametric statistics. Often, the data are not normally distributed, in which case a log-transformation or application of non-parametric statistics may be appropriate. Note that averages of analytical replicates are considered to be the measurement for that sample and subsequent statistics should be performed on biological replicate measurements.

Adapting to droplet digital PCR format

It is recognized that gene quantification technologies are continuing to evolve. Droplet digital PCR (ddPCR) promises to eliminate many of standardization variables that plague qPCR reporting; such as standard curves, melt curves analysis, and run variation, to yield more sensitive, precise, and reproducible measurements (Taylor et al., 2017). Quality assurance and quality control for ddPCR analysis is similar to that applied for traditional qPCR, including incorporation of triplicate reactions (Hindson et al., 2013). The digital MIQE guidelines as proposed by Huggett et al. (2013) require similar experimental design, sample concentration, and nucleic acid extraction as the traditional PCR MIQE guidelines (Bustin et al., 2009). However, in addition, the sequence accession number, amplicon length, location of each primer, and splice variants should also be provided. ddPCR is thought to be more tolerant to PCR inhibitors, but this should still be assessed in each sampling matrix of interest (Cao et al., 2015). Recovering the target amplicon is also possible with ddPCR, making it possible to further verify specificity by sequencing the resulting amplicons (Harringer & Alfreider, 2021).

High-throughput qPCR (HT-qPCR)

One emerging alternative to selecting one or a few ARGs to target is to instead apply a high throughput qPCR (HT-qPCR) array, which employs microfluidics or other commercial technology to simultaneously conduct multiple qPCR reactions in parallel (Ishii et al., 2013; Tourlousse et al., 2012; White et al., 2011; Zhu et al., 2013). HT-qPCR arrays can target at least 300 genes in one step (Sandberg et al., 2018) and have been applied to surface water, aquaculture, and wastewater samples (Ahmed et al., 2018; Bueno et al., 2019; Mathai et al., 2019; Quintela-Baluja et al., 2019). Sandberg et al. (2018) validated the efficacy of this approach in wastewater by targeting 39 ARGs simultaneously (48 genes overall). The findings aligned with past studies and traditional qPCR enumerations of ARGs in wastewater, but with 95% less time and materials. However, HT-qPCR has not been fully validated in terms of specificity (i.e., amplification of correct target) and is limited by lower sensitivity (i.e., higher detection limit) as a result of the small (nanoliter) reaction volumes (Stedtfeld et al., 2018; Waseem et al., 2019). Additional limitations to optimizing HT-qPCR for environmental monitoring stem from inability to simultaneously optimize annealing temperatures for multiple primers (Malhotra et al., 1998; Sipos et al., 2007). It is also not possible to collect the resulting amplicons and verify specificity via alternative means (e.g., DNA sequencing). The cost and need for specialized equipment is also a limiting factor for many labs. According to a Web of Science search, HT-qPCR is primarily used by a few research groups for ARG monitoring in the water environment (Figure S2, supplementary material). As commercial kits and laboratories enable HT-qPCR monitoring of ARGs, it is critical that they support reporting of traditional qPCR parameters and QA/QC criteria, such as the LOD/LOQ and efficiencies to support progress toward standardized ARG monitoring.

Conclusions

QPCR is a widely applied method for antibiotic resistance monitoring of various water environments. QPCR presents many advantages, especially in terms of specificity, sensitivity, and its quantitative capacity to inform various modeling efforts, including water treatment removal rates and human health risk assessment. Here we provide a critical evaluation of literature to date employing qPCR for the purpose of quantifying ARGs in surface water, recycled water and wastewater environments. In particular, we focused on *sul1*, *intI1*, *tetA*, *bla*CTX-M, and *vanA* as a candidate suite of genes that include targets that are readily detectable and sensitive to anthropogenic inputs and stressors, clinically-relevant, and indicative of multi-antibiotic resistance and mobility. Among the 117 papers identified in this study, 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. 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. Such collective efforts can help to guide investment in efforts for effectively mitigating the spread of waterborne sources and pathways of antibiotic resistance.

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